Antagonism of FOG-1 and GATA factors in fate choice for the mast cell lineage

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The zinc finger transcription factor GATA-1 requires direct physical interaction with the cofactor friend of GATA-1 (FOG-1) for its essential role in erythroid and megakaryocytic development. We show that in the mast cell lineage, GATA-1 functions completely independent of FOG proteins. Moreover, we demonstrate that FOG-1 antagonizes the fate choice of multipotential progenitor cells for the mast cell lineage, and that its down-regulation is a prerequisite for mast cell development. Remarkably, ectopic expression of *FOG-1* in committed mast cell progenitors redirects them into the erythroid, megakaryocytic, and granulocytic lineages. These lineage switches correlate with transcriptional down-regulation of *GATA-2*, an essential mast cell GATA factor, via switching of GATA-1 for GATA-2 at a key enhancer element upstream of the *GATA-2* gene. These findings illustrate combinatorial control of cell fate identity by a transcription factor and its cofactor, and highlight the role of transcriptional networks in lineage determination. They also provide evidence for lineage instability during early stages of hematopoietic lineage commitment.

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Abbreviations used: BMMC, bone marrow–derived mast cell; ChIP, chromatin immuno-precipitation; CtBP, C-terminal binding protein; eGFP, enhanced GFP; EKLF, erythroid Kruppel-like factor; EPO, erythropoietin; FcεRI, high affinity IgE receptor; FOG, friend of GATA; GMP, granulocyte/macrophage progenitor; GPIb, glycoprotein Ib; MC-CPA, mast cell carboxypeptidase A; MCP, mast cell progenitor; MEL, mouse erythroleukemia; MEP, megakaryocyte/erythroid progenitor; MMCP, mouse mast cell protease; SCF, stem cell factor; Srp, Serpent; TPO, thrombopoietin; Ush, U-shaped; YSMC, yolk sac–derived mast cell.

The zinc finger transcription factor GATA-1 serves as a useful paradigm for studying the role of lineage-specific factors in cell fate determination. Its expression is restricted to select cell types within the hematopoietic system, including erythroid, megakaryocytic, eosinophilic, and mast cells (1). Its only site of expression outside of the hematopoietic system is in Sertoli cells of the testis (2). Gene targeting studies in mice demonstrate an essential role for GATA-1 in erythroid and megakaryocytic terminal maturation (3–5). This activity requires direct physical interaction between GATA -1 and its cofactor, friend of GATA-1 (FOG-1; also known as *zfpm1*), a large multitype zinc finger–containing protein (6–10). In humans, germline missense mutations that disrupt the binding of GATA-1 to FOG-1 lead to severe thrombocytopenia and dyserthropoietic anemia, in some cases necessitating bone marrow transplantation (11–14).

Both GATA-1 and FOG-1 belong to families of proteins, whose members are expressed in overlapping patterns in diverse tissues. There are six known GATA factor genes (*GATA-1, -2, -3, -4, -5,* and *-6*) and two FOG genes (*FOG-1* and *-2*) in vertebrates. All GATA factor members contain highly conserved amino acid sequences within their amino zinc finger that mediate binding to FOG proteins (15). The requirement for direct interaction between GATA and FOG family members extends beyond GATA-1 and FOG-1. Interaction between GATA-2, another hematopoietic-expressed GATA factor, and FOG-1 is required during early megakaryopoiesis in the absence of GATA-1–FOG-1 binding (9). Interaction between GATA-4 and FOG-2, two nonhematopoietic-expressed family members, is required for normal cardiac and gonadal development (16–19). Thus, GATA–FOG protein interactions are required in broad developmental settings.

Despite the critical role that FOG proteins play in modulating GATA factor activity, their mechanism of action remains incompletely

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understood (for review see reference 20). To date, no sequence-specific high affinity DNA binding activity of FOG proteins has been identified. This infers that FOG proteins exert their influence via protein–protein interactions. Chromatin immuno-precipitation (ChIP) studies indicate that FOG-1 facilitates occupancy by GATA-1 at selected cis-regulatory chromatin elements in hematopoietic cells via a mechanism involving the switching of GATA-1 for GATA-2 (21, 22). However, the molecular details of this activity are unknown. FOG family proteins also interact with at least two transcriptional corepressor complexes: the C-terminal binding protein (CtBP) and the NuRD chromatin remodeling/histone deacetylase transcriptional complexes (23–27). The physiological significance of CtBP binding is not clear given that homozygous FOG-1 knock-in mice containing mutations that disrupt CtBP binding are viable, fertile, and have normal hematopoiesis (24). Interaction with the NuRD complex has not been fully examined in vivo as of yet.

Work over the past several years has shown that GATA-1 also plays a functional role in the terminal stages of mast cell maturation, particularly on expression of the high affinity IgE receptor (FcεRI) (28–32). A previous study from our group showed that FOG-1 expression is absent in cultured mast cell lines, suggesting a possible FOG-independent GATA-1 function in the mast cell lineage (6). In this study, we extend these findings using primary cells and gene-targeted mice to show that indeed GATA-1 functions independent of FOG protein in terminal mast cell maturation. Moreover, we show that FOG-1 potently represses cell fate choice for the mast cell lineage during early multipotent progenitor cell lineage commitment. Remarkably, ectopic expression of FOG-1 in prospectively isolated mast cell progenitors (MCPs) redirects them into erythroid, megakaryocytic, and granulocytic lineages. Collectively, these observations identify FOG-1 as a key negative regulator of mast cell lineage choice, and demonstrate combinational control of cell fate decisions by a transcription factor and its cofactor.

RESULTS
FOG-independent function of GATA-1 in mast cell development
Previously, we reported that FOG-1 expression is absent in the mouse mast cell lines P615 and HC.3 (6). However, its expression in primary mast cells has not been reported. We recently identified a bipotent basophil/MCP cell population that can be immunophenotypically isolated from the mouse spleen (Lin⁻c-kit⁺FcγRII/IIIʰⁱβ7ʰⁱFcεRIα⁻/ˡᵒ) (33). These cells develop exclusively into mast cells and basophils when cultured in a cocktail of cytokines that includes stem cell factor (SCF), IL-3, IL-5, IL-6, IL-7, IL-9, IL-11, GM-CSF, erythropoietin (EPO), and thrombopoietin (TPO). In the present study, we used this system to examine the expression patterns of FOG-1, GATA-1, and GATA-2 as primary cells commit to the mast cell/basophil lineage. As shown in Fig. 1, RT-PCR analysis reveals expression of GATA-1, GATA-2, and FOG-1 in hematopoietic stem cells, common myeloid progenitors, and megakaryocyte/

erythroid progenitors (MEPs), and low levels of FOG-1 in common lymphoid progenitors and granulocyte/macrophage progenitors (GMPs; along with low levels of GATA-2). In contrast, MCPs cultured for 3 d in the cytokine cocktail described earlier in this paragraph express relatively high levels of GATA-1 and GATA-2 but no detectable FOG-1. The same is true of primary peritoneal mast cells. Thus, FOG-1 expression is specifically down-regulated as multipotent progenitor cells commit to the mast cell lineage.

FOG-2, the only other known mammalian FOG gene, is thought to be expressed predominantly outside of the hematopoietic system (15, 34, 35). However, to rule out a possible role in mast cell development, we performed RT-PCR analysis for FOG-2 expression in primary mouse bone marrow–derived mast cells (BMMCs). As shown in Fig. 1 C, we found no detectable FOG-2 mRNA transcripts in these cells despite robust signals for the mast cell genes mouse mast cell protease 2 (MMCP2), MMCP-4, and mast cell carboxypeptidase A (MC-CPA) in the same sample, and amplification of FOG-2 mRNA transcripts from control heart tissue.

It is possible that FOG genes are expressed at levels below our detection limit or that yet additional FOG genes exist. To examine these possibilities, we analyzed yolk sac–derived mast cells (YSMCs) from knock-in mice containing substitution of valine 205 of GATA-1 by glycine (GATA-1^V205G). This mutation markedly impairs FOG-1 binding, resulting in lethal anemia and impaired megakaryopoiesis in mice (8, 9). A similar mutation (GATA-1^V205M) causes severe X-linked dyserythropoietic anemia and thrombocytopenia in humans, and the homologous mutation in GATA-4 (GATA-4^V217G) blocks binding to FOG-2 (11, 19). Because of the embryonic lethality of the mice, it was not possible to examine BMMCs. Instead, we cultured embryonic day 9.5 YSMCs in the presence of IL-3 and SCF for 6 wk, conditions typically used to generate BMMCs. These cells morphologically resemble BMMCs and express equivalent levels of the mast cell genes MC-CPA, MMCP-4, MMCP-5, tryptophan hydroxylase, and microphthalmia-associated transcription factor (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070544/DC1). As shown in Fig. 1 D, YSMCs cultured from wild-type embryos are highly granular in appearance and nearly all coexpress c-kit and FcεRI on their cell surface. In contrast, YSMCs from male GATA-1⁻ embryos are hypogranular and have markedly impaired FcεRI surface expression, consistent with previous reports of GATA-1ˡᵒʷ mast cells (28–31). A smaller population of c-kit⁻FcεRI⁻ cells is also present in these cultures. Importantly, cells cultured from GATA-1^V205G male embryo yolk sacs resemble those derived from wild-type, and not GATA-1⁻, embryos in morphology and FcεRI surface expression. We conclude that, in contrast to the erythroid and megakaryocyte lineages, GATA functions completely independent of FOG proteins in terminal mast cell development.

Inhibition of mast cell development by FOG-1 in vitro and in vivo
Could FOG-1 actually antagonize mast cell development? This possibility was suggested by the results of yolk sac colony
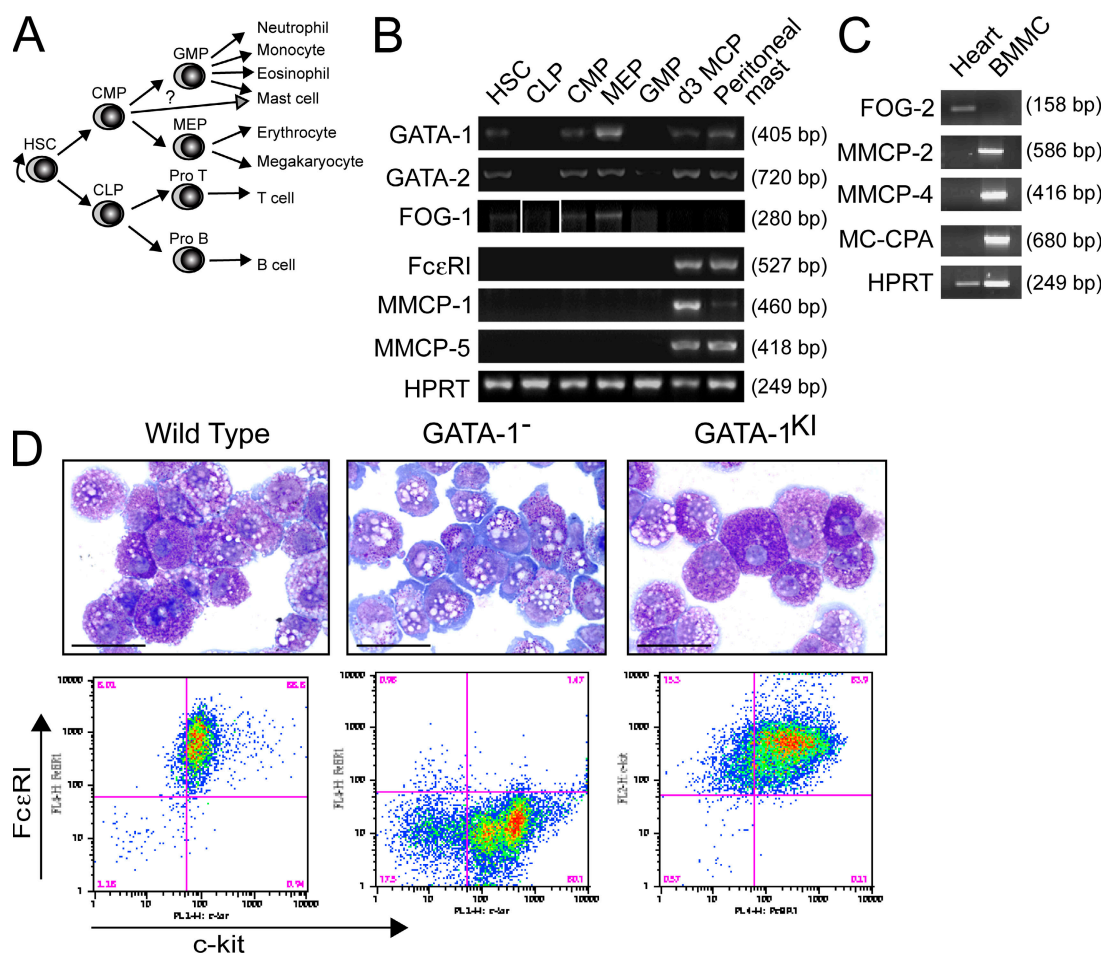
Figure 1. GATA–1 functions independent of FOG cofactors in mast cell development. (A) Schematic diagram showing hierarchical relationships of the hematopoietic progenitor populations examined in B. (B) RT-PCR analysis of *GATA-1*, *GATA-2*, and *FOG-1* expression in sorted progenitor cell populations, day 3 MCPs (d3 MCP), and peritoneal mast cells. *FcεRI*, *MMCP-1*, and *MMCP-5* are included as mast cell–specific marker genes. *HPRT* serves as the housekeeping gene control. White lines indicate that intervening lanes have been spliced out. (C) RT-PCR analysis of *FOG-2* expression in BMMCs (from 6-wk-old cultures) or whole mouse heart tissue. *MMCP-2*, *MMCP-4*, and *MC-CPA* are included as mast cell–specific markers. (D) May-Grunwald-Giemsa stains and FACS analysis for FcεRI and c-kit expression of YSMCs from wild-type, GATA-1^{−}, or GATA-1[V205G(KI)] male embryos. Bars, 10 μm.

assays from FOG-1^{−/−} mouse embryos. When yolk sac cells from embryonic day 9.5 FOG-1^{−/−} embryos are grown under conditions supporting multilineage growth (EPO, TPO, and SCF), there is a marked expansion of mast cell colonies (∼47-fold) compared with the wild type (Fig. 2 A). Identification of the colonies was based on cell morphology and positive Toluidine blue staining (Fig. 2 B). The absolute increase in mast cell colony numbers (473 ± 69 per 10[5] cells plated) is significantly greater than the additive loss of mixed, erythroid, and megakaryocytic colonies (48 ± 9 per 10[5] cells plated), suggesting a true expansion of MCPs rather than a simple defaulting of multipotent progenitors to the mast cell lineage. These results imply that FOG-1 represses mast cell lineage commitment, possibly at the level of common myeloid progenitors or GMPs.

To determine whether this inhibition requires direct physical interaction between FOG-1 and GATA-1 and/or GATA-2, we performed yolk sac colony assays from embryos containing knock-in mutations that disrupt the binding of FOG-1 to either GATA-1 (GATA-1[V205G]) and/or GATA-2 (GATA-2[V296G]) (9). As shown in Fig. 2 A, there is a slight expansion of mast cell colony numbers compared with the wild type in hemizygous GATA-1[V205G] males (∼1.9-fold) and a moderate expansion in GATA-2[V296G/V296G] embryos (∼6.2-fold), but a marked expansion in compound GATA-1[V205G], GATA-2[V296G/V296G] embryos (35-fold) to levels similar to those of FOG-1^{−/−} embryos. These results indicate that direct binding of FOG-1 to GATA factors is required for its inhibitory activity on mast cell lineage commitment, and that GATA-1 and GATA-2 play overlapping roles. This mirrors the overlapping FOG-dependent roles of GATA-1 and GATA-2 in early megakaryocytic development (9).

To test directly whether FOG-1 represses mast cell development, we enforced *FOG-1* expression in prospectively isolated
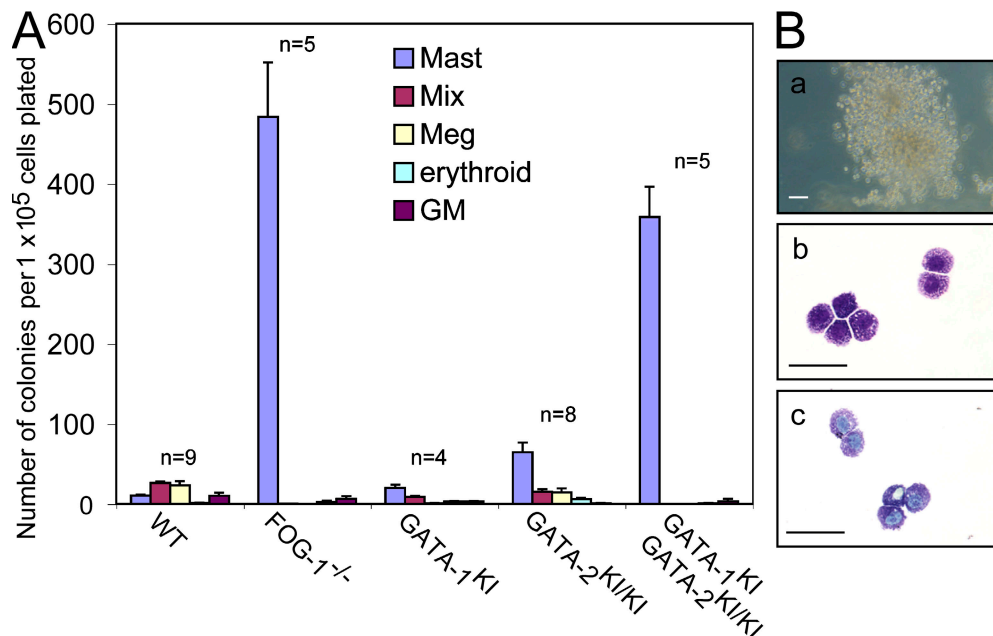
Figure 2. Loss of FOG–1 results in an increased number of YSMC progenitors in vivo. (A) Yolk sac cultures from WT, FOG-1$^{-/-}$, GATA-1$^{V205G(KI)}$ male, GATA-2$^{V296G/V296G(KI/KI)}$, and compound GATA-1^{KI}, GATA-2$^{KI/KI}$ male embryonic day 9.5 embryos. Shown are numbers of mast cell, mixed (Mix), megakaryocytic (Meg), erythroid, and GM colonies per 10^5 cells plated and cultured in 2 IU/ml EPO, 5 ng/ml TPO, and rat 50 ng/ml SCF for 7 d. The number of animals analyzed for each is indicated above the graphs. Error bars represent the SEM. (B) Colony and cell morphology of mast cell colonies from GATA-1^{KI}, GATA-2$^{KI/KI}$ male embryos. (a) Brightfield appearance of colony. Bar, 50 μm. (b) May-Grunwald-Giemsa stain of cytospun cells. Bar, 10 μm. (c) Toluidine blue stain of cytospun cells. Bar, 10 μm.

GMPs using retroviruses and examined the ability of the cells to form mast cells in vitro in a limiting dilution assay (Fig. 3). The retroviral vectors used coexpress enhanced GFP (eGFP) as a bicistronic mRNA, allowing for tracking of the transduced cells. As shown in Fig. 3 A, nearly all of the GFP$^+$ GMPs transduced with the empty control vector and cultured for 3 wk in the presence of IL-3, IL-6, IL-9, and SCF express high levels of FcεRI, contain granular cytoplasm, and lack expression of the granulocytic and monocyte/macrophage markers Gr-1 and CD11b. In contrast, GMPs transduced with *FOG-1*–expressing retroviruses (GFP$^+$) are almost entirely FcεRI$^-$, lack significant granularity, and express CD11b and, to a lesser extent, Gr-1 when cultured under identical conditions.

We also examined the effect of enforced *FOG-1* expression on mast cell development in vivo. A transgene containing ∼7 kb of DNA sequence upstream of the first erythroid exon of *GATA-1* is sufficient to drive expression in erythroid and megakaryocytic cells and, albeit somewhat weakly, in mast cells and eosinophils (36–38). Because no other well characterized mast cell–specific transgenic promoters have been reported, we chose to use this ∼7-kb *GATA-1* upstream sequence to examine the consequences of enforced *FOG-1* expression on mast cell lineage commitment (Fig. 3 B). We previously generated FOG-1 transgenic mice using this regulatory region and demonstrated that it rescues the hematopoietic defects of FOG-1$^{-/-}$ mice (39). In this study, we examined the same transgenic line for mast cell production. GMPs were isolated from bone marrow, plated under mast

cell growth conditions, and quantitated for mast cell growth by limiting dilution. An ∼2.5-fold greater number of GMPs was required to generate that same number of single cell mast cell clones from these transgenic mice compared with nontransgenic littermates, suggesting a reduction in the number of MCPs in the transgenic mice (Fig. 3 C). The somewhat modest effect, relative to the in vitro studies, is likely caused by relatively weak expression of the transgene in the mast cell lineage. Nonetheless, our data collectively indicate that down-regulation of *FOG-1* expression is a prerequisite for mast cell development.

Reprogramming of primary mast cells into alternate lineages by ectopic expression of FOG-1

In the preceding experiments, *FOG-1* expression was enforced in early multipotent progenitor cells. We next examined the consequences of ectopic *FOG-1* expression in cells already committing to the mast cell lineage. In the first set of studies, we generated primary mast cells by culturing whole bone marrow in the presence of IL-3 and SCF for 6 wk. FACS analysis of this starting cell population showed 100% of the cells were c-kit$^+$, Ter119$^-$ (erythroid marker), and 96% were FcεRI$^+$ (not depicted). We then infected them with retroviruses packaged with the empty vector, FOG-1, or a missense FOG-1 mutant with impaired GATA factor binding (m1,5,6,9) (40). We previously showed that the m1,5,6,9 FOG-1 protein is stable and properly targeted to the nucleus when expressed in a FOG-1–null hematopoietic
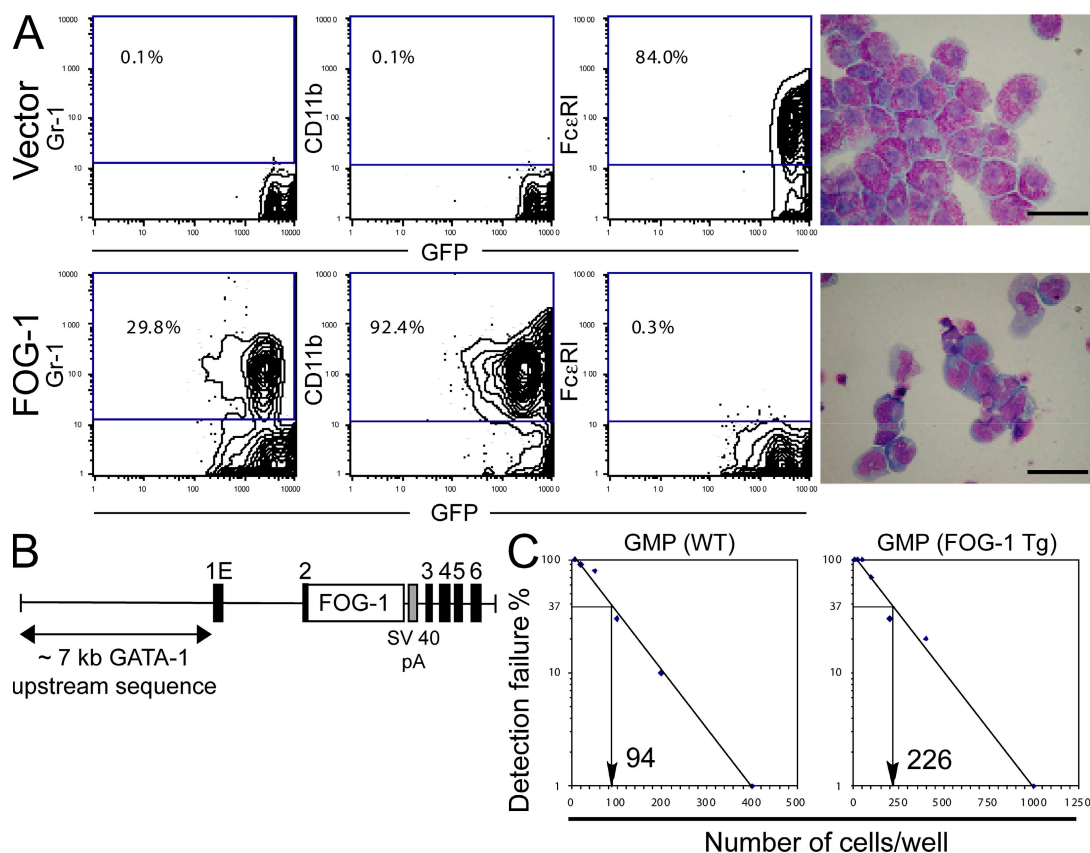
Figure 3. Enforced expression of FOG–1 blocks mast cell development. (A) FACS expression analysis for eGFP, Gr-1, CD11b, or FcεRI in GMPs transduced with retroviruses expressing eGFP alone (vector) or FOG-1–IRES-GFP (FOG-1), and cultured at limiting dilution in the presence of SCF, IL-3, IL-6, and IL-9 for 3 wk. Percentages of total cells that fall within the boxed FACS gates are shown. May-Grunwald-Giemsa stains of the GFP⁺ cells are shown on the right. Bars, 10 μm. (B) Schematic representation of the transgene used to express FOG-1 in the mast cell lineage. Approximately 7 kb of DNA 5′ to the first erythroid exon (1E) of *GATA-1* (noncoding) were used. Full-length FOG-1 cDNA was inserted into exon 2 of *GATA-1* upstream of the *GATA-1* start codon, followed by an SV40 polyA sequence (gray box). Exons are indicated by black boxes. (C) Limiting dilution analysis of bone marrow GMPs from FOG-1–transgenic mice or wild-type littermates. The percentage of wells that failed to generate mast cells was scored, and the number of cells per well leading to a 37% detection failure rate was calculated.

cell line (10). All of the constructs coexpress eGFP as a bicistronic mRNA. 2 d after retroviral infection, GFP⁺ cells were sorted and incubated in the presence of IL-3, SCF, EPO, and TPO for various time points. Cells transduced with the empty vector or the non-GATA–binding FOG-1 mutant retained nearly 100% viability throughout the first 5 d of culture, as measured by vital dye exclusion (Fig. 4 A). In contrast, only ∼70% of the FOG-1–transduced cells survived. Some of the cell death occurs via apoptosis, as indicated by an increased number of Annexin V⁺/7-amino-actinomycin D⁻ cells (41) in the FOG-1 compared with the empty vector and m1,5,6,9 FOG-1–transduced samples (7.1 vs. 1.8 vs. 2.1%, respectively) when examined 3 d after sorting (Fig. 4 B).

By 6 d after FACS sorting, cells transduced with either the empty vector or the non-GATA–binding FOG-1 mutant show typical mast cell morphology by May-Grunwald-Giemsa stains. They are highly granular, as indicated by prominent side scatter on FACS analysis, and nearly all express FcεRI on their cell surface (Fig. 4 C). In contrast, many of the FOG-1–transduced

cells appear hypogranular, with pale blue cytoplasm and large nuclei with an open chromatin pattern. Loss of granularity is evident by FACS analysis, which shows a marked reduction in cells with high side scattering compared with the control cells (after gating for viable cells). In addition, ∼25% of the viable FOG-1–transduced cells lack surface expression of FcεRI.

Remarkably, after culturing for an additional 5 d in the presence of EPO, TPO, IL-3, and SCF, cells resembling maturing erythrocyte, megakaryocytic, and granulocytic cells became evident in the FOG-1–transduced sample (Fig. 4 D). This was confirmed by staining with benzidine, which turns hemoglobinized cells a dark brown color, and for acetylcholinesterase, a marker of mouse megakaryocytes. Although the frequency of positive-staining cells was quite low (0.7 ± 0.4% benzidine positive and 0.5 ± 0.2% acetycholinesterase positive of 5,000 cells examined each), no positive cells were observed in >5,000 vector or m1,5,6,9-transduced cells examined.

One interpretation of these results is that ectopic FOG-1 expression reprograms "committed" MCPs into the erythroid,
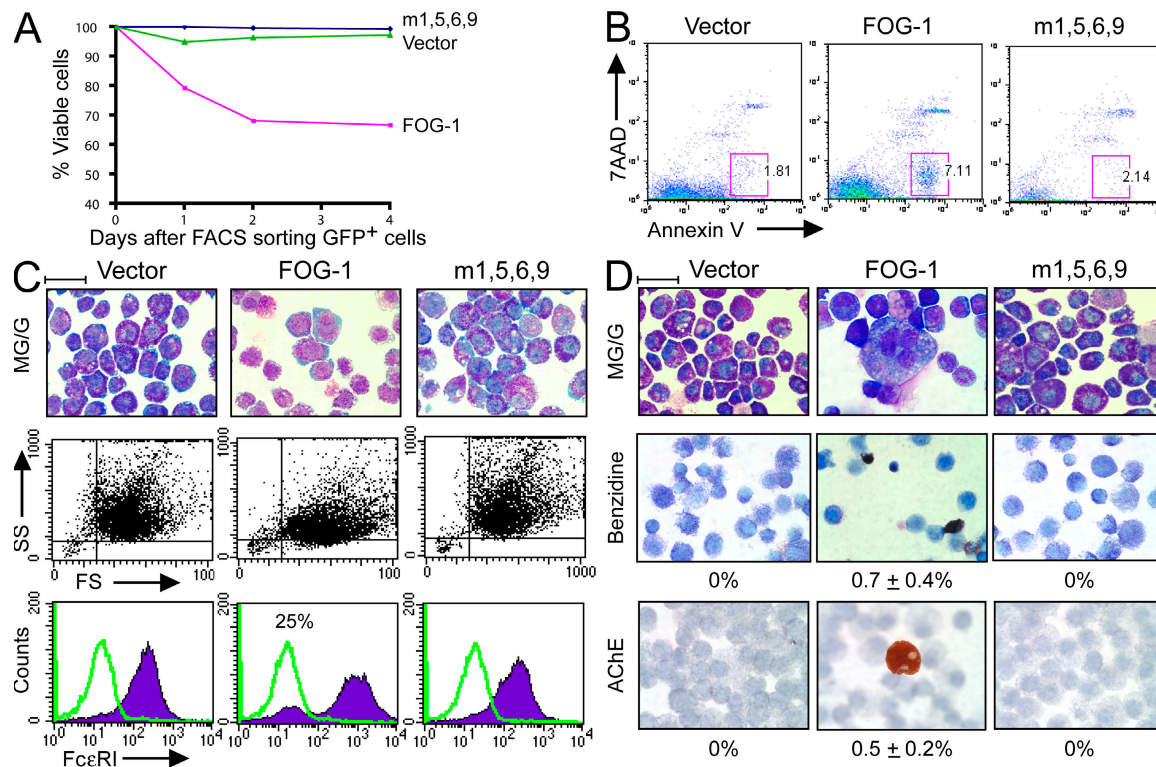
Figure 4. Ectopic expression of FOG-1 in BMMCs represses mast cell phenotype and produces alternate lineage cells. (A) Percentage of viable GFP+ primary BMMCs retrovirally transduced with eGFP alone (vector), FOG-1–IRES-eGFP (FOG-1), or m1,5,6,9 FOG-1–IRES-GFP (m1,5,6,9). Viability was determined by Trypan blue dye exclusion. Time refers to days after FACS sorting of eGFP+ cells (2 d after retroviral infection). (B) FACS analysis for Annexin V (apoptosis marker) and 7-amino-actinomycin D (necrosis marker) expression of primary BMMCs transduced with each of the retroviral vectors described in A and examined 3 d after FACS sorting of GFP+ cells. Percentages of total cells that fall within the boxed FACS gates are shown. (C) May-Grunwald-Giemsa stains, FACS analysis showing side-scatter (SS) and forward scatter (FS), and FcεRI expression of primary BMMCs transduced with each of the retroviral vectors shown in A, sorted for GFP after 2 d, and cultured for 6 d in 2 U/ml EPO, 5 ng/ml TPO, 10 ng/ml IL-3, and 50 ng/ml SCF. (bottom) The green lines represent negative control staining for FcεRI (no IgE added; see Materials and methods). The purple histograms represent staining for FcεRI (IgE added). The percentage in the middle panel represents the proportion of total stained cells present within the negative staining control peak area. Bar, 10 μm. (D) May-Grunwald-Giemsa, o-dianisidine (benzidine), and acetylcholinesterase (AChE) histochemical stains of the cultures in C incubated for an additional 5 d. Benzidine+ cells stain black/brown; AChE+ cells stain orange. The percentage of positive-staining cells is indicated (±SEM; $n = 2$; 5,000 cells examined for all samples). Bar, 10 μm.

megakaryocytic, and granulocytic lineages. However, based on the results so far, we could not exclude the possibility that our cultures contain rare contaminating multilineage progenitors, and that ectopic FOG-1 expression simply selects against cells committing to the mast cell lineage and for those committing to the erythroid, megakaryocytic, and granulocytic pathways. This seems unlikely because the starting cells were cultured for 6 wk in the presence of SCF and IL-3, conditions not expected to sustain multipotent progenitor cells. However, we took two approaches to address this possibility. First, we repeated the experiment using clonally derived primary mast cells. Because primary mast cells can be cultured for extended periods of time, it is possible to clone them at the single cell level. Whole bone marrow was cultured in the presence of IL-3 and SCF for 2 wk, and the cells were cloned by limiting dilution. This was the latest time point possible to begin the cloning and still generate enough cells for retroviral infection and analysis before they senesced. A single cell clone was

expanded in IL-3 and SCF for an additional 4 wk. These cells were then infected with the empty vector or FOG-1–expressing retroviruses, sorted for GFP expression after 2 d, and cultured in IL-3, SCF, EPO, and TPO for an additional 6 d. They were then analyzed by FACS analysis for surface expression of FcεRI and Ter-119, an erythroid specific marker. As shown in Fig. 5 A, essentially 100% of the vector-transduced cells were FcεRI+ Ter-119−, which was consistent with the starting population being a clonal mast cell line. In contrast, 22% of the FOG-1–transduced cells were FcεRI−, 12.6% were Ter-119+, and 2.5% were both FcεRI− and Ter-119+. Interestingly, a continuum of FcεRI–Ter-119–coexpressing cells was observed, suggesting an overlapping transition from FcεRI to Ter-119–expressing cells.

As a second approach, we prospectively isolated cell populations highly enriched in MCP cells (day 2 MCPs, which were GMPs cultured for 2 d in IL-3, SCF, and IL-6 and sorted for β7^hiCD11b− expression) and repeated the FOG-1
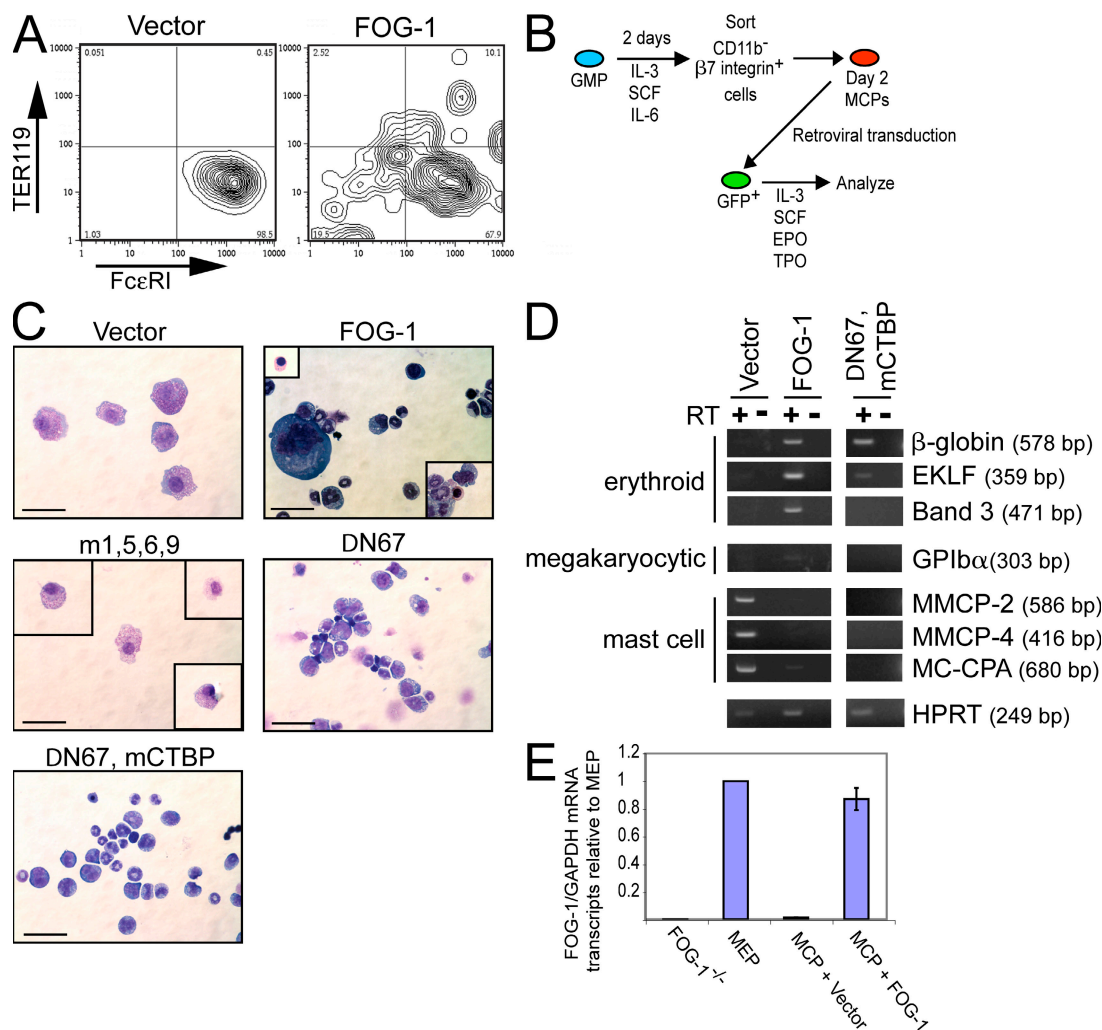
Figure 5. Ectopic expression of FOG–1 in MCPs blocks mast cell maturation and enables alternate lineage development. (A) FACS analysis for TER119 and FcεRI expression of clonal primary BMMCs retrovirally transduced with empty vector or FOG-1–IRES-eGFP–expressing retroviruses. GFP⁺ cells were sorted 2 d after retroviral infection and cultured in EPO, TPO, IL-3, and SCF for 6 d. (B) Scheme for isolation and retroviral infection of day 2 MCPs. (C) May-Grunwald-Giemsa stains of day 2 MCPs transduced with empty vector, FOG-1, or the FOG-1 mutants m1,5,6,9, ΔN67, and ΔN67, mCTBP expressing retroviruses; cultured in mast cell media containing EPO, TPO, SCF, and IL-3 for 4 d; sorted for GFP expression (GFP⁺ cells); and cultured for an additional of 5 d in mast cell media containing EPO, TPO, SCF, and IL-3. Boxes indicate cells seen in additional fields. Bars, 10 μm. (D) RT-PCR analysis for erythroid, megakaryocytic, and mast cell marker gene expression in the cells shown in C (harvested 5 d after sorting GFP⁺ cells). (E) Quantitative RT-PCR analysis for *FOG-1* mRNA transcript levels in day 2 MCPs retrovirally transduced with the empty vector or FOG-1, sorted for GFP⁺ expression after 4 d, and cultured for an additional 5 d in the presence of SCF, IL-3, EPO, and TPO. Levels are displayed relative to those determined in parallel from sorted mouse MEPs and normalized to *GAPDH* mRNA transcript levels. The relative signal from FOG-1⁻/⁻ cells (reference 10) is shown as an indicator of experimental background. All measurements were made in triplicate. The error bars represent the SEM.

transduction experiment (Fig. 5 B). GFP⁺ cells were sorted after 4 d, and the cells were cultured for an additional 5 d in the presence of SCF, IL-3, EPO, and TPO. As shown in Fig. 5 (C and D), cells transduced with the empty vector have a granular appearance, express the mast cell–specific genes *MMCP-2*, *MMCP-4*, and *MC-CPA*, and lack expression of the erythroid– and megakaryocyte-specific genes *β-globin*, *band 3*, *erythroid Kruppel-like factor* (*EKLF*), and *glycoprotein Ibα* (*GPIbα*) by RT-PCR analysis. Consistent with our earlier observation (Fig. 4), most of the cells transduced with the FOG-1–expressing retroviruses lack typical mast cell morphology, and many resemble

cells maturing along the erythroid, megakaryocytic, and granulocytic lineages. RT-PCR analysis shows markedly diminished expression of *MMCP-2*, *MMCP-4*, and *MC-CPA*, and activation of *β-globin*, *band 3*, *EKLF*, and *GPIbα*. Approximately 4% of the cells were benzidine positive, and 5% expressed acetylcholinesterase based on histochemical staining (not depicted). These higher levels compared with those from 6-wk-old primary BMMCs (Fig. 4) are likely caused by an increased proportion of more immature MCPs, which may be more amenable to lineage reprogramming. Quantitative RT-PCR analysis of the retrovirally transduced MCPs shows *FOG-1*

expression levels close to those found physiologically in MEPs (Fig. 5 E). As expected, only background levels of the FOG–1 signal were detectable in the MCPs transduced with the empty vector based on comparison to FOG–1$^{-/-}$ cells. Collectively, these data strongly suggest that ectopic expression of FOG–1 redirects committed MCP cells into alternate lineages.

Requirement for interaction with GATA factors but not CtBP and NuRD corepressor complexes

Next, we wanted to explore the mechanism of FOG–1–mediated mast cell repression and lineage reprogramming. First, we tested whether this activity requires direct binding of FOG–1 to GATA factors. As shown in Fig. 5 C, the non–GATA–binding FOG–1 mutant m1,5,6,9 was inactive in day 2 MCP reprogramming, which was consistent with our earlier observations in YSMCs (Fig. 2) and bulk BMMC cultures (Fig. 4). Thus, direct binding to GATA factors appears necessary. We next tested whether interaction between FOG–1 and one or both of its known transcriptional corepressor partners, the

NuRD and CtBP complexes, is required. We first examined a truncation FOG–1 mutant molecule (FOG–1 ΔN67) that lacks the N-terminal 67 amino acids, a region that includes the entire NuRD interaction motif (25). This molecule behaved similarly to the full-length protein when expressed in the day 2 MCPs, although megakaryocytic development was not observed, as expected (Fig. 5 C) (10). We then tested a double mutant that also substitutes the CtBP interaction motif PIDLS with the nonbinding sequence PIASS (ΔN67, mCTBP) (24). This construct also blocks mast cell development and leads to activation of some erythroid-specific genes (Fig. 5, C and D). Although expression levels of these mutants were considerably higher than endogenous *FOG-1* levels in MEPs (~18-fold higher for ΔN67 and ~150–fold higher for ΔN67, mCTBP as measured by quantitative RT-PCR analysis), these results suggest that mechanisms other than or overlapping with transcriptional corepression via the NuRD and CtBP complexes are responsible for FOG–1's antagonistic role in mast cell development.
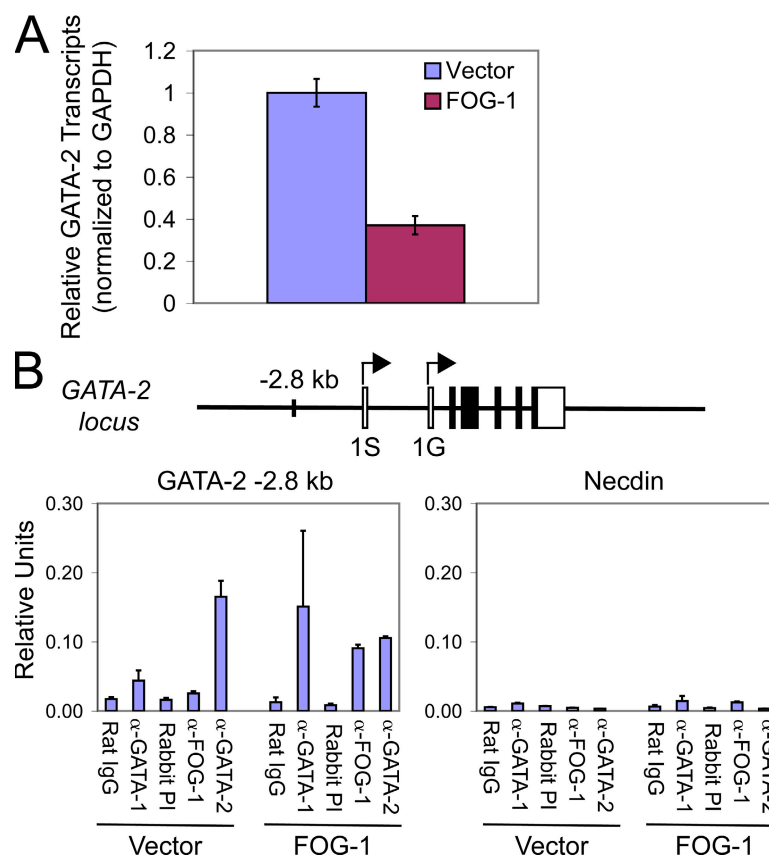

Figure 6. FOG–1–mediated down–regulation of *GATA–2* expression correlates with GATA factor switching at the −2.8–kb *GATA–2* enhancer element in mast cells. (A) Quantitative RT-PCR analysis of *GATA-2* mRNA transcript levels in GFP+ GMPs infected with retroviruses expressing either the empty vector (IRES-GFP) or FOG–1–IRES-GFP, sorted after 2 d, and analyzed immediately. Error bars represent SEM (*n* = 3). (B) ChIP assay examining occupancy of the *GATA-2* −2.8-kb enhancer or the *Necdin* promoter by GATA-1, GATA-2, and/or FOG-1 in 4-wk-old BMMCs retrovirally transduced with either the empty vector (IRES-GFP) or FOG-1–IRES-GFP and sorted for GFP+ cells after 2 d. A schematic drawing of the mouse *GATA-2* locus with the −2.8-kb enhancer element is indicated. 1S and 1G refer to different transcriptional start sites. Normal pooled rat IgG was used as the control for GATA-1 ChIP, and FOG-1 preimmune rabbit serum served as the control for FOG-1 and GATA-2 ChIPs. The level of detected amplicon was normalized to a standard curve of input chromatin and is indicated as relative units. Error bars represent the SEM (*n* = 2).

FOG–1–mediated down–regulation of GATA–2 in mast cells

High level *GATA-2* expression is required for mast cell development (28, 42). Grass et al. have studied the transcriptional regulation of *GATA-2* and identified a critical enhancer element located ∼2.8 kb upstream of the 1S promoter that contains phylogenetically conserved GATA consensus binding sequences (43). Based on ChIP and transcriptional studies, they proposed a model in which GATA–2 occupies this site in early multipotent progenitor cells and autoactivates *GATA-2* gene transcription. As cells mature along the erythroid pathway and GATA–1 levels increase, occupancy of this enhancer element switches from GATA–2 to GATA–1, leading to repression of *GATA-2* transcription. In collaborative studies, we showed that this GATA factor switch depends on the presence of FOG–1 (21). Moreover, FOG–1 rapidly and potently represses *GATA-2* expression in reconstituted FOG–1$^{-/-}$ cells, and the non-FOG–binding GATA–1 mutant GATA–1^{V205G} fails to repress *GATA-2* transcription in reconstituted GATA–1–null erythroid cells (8, 21).

Given GATA–2's essential role in mast cell development, we hypothesized that FOG–1's antagonist activity in mast cell development could be in part caused by GATA factor switching at the *GATA-2* −2.8-kb enhancer and subsequent repression of *GATA-2* transcription (42, 44). To test this, we examined changes in *GATA-2* mRNA transcript levels at the earliest time point possible after FOG–1 retroviral overexpression in GMPs. As shown in Fig. 6 A, we observed a 2.7-fold reduction in *GATA-2* transcripts by quantitative RT-PCR in FOG–1– versus empty vector–transduced GMPs sorted 2 d after retroviral infection for GFP expression and analyzed immediately. Because there are insufficient numbers of cells to perform ChIP assays in GMPs, we expressed FOG–1 or the empty vector in BMMCs and examined occupancy of the −2.8-kb region immediately after sorting GFP$^+$ cells 2 d after infection. As shown in Fig. 6 B, control cells (empty vector) contain high levels of GATA–2, low levels of GATA–1, and no detectable FOG–1 (over background) at the −2.8-kb region, as expected. In contrast, significant levels of FOG–1 are detectable at the −2.8-kb site in the FOG–1–transduced cells, and there is a marked increase in GATA–1 and a decrease in GATA–2 occupancy compared with control cells. Only background levels of GATA–1, GATA–2, and FOG–1 were found at the control Necdin promoter, which does not contain GATA binding sites, in both vector– and FOG–1–transduced cells. Thus, FOG–1–mediated GATA factor switching at the −2.8-kb *GATA-2* enhancer occurs as an early event after ectopic expression in mast cells. Consequential down–regulation of *GATA-2* expression may therefore explain, at least in part, the mechanism of FOG–1's antagonistic activity in mast cell lineage commitment.

DISCUSSION

In this study, we use both loss- and gain–of–function approaches to demonstrate that FOG–1 acts in a combinatorial manner with GATA factors to specify the cell fate of multipotent hematopoietic progenitor cells (Fig. 7). Although FOG–1 acts cooperatively with GATA factors in erythroid and megakaryocytic differentiation, it potently antagonizes GATA function in mast cell lineage commitment. This requires direct GATA factor binding and correlates with transcriptional down–regulation of *GATA-2* via a GATA factor switching mechanism. Moreover, we show that FOG–1, as a single factor, is capable of redirecting committed primary MCP cells into alternate lineage fates. This provides evidence for lineage plasticity during at least the early stages of hematopoietic lineage commitment.

Down–regulation of FOG–1 in eosinophil development

Our results are strikingly similar to those reported by Querfurth et al. in their studies of eosinophil development (45). Both eosinophils and mast cells require GATA–1 for complete maturation (46). Like mast cells, *FOG-1* expression is extinguished as eosinophils differentiate from multipotent progenitor cells, and this is a prerequisite for their development. Moreover, ectopic expression of FOG–1 in an eosinophilic cell line changes it to a more primitive state. These findings highlight important parallels in the transcriptional control of eosinophil and mast cell development (Fig. 7).

Combinatorial control of hematopoietic cell fate decisions by the *Drosophila* GATA factor *Serpent* (*Srp*) and the FOG orthologue *U–shaped* (*Ush*)

The combinatorial control of hematopoietic cell fate determination by GATA and FOG described in this paper is also reminiscent of that reported in studies of *Drosophila* hematopoiesis. The *Drosophila* hematopoietic system consists of multipotent hemocyte progenitor cells that give rise to one of three specialized cell types: plasmatocytes, crystal cells, and lamellocytes, all of which function in immune responses (for review see reference 47). Remarkably, most of the same
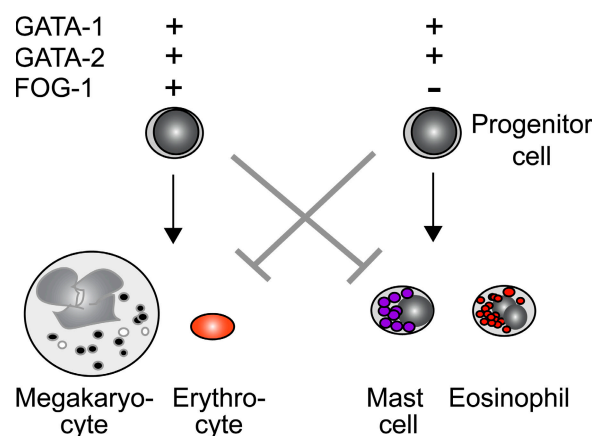

Figure 7. Model of combinatorial control of erythrocyte/ megakaryocyte versus mast cell/eosinophil lineage commitment by GATA–1 and GATA–2 depending on the presence of FOG–1. + and − refer to the presence or absence, respectively, of expression of each gene. The arrows represent the favored differentiation pathway, and the gray "T" bars represent the blocked differentiation pathway.

transcription factors required for mammalian hematopoiesis also play essential roles in *Drosophila* blood development (48). The GATA orthologue *Srp* is required for hemocyte precursor, crystal cell, and plasmatocyte development. The FOG orthologue *Ush* is required for hemocyte precursor formation and plasmatocyte differentiation. However, it is selectively down-regulated during crystal cell differentiation (49). This is essential for crystal cell formation, because enforced *Ush* expression ablates crystal cell development, paralleling our findings of blocked mast cell development by enforced *FOG-1* expression (Fig. 3). Conversely, loss-of-function *Ush* mutants contain marked expansion of crystal cells and a reduced number of plasmatocytes, similar to the enhanced mast cell and impaired erythroid/megakaryocyte production we observe in FOG-1$^{-/-}$ yolk sac cultures (Fig. 2). As in our findings, the effects in *Drosophila* require direct binding of Ush to Srp, because an isoform of Srp that lacks the equivalent N-terminal zinc finger of vertebrate GATA factors is immune to the inhibitory effects of Ush (50).

Collectively, these studies underscore how combinations of GATA and FOG family members control hematopoietic cell fate decisions. Given the broad developmental requirement for interaction between GATA and FOG family members, such as GATA-4 and FOG-2 in cardiac and gonadal development, it is tempting to speculate that combinatorial GATA–FOG control mechanisms are involved in nonhematopoietic cell fate decisions as well.

Combinatorial control of cell fate decisions by transcription factors and cofactors

Transcription factor combinatorial control mechanisms of cell fate decisions have been appreciated for some time. However, few examples exist involving a transcription factor and a cofactor (i.e., transcription factor protein–protein interactions). One could imagine several possible mechanisms by which such interactions could affect gene programs in a lineage-specific manner: (a) the cofactor could alter the DNA affinity and/or sequence specificity of the transcription factor; (b) the cofactor could enzymatically alter the local chromatin structure, affecting the accessibility of other transcription factors; (c) the cofactor could alter the locus conformation by bridging the transcription factor to another sequence-specific DNA binding protein; and (d) the cofactor could sequester the transcription factor.

Our data are consistent with a novel mechanism in which FOG-1 mediates the switching of two transcription factor family members at specific loci to yield distinct transcriptional outcomes. The molecular details of this switching event remain to be determined. Our data do not exclude the possibility of additional mechanisms operating. Vakoc et al. recently used chromosome conformation capture to show that interaction between GATA-1 and FOG-1 is required for spatial proximity of the distant upstream *β-globin* locus control region to the *β-globin* gene proximal promoter during erythroid development (51). This DNA looping event correlates with the onset of *β-globin* transcription. The absence of FOG-1 in developing mast cells would preclude such a locus reconfiguration and prevent efficient *β-globin* gene expression.

FOG proteins interact with at least two transcriptional corepressor complexes, NuRD and CtBP. Rodriguez et al. demonstrated promoter cooccupancy of the eosinophilic-specific gene *MBP* by GATA-1, FOG-1, and the NuRD component MBD3 in induced mouse erythroleukemia (MEL) cells, which was consistent with FOG-1–mediated repression of eosinophil-specific genes in erythroid cells via NuRD (26). We hypothesized that FOG-1's repressive activity in mast cell development involves its recruitment of one or both of these complexes. However, a double mutant molecule incapable of binding either the NuRD or CtBP complexes was as potent as the wild-type molecule in repressing mast cell development from MCPs (Fig. 5). Our results are consistent with a recent report showing that a double ΔN, ΔCtBP FOG-1 mutant represses GATA-1–dependent activation of the *FcεRI-β* promoter as well as wild-type FOG-1 in transient transfection reporter assays in the PT18 mast cell line (52). These results suggest that mechanisms other than recruitment of the NuRD and/or CtBP complexes are responsible for FOG-1's inhibitory activity on mast cell development.

Regulation of FOG–1 expression

Regardless of the mechanism, our data, in combination with that of Querfurth et al., indicate that repression of *FOG-1* expression is critical for mast cell and eosinophil differentiation from multipotent progenitors (45). This begs a question: what turns off FOG-1 during the development of these lineages? The transcriptional regulation of *FOG-1* is just beginning to be explored. Welch et al. used bioinformatics to predict critical cis–regulatory elements controlling *FOG-1* expression (53). They showed that one such region, predicted cis–regulatory module 1, is occupied by GATA-1 in erythroid cells, which is consistent with their data indicating transcriptional up-regulation of *FOG-1* by GATA-1. We examined this same region in primary mast cells and instead found relatively low GATA-1 but high GATA-2 occupancy compared with induced MEL cells (unpublished data). In addition, histone 3 is markedly hypoacetylated in this region, compared with induced MEL cells, consistent with epigenetic silencing of FOG-1 in mast cells. Whether high level GATA-2 occupancy at predicted cis–regulatory module 1 is responsible for *FOG-1* down-regulation will require further studies. However, given our findings that FOG-1 represses *GATA-2* expression (Fig. 6) (8, 21), it is possible that cross-antagonistic regulatory loops exist between GATA-2 and FOG-1. In this model, high GATA-2 levels silence *FOG-1* expression in maturing mast cells, leading to enhanced *GATA-2* expression and further *FOG-1* repression. Conversely, high FOG-1 levels in maturing erythroid/megakaryocytic cells inhibit *GATA-2* expression, leading to further elevation of FOG-1 and reduced *GATA-2* expression.

Mast cells in human disease

Mast cells play important roles in human disease, including asthma and type I hypersensitivity reactions. They have also recently been implicated as key intermediaries of regulatory

T cell–mediated immune tolerance (54). Our data provide new insights into the molecular control of mast cell development and suggest possible novel approaches to manipulating mast cell–related processes. In theory, derepression of *FOG-1* in maturing MCPs should inhibit their production and, possibly, alleviate the secondary effects of mast cell release.

Mastocytosis refers to a heterogeneous group of disorders associated with inappropriate or nonatopic expansion of mast cells. It ranges in clinical severity from localized cutaneous mastocytosis to aggressive systemic mastocytosis. Activating mutations in c-kit or interstitial chromosome 4 deletions that generate a constitutively active FIP1LI–PDGFA fusion receptor tyrosine kinase have been identified in many, but not all, cases of systemic mastocytosis (55–57). Based on our data, it is intriguing to speculate that acquired FOG-1 loss-of-function mutations in early multipotent progenitor or stem cells might also cause or contribute to clonal mast cell expansion in some cases, particularly those with associated eosinophilia and/or myelodysplasia. Examination of such a possibility seems warranted.

Lineage plasticity during the early stages of hematopoietic differentiation

The classical view of hematopoietic development is one in which multipotent progenitor cells irreversibly commit to distinct lineage types during differentiation. However, beginning with the work of Boyd and Schrader (58), the irreversibility of these decisions has been questioned (59–63). Although most of the early studies used transformed cell lines, more recent work using primary cell and in vivo systems support the initial finding (64–69). Our results provide further evidence for lineage plasticity during at least the early stages of hematopoietic cell fate commitment. Although we cannot completely exclude the possibility of selection of rare contaminating multipotent progenitors cells in our system, several aspects of our findings suggest that the FOG-1–mediated block in mast cell development and the appearance of alternate lineage cells represents true "reprogramming." First, MCPs were prospectively isolated in two rounds of immunophenotypic cell sorting before FOG-1 transduction. Second, FOG-1 reprogramming activity was observed in clonal primary mast cells (and the parent clone was 100% FcεRI positive and Ter119 negative after culture in multilineage-supporting cytokine cocktails). Third, the block in mast cell development and the appearance of abundant alternate lineage cells appears rapidly (over 6–11 d), which seems unlikely if it represents the selection of a very rare contaminating multipotent progenitor cell in the original retrovirally infected cell population.

Although it seems unlikely that FOG-1–induced lineage switching of MCPs occurs physiologically, the findings demonstrate the potential for such plasticity. Whether this occurs via direct transdifferentiation of MCPs to erythroid/megakaryocytic cells or via dedifferentiation followed by redifferentiation along erythroid/megakaryocytic pathways remains to be determined.

Our results, collectively with those of others described in this section, support a model of "lineage instability" during the early stages hematopoietic cell fate determination. In this developmental window, the term "commitment" should per-

haps be considered more relative, rather than absolute, reflecting what the cell would do if left unperturbed. As the cell matures, lineage choices may become increasingly stabilized by transcriptional networks involving positive autoregulation and cross-antagonism of alternate lineage transcription factors and cofactors. Is there a point of no return? That is, is there a point in differentiation when reprogramming is not possible? When we attempted to reprogram more mature BMMCs (sorted for FcεRI surface expression), as opposed to MCPs, we observed mostly cell death (unpublished data). Further understanding the mechanisms that mediate the transition from "reversible" to "irreversible" lineage commitment will be of interest and, possibly, clinical utility in the future.

MATERIALS AND METHODS

Materials and constructs. All cytokines were recombinant preparations purchased from R&D Systems unless otherwise stated. Antibodies for FACS staining were obtained from BD Biosciences unless otherwise stated. The mutant FOG-1 constructs have been previously described (10). The ΔN67, mCTBP FOG-1 double mutant was constructed by recombining the ΔN67 and mCtBP FOG-1 mutants.

Mouse strains. Previous papers have described the generation of the GATA-1⁻ (3), FOG-1⁻/⁻ (7), GATA-1^{V205G} (9), GATA-2$^{V296G/V296G}$ (9), and FOG-1 transgenic mice (39) used in this study. All experiments involving mice were approved by the Children's Hospital Boston Animal Care and Use Committee and conform to institutional regulatory standards.

YSMCs. Collagenase-released yolk sac cells were cultured in methylcellulose (StemCell Technologies Inc.) in the presence of 5 ng/ml mTpo, 2 IU/ml hEpo, and 50 ng/ml of rat SCF (Amgen), as previously described (9). Colonies were scored after 7 d of culture. Cytospins of colonies were performed and analyzed by May-Grunwald-Giemsa and Toluidine blue staining to confirm colony type.

Generation of YSMCs and BMMCs. For YSMCs, pregnant females were killed at 9.5 d after coitus. Yolk sacs were dissected away from the embryos, digested with 0.1% (wt/vol) collagenase (Worthington Biochemical Corp.) in 20% FCS/calcium and magnesium–free 1× PBS for 90 min at 37°C with agitation, passaged 8× through an 18-gauge needle, and placed in IMDM (Invitrogen) containing 15% FCS. The suspension was centrifuged, and the cell pellets were cultured in IMDM containing 15% FCS, penicillin/streptomycin, gentamicin, 10 ng/ml of mouse IL-3, and 50 ng/ml of mouse SCF (mast cell growth media) for 6 wk (unless stated otherwise in the figure legends), splitting the cell cultures as needed.

For BMMCs, adult male C57/BL6 mice were killed and both femurs were removed. The marrow cavity was flushed with IMDM containing 15% FCS. The samples were vortexed and allowed to sit at room temperature for 5 min to allow large debris to settle. The supernatant was centrifuged, and the cell pellet was resuspended in mast cell growth media. Cells were cultured in mast cell growth media for 6 wk (unless stated otherwise in the text), splitting the cell cultures as needed.

Limiting dilution analysis of MCPs from transgenic mice. GMPs were purified from wild-type or FOG-1–transgenic mice, as previously described (70), and plated on 96-well culture plates containing mast cell growth media at graded cell numbers using an automatic cell deposition unit system. Cells were cultured for 4 wk and examined in cytospin preparations. Wells positive for mast cell growth were determined morphologically by May-Grunwald-Giemsa and Toluidine blue staining.

Isolation of MCP cells. For day 2 MCP sorting, purified GMPs were cultured for 48 h in IMDM containing 20% FBS in the presence of 50 ng/ml

mSCF, 20 ng/ml mIL–3, and 20 ng/ml mIL–6. Cells were then stained with FITC-conjugated anti–β7 integrin (M293) and PE-conjugated anti-CD11b (M1/70; BD Biosciences). Day 2 MCPs were sorted as CD11b⁻ β7 integrin⁺ cells.

Retroviral infection of primary mast cells and cell sorting. Constructs were inserted between the viral ATG and an internal ribosome start site (IRES)–GFP cassette in the mouse myeloproliferative retroviral vector, and retroviral particles were packaged in 293GPG cells and concentrated by ultracentrifugation, as previously described (10, 71). BMMCs, GMPs, or day 2 MCPs were incubated with concentrated retroviral supernatants in mast cell growth media containing 4 μg/ml polybrene, 20 ng/ml mIL–3, 50 ng/ml SCF, 20 ng/ml mIL–6, 5 ng/ml mTPO, and 2 IU/ml hEpo for 12–16 h in a tissue culture incubator at 37°C/5%CO$_2$. After incubation, cells were washed and resuspended in mast cell growth media containing the cytokines listed earlier in this paragraph. After 2 d of additional culture, GFP⁺ cells were sorted using high speed FACS sorters (Altra or Aria; Becton Dickinson). Reanalysis of sorted cells revealed GFP⁺ purities of at least 95%. The GFP⁺ cells were placed in mast cell growth media containing 20 ng/ml mIL–3, 50 ng/ml SCF, 5 ng/ml mTPO, and 2 IU/ml hEPO at a starting concentration of 10⁵ cells per milliliter, and incubated for various amounts of time, as indicated in the figures. Cultures were expanded as necessary.

FACS analysis. FACS staining for FcεRI surface expression was performed as previously described (72). In brief, all incubations were done at 4°C in DMEM (Invitrogen) containing 2% FCS. Cells were preincubated with monoclonal anti–CD3 (B3B4) and anti–FcγRII/III (clone 2.42G) mAbs for 15 min to block the low affinity IgE and FcγRII/III receptors, respectively. Mouse IgE (anti-DNP) mAb (clone SPE-7; Sigma-Aldrich) was added, and the cells were incubated for an additional 1 h. After washing in DMEM/2%FCS, the cells were incubated with biotinylated anti–mouse IgE (BD Biosciences) for 30 min. The cells were washed again and simultaneously stained with PE–anti–c-kit and allophycocyanin-streptavidin for 30 min. After final washes, the cells were analyzed using a FACSCalibur (Becton Dickinson). Standard FACS staining was used for the analysis of Gr-1, CD11b, and Ter-119 expression.

RT-PCR analysis of gene expression. Total RNA was extracted from the cells or whole organ (heart) using TRIᴢOʟ (Invitrogen), according to the manufacturer's instructions. 5 μg of RNase-free glycogen was included as a carrier during RNA precipitation. First-strand cDNA synthesis was performed at 37°C for 1 h using oligodT$_{15}$ as a primer and MMLV-RT (Roche), according to manufacturer's instructions. PCRs were performed for 22–34 cycles using the Advantage 2 kit (BD Biosciences), according the manufacturer's instructions, and analyzed by electrophoresis in 2% agarose gels containing ethidium bromide. Primer sequences for β-*major globin*, *EKLF*, *HPRT* (73), *GPIbα* (5), *GATA-1*, *GATA-2*, *FOG-1*, *FcεRI*, *MMCP-1*, *MMCP-5* (38), and *FOG-2* (exons 3/4) (74) have been previously described. Other primer sequences are as follows: *MMCP-2*, (forward) 5′-GTGATGACTGCTGCACACTG-3′ and (reverse) 5′-CTTGAAGAGTCTGACTCAGG-3′; *MMCP-4*, (forward) 5′-GTAATTCCTCTGCCTCGTCCT-3′ and (reverse) 5′-CCCAAGGG-TTATTAGAAGAGCTC-3′; *MC-CPA*, (forward) 5′-ACACAGGATC-GAATGTGGAG-3′ and (reverse) 5′-TAATGCAGGACTTCATGAGC-3′; and *band 3*, (forward) 5′-GGCACCTTCCTTCTGGGTCTGGC-3′ and (reverse) 5′-GTCGACATGCGGAGCCTCAGGTC-3′. For quantitative PCR analysis, reactions were performed using SYBR green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions and analyzed on a real-time PCR machine (MyCycler; Bio-Rad Laboratories). *GATA-2* or *FOG-1* transcript levels were calculated using the $2^{\Delta\Delta C(t)}$ method, normalizing to transcript levels of the housekeeping gene *GAPDH*. Melt curves demonstrated a single product species for all reactions. Sequences of the real-time PCR primers for *GATA-2* (exons 3/4), *FOG-1* (exons 5/6), and *GAPDH* are as previously described (21).

ChIP assays. ChIP assays were performed as previously described (21). In brief, BMMCs cultured for 6 wk in mast cell growth media were treated with 1% formaldehyde (final concentration) for 10 min at room temperature, and the reaction was stopped by adding excess glycine. Nuclei were lysed and the chromatin was sonicated to generate ∼500–1,000-bp fragments. Immuno-precipitation was performed using anti-GATA-1 (N6; Santa Cruz Biotechnologies), followed by rabbit anti–rat IgG (Jackson ImmunoResearch Laboratories), anti-GATA-2 (H-116; Santa Cruz Biotechnologies), or anti–FOG-1 polyclonal antisera (6), or the equivalent protein amount of pooled normal rat IgG (Sigma-Aldrich) or FOG-1 preimmune sera. After washing, the cross-links were reversed and the released DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation. Recovery of specific DNA fragments was assessed by real-time PCR (My-Cycler or iCycler; Bio-Rad Laboratories) and normalized to a serial dilution standard curve of input chromatin for each reaction. PCR primer sets are as follows: *Necdin* promoter, 5′-GGTCCTGCTCTGATCCGAAG-3′ and 5′-GGGTCGCT-CAGGTCCTTACTT-3′; and *GATA-2* −2.8 kb, 5′-GCATGGCCCTG-GTAATAGCA-3′ and 5′-CAGCCGCACCTTCCCTAA-3′.

Online supplemental material. Fig. S1 examines the morphology and marker gene expression of YSMCs and BMMCs. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070544/DC1.

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