Transcriptional Mechanisms Underlying Hemoglobin Synthesis

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The physiological switch in expression of the embryonic, fetal, and adult β -like globin genes has garnered enormous attention from investigators interested in transcriptional mechanisms and the molecular basis of hemoglobinopathies. These efforts have led to the discovery of cell type-specific transcription factors, unprecedented mechanisms of transcriptional coregulation, genome biology principles, unique contributions of nuclear organization to transcription and cell function, and promising therapeutic targets. Given the vast literature accrued on this topic, this article will focus on the master regulator of erythroid cell development and function GATA-1, its associated proteins, and its frontline role in controlling hemoglobin synthesis. GATA-1 is a crucial regulator of genes encoding hemoglobin subunits and heme biosynthetic enzymes. GATA-1-dependent mechanisms constitute an essential regulatory core that nucleates additional mechanisms to achieve the physiological control of hemoglobin synthesis.

The transcriptional switch from embryonic to fetal, and subsequently to adult globin gene transcription constitutes an elegant mechanism to meet the unique developmental requirements for oxygenation of cells and tissues during gestation (Blau and Stamatoyannopoulos 1994; Orkin and Higgs 2010; Sankaran and Orkin 2013). Mechanistic studies on this problem have a dual motivation. Investigators studying transcriptional mechanisms in erythroid cells have accrued an impressive track record with respect to forging principles of gene and chromosome regulation that have broad relevance to diverse systems. Furthermore, preva-

lent hemoglobin-associated pathologies (sickle cell anemia, additional anemias, thalassemias, and developmental disorders) constitute a major public health problem worldwide (Weatherall 2010). Elevating expression of the developmentally silenced γ -globin gene can supplant mutant or inadequate levels of β -globin in human disease states, thereby suppressing the associated symptoms (Rodgers et al. 1989; Bunn 1999; Atweh and Schechter 2001; Orkin and Higgs 2010). Thus, dissecting mechanisms controlling hemoglobin biosynthesis in exquisite detail is expected to yield novel molecular strategies for the treatment of hemoglobinopathies.

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Other articles in this collection have highlighted the molecular biology of genes encoding hemoglobin subunits (Hardison 2012), hemoglobinopathies (Nienhuis and Nathan 2012; Higgs 2013), and human genetic studies that revealed an extremely important new regulator of hemoglobin switching, the transcriptional coregulator BCL11A (Lettre 2012; Sankaran and Orkin 2013). Undoubtedly, BCL11A is a crucial component of the hemoglobin switching machinery, and major efforts are under way to elucidate how BCL11A functions in physiological and pathological contexts. As transcriptional coregulators almost invariably function in large heteromeric protein complexes, one would expect a host of established and undiscovered BCL11A interactors (either direct or indirect) to have integral roles in hemoglobin switching. Independent of BCL11A, additional factors (e.g., the master regulator of erythrocyte development and function GATA-1) (Evans and Felsenfeld 1989; Tsai et al. 1989; Yamamoto et al. 1990; Zon et al. 1990), regulate hemoglobin synthesis, and initial studies imply potentially important functional links to BCL11A-dependent mechanisms.

Whereas the exciting BCL11A work (Menzel et al. 2007a; Lettre et al. 2008; Sankaran et al. 2008, 2009, 2010b; Uda et al. 2008; Xu et al. 2010) has inarguably energized this extremely important field, more than a decade of studies have highlighted the essential role of GATA-1 as a critical determinant of hemoglobin synthesis. GATA-1 directly regulates expression of genes encoding hemoglobin subunits and heme biosynthetic enzymes (Johnson et al. 2002; Cheng et al. 2009; Fujiwara et al. 2009; Yu et al. 2009). Elucidating mechanisms underlying GATA-1 function/regulation is essential to achieve a holistic perspective of hemoglobin synthesis, which will naturally lead to rational approaches to therapeutically modulate hemoglobin switching. The transcriptional control of hemoglobin synthesis has multiple regulatory layers, all contributing essential functions. GATA-1 establishes the erythroid-specific chromatin structure of the α - and β -like globin gene clusters (Stamatoyannopoulos et al. 1995; Pomerantz et al. 1998; Goodwin et al. 2001; Letting et al. 2003;

Anguita et al. 2004; Im et al. 2005; Kim et al. 2007; Fujiwara et al. 2009; Wu et al. 2011) and participates in various mechanistic steps to ensure expression of the globin genes in a developmental stage-appropriate manner. Thus, GATA-1 creates a regulatory core, on which additional mechanisms (e.g., those involving BCL11A), are seamlessly integrated to yield the complete regulatory system. Without GATA-1, the active loci would likely revert into repressive chromatin structures resembling nonerythroid cells, and erythroblasts would lose the capacity to survive.

GATA FACTOR MECHANISMS AND BIOLOGY

Efforts to understand globin gene regulation led to the discovery of a simple DNA sequence, (A/T)GATA(A/G) (WGATAR = GATA motif), that commonly resides at *cis*-regulatory elements of globin promoters and enhancers (Evans et al. 1988). This discovery paved the way for the cloning of the erythroid protein that binds this sequence, the founding member of the GATA factor family, GATA-1 (Evans and Felsenfeld 1989; Tsai et al. 1989; Yamamoto et al. 1990). Impressively, GATA-1 regulates hundreds of genes (Welch et al. 2004; Fujiwara et al. 2009; Yu et al. 2009) that mediate the development and function of erythroid cells (Pevny et al. 1991, 1995; Simon et al. 1992; Fujiwara et al. 1996). The cloning of GATA-1 ushered in the discovery of related GATA factors (GATA-2-6) (Wilson et al. 1990; Yamamoto et al. 1990; Ho et al. 1991; Lee et al. 1991; Dorfman et al. 1992; Arceci et al. 1993; Ito et al. 1993; Kelley et al. 1993; Tsai et al. 1994; Morrissey et al. 1996, 1997; Kuo et al. 1997; Molkenstein et al. 1997), which almost invariably differ from GATA-1 in expression patterns and functions in systems including the brain, heart, vasculature, and skin. A hallmark attribute of GATA factors is their diagnostic dual zinc finger motif (Fig. 1) (Liew et al. 2005; Gamsjaeger et al. 2007). The zinc finger closest to the carboxyl terminus of GATA-1 (carboxyl finger) mediates sequence-specific DNA binding (Martin and Orkin 1990; Ko and Engel 1993; Merika and Orkin 1993), whereas the zinc finger closest to the GATA-1 amino terminus (amino finger)

Figure 1. The molecular basis of GATA-1 action. (A) Diagram of GATA-1 protein organization illustrating the amino and carboxyl fingers and posttranslational modification sites. The amino finger mediates FOG-1 binding, whereas the carboxyl finger mediates sequence-specific DNA binding to GATA motifs. M83 is an alternative translation start site of a leukemogenic form of GATA-1 in acute megakaryoblastic leukemia (Wechsler et al. 2002; Crispino 2005). V205 is mutated in dyserythropoietic anemia and thrombocytopenia (Nichols et al. 2000), and facilitates FOG-1 binding (Crispino et al. 1999). Phosphorylation (P) (Crossley and Orkin 1994; Towatari et al. 2004; Kadri et al. 2005; Yu et al. 2005; Zhao et al. 2006) and acetylation (Ac) (Hung et al. 1999; Lamonica et al. 2006, 2011) sites are depicted, as well as a sumoylated site (Collavin et al. 2004; Lee et al. 2009). (B) Multiple modes of GATA-1 function. GATA-1 activates or represses its target genes with or without FOG-1. (Legend continues on following page.)

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binds the cell type-restricted transcriptional coregulator Friend of GATA-1 (FOG-1) (Tsanget al. 1997, 1998; Crispino et al. 1999). The amino fingers of other GATA factors (e.g., GATA-4 and GATA-6) bind the FOG-1-related coregulator FOG-2 (Lu et al. 1999; Svensson et al. 1999; Tevosian et al. 1999, 2000). FOG-1 and FOG-2 each contain nine zinc fingers, none of which have been shown to mediate DNA binding (Gamsjaeger et al. 2007). However, four zinc fingers of FOG-1 are implicated in binding the GATA-1 amino finger (Fox et al. 1999; Cantor et al. 2002). FOG-1 interaction partners include the chromatin remodeling/modifying nucleosome remodeling and histone deacetylase (NuRD) complex (Hong et al. 2005; Gregory et al. 2010; Miccio et al. 2010), the corepressor CtBP (Holmes et al. 1999; Katz et al. 2002; Snow et al. 2010a), and the centrosomal protein transforming acidic coiled coiled 3 (TACC3) (Garriganut and Orkin 2004; Simpson et al. 2004).

The intimation that GATA-1 would be a universally important regulator of erythroid gene expression was definitively established through loss-of-function studies in embryonic stem cells and mice (Pevny et al. 1991, 1995; Simon et al. 1992; Weiss et al. 1994; Weiss and Orkin 1995; Fujiwara et al. 1996) and via human disease-associated GATA-1 mutations (Nichols et al. 2000; Crispino 2005). The targeted deletion of GATA-1 yielded embryonic lethality at E10.5–11.5 and a striking disruption of erythroid cell development and maturation (Fujiwara et al. 1996). Consistent with its expression in select additional hematopoietic lineages, GATA-1 is also required for the development and maturation of megakaryocytes, eosinophils, and mast cells (Zon et al. 1991; Vyas et al. 1999; Hirasawa et al. 2002; Yu et al. 2002; Migliaccio et al. 2003; Humbles et al. 2004; Cantor et al. 2008; Sugiyama et al.

2008; Huang et al. 2009). The critical importance of FOG-1 as a GATA-1 coregulator was also established through gene-targeting studies in the mouse. FOG-1 nullizygous mouse embryos die at an embryonic stage similar to that of GATA-1 nullizygous embryos (E10.5–E11.5) and show defective erythropoiesis and megakaryopoiesis (Tsang et al. 1998). Furthermore, a V205M mutation in the GATA-1 amino finger disrupts the GATA-1–FOG-1 interaction, resulting in familial dyserythropoietic anemia and thrombocytopenia (Nichols et al. 2000).

The extraordinarily important biological functions of GATA-1 and FOG-1 provided the impetus for intense efforts to elucidate their mechanisms of action. Analogous to numerous *trans*-acting factors, GATA factors can activate and repress transcription (Kim and Bresnick 2007; Bresnick et al. 2010). Mechanistic studies to drill deeply into GATA-1-dependent transcriptional mechanisms are ongoing. FOG-1 mediates the majority of GATA-1-dependent transcriptional responses (Johnson et al. 2007; Chlon et al. 2012), and FOG-2 is used by other GATA factors. Although many questions remain unanswered regarding FOG-1 and FOG-2 mechanisms, FOG-1 has a chromatin occupancy facilitator activity (Letting et al. 2004; Pal et al. 2004) that increases GATA-1 chromatin occupancy at certain, but not all, chromatin sites and suppresses GATA-1 from accessing another cohort of sites (Johnson et al. 2006; Chlon et al. 2012). GATA-2 frequently occupies chromatin sites before GATA-1 during hematopoiesis (Grass et al. 2003, 2006; Bresnick et al. 2005, 2010, 2012; Martowicz et al. 2005; Kim and Bresnick 2007; Lugus et al. 2007; Fujiwara et al. 2009; Dore et al. 2012). When GATA-1 levels increase during erythropoiesis, it displaces GATA-2 from chromatin, including sites at the *Gata2* gene.

Figure 1. (Continued) The precise mode of transcriptional control is locus-specific, and representative target genes that conform to each regulatory mode are depicted (Bresnick et al. 2012). (C) GATA switch model. GATA switches are defined as a molecular transition in which one GATA factor replaces another from a chromatin site, which is often associated with an altered transcriptional output. In hematopoietic stem cells, select progenitors, and early-stage erythroblasts, GATA-2 occupies target loci. During erythropoiesis, poorly understood signals induce GATA-1 expression, and GATA-1 acquires the capacity to displace GATA-2 from chromatin, including sites at the *Gata2* locus, which results in transcriptional repression. FOG-1 facilitates GATA-1 occupancy at select chromatin sites and GATA switches.

This “GATA switch” is often associated with altered transcriptional outputs of the respective genes (Bresnick et al. 2010, 2012). For example, GATA switches occur at five sites of the *Gata2* locus and were initially correlated with *Gata2* repression (Grass et al. 2003, 2006; Martowicz et al. 2005). However, targeted deletions of three of these sites individually revealed qualitatively distinct activities. The -1.8 site was essential to maintain *Gata2* repression in late-stage erythroblasts (Snow et al. 2010b). The -2.8 and -1.8 sites conferred maximal *Gata2* expression in hematopoietic precursors (Snow et al. 2010b, 2011). The most interesting of the sites, the intronic $+9.5$ site, was essential for *Gata2* expression in the fetal liver, for fetal liver hematopoietic stem and progenitor cell activity, and for conferring vascular integrity (Johnson et al. 2012). This *cis*-element targeting study provided the first example of an exceedingly important function for a GATA switch site in vivo. Similar results were obtained using a $+9.5$ site-driven Cre recombinase to conditionally delete a floxed *Gata2* allele (Lim et al. 2012).

FOG-1 facilitates the GATA switch at the *Gata2* locus, required for GATA-1 to repress *Gata2* (Pal et al. 2004), which is important to ensure normal erythrocyte development and therefore normal hemoglobin synthesis. Constitutive overexpression of GATA-2 in bone marrow represses hematopoiesis (Persons et al. 1999). Analysis of a mutant mouse strain containing a knock-in FOG-1 allele defective in NuRD binding indicated that this interaction is important for activation and repression (Miccio et al. 2010). Furthermore, the FOG-1-NuRD interaction appears to be important for the GATA switch at the *Gata2* locus (Gao et al. 2010). The GATA switch mechanism is applicable to the control of numerous erythroid genes (Pal et al. 2004; Bresnick et al. 2010; Dore et al. 2012), and as discussed later in this review, this mechanism has been implicated in γ -globin transcriptional regulation (Costa et al. 2012; Zhu et al. 2012).

A limited ensemble of direct GATA-1 target genes (occupied by endogenous GATA-1 [Cheng et al. 2009; Fujiwara et al. 2009; Yu et al. 2009; Kang et al. 2012] and regulated by GATA-1 in a

genetic complementation assay in GATA-1-null erythroid precursor cells [Weiss et al. 1997]) are not particularly sensitive to a GATA-1 mutation that reduces FOG-1 binding (e.g., V205G or V205M) or to reducing the FOG-1 level (e.g., in a FOG-1 nullizygous hematopoietic precursor cell line [Cantor et al. 2002], or on siRNA-mediated knockdown of FOG-1) (Crispino et al. 1999; Johnson et al. 2006, 2007; Lee et al. 2009). We have referred to these genes as “FOG-1-independent,” but examples exist in which there appears to be a partial FOG-1 requirement, rather than absolute FOG-1 independence. This differs from the genes conventionally deemed to be “FOG-1-dependent,” such as *Gata2* (repressed) and β *major* (activated), as GATA-1 is largely incapable of regulating these genes without FOG-1. Given the apparent spectrum of FOG-1 sensitivities, we now refer to FOG-1-sensitive and -insensitive, rather than an absolute dependence or independence. The magnitude of the gene expression changes at FOG-1-insensitive genes can be considerable, and these genes include *Lyl1* (repressed) (Johnson et al. 2007), which encodes an important transcriptional regulator of HSCs (Souroullas et al. 2009; Zohren et al. 2012) and *EPB49* (activated) (Kim et al. 2007), which encodes an important component of the red cell cytoskeleton, dematin (Rana et al. 1993; Khanna et al. 2002; Mohseni and Chishti 2008). Whereas it is likely that this mode of GATA-1 transcriptional control has functional importance, why certain target genes lack the crucial FOG-1 requirement remains a black box. Of greatest importance for this review is the FOG-1-sensitive mechanism, as GATA-1 uses FOG-1 to regulate the α - and β -like globin genes (Kim and Bresnick 2007).

Consistent with the conventional mechanism of metazoan transcriptional regulators, GATA factors control chromatin remodeling and modification. GATA-1 binds and recruits the histone/protein acetyltransferase CREB-binding protein (CBP/p300) to chromatin, inducing both GATA-1 acetylation and histone acetylation (Blobel et al. 1998; Hung et al. 1999). GATA-1 recruits the BRG1 subunit of the ATP-dependent chromatin remodeling complex SWI/SNF (Im et al. 2005; Kim et al. 2007,

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2009a,b), and *Brg1* hypomorphic mutant mice show significant anemia and impaired erythropoiesis (Bultman et al. 2005). In contrast to CBP/p300 and BRG1, GATA-1 interacts with a component of the mediator complex Med1 (Crawford et al. 2002; Stumpf et al. 2006), which is not known to have chromatin remodeling and/or modifying activity. Med1 is a core component of the broadly expressed 1–2 MDa mediator complex, which consists of more than 30 subunits, the vast majority of which have no ascribed biochemical function (Conaway et al. 2005; Kornberg 2005). Med1 knockout mouse embryos are severely anemic and show reduced progenitor activity in the erythroid, but not myeloid, lineage (Crawford et al. 2002; Stumpf et al. 2006). A conditional Med1 knockout confirmed the cell-autonomous nature of the erythroid requirement for Med1 (Stumpf et al. 2010). Although GATA-1 recruits Med1 and FOG-1 similarly, Med1 and FOG-1 differ quantitatively in their contributions to GATA-1 function. Knockdown studies indicate that Med1 amplifies GATA-1 target gene activation, whereas FOG-1 contributes to a much greater extent to GATA-1 function (Pope and Bresnick 2010).

As an important manifestation of its molecular activities, GATA-1 bound to distal *cis*-regulatory elements can induce chromatin looping, thus bringing a distal site in close proximity to a promoter (Vakoc et al. 2005). GATA-1 binding to the β -globin LCR repositions the LCR such that its proximity to the β *major* promoter increases considerably (Vakoc et al. 2005). GATA-1 can also induce higher-order chromosomal transitions, in which a target locus is expelled from the nuclear periphery, freeing the locus from potential inhibitory constraints imposed by the unique subnuclear environment at the periphery (Lee et al. 2009, 2011). FOG-1 promotes both looping (Vakoc et al. 2005; Kim et al. 2007) and subnuclear relocalization of loci (Lee et al. 2009, 2011). BRG1 (Kim et al. 2009b) and the Lim domain protein Ldb1 (Song et al. 2007) are also required to establish the β -globin locus LCR-promoter loop. Tiling the chromosomal region surrounding the β -globin locus with bacterial artificial chromosome probes and conducting three-dimensional (3D) immunofluo-

rescence in situ hybridization (immunoFISH) revealed that GATA-1 expels the β -globin locus, but not the neighboring chromosomal sequences, from the nuclear periphery (Lee et al. 2011). Both FOG-1 and K137-sumoylated GATA-1 are required for this GATA-1 activity, and it is facilitated by the erythroid Krüppel-like factor KLF1 (Miller and Bieker 1993; Perkins et al. 1995) and the NuRD complex subunit Mi2 β (Lee et al. 2011). KLF1 organizes numerous target genes within nuclear neighborhoods (Drissen et al. 2004; Schoenfelder et al. 2010). Looping and subnuclear relocalization of the β -globin locus, as measured by chromosome conformation capture (3C) and 3D-immunoFISH assays, may be related or identical processes. Although it is not our intent to comprehensively review the precise details of GATA-1-dependent molecular and supramolecular transitions, many of which remain unresolved, dissecting these mechanisms in exquisite detail is expected to continue to yield pivotal insights into the control of hemoglobin synthesis and to uncover targets for therapeutic modulation of this crucial process.

EVIDENCE LINKING GATA FACTORS TO THE CONTROL OF HEMOGLOBIN SYNTHESIS

Studies on how GATA-1 regulates genes encoding hemoglobin subunits and heme biosynthetic enzymes have forged principles of GATA factor function and revealed important insights into mechanisms controlling hemoglobin synthesis. The presence of GATA motifs (WGATAR) at globin gene promoters and enhancers, as well as transient transfection studies with globin promoter and enhancer reporter genes, led to the view that GATA-1 controls hemoglobin synthesis (Evans et al. 1990; Orkin 1990). The application of ChIP technology to measure protein occupancy at endogenous loci revealed GATA-1 occupancy at loci encoding globin subunits and heme biosynthetic enzymes (Horak et al. 2002; Johnson et al. 2002; Kiekhäfer et al. 2002; Im et al. 2005; Cheng et al. 2009; Fujiwara et al. 2009; Yu et al. 2009). In a genetic complementation assay in GATA-1-null erythroid precursor cells (Weiss et al. 1997), GATA-1 occupancy of LCR sites precedes occupancy

at the adult β major promoter (Im et al. 2005; Kim et al. 2007). GATA-1 recruits RNA polymerase II (Pol II) to the LCR and subsequently to the promoter, which occurs concomitantly with transcriptional activation (Johnson et al. 2001, 2002, 2003; Im et al. 2005; Kim et al. 2007). Strikingly, however, numerous genomic sites harbor GATA motifs that bind GATA factors with high affinity in the context of naked DNA, but these sites are not occupied in cells (Bresnick et al. 2006). Despite the ~ 7 million GATA motifs (WGATAR) in the human genome, genome-wide ChIP-seq analysis showed that GATA-1 discriminates exquisitely among these sites (Fujiwara et al. 2009; Kang et al. 2012). Occupancy was detected at only 0.07% of sites containing WGATAR. Thus, the presence of a GATA motif at a chromosomal site, even when evolutionarily conserved, appears to have little to no predictive value as to whether the associated gene will be bound to or regulated by GATA-1.

Given the low frequency of GATA-1 occupancy at sites containing the conventional GATA motif WGATAR, the sequence requirements for occupancy in vivo might vary from those required for naked DNA binding, and/or additional parameters beyond the presence of a high-affinity GATA motif may dictate GATA motif access in vivo. Genome-wide ChIP-seq studies provided evidence for the existence of a more complex GATA motif at GATA-1-occupied sites in erythroid cells [(C/G)(A/T)GATAA(G/A/C)(G/A/C)], which exists at 297,124 copies in the human genome (Fujiwara et al. 2009). Although the frequency of occupancy at these sites is an order of magnitude greater than at WGATAR, it remains quite low (0.7%). GATA motifs often reside in close proximity to other *cis*-regulatory elements, and it is likely that the neighboring elements constitute an important parameter that dictates GATA motif access in chromatin. However, interrogation of GATA-1-bound DNA sequences isolated by ChIP using de novo motif finding methodology has not revealed individual or combinations of sites with significant utility for predicting GATA-1 occupancy (Fujiwara et al. 2009; Kang et al. 2012). In megakaryocytes (Dore et al. 2012) and endothe-

lial cells (Linnemann et al. 2011), additional *cis*-regulatory elements show highly statistically significant enrichments at GATA-2-occupied sites, respectively, although it remains unclear whether the combinatorial usage of the multiple elements is an important determinant of GATA factor chromatin occupancy.

Analogous to the chromatin site discrimination problem, major work is required to unravel the determinants of whether GATA-1 binding to chromatin activates or represses one or more neighboring target genes. GATA-1 and GATA-2 often colocalize on chromatin with the E-protein Scl/TAL1 (Wozniak et al. 2008), and Scl/TAL1 colocalization correlates with activation in certain contexts (Cheng et al. 2009; Tripic et al. 2009; Yu et al. 2009); however, exceptions to this correlation exist. As noted above, FOG-1 mediates both activation and repression (Crispino et al. 1999). This is a particularly challenging issue, as transient transfection assays do not faithfully recapitulate the activation and repression functions of endogenous GATA-1-associated *cis*-regulatory elements. Sites that confer enhancer activity with reporter genes in transiently transfected cells and in transgenic mice do not invariably exert crucial activities at endogenous loci (Snow et al. 2010b, 2011).

Analyses of the β -globin LCR, a far upstream strong enhancer element consisting of four DNaseI hypersensitive sites (HSs) (Tuan and London 1984; Forrester et al. 1986, 1987; Grosfeld et al. 1987), have been particularly instructive for understanding long-range transcriptional mechanisms in which clusters of *cis*-regulatory elements control distant promoters (Fig. 2). Targeted deletion of the four HSs of the LCR from the endogenous mouse genome severely impairs β -like globin gene expression (Epner et al. 1998; Reik et al. 1998; Bender et al. 2000a). Although GATA motifs confer DNaseI hypersensitivity and contribute to enhancer activities of the HSs (Philipsen et al. 1990, 1993; Pruzina et al. 1991, 1994; Ellis et al. 1993; Stamatoyannopoulos et al. 1995; Pomerantz et al. 1998; Goodwin et al. 2001), targeted deletion of the individual HSs, each containing GATA motifs and additional *cis*-regulatory elements, only incrementally influence β -like glo-

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Figure 2. (Top) Organization of the human and murine β -globin loci. The human locus contains β -globin genes expressed during embryogenesis (ϵ), fetal development ($G\gamma$ and $A\gamma$), and the adult (δ and β). The mouse locus contains genes expressed during embryogenesis ($\epsilon\gamma$ and $\beta H1$) and adult (β major and β minor). The regions identified in Hispanic thalassemia and hereditary persistence of fetal hemoglobin (HPFH) mutations are described. The genomic structure of the human and murine β -globin and α -globin loci. Multiple DNaseI hypersensitive sites (HSs) are indicated by red bars. The human β -globin locus has five proximal HSs and additional HSs. The mouse locus has six proximal HSs and additional HSs. Among these HSs, HS1–4 are termed the locus control region (LCR). The LCR mediates high-level transcription of the β -globin-like genes. A large deletion of the LCR characterizes Hispanic thalassemia (Driscoll et al. 1989; Forrester et al. 1990). (Bottom) Organization of the human and murine α -globin loci. The human locus contains α -globin genes expressed during embryogenesis (ζ) and the adult ($\alpha 1$, $\alpha 2$, and θ). Similarly, the mouse locus contains α -globin genes expressed during embryogenesis (ζ) and the adult ($\alpha 1$ and $\alpha 2$). Analogous to the β -globin locus, there are several DNaseI HSs at the α -globin locus. A large deletion including these HSs and ζ has been identified in several patients (Hatton et al. 1990). Among these HSs, HS-40 (corresponding to HS-26 in the mouse locus) is considered to be functionally important.

bin gene transcription (Fiering et al. 1995; Hug et al. 1996; Bender et al. 2000b). The targeted deletion studies indicated that multiple HSs function additively in vivo (Bender et al. 2001), which was surprising given the qualitatively and quantitatively distinct activities of the individual HSs in reporter gene and transgenic mouse assays (Ryan et al. 1989; Caterina et al. 1994; Bungert et al. 1995, 1999; Pawlik and Townes 1995; Ellis et al. 1996; Peterson et al. 1996; Navas et al. 1998, 2001). It is unclear whether the HS activities are considerably more important in promiscuous reporter assays and at ectopic chromosomal sites versus the endogenous locus, whether human and mouse LCR sequences have distinct activities, or whether compensatory mechanisms

mask their important functions when they are removed from the genome, and all are potentially important possibilities to rigorously explore. Regardless, GATA-1 occupies multiple sites at the endogenous β -globin and α -globin loci (Johnson et al. 2002; Anguita et al. 2004; Im et al. 2005; De Gobbi et al. 2007; Fujiwara et al. 2009), and it seems highly likely that some of these sites control endogenous globin gene transcription. Strikingly, a single nucleotide polymorphism upstream of the α -globin locus generates a GATA motif that confers promoter activity, thereby disrupting regulation of the downstream α -globin genes (De Gobbi et al. 2006).

Studies with β -globin locus transgenes have provided further evidence to support a model

in which the core GATA-1 regulatory function is a critical determinant of hemoglobin switching. A GATA motif at the ϵ -globin promoter (-208) suppresses activity of the promoter in a β -globin locus transgene in adult mice (Raich et al. 1995). Similarly, a GATA motif at -566 and -567 relative to the $A\gamma$ and $G\gamma$ globin genes participates in γ -globin silencing in definitive erythroid cells (Harju-Baker et al. 2008). A hereditary persistence of fetal hemoglobin (HPFH) mutation at the -566 site prevents assembly of the complex and sustains γ -globin expression at developmental stages in which γ -globin should be silenced. Taken together with evidence that GATA-1, FOG-1, and Mi2 β occupy this site, and that an erythroid cell-specific Mi2 β knockout elevates γ -globin expression from a β -YAC in mice (Costa et al. 2012), these factors assemble a repressive complex that controls hemoglobin switching. In contrast to these results with β -globin locus transgenes, a knock-in mouse strain expressing a mutant FOG-1 defective in NuRD complex binding from the endogenous FOG-1 locus crossed with a human β -globin locus transgenic line shows normal γ -globin silencing (Miccio and Blobel 2010). Thus, the FOG-1-NuRD interaction is apparently not required for silencing the γ -globin genes, although one cannot rule out the possibility that NuRD, and/or specific components of NuRD, are recruited to the locus independent of FOG-1. Studies in the K562 erythroleukemia cell model system and primary erythroid cells indicate that GATA-2 and the CCAAT box binding factor NF-Y participate in γ -globin gene activation, whereas GATA-1, BCL11A, and COUP-TFII confer repression through the same site (Zhu et al. 2012). This finding conforms to the GATA switch mechanism (Bresnick et al. 2005, 2010), in which GATA-1 replacement of GATA-2 from chromatin induces a distinct transcriptional output. In summary, endogenous GATA-1 chromatin occupancy, induction of β -globin gene expression in a genetic complementation assay, and molecular/cellular studies using diverse systems support a role for GATA-1 in establishing a physiological pattern of hemoglobin synthesis.

INTEGRATING GATA FACTOR-DEPENDENT MECHANISMS WITH OTHER MODES OF CONTROLLING HEMOGLOBIN SYNTHESIS

Variations in the level of fetal hemoglobin (HbF) in sickle cell disease (SCD) patients paved the way for the discovery of genetic modulators of hemoglobin switching. Genome-wide association studies identified SNPs that correlate with higher levels of HbF, including QTLs near the *HBS1L-MYB* locus and the β -globin gene cluster, which were described previously, along with several QTLs at the *BCL11A* locus (Menzel et al. 2007b; Lettre et al. 2008; Uda et al. 2008). The *HBS1L-MYB* intergenic region associated with high HbF has HSs that are occupied by GATA-1 in human erythroid precursor cells (Wahlberg et al. 2009). In addition, GATA-1 down-regulates *Myb* expression (Bartunek et al. 2003; Welch et al. 2004). Reduction of *MYB* mRNA by miRNA-15a and -16-1 in primary human erythroid cells results in elevated ϵ - and γ -globin synthesis (Sankaran et al. 2011). In comparison to the SNPs at the *HBS1L-MYB* and *β -globin* loci, the high-HbF-associated allele at rs4671393 in the *BCL11A* locus was the strongest indicator of fetal hemoglobin levels in a Brazilian cohort with SCD (Lettre et al. 2008). These studies led to the hypothesis that disruption of *BCL11A* modulates HbF levels, and therefore BCL11A represents a target for therapeutic intervention in SCD and β -thalassemia.

Recent studies have made the exceptionally interesting link between BCL11A activity to control hemoglobin switching and GATA-1 function. Proteomic analysis revealed interactions between expressed BCL11A and GATA-1, FOG-1, and the NuRD complex in cultured erythroid cells (Sankaran et al. 2008). BCL11A also interacts with the transcription factor Sox6, a regulator of γ -globin transcription (Yi et al. 2006; Xu et al. 2010). ChIP-chip analysis in primary human erythroid precursors revealed BCL11A occupancy at HSs of the β -globin LCR, which are also occupied by GATA-1 and Pol II. 3C analysis of the human β -globin locus in a β -YAC transgenic mouse crossed to a wild-type or *Bcl11a*^{-/-} mouse indicated that

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deletion of *BCL11A* reconfigures the higher-order chromatin structure of the locus (Xu et al. 2010). The LCR-dependent chromatin loop normally positions the LCR in close proximity to the β -globin promoter, but in this case, the LCR engages the $G\gamma$ and $A\gamma$ promoters. Although this intriguing mechanism has not been described in the context of the endogenous β -globin locus, these studies indicate that *BCL11A* can interact with GATA-1, and both factors regulate chromatin looping at the β -globin locus.

The conditional knockout of *Bcl11a* using *EpoR-Cre*⁺ mice crossed to β -YAC transgenic mice prevented complete silencing of human γ -globin from the β -YAC (Xu et al. 2011). Genome-wide analysis indicates that this modulation of fetal globin expression is exquisitely specific, as the vast majority of genes were unaffected. Importantly, adult β -globin expression and other regulators of β -like globin genes, including KLF1 (Miller and Bieker 1993; Perkins et al. 1995), are unaffected by loss of *BCL11A*. *Bcl11a* knockout synergizes with HbF modulators, including the DNA methylation inhibitor 5-aza-2'-deoxycytidine (DAC), and the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA), to further increase γ -globin expression. Crossing the conditional *Bcl11a* knockout to the Berkeley SCD mouse model increases HbF expression, and corrects multiple SCD clinical parameters.

Genome-wide association studies in 27 Maltese family members with HPFH identified a locus with strong association to high HbF levels at 19p13.12–13, which contains the gene encoding the erythroid Krüppel-like factor EKLF (*KLF1*) (Borg et al. 2010). Genomic sequencing of this cohort revealed a deletion that disrupts the zinc finger domain of KLF1, rendering it defective for DNA binding. The resulting KLF1 haploinsufficiency was accompanied by a decrease in *BCL11A* expression and an increase in the γ -globin to α -globin ratio. A knockout of the HS1 enhancer upstream of the *Klf1* gene greatly reduced KLF1 and *BCL11A* expression (Zhou et al. 2010). KLF1 occupies the murine *Bcl11a* promoter in bone marrow-derived Ter119⁺ cells, and the human *BCL11A* promoter

in erythroid progenitors isolated from peripheral blood (Borg et al. 2010; Zhou et al. 2010). KLF1 does not occupy the human *BCL11A* promoter in fetal liver erythroid progenitors, and KLF1 directly binds and activates the *HBB* gene in adult but not fetal cells. KLF1 expression is elevated in adult erythroid precursors versus fetal erythroid precursors (Zhou et al. 2006). In aggregate, these data suggest that KLF1 directly regulates the switch from fetal to adult hemoglobin by binding to and activating the *HBB* gene and indirectly triggers the switch via activating *BCL11A*, which in turn suppresses fetal globin gene expression. As GATA-1 can directly induce KLF1 expression (Crispino et al. 1999; Kim et al. 2007), this constitutes another critical link between the core GATA-1 regulatory function and hemoglobin switching.

In addition to the obviously crucial KLF1-*BCL11A* axis for regulating hemoglobin switching, additional factors and mechanisms are implicated in controlling this crucial process (Jane et al. 1995; Filipe et al. 1999; Lopez et al. 2002; Tanabe et al. 2002, 2007; Pace et al. 2003; Basu et al. 2005; Bank 2006; Rupon et al. 2006, 2011; Sangerman et al. 2006; Yi et al. 2006; Bottardi et al. 2009; Sripichai et al. 2009; Yao et al. 2009; Sankaran et al. 2010a; Campbell et al. 2011; Wilber et al. 2011; Funnell et al. 2012; Ross et al. 2012). Many important questions remain, however, regarding the underlying mechanisms and biology, including whether the mechanisms are intricately linked to, or perhaps independent of, GATA-1 and KLF1-*BCL11A*. Furthermore, it will be important to elucidate the relevance of specific components of the switching machinery to existing and new preclinical and clinical approaches for therapeutically modulating γ -globin expression (Fibach et al. 1993; Rodgers et al. 1993; Fucharoen et al. 1996; Watanapokasin et al. 2005; Weinberg et al. 2005; Sangerman et al. 2006; Lavelle et al. 2008; Mabaera et al. 2008; Macari and Lowrey 2011; Wilber et al. 2011; Kutlar et al. 2012; Macari et al. 2012). Finally, almost undoubtedly, new mechanistic insights will launch leveraging opportunities to develop novel molecularly targeted therapies, although this will require sustained intense experimental efforts, while being particularly cautious not

to fast-forward such initiatives in the absence of compelling mechanistic, biological, and/or pharmacological foundations. Clearly, the mechanistic infrastructure is insufficient at the present time to divert major efforts into applied therapeutics development and clinical optimization of existing strategies that are often less efficacious than desired.

As BCL11A has unequivocally emerged as an extremely important constituent in the regulation of hemoglobin switching, it represents an attractive target for therapeutic modulation of this process. However, developing efficacious BCL11A targeting strategies is not without significant challenges. Challenges include those intrinsic to drug design, as BCL11A is a zinc finger transcription factor without obvious binding pockets that can accommodate inhibitory drug docking. Second, as mentioned previously, the whole body knockout of BCL11A results in postnatal death (Liu et al. 2003). Although BCL11A deletion does not affect development of macrophage-granulocyte or erythroid lineages, it severely disrupts lymphopoiesis. Transplantation of *Bcl11a*^{-/-} fetal liver cells into irradiated mice results in T-cell leukemia and almost no donor-derived B-cell production. Conditional *Bcl11a* targeting in the adult also revealed a crucial role in controlling lymphopoiesis (Yu et al. 2012). Thus, an additional challenge relates to establishing efficacy in erythroid cells, while minimizing potential deleterious actions on immune cell function. However, with modern approaches to drug design, potential opportunities for developing BCL11A targeting strategies indirectly through upstream factors, and fundamental pharmacological development, there is no reason to believe that these challenges will be insurmountable. Given the emerging rich flavor of the core GATA-1 regulatory function, in conjunction with additional regulatory layers that will almost certainly implicate previously undiscovered mechanisms, rationale is high for continuing to drill deeply into these mechanisms. This type of work will undoubtedly yield further payoffs vis-a-vis innovative mechanistic insights, attractive therapeutic targets that lead to efficacious therapies for hemoglobinopathies, and serendipitous dis-

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coveries that advance science and medicine in disparate fields.

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Mechanisms Underlying Hemoglobin Synthesis

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Transcriptional Mechanisms Underlying Hemoglobin Synthesis

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