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Megakaryocytic Programming by a Transcriptional Regulatory Loop: A Circle Connecting RUNX1, GATA-1, and P-TEFb

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ABSTRACT

Transcription factors originally identified as drivers of erythroid differentiation subsequently became linked to megakaryopoiesis, reflecting the shared parentage of red cells and platelets. The divergent development of megakaryocytic and erythroid progenitors relies on signaling pathways that impose lineage-specific transcriptional programs on non-lineage-restricted protein complexes. One such signaling pathway involves RUNX1, a transcription factor upregulated in megakaryocytes and downregulated in erythroid cells. In this pathway, RUNX1 engages the erythro-megakaryocytic master regulator GATA-1 in a megakaryocytic transcriptional complex whose activity is highly dependent on the P-TEFb kinase complex. The implications of this pathway for normal and pathological megakaryopoiesis are discussed. J. Cell. Biochem. 107: 377–382, 2009.

KEY WORDS: MEGAKARYOCYTE; GATA-1; RUNX1; P-TEFb; ERYTHROID

At a phenotypic level, megakaryocytes and erythroblasts are polar opposites, but several studies have proven that they have a common origin in the bipotent MEP progenitor cell [Goldfarb, 2007]. Consistent with their shared heritage, striking similarities have been found in the transcriptional regulators programming each of these lineages. Transcription factors originally associated with highly specialized red cell functions, that is, regulation of hemoglobin synthesis, were subsequently found to participate in normal megakaryocytic development. In fact, the majority of erythroid and megakaryocytic promoters have been found to recruit a common array of factors that includes GATA-1, FOG-1, SCL/tal, LMO2, Ldb-1, and NF-E2 [Goldfarb, 2007]. Thus, divergence in erythroid and megakaryocytic transcriptional programs involves tight and complex regulation superimposed on highly overlapping transcriptional complexes. One pivotal element in this lineage divergence consists of RUNX1 and its cofactor CBFβ, which undergo upregulation early in megakaryocytic differentiation and downregulation early in erythroid differentiation [Kundu et al., 2002; Elagib et al., 2003; Lorsbach et al., 2004]. This article will discuss how the interaction of RUNX1 with GATA-1 provides a critical signal in the establishment and maintenance of the megakaryocytic transcriptional program. A model is presented in which the P-TEFb kinase complex, originally associated with transcriptional elongation, functions as a critical integrating element in this developmental signaling pathway.

RUNX1 AND GATA-1 IN MEGAKARYOPOIESIS: LESSONS FROM HUMAN DISEASE

Human diseases have provided important information on the roles of RUNX1 and GATA-1 in megakaryopoiesis. Both human germline mutations of *RUNX1* and *GATA-1* have been identified in hereditary thrombocytopenia. In the case of *RUNX1*, *f*amilial *p*latelet *d*isorder with predisposition to *a*cute *m*yeloid *l*eukemia (FPD/AML) manifests as an autosomal dominant thrombocytopenia with impaired platelet function, associated with mutations that predominantly impair the DNA-binding function of the runt domain [Song et al., 1999; Michaud et al., 2002]. Marrows of patients with FPD/AML show increased numbers of small hypolobulated megakaryocytes [Heller et al., 2005] and impaired megakaryocytic colony formation [Song et al., 1999]. FPD/AML platelets express markedly diminished levels of the c-Mpl receptor [Heller et al., 2005] and show defects in α-granule contents [Sun et al., 2004]. In the case of *GATA-1*, X-linked thrombocytopenia (XLT) presents with moderate-to-severe thrombocytopenia, impaired platelet function, and variable degrees

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of concomitant anemia, associated with missense mutations affecting the GATA N-finger [Nichols et al., 2000; Mehaffey et al., 2001]. Marrows from these patients show a moderate to marked increase in megakaryocytes, which may be small and hypolobulated [Nichols et al., 2000; Mehaffey et al., 2001]. Paradoxically, frequent erythroblasts in these patients show enlargement with multinucleation [Nichols et al., 2000]. EM on XLT marrows has revealed abnormal megakaryocytic ultrastructure, with diminished granulation and demarcation membranes [Nichols et al., 2000]. A single kindred has recently been described with a germline mutation in exon 2 of *GATA-1*, associated with exclusive expression of a short isoform, GATA-1s, which lacks amino acids 1–83; the clinical features in this kindred included X-linked macrocytic anemia and leukopenia (XL anemia/leukopenia); platelets were normal in number but abnormal in function [Hollanda et al., 2006]. The marrows displayed hypocellularity with increased megakaryocytes that were small and hypolobulated, as well as erythroblasts with enlargement and multinucleation. EM on the platelets revealed diminished granulation and a round shape. As discussed below, acquired somatic exon 2 mutations causing GATA-1s expression are a hallmark of megakaryocytic proliferative disorders in Down syndrome [Gurbuxani et al., 2004]. However, none of the eight patients with germline *GATA-1* exon 2 mutation has shown development of a proliferative disorder.

Acquired, somatic mutations of *RUNX1* and *GATA-1* occur consistently in human leukemias. *RUNX1* and *CBF*β collectively represent the genetic loci most frequently targeted for chromosomal translocations in acute leukemia [Downing and Shannon, 2002]. In addition, point mutations within *RUNX1* frequently occur in myelodysplasia and occasionally in AML [Harada et al., 2004]. In cases of acute megakaryoblastic leukemia, FISH analysis has detected allelic loss or translocation of the *RUNX1* locus in 25% of cases (4/15) [Berger et al., 2006]. Mutations of *GATA-1* exon 2, leading to GATA-1s expression, occur in virtually all cases of the Down syndrome megakaryocytic proliferative disorders [Gurbuxani et al., 2004]. These disorders include the transient myeloproliferative disorder of Down syndrome and acute megakaryoblastic leukemia (DS-AMKL). These *GATA-1* mutations do not occur in remission marrows of Down syndrome patients and have generally not been found in non-Down syndrome megakaryocytic leukemias (non-DS-AMKL). Microarray gene expression profiling has been exploited to compare DS-AMKL cells with non-DS-AMKL cells from age-matched patients [Bourquin et al., 2006; Ge et al., 2006]. Two independent microarray studies have found markedly increased erythroid gene expression in DS megakaryoblasts, suggesting that GATA-1s is defective in repressing the erythroid program during megakaryocytic differentiation. Bourquin et al. [2006] also identified upregulation of *GATA-2* and downregulation of *RUNX1* as specific features of DS-AMKL. Table I highlights the similarities of human diseases with *GATA-1* and *RUNX1* mutations.

RUNX1 AND GATA-1 IN MEGAKARYOPOIESIS: LESSONS FROM MOUSE MODELS

Knockout mice have also provided critical information on the contributions of RUNX1 and GATA-1 to megakaryopoiesis. Inducible deletion of *RUNX1* in adult mice, $RUNX1^{\Delta/\Delta}$, causes a rapid and prolonged fivefold drop in peripheral blood platelet counts, with minimal effects on red cell or neutrophil numbers [Ichikawa et al., 2004; Growney et al., 2005]. Marrows from $RUNX1^{\Delta/\Delta}$ mice show virtual absence of normal-appearing megakaryocytes but a striking increase in overall numbers of megakaryocytes, as assessed by acetylcholinesterase staining. RUNX1-deficient megakaryocytes are small and hypolobulated, showing a marked decrease in ploidy as compared with normal megakaryocytes [Ichikawa et al., 2004; Growney et al., 2005]. $RUNX1^{\Delta/\Delta}$ marrows yield a threefold increase in numbers of CFU-MK as well as a marked increase in individual colony size [Ichikawa et al., 2004; Growney et al., 2005]. EM has identified ultrastructural disruption consisting of poorly developed demarcation membranes [Ichikawa et al., 2004].

In vivo assessment of GATA-1 function in megakaryopoiesis has relied on the *GATA-1Lo* mouse strain (also known as ΔneoΔHS *Gata-1*) in which knockout of an upstream regulatory element has led to megakaryocyte-specific loss of GATA-1 expression [Shivdasani et al., 1997]. These mice show diminished platelet counts at 15% of normal, coupled with a striking increase in marrow megakaryocytes. GATA-1-deficient megakaryocytes are also small and hypolobulated, with diminished ploidy on flow cytometry [Vyas et al., 1999]. Ultrastructural abnormalities include poorly developed demarcation membranes and diminished granulation [Shivdasani et al., 1997]. In ex vivo culture, GATA-1-deficient megakaryocytes are hyperproliferative, forming extremely large colonies in methylcellulose [Kuhl et al., 2005]. Interestingly, knock-in mice expressing only GATA-1s (GATA1ΔN strain) display no abnormalities as adults but do manifest a proliferative burst of megakaryocytic progenitors in fetal yolk sac and liver from E9.5-E16.5 [Li et al., 2005]. These fetal *GATA-1s* megakaryocytic progenitors also display a hyperproliferative phenotype in ex vivo cultures but retain a capability for maturation. Table II highlights similarities between *RUNX1* and *GATA-1* knockout strains.

TABLE I. Human Diseases With Either GATA-1 or RUNX1 Mutations

Gene	Transmission	Disease	Mutation	Platelets	Megakaryocytes
RUNX1	Autosomal dominant	FPD/AML	Loss of function, runt domain	Decreased, defective aggregation	Increased, small, hypolobulated, decreased granules
GATA-1	X-linked	XLT	Loss of function, N-finger	Decreased, defective aggregation	Increased, small, hypolobulated, decreased granules
GATA-1	X-linked	XL anemia/ leukopenia	Loss of function, GATA-1s expression	Nl number, defective aggregation	Increased, small, hypolobulated, decreased granules
RUNX1	Acquired	Non-DS-AMKL	Allelic loss (25% of cases)		
GATA-1	Acquired	DS-AMKL	Loss of function, GATA-1s		

TABLE II. Mouse Strains With Either *RUNX1* or *GATA-1* Mutations

Strain	Overall phenotype	Platelets	Megakaryocytes
GATA-1−/−	Embryonic lethal E10.5–11.5	Not evaluable	Not evaluable
RUNX1−/−	Embryonic lethal E11.5–12.5	Not evaluable	Not evaluable
GATA-1Lo (*ΔneoΔHS*)	Viable adult, shortened lifespan	Sixfold decrease	Small, hypolobulated, decreased ploidy, hyperproliferative, poorly formed demarcation membranes
RUNX1$^{f/f}$:Mx1-CRE (pIpC-induced deletion)	Viable adult, shortened lifespan	Fivefold decrease	Small, hypolobulated, decreased ploidy, hyperprolferative, poorly formed demarcation membranes
GATA-1s (GATA1*ΔN*)	Normal adult. Transient expansion of embryonic megakaryocyte progenitors	Normal	Hyperproliferative fetal megakaryocytes

OVERLAPPING TRANSCRIPTIONAL PROGRAMS OF GATA-1 AND RUNX1 IN MEGAKARYOPOIESIS

Gene expression profiling of normal and GATA-1-deficient murine megakaryocytes has identified several positive and negative target genes [Muntean and Crispino, 2005]. Retroviral restoration of GATA-1 in GATA-1Lo fetal liver megakaryocytes demonstrated rescued expression of several positive target genes including *GPIIβ*, *GPIbα*, and *JAK2*. However, retrovirally expressed GATA-1 failed to repress the negative target genes *GATA-2* and *Ets1*. Gene expression profiles of RUNX1-deficient megakaryocytes have not been published, but in one study *RUNX1−/−* murine ES cells subjected to in vitro megakaryocytic cultures showed loss of expression of *GPIbβ* and *GPIIIα* [Liu et al., 2006a]. In addition, expression of a RUNX1 inhibitor, AML1-ETO, in murine Lin$^-$ marrow cells caused downregulation of *JAK2* and *GPIIβ*, implicating these genes as positive targets of wild-type RUNX1 [Liu et al., 2006b]. Mice bearing hypomorphic alleles of *CBFβ*, a critical cofactor for RUNX1, have shown markedly decreased platelet expression of CD41 (GPIIbIIIα) [Talebian et al., 2007]. In vitro studies have identified CD41 and *GPIbα* as direct RUNX1 targets based on chromatin immunoprecipitation (ChIP) and reporter assays [Xu et al., 2006; Zhao et al., 2008]. In vivo evidence has implicated *C-MPL* as a shared RUNX1/GATA-1 target gene in that platelets from FPD/AML patients show diminished Mpl protein and mRNA [Heller et al., 2005], and megakaryocytes from *GATA-1Lo* mice show decreased *MPL* transcript levels [Vyas et al., 1999]. Thus, deficiencies of RUNX1 and of GATA-1 produce similar phenotypes in megakaryocytes and appear to affect a common pool of target genes, which include *GPIIβ*, *GPIbα*, *JAK2*, and *C-MPL*.

GATA-2 CANNOT COMPENSATE FOR GATA-1 IN MEGAKARYOPOIESIS

GATA-2 bears a high degree of homology to GATA-1 but has been associated with driving expansion of early multipotent hemaopoietic progenitors [Goldfarb, 2007]. The degree of functional redundancy between these two factors remains the subject of active investigation. Loss of GATA-1 expression in megakaryocytic and erythroid lineages leads to markedly increased *GATA-2* mRNA levels, reflecting the role of GATA-1 as a critical repressor of the *GATA-2* locus [Welch et al., 2004]. Primary purified GATA-1-deficient megakaryocytes, despite a fivefold increase in *GATA-2* mRNA, manifest multiple abnormalities in growth control and differentiation [Muntean and Crispino, 2005]. One complicating issue is that GATA-2 protein is much less stable than GATA-1, raising questions about the relevance of mRNA levels [Minegishi et al., 2005]. A recent study addressed this issue by breeding a *GATA-2* transgene onto a *GATA-1* null background such that the protein levels and spatiotemporal patterns of GATA-2 expression closely recapitulated those of normal GATA-1 [Ferreira et al., 2007]. Unexpectedly, these mice, and similar strains with GATA-3 replacement of GATA-1, showed almost complete rescue of erythropoiesis. However, both the GATA-2 and GATA-3 replacement strains displayed significant thrombocytopenia, with two- to threefold reductions in platelet counts. Thus, the most carefully controlled in vivo experiments to date indicate that the hematopoietic GATA factors are functionally redundant with regard to erythropoiesis, but that the GATA-1 protein exerts a unique function in megakaryopoiesis.

RUNX–GATA–FOG INTERACTIONS: A PHYLOGENETICALLY CONSERVED PATHWAY

Transcriptional cooperativity of RUNX1 and GATA-1 in the activation of megakaryocytic promoters has been established by reporter assays using transient transfection of several mammalian cell lines [Elagib et al., 2003; Xu et al., 2006]. These studies have also demonstrated physical interaction of the two factors via their conserved DNA-binding domains. Interestingly, DS-AMKL derived GATA-1 mutants fully retain physical interaction with RUNX1 but manifest diminished transcriptional cooperation [Xu et al., 2006]. Strong phylogenetic conservation of this pathway is evident in that *Drosophila* counterparts of RUNX1 and GATA-1, Lozenge (Lz), and Serpent (Sp), cooperate in programming fly hematopoietic development, promoting crystal cell lineage commitment [Fossett et al., 2003; Waltzer et al., 2003]. The *Drosophila* homolog of FOG, U-shaped, blocks transcriptional cooperation of Lz and Sp and crystal cell development, in a manner dependent on its recruitment by the Sp amino terminal zinc finger module [Fossett et al., 2003; Waltzer et al., 2003]. In a striking correspondence, human GATA-1 mutants (ΔNF) defective in FOG1 binding show marked enhancement of its transcriptional cooperativity with RUNX1 [Xu et al., 2006]. The mechanism for U-shaped/FOG interference with Lz–Sp/RUNX–GATA cooperation remains unknown but does not rely on FOG engagement of the NuRD corepressor complex as U-shaped lacks a NuRD recruitment domain [Hong et al., 2005].

P-TEFb KINASE COMPLEX MEDIATES RUNX–GATA COOPERATION AND MEGAKARYOCYTIC DEVELOPMENT

While analyzing transcriptional cooperation of RUNX1 and GATA-1, we found via multiple approaches an essential role for the kinase P-TEFb [Elagib et al., 2008]. In particular, pharmacologic P-TEFb inhibitors (roscovitine, flavopiridol, DRB), a dominant-negative kinase mutant, and the endogenous inhibitory protein HEXIM1 all interfered with the combined transcriptional activity of RUNX1 plus GATA-1 but did not affect the activity of each factor alone. Furthermore, induction of megakaryocytic differentiation tightly correlated with activation of P-TEFb, while inhibition of P-TEFb blocked megakaryocytic differentiation both in vitro and in vivo [Elagib et al., 2008].

The functional core of P-TEFb consists of the cyclin-dependent kinase Cdk9 in association with the regulatory subunit cyclin T. The majority of P-TEFb in cells resides within a large inactive complex containing HEXIM1, the small nuclear RNA (snRNA) 7SK, and numerous additional proteins and ribonucleoproteins [Price, 2008]. Activating signals release the Cdk9/cyclin T dimer from the large complex, permitting the latter to activate promoter-specific transcriptional elongation through phosphorylation of RNA polymerase II carboxy terminal domain serine 2 (RNAP II CTD S2), as well as the negative elongation factors DSIF and NELF [Peterlin and Price, 2006].

P-TEFb signaling specifically contributes to the developmental regulation of genes during cellular differentiation and is not required for maintenance of cell growth and survival. In fact, a functional shRNA screen in 21 different cell lines examining 85% of all human kinases has distinguished Cdk9 as one of the most dispensable factors in the entire kinome [Grueneberg et al., 2008]. In vivo knockdown of Cdk9, applying morpholino oligonucleotides to zebrafish embryos, specifically blocked hematopoietic development with no obvious effects on non-hematopoietic tissues [Meier et al., 2006]. In mammalian systems, P-TEFb activity specifically participates in myogenic, cardiac hypertrophic, and megakaryocytic differentiation programs [Sano et al., 2002; Simone et al., 2002; Elagib et al., 2008]. The contribution of P-TEFb to cellular differentiation may derive in part from its gene-specific enhancement of transcriptional elongation but may also arise from its direct phosphorylation of other nuclear factors [Simone et al., 2002].

Earlier studies have suggested that both GATA-1 and RUNX1 regulate transcription through functional and physical interactions with the P-TEFb kinase complex. RUNX1 has been shown negatively to regulate P-TEFb function through its sequestration of cyclin T1 into inactive chromatin loops [Jiang et al., 2005; Jiang and Peterlin, 2008]. In this manner, RUNX1 can retain target genes in an inactive but transcriptionally "poised" state. These target genes may then become rapidly activated through remodeling of chromatin loops and release of activated P-TEFb/RNAP II [Peterlin and Price, 2006; Jiang and Peterlin, 2008]. By contrast, GATA-1 has been shown to activate target genes by promoting transcriptional elongation [Dore et al., 2008]. This function most likely arises from the ability of GATA-1 to physically interact with P-TEFb. Importantly, the formation of GATA-1-P-TEFb complexes is highly dynamic, with complex assembly occurring during megakaryocytic induction and disassembly occurring during erythroid differentiation [Meier et al., 2006; Elagib et al., 2008]. A related biochemical function of GATA-1 consists of its ability to induce remodeling of chromatin loops [Jing et al., 2008]. Thus, P-TEFb serves as a common cofactor for both RUNX1 and GATA-1, most likely acting to integrate signals provided by these two transcriptional factors during megakaryocytic differentiation.

A MODEL FOR INTEGRATIVE DEVELOPMENTAL PROGRAMMING: RUNX1 BOOKMARKING

Megakaryocytic lineage commitment from bipotent MEP cells requires the precisely coordinated activation of megakaryocytic genes in a temporally appropriate sequence, while all erythroid genes are actively maintained in a repressed state. One of the earliest events distinguishing megakaryocytic and erythroid lineages consists of differential RUNX1 expression [Elagib et al., 2003; Lorsbach et al., 2004]. However, RUNX1 alone does not suffice for activation of megakaryocytic genes and in fact initially acts to repress *C-MPL* prior to the onset of megakaryocytic differentiation [Satoh et al., 2008]. We postulate that as an initial step in megakaryocytic differentiation, RUNX1 is recruited to megakaryocytic promoters, assembling inactive P-TEFb and paused RNAPII, a process consisting of "bookmarking for future reading." As megakaryocytic differentiation proceeds, GATA-1 expression replaces that of its more primitive counterpart GATA-2 [Muntean and Crispino, 2005]. This "GATA switch" is proposed to promote remodeling of P-TEFb into an active complex, releasing RNAPII for transcription of megakaryocytic target genes. Two critical target genes known to be highly responsive to P-TEFb activity are *RUNX1* itself and *HEXIM1* [He et al., 2006; Meier et al., 2006; Elagib et al., 2008]. Emergence of a positive loop for RUNX1 expression is postulated to reinforce megakaryocytic lineage commitment, while HEXIM1 upregulation acts in a contrary manner to buffer P-TEFb activity.

We further postulate a central role for the GATA–RUNX–P-TEFb pathway in maintaining erythroid genes in a repressed state. Initial evidence for this notion derived from experiments over a decade ago by Hensold and colleagues in which DRB, a specific P-TEFb inhibitor, induced erythroid differentiation of MEL cells, including upregulated transcription of β-*GLOBIN* and *BAND 3*, very paradoxical given the role of P-TEFb in transcriptional elongation! Subsequent experiments have documented a correlation between chemical induction of HEXIM1 expression and erythroid lineage commitment in MEL cells [He et al., 2006; Turano et al., 2006]. Megakaryoblasts in DS-AMKL display aberrant coexpression of several erythroid markers including CD71, suggesting a defect in this repressive pathway potentially arising from a failure of the mutant GATA-1s to properly engage P-TEFb [Bourquin et al., 2006; Ge et al., 2006]. In vivo P-TEFb blockade by flavopiridol administration induces a megakaryoproliferative disorder in *GATA-1Lo* mice characterized by the expansion of $CD71^{+}$ megakaryoblasts, again implicating GATA-1 cross-talk with

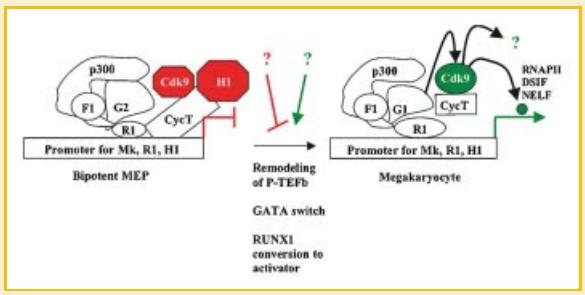

Fig. 1. A hypothetical model of RUNX1 (R1), GATA-1 (G1), and P-TEFb interactions during megakaryocytic differentiation. In the bipotent megakaryocyte erythroid progenitor (MEP), key target genes are "bookmarked for future reading" via RUNX1 (R1) recruitment of inactive P-TEFb, as well as RNA polymerase II (RNAPII). The key target genes include megakaryocytic genes (Mk), as well as RUNX1 (R1) and HEXIM1 (H1). The positive and negative signals regulating P-TEFb remodeling remain unknown but are somehow coupled to the GATA switch in which GATA-1 (G1) replaces GATA-2, and possibly to the conversion of RUNX1 from a repressor to activator. Once activated, P-TEFb promotes transcriptional elongation through phosphorylation of RNAPII and the negative regulators of elongation, DSIF and NELF. However, it is likely that P-TEFb also phosphorylates other factors, possibly even those involved in its own remodeling. Additional players include FOG1 (F1), which negatively regulates RUNX1–GATA-1 cooperation, and p300, which can serve as a coactivator for GATA-1 and RUNX1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

P-TEFb in repression of the erythroid program during megakaryopoiesis [Elagib et al., 2008].

A model is shown in Figure 1 depicting how the RUNX1–GATA-1–P-TEFb signaling axis may function in programming megakaryocytic lineage commitment. Key remaining questions include: (1) What factors mediate the remodeling of P-TEFb from an inactive to an active complex during megakaryocytic development? (2) What are the relevant downstream targets of P-TEFb? (3) How do RUNX1 and GATA-1 exert their influence on P-TEFb? (4) How do signals that negatively regulate megakaryocytic development, for example, cAMP signaling [Freson et al., 2008], interact with the RUNX1–GATA-1–P-TEFb signaling axis?

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