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Genetic Analysis of Hierarchical Regulation for *Gata1* and *NF-E2 p45* Gene Expression in Megakaryopoiesis▽

Mariko Takayama,[1,2]† Rie Fujita,[1]† Mikiko Suzuki,[1]† Ryuhei Okuyama,[2] Setsuya Aiba,[2] Hozumi Motohashi,[1,3]* and Masayuki Yamamoto[1]*

Department of Medical Biochemistry,[1] Department of Dermatology,[2] and Center of Radioisotope Sciences,[3] Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

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GATA1 and NF-E2 p45 are two important regulators of megakaryopoiesis. Whereas GATA1 is known to regulate the *p45* gene, details of the GATA1 contribution to the spatiotemporal expression of the *p45* gene remain to be elucidated. To clarify the relationship between GATA1 and p45, we performed genetic complementation rescue analysis of p45 function in megakaryocytes utilizing the hematopoietic regulatory domain of the *Gata1* gene (*G1HRD*). We established transgenic mouse lines expressing p45 under *G1HRD* regulation and crossed the mice with *p45*-null mice. Compound mutant mice displayed normal platelet counts and no sign of hemorrhage, indicating that *G1HRD* has the ability to express p45 in a spatiotemporally correct manner. However, deletion of 38 amino acids from the N-terminal region of p45 abrogated the p45 rescue function, suggesting the presence of an essential transactivation activity in the region. We then crossed the *G1HRD*-p45 transgenic mice with megakaryocyte-specific *Gata1* gene knockdown (*Gata1*$^{\Delta neo\Delta HS}$) mice. The *G1HRD*-p45 transgene was insufficient for complete rescue of the *Gata1*$^{\Delta neo\Delta HS}$ megakaryocytes, suggesting that GATA1 or other factors regulated by GATA1 are required to cooperate with p45 for normal megakaryopoiesis. This study thus provides a unique *in vivo* validation of the hierarchical relationship between GATA1 and p45 in megakaryocytes.

Cell differentiation is uniquely regulated by lineage-specific transcription factors. Efforts have been made to understand the function of each transcription factor from the viewpoint of context-dependent and hierarchical relationships with other transcription factors. Recent studies have revealed critical regulatory networks among key transcription factors regulating differentiation of hematopoietic cells (8, 51). However, such interrelations between transcription factors are usually validated in cell culture but not by *in vivo* experimental systems. Recent studies have shown that the latter approach is more informative than *in vitro* or *in transfecto* approaches because various physiological parameters are present for validation.

Several key transcription factors regulating megakaryocytic differentiation have been described. c-Myb is a critical regulator at the bifurcation of erythroid and megakaryocytic differentiation from the megakaryocytic-erythroid bipotential progenitor (MEP). Decreased c-Myb activity in MEP was found to enhance megakaryocytic differentiation (27). The other factors regulating megakaryocytic differentiation, especially after lineage commitment, include GATA1, GATA2, SCL/Tal1, Runx1, and the Ets family factors Fli-1 and TEL (32, 38, 41). In contrast, terminal maturation of megakaryocytes depends heavily on NF-E2, a heterodimer of the cap'n'collar (CNC) transcription factor p45 and a small Maf protein (24, 31, 34, 36).

GATA1 has been shown to be an indispensable regulator of erythroid and megakaryocytic cell differentiation. Disruption of the *Gata1* gene in mouse results in lethality at the midgestation stage due to the failure of primitive hematopoiesis (10, 42). In contrast, a megakaryocyte-specific knockdown of the *Gata1* gene (*Gata1*$^{\Delta neo\Delta HS/Y}$) was reported not to be lethal but to result in severe thrombocytopenia and accumulation of immature megakaryocytes (37). The temporal and spatial expression pattern of *Gata1* conforms with these contributions of GATA1 to erythroid and megakaryocytic cell differentiation (43). Regulatory regions recapitulating endogenous *Gata1* gene expression in hematopoietic cells have been delineated (30), and we refer to the regulatory region as the *Gata1* gene hematopoietic regulatory domain (*G1HRD*).

It has been shown that impaired function of NF-E2 affects terminal maturation of megakaryopoiesis, resulting in the accumulation of mature megakaryocytes with higher ploidy (34, 36) and defective proplatelet formation (16, 31). As the small Maf proteins lack any canonical transactivation domains, the transcription activation ability of NF-E2 resides solely in the p45 subunit (3, 24, 29), suggesting that p45 abundance may be a primary determinant of NF-E2 activity. In this regard, the N-terminal half of p45 has been identified as a transactivation domain (3, 29), which recruits $TAF_{II}130$ and CBP (1, 12). Another structural feature of the p45 N-terminal region is the presence of two WW domain-binding motifs (or PPXY motifs), which are necessary for β-*Globin* gene transcription (15, 22). In addition to the PPXY motifs, the very end of the N-terminal region is also necessary for β-*Globin* gene transcription (3). It should be noted that all of these studies exam-

* Corresponding author. Mailing address for Hozumi Motokashi: Center of Radioisotope Science, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan. Phone: 81 22 717 8084. Fax: 81 22 717 8090. E-mail: hozumim@m.tains.tohoku.ac.jp. Mailing address for Masayuki Yamamoto: Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan. Phone: 81 22 717 8084. Fax: 81 22 717 8090. E-mail: masiyamamoto@m.tains.tohoku.ac.jp.

† These authors contributed equally to this work.

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ined the domain function of p45 in immortalized erythroid cells but not in the megakaryocytic lineage.

Three lines of evidence suggest that GATA1 directly activates *p45* gene expression in megakaryocytes. First, genetic analyses revealed that GATA1 dysfunction causes a reduction of p45 expression in megakaryocytes (35, 49). Second, a well-conserved tandem palindromic GATA-binding site has been found in the *p45* gene 1b promoter, which was shown to be functional in a reporter assay using K562 cells (21). Third, GATA1 appears to be required at earlier stages of megakaryopoiesis than p45 (5, 36, 37, 49). In addition to GATA1, it has also been shown that other factors, such as GATA2 and SCL, participate in *p45* gene regulation in megakaryocytes (4, 19).

To validate the GATA1-p45 regulatory axis in megakaryocytes, we adopted a transgenic complementation rescue approach. We examined whether *G1HRD* directs sufficient expression of p45 to sustain normal megakaryopoiesis. We generated transgenic mouse lines expressing p45 under the control of *G1HRD*, and these lines were crossed into the *p45*-null background. The compound mutant mice showed normal platelet counts and no sign of hemorrhage, indicating that *G1HRD*-driven p45 rescued the defective megakaryopoiesis and thrombogenesis of *p45*-null mice. In contrast, a p45 mutant lacking 38 amino acids of the N-terminal region could not rescue thrombogenesis in *p45*-null mice, suggesting the presence of essential transactivation activity in this region. We also evaluated the contribution of p45 to the GATA1-directed regulatory hierarchy by crossing the *G1HRD*-p45 mice with *Gata1*$^{\Delta neo\Delta HS}$ mice. Whereas *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes express reduced amounts of p45, the p45 transgene rescued p45 levels to normal levels in megakaryocytes from compound mutant mice. Of the phenotypes observed in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes, decreased expression of platelet genes and an abnormal increase in immature megakaryocytes were partially restored by the transgene-derived p45 expression, but thrombocytopenia in peripheral blood was not substantially improved. This study is a unique *in vivo* validation of the hierarchical relationship between GATA1 and p45, demonstrating that the GATA1-p45 regulatory axis is operative in megakaryopoiesis.

MATERIALS AND METHODS

Generation of transgenic mouse lines and mating with *p45*-null and *Gata1* knockdown mice. *G1HRD*-polyAH was first generated to construct *G1HRD*-p45. A XhoI-NotI fragment of IE3.9int-LacZ (30) was inserted into the XhoI-NotI site of pIM-LacZ (23) to make *G1HRD*-polyAH. The mouse p45 cDNA was inserted into the NotI site of *G1HRD*-polyAH to generate *G1HRD*-p45. To construct *G1HRD*-ΔN38 p45, a DNA fragment encoding ΔN38 p45 (deletion of amino acids 2 to 38) was amplified by PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI). The NotI-NotI fragment containing the cDNA encoding ΔN38 p45 was then inserted into the NotI site of *G1HRD*-polyAH. The XhoI-HindIII fragments of *G1HRD*-p45 and *G1HRD*-ΔN38 p45 were purified and injected into fertilized eggs using standard procedures as described previously (23).

The *G1HRD*-p45 and *G1HRD*-ΔN38 p45 transgenes were identified by PCR using the primer pair 5′-TGT CTC ACA ACC CTT TCT GTC C-3′ and 5′-GCT TGG GTT GCC CCA CTG GC-3′ to amplify the transgene. *p45* heterozygous mice (36) and *Gata1*$^{\Delta neo\Delta HS}$ (20) mating pairs were purchased from Jackson Labs (Bar Harbor, ME). *p45* heterozygous mice were mated with *G1HRD*-p45 and *G1HRD*-ΔN38 p45 transgenic mice to generate *p45*$^{+/-}$:*G1HRD*-p45 (*p45*$^{+/-}$:Tg) and *p45*$^{+/-}$:*G1HRD*-ΔN38 p45 (*p45*$^{+/-}$:ΔNTD Tg) mice, respectively. *p45*$^{+/-}$:Tg and *p45*$^{+/-}$:ΔNTD Tg mice were further crossed with *p45*$^{+/-}$ mice to obtain *p45*$^{-/-}$:Tg mice and *p45*$^{-/-}$:ΔNTD Tg mice, respectively. Genotypes were determined by 2 weeks after birth. *Gata1*$^{\Delta neo\Delta HS/X}$ female mice were mated with *G1HRD*-p45 transgenic male mice to generate *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg mice. Genotyping of the *Gata1* locus for the ΔneoΔHS allele was performed as previously described (20). All analyses using transgenic mice were performed with two independent lines, and results obtained from one line are shown as representative. All animal experiments were carried out under the permission of the animal center of Tohoku University.

Hematological analysis. Whole blood was collected from the retro-orbital sinuses of anesthetized mice, and hematopoietic indices were measured using an automatic blood cell analyzer (Nihon Koden, Tokyo, Japan). Peripheral blood smears were stained with Wright-Giemsa stain.

Histological analysis. Spleens were fixed in 3.7% formalin in phosphate-buffered saline (PBS). The samples were embedded in paraffin and stained with either hematoxylin and eosin (HE) or silver impregnation. The microscopic images were captured with a DP71 digital camera system (Olympus, Tokyo, Japan).

Proplatelet formation. Megakaryocytes were isolated from the bone marrow of 2-month-old mice, and the projection of proplatelets was observed as described previously (31).

Primary culture of megakaryocytes. Whole livers were collected from mouse fetuses at embryonic days 13.5 (E13.5) and E14.5, and single-cell suspensions were prepared by successive passage through 25-gauge needles. Fetal liver cells were maintained in RPMI 1640 (Wako, Osaka, Japan) supplemented with 20% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml recombinant human thrombopoietin (TPO) (generously provided by Kyowa Hakko Kirin Pharma). From a day 3 culture, megakaryocytes were harvested for RNA purification. All samples processed from primary megakaryocytes were compared within littermates.

RNA purification and quantitative reverse transcription (RT)-PCR. CD41$^+$ cells, Ter119$^+$ cells, and Mac-1$^+$ cells were selected from a day-3 culture of E14.5 fetal liver cells and bone marrow cells of adult wild-type mice using Dynabeads (Dynal Biotech ASA, Oslo, Norway). Fetal liver cultures and bone marrow cells were incubated with biotinylated anti-CD41 antibody (clone MWReg30; Serotec), biotinylated anti-Ter119 antibody (Ly-76; BD Pharmingen), and biotinylated anti-Mac-1 antibody (M1/70; BD Pharmingen), followed by incubation with streptavidin-coupled Dynabeads. Total RNA was purified from the sorted cells using RNAiso (Takara), and cDNA was synthesized from the RNA using random hexamers. Real-time PCR was performed using an ABI7300 sequence detection system. The reaction was carried out for 40 to 60 cycles of 30 s at 95°C and 1 min at 60°C using qPCR Mastermix (Eurogentec). rRNA control reagents (Applied Biosystems) were used as an internal control. To examine the molar ratio of 1b transcript against 1a transcript, quantitative real-time PCR was performed using qPCR Mastermix for SYBR green I (Eurogentec). The molar ratio was calculated by the following equation (44): molar ratio = $[L_a \times (1 + E_a)^{CTa}]/[L_b \times (1 + E_b)^{CTb}]$. L_a and L_b indicate lengths of the amplicon for 1a and 1b transcripts, respectively. E_a and E_b indicate the amplification efficiency of a primer set for 1a and 1b transcripts, respectively. CT_a and CT_b indicate the numbers of threshold cycles for the 1a and 1b transcripts, respectively. Sequences of primers and probes used for quantitative real-time PCR are shown in Table 1.

FACS analysis. Primary megakaryocytes cultured from fetal livers were stained with biotinylated anti-CD41 antibody, allophycocyanin (APC)-conjugated streptavidin, and phycoerythrin (PE)-conjugated anti-CD61 antibody (BD Pharmingen, San Diego, CA). The cells were analyzed using a FACSAria cell sorter (Becton Dickinson, San Jose, CA).

Immunoblot analysis. Whole-cell extracts were prepared from bone marrow megakaryocytes. To purify megakaryocytes, bone marrow cells were incubated with biotinylated anti-CD41 antibody (clone MWReg30; Serotec), followed by incubation with streptavidin-coupled Dynabeads. Whole-cell extracts were examined by immunoblot analysis using anti-p45 antibody (sc-291X; Santa Cruz) and anti-lamin B (sc-6217; Santa Cruz). Whole-cell extracts were prepared from 293T cells 24 h after transfection and examined in the same way.

Plasmid construction. To generate pGL3-TATA-*p45* 1b, a 160-bp fragment of the mouse *p45* 1b promoter containing a tandem GATA site (from nucleotide position −163 to −4 relative to the transcription initiation site, set as +1) was amplified by PCR using the primers 5′-GGG ACA TGA CCA AAT GAC CT-3′ and 5′-TAG CAA CCC TTC CCT CTC CT-3′. The fragment was cloned into pGEM-T Easy (Promega, Madison, WI), and a NotI-NotI fragment was inserted into the SmaI site of pGL3-TATA. pGL3-TATA was constructed by replacing the BglII-HindIII region containing the simian virus 40 (SV40) promoter of the pGL3 promoter vector with a minimal TATA promoter derived from the rabbit β-globin gene (53). To generate pGL3-TATA-*p45* 1b-mutGATA, a double GATA site, TGATAA ACCCC TTATCT, was mutated to TttTAA ACCCC TTAaaT by PCR. To generate mammalian expression plasmids of p45 and its

TABLE 1. Sequences of primers and probes used for quantitative real-time PCR

Primer name	Sequence
p45-F	5′-TTGGCACAGTATCCGCTAAC-3′
p45-R	5′-TCCAGTTTTCTCTTGCGACA-3′
p45-P	5′-FAM-TTCGGGACATCCGTCGAC-TAMRA-3′
Txas-F	5′-AATTGGAACTCCGAGAGCGA-3′
Txas-R	5′-GACAACGTGCATCCGACG-3′
Txas-P	5′-FAM-ACGGGCCTCTGTGTGGGTACTATCTTGG-TAMRA-3′
Slc6a4-F	5′-AGACGTGTCCGAGGTGGC-3′
Slc6a4-R	5′-TGCCTCCGCATATGTGATGA-3′
Slc6a4-P	5′-FAM-AGACGCGGGCCCCAGCCTC-TAMRA-3′
Gp6-F	5′-AGCCCCGAGTGACCCTCTA-3′
Gp6-R	5′-TCCGTGGGTACCTGGCTG-3′
Gp6-P	5′-FAM-TGCTTGTGGTTACTGGACTCTCTGCCAC-TAMRA-3′
Selp-F	5′-ACTGGTCAGATGCCATGCC-3′
Selp-R	5′-ACCCAAGTACGTCAGAGCTTCC-3′
Selp-P	5′-FAM-CTGCCAAGCAGGGACACTGACAATCC-TAMRA-3′
Slamf1-F	5′-AGCCTGCCCCAGAGTCTGT-3′
Slamf1-R	5′-TATGGGTCAGCTCTCTGGCAGT-3′
Slamf1-P	5′-FAM-CAGGAACCAAACCCCACCACAGTTTATG-TAMRA-3′
F5-F	5′-GGCATGCAAACGCCATTT-3′
F5-R	5′-ATATTCCGAAGCCTTGATCTGTG-3′
F5-P	5′-FAM-TGCCAATGGGACTAAGCACTGGTGTCA-TAMRA-3′
p45 promoter 1a-F	5′-CACAGGTGCCTGAAAGGTTG-3′
p45 promoter 1b-F	5′-TGGGAAGAAAGGGCTGAAAT-3′
p45 promoter-R	5′-ACCTGTTCCTGTTCTGCTGA-3′
Gata1-F	5′-CAGAACCGGCCTCTCATCC-3′
Gata1-R	5′-TAGTGCATTGGGTGCCTGC-3′
Gata1-P	5′-FAM-CCCAAGAAGCGAATGATTGTCAGCAAA-TAMRA-3′

truncated mutants, cDNA fragments containing His_6-tagged wild-type p45, ΔN38 p45 (deletion of amino acids 2 to 38), ΔN109 p45 (deletion of amino acids 2 to 109), ΔN153 p45 (deletion of amino acids 2 to 153), and ΔN225 p45 (deletion of amino acids 2 to 225) were amplified by PCR, and their NcoI-SalI fragments were replaced with that of pEF-p45 (13). To generate pIM-His-MafK XB, pET15b-MafK was first generated by inserting a full-length mouse MafK cDNA between the NdeI and BamHI sites of pET15b. The XbaI-BamHI fragment of pET15b-MafK was inserted into the NotI-NotI vector fragment of pIM-lacZ (25).

Transient transfection and reporter assay. MEG01 cells were maintained in RPMI 1640 (Wako) supplemented with 10% charcoal-stripped FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To attain cell differentiation, 10 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added to the subconfluent MEG01 culture. At 24 h after the addition of TPA, either pGL3-TATA, pGL3-TATA-*p45* 1b, or pGL3-TATA-*p45* 1b-mutGATA was electroporated using a MicroPorator-mini instrument (Digital Bio). 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Wako) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. pRBGP2, a previously described reporter plasmid (13), was introduced into 293T cells along with expression vectors encoding p45 and its truncated mutants and the MafK expression vector pIM-His-MafK XB using the FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol. For both experiments, luciferase activity was measured 24 h after transfection. The firefly luciferase activities of the reporter genes were normalized against the cotransfected sea pansy luciferase activities driven by the elongation factor 1α promoter. The expression of both firefly and sea pansy luciferases were quantified using the dual-luciferase reporter assay system (Promega, Madison, WI) and a luminometer (Berthold Japan, Tokyo, Japan). All samples were prepared in triplicate, and means and standard deviations were calculated.

RESULTS

The *p45* gene utilizes both 1a and 1b promoters in megakaryocytes. The *p45* gene possesses two alternative first exons with affiliated promoters, which produce transcripts with different untranslated sequences (21, 33, 45). The distal and proximal first exons are designated 1a and 1b (or 1f) exons, respectively, and organization of the exons is conserved between human and mouse (Fig. 1A). There is a tandem palindromic GATA binding motif within the 1b promoter, and this is conserved between the human and mouse *p45* genes. Previous studies have demonstrated that the 1b promoter is utilized in erythroid cells but not in myeloid cells (21), while in contrast, the 1a promoter is widely utilized, including in myeloid cells. These observations imply that the 1a and 1b promoters are differentially regulated.

To gain insight into the transcriptional regulatory mechanisms of the *p45* gene in megakaryocytes, we first examined which promoter is utilized in megakaryocytes (Fig. 1B). The 1a exon was transcribed in erythroid and myeloid cells, while the 1b exon was transcribed in erythroid cells but not myeloid cells, which agrees with a previous report (21). In megakaryocytes, the 1b exon was transcribed in addition to the 1a exon.

The critical contribution of the tandem GATA site to 1b promoter activity was shown in an erythroleukemic cell line, K562 (21). The recruitment of GATA1 and GATA2 to the 1b promoter region in K562 cells was also shown in a recent report (9a). To examine the activity of the 1b promoter in megakaryocytes, we used MEG01 cells, which are a human megakaryoblastic cell line and can be induced to differentiate by TPA into a megakaryocytic lineage (28). The 1b promoter was active in TPA-treated MEG01 cells, and mutation of the tandem GATA site completely abrogated the activity (Fig. 1C). Thus, the *p45* gene is regulated, at least in part, through a GATA-dependent mechanism.

We found that the 1a transcript is expressed more abundantly than the 1b transcript in erythroid, megakaryocytic, and myeloid lineages (Fig. 1D, left panel). The contribution of 1b is larger in megakaryocytes and erythroid cells than in myeloid cells (Fig. 1D, right panel). While GATA1-deficient megakaryocytes from *Gata1*$^{\Delta neo\Delta HS/Y}$ mice displayed dramatic reduction of the 1b transcript compared to the 1a transcript (Fig. 1E, right panel), the amount of 1a transcript was also decreased in the absence of GATA1 (Fig. 1E, left panel). These results demonstrate that GATA1 is a major activator of the 1b promoter and that GATA1 partially contributes to 1a promoter activity, although GATA sites are not found in proximity to the 1a promoter. Since a substantial amount of the 1a transcript was produced in the absence of GATA1, there seems to be a GATA1-independent regulation of the *p45* gene in megakaryocytes.

Generation of transgenic mouse lines expressing p45 under regulation of *G1HRD*. To address the question of whether GATA1-dependent regulation is sufficient to support p45 function *in vivo*, we performed a transgenic complementation rescue experiment with *p45*-null mice with a transgene producing p45 under the regulatory influences of the *Gata1* gene. Our rationale was that if GATA1 is the major activator of the *p45* gene, the time and place of p45 expression should be contained within those for *Gata1* gene expression and the defective

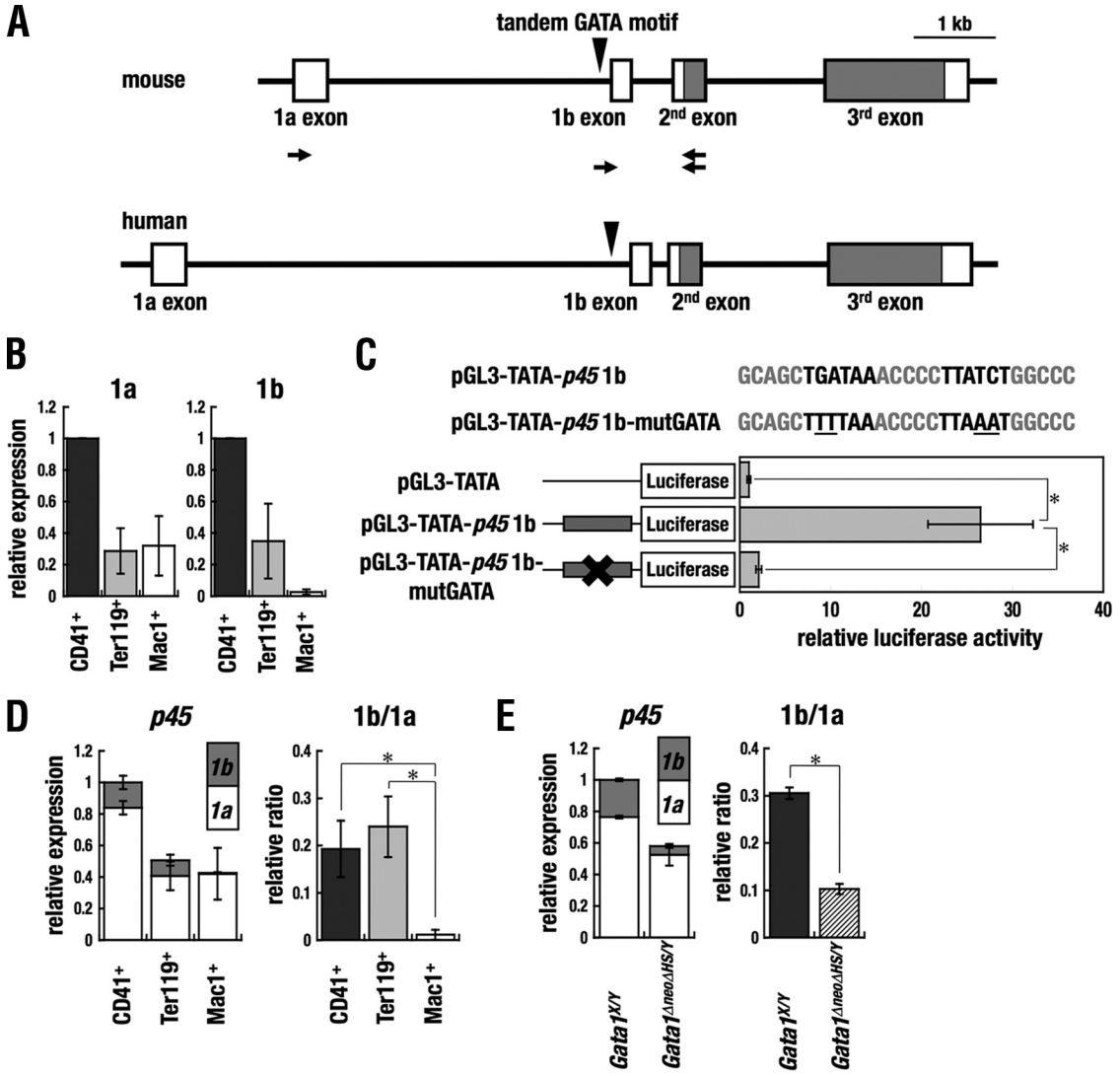

FIG. 1. Promoter utilization of the *p45* gene in megakaryocytes and contribution of a tandem GATA site to 1b promoter activity. (A) The structures of mouse and human *p45* genes. White and gray boxes indicate untranslated regions and protein-coding regions, respectively. The tandem GATA motifs are indicated with arrowheads. Arrows indicate PCR primers for specific detection of each transcript variant. Two different primers corresponding to each first exon were coupled with a common primer corresponding to the second exon. The bar corresponds to 1 kb. (B) Relative abundance of the transcript variants derived from 1a (left panel) and 1b (right panel) promoters. cDNA synthesized from total RNA of CD41-positive, Ter119-positive, and Mac1-positive cells isolated from bone marrow cells was examined. The specific primer sets depicted in panel A were used for detecting the 1a and 1b transcripts. $CD41^+$, $n = 4$; $Ter119^+$, $n = 4$; and $Mac1^+$, $n = 4$. Each value was normalized to rRNA expression. Average values are expressed, and error bars indicate standard deviations. (C) 1b promoter activity of the *p45* gene was examined in TPA-induced MEG01 cells. The tandem GATA site contained in pGL3-TATA-*p45* 1b and the mutated tandem GATA site contained in pGL3-TATA-*p45* 1b-mutGATA are shown in boldface, and the mutated bases are underlined (top panel). Relative luciferase activities are shown in the bar graph (bottom panel). The average values are shown with standard deviations. A representative result from three independent experiments is shown. The horizontal axis indicates relative luciferase activity. Firefly luciferase activity with the control reporter plasmid pGL3-TATA is set to 1. The Student *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$. (D and E) Comparison of the molar abundances of 1a and 1b transcripts between $CD41^+$ ($n = 4$), $Ter119^+$ ($n = 4$), and $Mac1^+$ ($n = 4$) cells of wild-type mice (D) and between $CD41^+$ cells of $Gata1^{X/Y}$ mice ($n = 3$) and those of $Gata1^{\Delta neo\Delta HS/Y}$ mice ($n = 4$) (E). The stacked bar graph (left panels) shows the relative abundances of 1a and 1b transcripts within the total p45 transcript. The molar ratio of the 1b transcript against the 1a transcript was calculated and is shown in the bar graph (right panels). Average values are expressed, and error bars indicate standard deviations. The Student *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$.

megakaryopoiesis observed in *p45*-null mice would be rescued by p45 expressed under the regulation of the *Gata1* gene.

Regulatory domains mediating tissue- and stage-specific expression of the *Gata1* gene have been well analyzed in transgenic mouse reporter experiments (30, 39, 40, 43). To express p45 in mice, we utilized *G1HRD*, an 8.0-kb genomic fragment of the *Gata1* gene that is sufficient for recapitulation of endogenous GATA1 expression in erythroid and megakaryocytic lineages. We generated two independent lines of transgenic mice expressing p45 driven by *G1HRD*

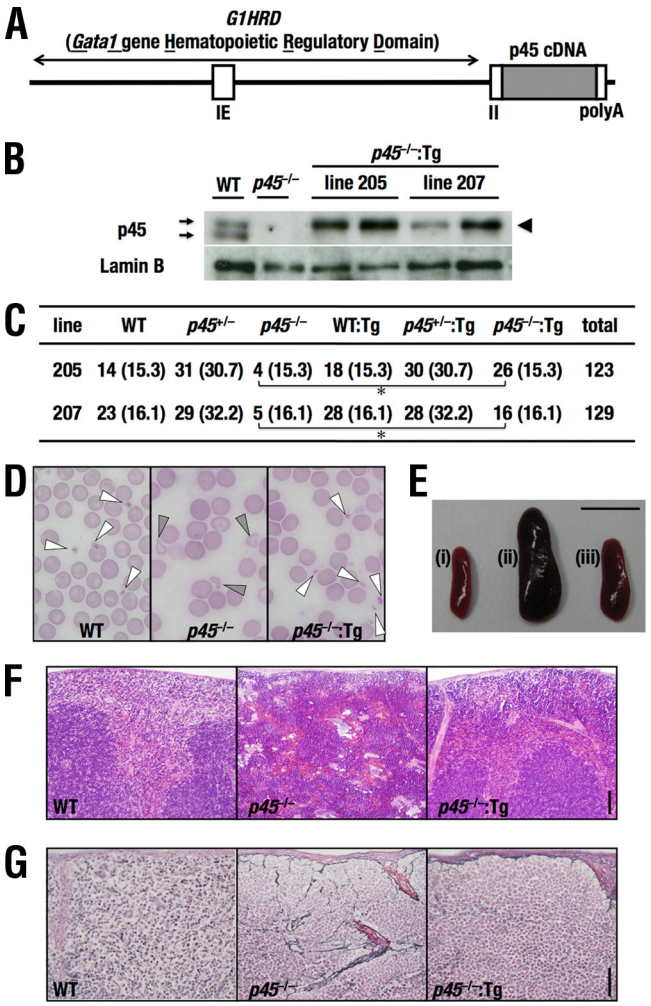

FIG. 2. *G1HRD*-driven p45 corrected thrombocytopenia and defective megakaryopoiesis of *p45*-null mice. (A) The structure of the *G1HRD*-p45 transgene. *G1HRD* contains the *Gata1* genomic locus spanning from the 3.9-kb upstream region of the hematopoietic IE exon to the second exon (II). A cDNA fragment encoding p45 was fused to the 5′ half of the second exon. (B) Expression of p45 in wild-type and $p45^{-/-}$:Tg mice. Whole-cell extracts were prepared from bone marrow megakaryocytes and subjected to immunoblot analysis with anti-p45 antibody. Lamin B was used as a loading control. Arrows indicate a doublet of endogenous p45, and an arrowhead indicates transgene-derived p45. (C) Number of pups of each genotype born from matings between $p45^{+/-}$ mice and $p45^{+/-}$:Tg mice. Genotypes were determined at 2 weeks after birth. Two independent transgenic lines (205 and 207) were examined. The expected numbers calculated from Mendelian inheritance are shown in parentheses. Comparison of two proportions was performed to calculate statistical significance (*P*) between the birth frequencies of $p45^{-/-}$ pups and $p45^{-/-}$:Tg pups. *, $P < 0.05$. WT, wild type. (D) Wright-Giemsa staining of peripheral blood smears drawn from wild-type (left), *p45*-null (middle), or $p45^{-/-}$:Tg (right) mice. White and gray arrowheads indicate platelets and poikilocytes, respectively. A result from line 205 is shown as a representative. (E) Macroscopic observation of spleens: (i) wild-type, (ii) *p45*-null, and (iii) $p45^{-/-}$:Tg mice at 2 months of age are shown. The scale bar corresponds to 1 cm. Results from line 207 are shown as representative data. (F and G) Hematoxylin and eosin staining (F) and silver impregnation (G) of spleens from wild-type (left), *p45*-null (middle), and $p45^{-/-}$:Tg mice (right). Scale bars correspond to 100 μm (F) and 50 μm (G). Results from line 205 are shown as representative data (F and G).

(Fig. 2A). *G1HRD*-p45 transgenic mice (Tg mice) were viable and fertile and did not show apparent hematological abnormalities (data not shown).

***G1HRD*-driven p45 sustains thrombogenesis in the absence of endogenous p45.** We crossed the Tg mice into a *p45*-null background, and $p45^{-/-}$:Tg mice were obtained from the mating pairs of $p45^{+/-}$ and $p45^{+/-}$:Tg mice. The expression level of the transgene was examined by an immunoblot analysis of whole-cell extracts prepared from bone marrow megakaryocytes (Fig. 2B). For both lines 205 and 207, transgene expression in each mouse varies to some extent even though they were obtained from the same line, which was interpreted as position effect variegation of the transgene. The fluctuation of the expression levels of the transgene did not affect the hematological and histological phenotypes of the compound mutant mice (see below). The results of gene expression analysis were interpreted by considering the abundance of transgene-derived p45 (Fig. 3). While wild-type mice contain two endogenous p45 bands, the size of transgenic p45 coincides with the more slowly migrating band. The molecular mechanism for this phenomenon is unknown at present.

It has been reported that more than 90% of *p45*-null mice die due to extensive hemorrhage during delivery (36). To examine whether the fatal perinatal hemorrhage of *p45*-null mice was rescued in $p45^{-/-}$:Tg mice, we genotyped an entire litter by 2 weeks after birth (Fig. 2C). Showing excellent agreement with the previous report, the number of *p45*-null mice born alive was significantly less than that of wild-type mice. In contrast, the number of $p45^{-/-}$:Tg mice born alive was similar to that expected from Mendelian inheritance for both Tg lines (Fig. 2C).

We found that neonates of $p45^{-/-}$:Tg mice were indistinguishable among their littermates, while $p45^{-/-}$ pups were easily recognized due to subcutaneous hemorrhage that occurred during delivery (data not shown). When the peripheral blood count was examined, the severe thrombocytopenia of *p45*-null mice was rescued in the compound mutant mice (Table 2). Platelets were observed consistently in the blood smears from wild-type and $p45^{-/-}$:Tg mice but not in those from *p45*-null mice (Fig. 2D).

We further examined histological alterations of the spleen. Surviving *p45*-null mice displayed significant splenomegaly by 2 months of age, whereas spleens of $p45^{-/-}$:Tg mice appeared normal and were macroscopically indistinguishable from those of wild-type mice (Fig. 2E). Microscopic examination revealed remarkable improvement of histological abnormalities in the $p45^{-/-}$:Tg spleen compared with results for the *p45*-null spleen. In *p45*-null mice, enlarged megakaryocytes were accumulated and dispersed all over the spleen, and the red pulp/white pulp organization of the spleen was completely disrupted (Fig. 2F, middle panel). In addition, severe fibrosis was apparent in the $p45^{-/-}$ spleen (Fig. 2G, middle panel). In contrast, neither abnormal megakaryocytic accumulation nor fibrotic change was observed in the rescued $p45^{-/-}$:Tg mouse spleen, and spleen organization appeared to be similar to that of wild-type mice (Fig. 2F and G, left and right panels).

To assess the platelet production ability of the megakaryocytes, a proplatelet formation (PPF) assay was performed using megakaryocytes isolated from bone marrow. While *p45*-null megakaryocytes completely lacked PPF ability, megakaryo-

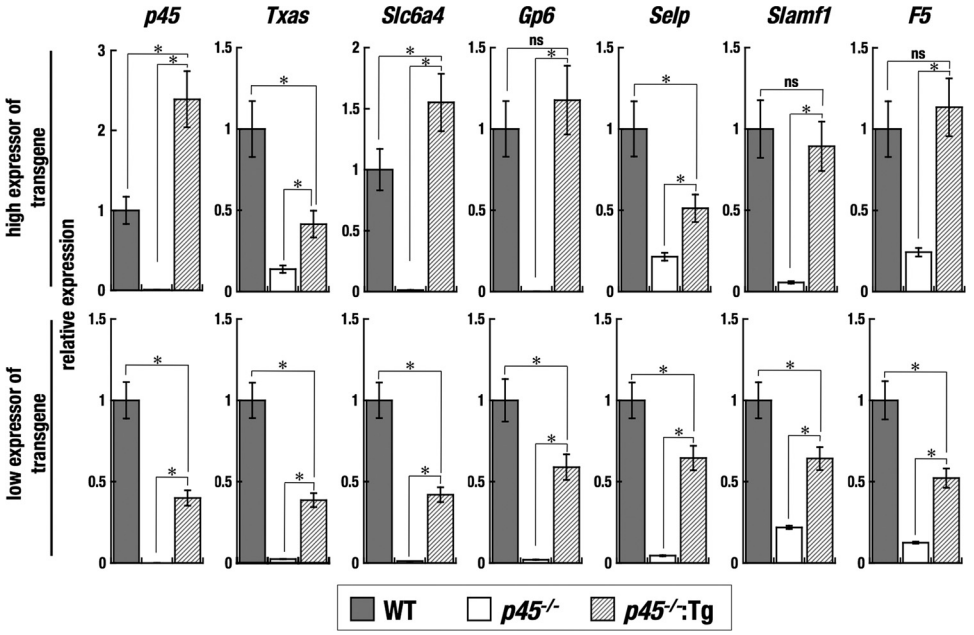

FIG. 3. *G1HRD*-driven p45 contributes to activation of platelet genes. Expression levels of platelet genes in primary megakaryocytes, including *Txas*, *Slc6a4*, *Gp6*, *Selp*, *Slamf1*, and *F5*, were examined by quantitative RT-PCR. Results of two representative sets of experiments are shown according to the abundance of transgene-derived p45 mRNA. Upper and lower panels are the results of high and low expressors of the transgene, respectively. Average values are shown, and error bars indicate standard deviations. The average values for wild-type megakaryocytes are set to 1. Results from lines 205 and 207 are shown as representative data for high expressors of the transgene (upper panels) or low expressors of transgene (lower panels), respectively. The Student *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$. ns, not significant.

cytes from $p45^{-/-}$:Tg mice generated proplatelets. The frequency of PPF in $p45^{-/-}$:Tg mice was nearly equal to that for wild-type mice (Table 3).

***G1HRD*-driven p45 activates platelet genes in primary megakaryocytes.** We examined gene expression in megakaryocytes cultured from $p45^{-/-}$:Tg fetal livers. We recently identified several platelet genes that are repressed in *p45*-null megakaryocytes (26). These genes include *Thromboxane A synthetase 1* (*Txas*), *Solute carrier family 6* (*neurotransmitter transporter*, *serotonin*) *member 4* (*Slc6a4*), *Glycoprotein 6* (*Gp6*), *Selectin P* (*Selp*), *Signaling lymphocytic activation molecule family member 1* (*Slamf1*), and *Factor 5* (*F5*), all of which are assumed to be downstream effectors of p45. When primary megakaryocytes were cultured and purified from fetal livers and their cDNAs were examined by quantitative RT-PCR, the expression of the platelet genes was consistently higher in $p45^{-/-}$:Tg megakaryocytes than in *p45*-null cells (Fig. 3). In contrast, the relative expression of the platelet genes in $p45^{-/-}$:Tg megakaryocytes compared to those in the wild-type cells varied from embryo to embryo, probably due to position effect variegation of the transgene. The variation amplitude in the amount of p45 mRNA was similar to that of the p45 protein (see Fig. 2B; also data not shown). Platelet gene expression was lower in the $p45^{-/-}$:Tg megakaryocytes expressing smaller amounts of p45 (Fig. 3, lower panels), while the genes were fully activated in those expressing higher levels of p45 (Fig. 3, upper panels). Thus, *G1HRD* appears to confer spatiotemporal specificity of *p45* gene expression, supporting the notion that GATA1 is one of the important upstream regulators of p45 in megakaryocytes.

It should also be noted that the mild anemia observed in *p45*-null mice was not apparent in $p45^{-/-}$:Tg mice (Table 2), and the poikilocytosis observed in *p45*-null mice was not present in the compound mutant mice (Fig. 2D), suggesting that transgene-derived p45 also compensates for the endogenous function of p45 in erythroid cells. Taken together, we

TABLE 2. Blood count measurement for compound mutant mice in *p45*-null background[a]

Genotype (n[b])	Blood count[c]			
	RBC ($10^4/\mu l$)	Hb (g/dl)	Htc (%)	Plt ($10^4/\mu l$)
WT (7)	971 ± 51	14.6 ± 0.4	46.6 ± 2.2	92.1 ± 11.7
$p45^{-/-}$ (3)	875 ± 59	12.7 ± 1.1	40.6 ± 3.1	10.1 ± 2.6
$p45^{-/-}$:Tg (5)	959 ± 98	13.8 ± 1.5	43.5 ± 4.1	70.4 ± 12.0
$p45^{-/-}$:ΔNTD Tg (3)	994 ± 26	13.7 ± 0.6	41.8 ± 1.1	32.2 ± 19.3

[a] Wild-type, *p45*-null, $p45^{-/-}$:Tg and $p45^{-/-}$:ΔNTD Tg mice were examined at 2 months of age. Results from lines 207 and 340 are shown as representatives of the $p45^{-/-}$:Tg and $p45^{-/-}$:ΔNTD Tg genotypes, respectively. Values are expressed as means ± standard deviations.

[b] n, no. of mice.

[c] RBC, red blood cells; Hb, hemoglobin; Htc, hematocrit; Plt, platelets.

TABLE 3. PPF frequencies of megakaryocytes purified from the bone marrow of compound mutant mice

Genotype	PPF (%)[a]
WT	34.0 ± 5.3
p45$^{-/-}$	0.0 ± 0.0
p45$^{-/-}$:Tg	31.3 ± 7.6
p45$^{-/-}$:ΔNTD Tg	0.0 ± 0.0

[a] The ratios of megakaryocytes forming proplatelets to total megakaryocytes are indicated. Results from lines 205 and 340 are shown as representative data for *p45*$^{-/-}$:Tg and *p45*$^{-/-}$:ΔNTD Tg mice, respectively. Values are expressed as means ± standard deviations.

conclude that the defective megakaryopoiesis, platelet production, and erythropoiesis observed in the *p45*-null mice were fully rescued in the *p45*$^{-/-}$:Tg compound mice. These results indicate that *G1HRD*-driven p45 substantially compensates for the endogenous function of p45.

The N-terminal region of p45 is necessary for reporter gene activation. To corroborate that the transactivation ability of p45 is actually required for the rescue of defective megakaryopoiesis and platelet production of *p45*-null mice by *G1HRD*-driven p45, we tested a transactivation-defective p45 protein in the same set of transgenic rescue experiments. We first made a series of p45 deletion mutants and transfected them into 293T cells (Fig. 4A). The expression levels of full-length p45 and of each mutant were comparable (Fig. 4B). The expression vectors were introduced into 293T cells together with a MafK expression vector, and the luciferase reporter gene was connected to three copies of the Maf recognition element (MARE), a high-affinity binding sequence of the p45-MafK heterodimer (13). Whereas full-length p45 increased luciferase activity, deletion of 38 amino acids from the N terminus greatly decreased reporter activity. Further deletion of the N-terminal region substantially deprived p45 of its transactivation ability (Fig. 4C). These results are consistent with previous reports stating that the N-terminal half of p45 possesses transactivation ability (3, 29).

The N-terminal 38 amino acids are required for p45 function in thrombogenesis. Because the reporter assay indicated that deletion of 38 amino acids from the N terminus mostly abolished the transactivation ability of p45, we chose the truncated mutant ΔN38 p45 to confirm that the transgenic rescue experiment actually depends on p45 activity. For simplicity, the N-terminal 38 amino acids and ΔN38 p45 are designated the N-terminal domain (NTD) and ΔNTD, respectively.

We generated transgenic mice carrying *G1HRD*-ΔN38 p45 (Fig. 5A) and obtained two independent mouse lines. *G1HRD*-ΔN38 p45 transgenic mice (ΔNTD Tg mice) were viable and fertile and did not show apparent hematological abnormalities (data not shown). These mice were crossed into a *p45*-null background, and *p45*$^{-/-}$:ΔNTD Tg mice were generated. Transgene expression was examined using the compound mutant mice generated from each line by immunoblot analysis of whole-cell extracts prepared from bone marrow megakaryocytes (Fig. 5B). The transgene-derived ΔNTD expression was observed in both lines 311 and 340 and varied from mouse to mouse to some extent even though the mice were obtained from the same line. Similar to the case with the compound mutant mice expressing p45, the fluctuation of the transgene-derived ΔNTD expression did not affect the hematological and histological phenotypes of the compound mutant mice. Gene expression was analyzed separately depending on the abundance of the transgene-derived ΔNTD (see below) (Fig. 6). Because the anti-p45 antibody used in the immunoblot analysis recognizes the C-terminal region of p45, the immunoreactivity with the antibody should be the same between full-length p45 and ΔNTD, which made it possible to compare the molar abundances of p45 and ΔNTD. The variations in p45 protein levels in *p45*$^{-/-}$:Tg mice and ΔNTD in *p45*$^{-/-}$:ΔNTD Tg mice were within a similar range, judging from the band intensities (see Fig. 2B and 5B).

To examine whether lethal perinatal hemorrhage of *p45*-null mice was observed in *p45*$^{-/-}$:ΔNTD Tg mice, we genotyped an entire litter at 2 weeks after birth. In contrast to the number of *p45*-null mice born alive, the number of *p45*$^{-/-}$:ΔNTD Tg mice born alive was similar to that expected from Mendelian inheritance for both lines (Fig. 5C). The NTD was dispensable as far as the avoidance of perinatal lethality was concerned.

However, we found that the platelet counts of *p45*$^{-/-}$:ΔNTD Tg mice were much lower than those of wild-type mice,

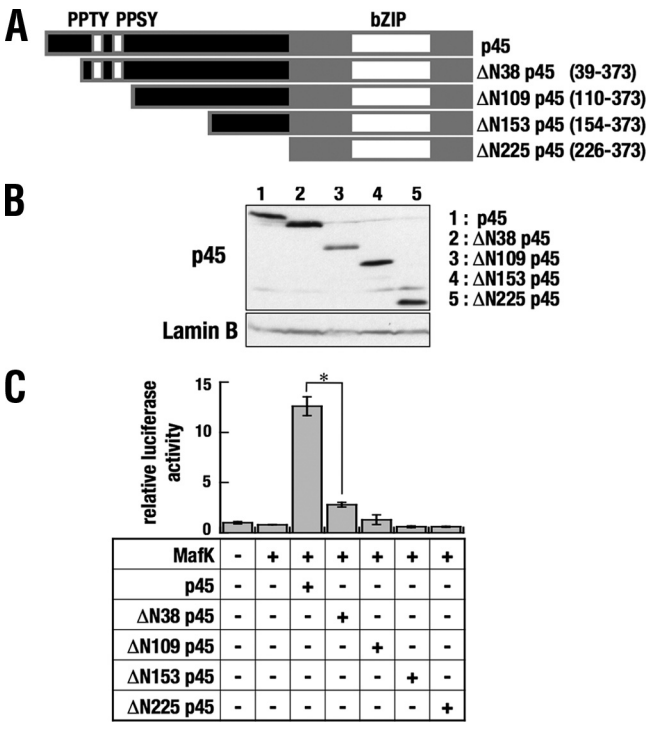

FIG. 4. The N-terminal region of p45 is important for transactivation. (A) A series of N-terminal truncations of p45 is shown. PPXY and bZIP motifs are indicated. (B) Transient overexpression of p45 deletion mutants in 293T cells. Whole-cell extracts were examined by immunoblot analysis using anti-p45 antibody. Lamin B is used as a control. (C) A reporter assay with a luciferase reporter gene in 293T cells. Expression vectors for the N-terminally truncated forms of p45 shown in panel A are introduced into 293T cells with the pRBGP2 reporter plasmid. The average values are shown with standard deviations. A representative result from three independent experiments is shown. The vertical axis indicates relative luciferase activity. Firefly luciferase activity in the absence of effector plasmids is set to 1. The Student *t* test was used to calculate statistical significance (*P*). *, $P < 0.05$.

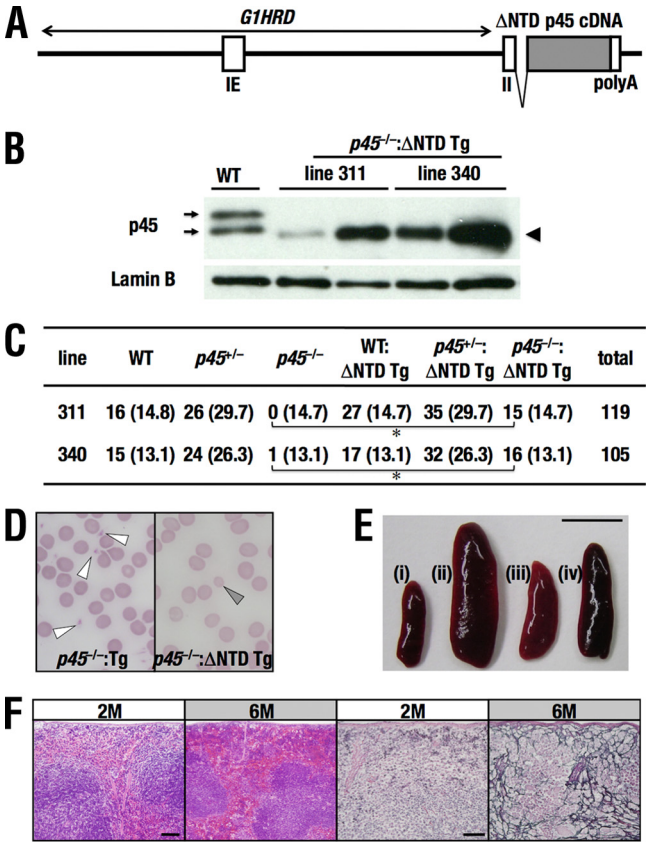

FIG. 5. The N-terminal 38 amino acids are necessary for p45 function in megakaryopoiesis. (A) The structure of the *G1HRD*-ΔNTD transgene. A cDNA fragment encoding ΔN38 p45 (ΔNTD) was fused to the 5′ half of the second exon of the *Gata1* gene. (B) Comparison of expression levels of transgene-derived ΔNTD and endogenous p45. Whole-cell extracts were prepared from bone marrow megakaryocytes and subjected to immunoblot analysis with anti-p45 antibody. Lamin B was used as a loading control. Arrows indicate a doublet of endogenous p45, and an arrowhead indicates the transgene-derived ΔNTD. (C) Number of pups of each genotype born from mating between $p45^{+/-}$ mice and $p45^{+/-}$:ΔNTD Tg mice. Genotypes were determined by 2 weeks after birth. Two independent transgenic mouse lines (311 and 340) were examined. Expected numbers calculated from Mendelian ratios are shown in parentheses. Comparison of two proportions was performed to calculate statistical significance (*P*) between birth frequency of $p45^{-/-}$ pups and that of $p45^{-/-}$:ΔNTD Tg mice. *, $P < 0.05$. (D) Wright-Giemsa staining of peripheral blood smears drawn from $p45^{-/-}$:Tg mice (left) and $p45^{-/-}$:ΔNTD Tg mice (right). White and gray arrowheads indicate platelets and poikilocytes, respectively. Results from lines 205 and 311 are shown as representative data for $p45^{-/-}$:Tg and $p45^{-/-}$:ΔNTD Tg mice, respectively. (E) Macroscopic observation of spleens: (i) wild-type, (ii) *p45*-null, (iii) $p45^{-/-}$:Tg, and (iv) $p45^{-/-}$:ΔNTD Tg mice at 6 months of age. The bar corresponds to 1 cm. Results from lines 207 and 340 are shown as representative data for $p45^{-/-}$:Tg and $p45^{-/-}$:ΔNTD Tg mice, respectively. (F) Hematoxylin and eosin (HE) staining (left two panels) and silver impregnation (right two panels) of spleens from $p45^{-/-}$:ΔNTD Tg mice at 2 and 6 months of age. The scale bar corresponds to 100 μm and 50 μm in HE and silver impregnation, respectively. Results from line 340 are shown as representative data for $p45^{-/-}$:ΔNTD Tg mice.

albeit higher than those of *p45*-null mice (Table 2). In the blood smears, while platelets were observed in $p45^{-/-}$:Tg mice, very few platelets were observed in $p45^{-/-}$:ΔNTD Tg mice (Fig. 5D). Poikilocytosis was also observed in $p45^{-/-}$:ΔNTD Tg mice. Thus, ΔNTD only partially rescued the hematological defects in *p45*-null mice.

We then examined histological alterations of the spleen. $p45^{-/-}$:ΔNTD Tg mice did not display apparent abnormalities of the spleen at 2 months of age, by which time *p45*-null mice had already developed significant splenomegaly and fibrosis. However, by 6 months of age, mild splenomegaly became recognizable in the rescued $p45^{-/-}$:ΔNTD Tg mice (Fig. 5E) and was accompanied by mild disorganization of splenic architecture, accumulation of megakaryocytes, and severe fibrosis (Fig. 5F and data not shown). The spleen phenotypes were mitigated but not completely corrected in the $p45^{-/-}$:ΔNTD Tg mice.

Importantly, PPF was severely affected by deletion of the NTD (Table 3). Whereas the PPF frequency of $p45^{-/-}$:Tg mice was similar to that of wild-type mice, practically no PPF was observed in megakaryocytes isolated from the bone marrow of $p45^{-/-}$:ΔNTD Tg mice. These results authenticate that rescue of *p45*-null mice by the *G1HRD*-p45 transgene is attributable to the activity of transgene-derived p45, for which the NTD is critical.

The NTD is essential for expression of selected genes regulated downstream of p45. We next examined the expression levels of platelet genes in the $p45^{-/-}$:ΔNTD Tg mice. Expression of *Txas*, *Slc6a4*, and *Gp6* was examined in primary megakaryocytes cultured from fetal livers (Fig. 6). The expression levels of *Txas*, *Slc6a4*, and *Gp6* in $p45^{-/-}$:ΔNTD Tg megakaryocytes were closer to those of *p45*-null megakaryocytes, regardless of the mRNA abundance of transgene-derived ΔNTD. When the high transgene expressors were compared, the expression levels of *Txas*, *Slc6a4*, and *Gp6* in $p45^{-/-}$:ΔNTD Tg megakaryocytes were consistently less than 15% of those in wild-type cells (Fig. 6, top panel), while their expression levels in $p45^{-/-}$:Tg megakaryocytes were comparable to those in wild-type cells, except for *Txas* (Fig. 3, top panel). When the low transgene expressors were compared, platelet gene levels relative to the p45 level were lower in $p45^{-/-}$:ΔNTD Tg cells (Fig. 6, bottom panel) but comparable in $p45^{-/-}$:Tg cells (Fig. 3, bottom panel). Thus, the NTD is required for expression of the *Txas*, *Slc6a4*, and *Gp6* genes.

Interestingly, we observed a distinct effect of NTD deletion on expression of the *Selp*, *Slamf1*, and *F5* genes. ΔNTD, when expressed at a higher level, supported the expression of these genes in the absence of endogenous p45 (Fig. 6, top panel). Thus, the requirement of the NTD was not uniform, and the NTD seemed to be dispensable for expression of some platelet genes.

It should be noted that the NTD does not harbor any canonical functional motifs. Compared with the well-characterized PPXY motifs (15, 22), the functional significance of this region residing more N-terminal to the PPXY motifs is not yet fully understood, especially in megakaryocytes. These results imply a novel mechanism of transcriptional activation by p45 within the GATA1-p45 regulatory axis *in vivo*.

***G1HRD*-driven p45 partially corrects defective gene expression and maturation arrest in GATA1-deficient megakaryocytes.** Finally, we assessed how p45 contributes to the GATA1-directed transcriptional hierarchy. Because GATA1 is an upstream regulator of p45, GATA1 function during megakaryopoiesis should at least in part be executed via p45 func-

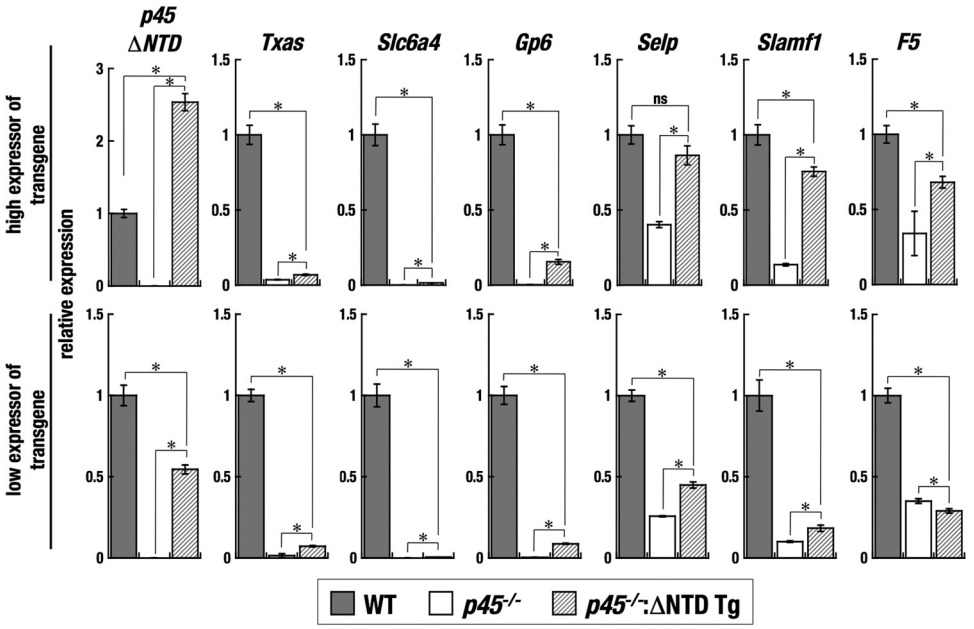

FIG. 6. Essential contribution of the N-terminal 38 amino acids to platelet gene activation in megakaryocytes. Expression levels of platelet genes in primary megakaryocytes, including *Txas*, *Slc6a4*, *Gp6*, *Selp*, *Slamf1*, and *F5*, were examined by quantitative RT-PCR. Results of two representative sets of experiments are shown according to the abundance of transgene-derived ΔNTD mRNA. Upper and lower panels are the results from high and low expressors of the transgene, respectively. Average values are shown, and error bars indicate standard deviations. The average values of wild-type megakaryocytes are set to 1. Results from line 340 are shown as representative data. The Student *t* test was used to calculate statistical significance (*P*). ∗, $P < 0.05$. ns, not significant.

tion. To address this issue, we examined whether *G1HRD*-driven p45 was able to rescue a GATA1 deficiency in megakaryocytes. It has been reported that *Gata1*$^{\Delta neo\Delta HS}$ mice display marked thrombocytopenia as a result of proliferation of immature megakaryocytes and impairment of platelet production, accompanied by reduced expression of p45 (37, 49). Therefore, we crossed *G1HRD*-p45 male mice with heterozygous female (*Gata1*$^{\Delta neo\Delta HS/X}$) mice, expecting that supplementation of p45 would assist *Gata1*$^{\Delta neo\Delta HS/Y}$ mice in generating platelets. Because the *Gata1* gene is located on the X chromosome, we analyzed male mice (*Gata1*$^{X/Y}$, *Gata1*$^{X/Y}$:Tg, *Gata1*$^{\Delta neo\Delta HS/Y}$, and *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg) at E13.5 and adult (2 months old) stages.

GATA1 expression levels in *Gata1*$^{\Delta neo\Delta HS/Y}$ and *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg megakaryocytes were reduced to 1.2% and 1.1% of those in *Gata1*$^{X/Y}$ megakaryocytes, respectively, showing good agreement with previous reports (Fig. 7A) (37, 49). As shown in Fig. 1E, the expression level of p45 in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes was decreased to approximately half of that in *Gata1*$^{X/Y}$ megakaryocytes. Expression of all platelet genes examined was generally reduced in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes (Fig. 7A).

p45 expression in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg megakaryocytes was comparable to that in *Gata1*$^{X/Y}$ megakaryocytes (Fig. 7A). This result indicates that *G1HRD* is active in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes. Although expression levels of platelet genes tended to be higher in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg megakaryocytes than those in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes, the effects were modest, especially for *Slamf1* and *F5*. In the absence of GATA1, the contribution of p45 to the activation of platelet genes appears to be limited.

To examine whether *G1HRD*-driven p45 restores the abnormal increase in *Gata1*$^{\Delta neo\Delta HS/Y}$ megakaryocytes at an immature stage (37), we performed flow cytometry analysis and characterized megakaryocytes cultured from fetal livers. In *Gata1*$^{\Delta neo\Delta HS/Y}$ cultures, CD41^{low} CD61^{low} megakaryocytes (Fig. 7B, fraction I) were relatively abundant compared with CD41^{high} CD61^{high} megakaryocytes (Fig. 7B, fraction II). The ratio of these two fractions (II/I) was lower in *Gata1*$^{\Delta neo\Delta HS/Y}$ cultures and slightly higher in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg cultures, but the latter ratio was not as high as that in *Gata1*$^{X/Y}$ cultures (Fig. 7C). These results indicate that transgene-derived p45 partially restored the number of mature megakaryocytes in the *Gata1*$^{\Delta neo\Delta HS/Y}$ background. At the adult stage, platelet production remained low and severe thrombocytopenia was observed in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg mice (Table 4). These results suggest that p45 expression is not sufficient in fully mature GATA1-deficient megakaryocytes for normal platelet production and that GATA1 needs additional downstream effectors other than p45. Taken together, GATA1 is one of the important upstream regulators of p45, and p45 promotes megakaryopoiesis in cooperation with other factors under the control of GATA1 or GATA1 itself.

DISCUSSION

In megakaryocytes, GATA1 deficiency reduces p45 expression by approximately 50%, indicating the presence of GATA1-dependent and -independent regulation of the *p45* gene. In this study, we have revealed that the GATA1-p45 regulatory axis is one of the hierarchies of transcription factors regulating megakaryopoiesis *in vivo* based on two contrasting

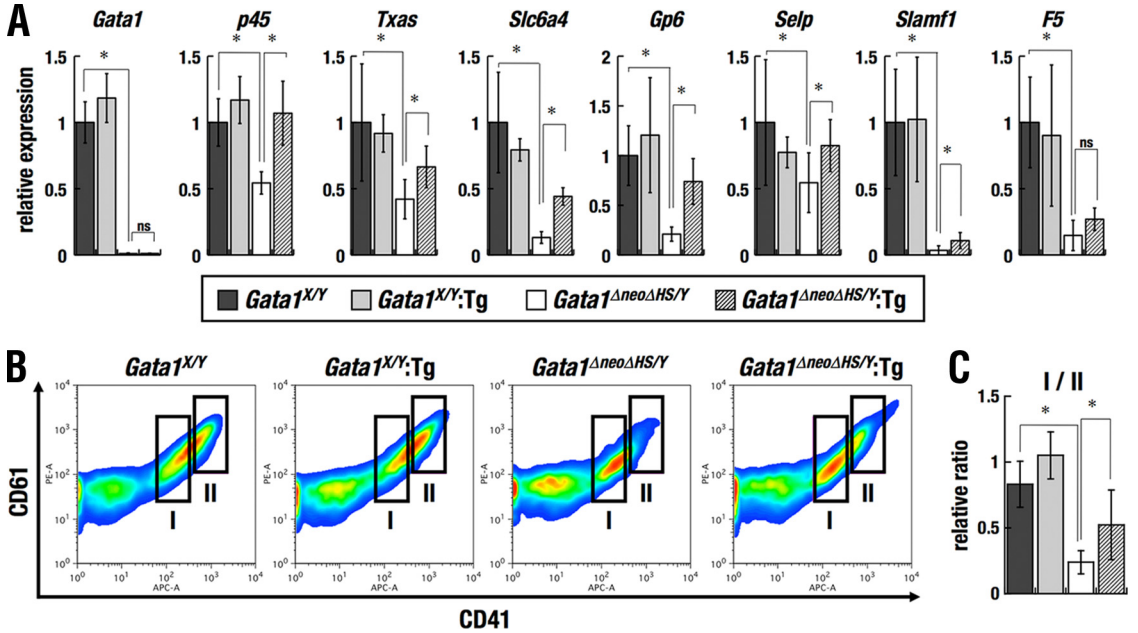

FIG. 7. *G1HRD*-driven p45 does not fully rescue the defective megakaryopoiesis caused by GATA1 deficiency. (A) *Gata1*, *p45*, and platelet gene (*Txas*, *Slc6a4*, *Gp6*, *Selp*, *Slamf1*, and *F5*) expression levels of primary megakaryocytes cultured from fetal livers were examined by quantitative RT-PCR. *Gata1*$^{X/Y}$, $n = 9$; *Gata1*$^{X/Y}$:Tg, $n = 6$; *Gata1*$^{\Delta neo\Delta HS/Y}$, $n = 11$; *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg, $n = 4$. Average values are expressed, and error bars indicate standard deviations. The Student t test was used to calculate statistical significance (P). *, $P < 0.05$. ns, not significant. (B) Primary megakaryocytes in cultures from *Gata1*$^{X/Y}$ ($n = 4$), *Gata1*$^{X/Y}$:Tg ($n = 5$), *Gata1*$^{\Delta neo\Delta HS/Y}$ ($n = 7$), and *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg ($n = 5$) fetal livers were analyzed by flow cytometry for expression of CD41 and CD61. Results from line 205 are shown as representative data. The CD41$^+$ CD61$^+$ cells were divided between regions I and II, with the former and latter representing cell populations with intermediate and high expression levels of the two surface markers, respectively. The ratios of cell numbers in region II to those in region I are shown in panel C. (C) Average ratios of cell numbers in region II to those in region I from *Gata1*$^{X/Y}$ ($n = 5$), *Gata1*$^{X/Y}$:Tg ($n = 5$), *Gata1*$^{\Delta neo\Delta HS/Y}$ ($n = 4$), and *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg ($n = 7$) mice. The Student t test was used to calculate statistical significance (P). *, $P < 0.05$.

approaches. The first approach revealed that *G1HRD*-driven p45 substantially rescued the defective megakaryopoiesis in *p45*-null mice, demonstrating that *G1HRD* retains the ability to direct spatiotemporally accurate expression of p45 and implying that GATA1-dependent regulation of the *p45* gene substantially contributes to the function of p45 in megakaryopoiesis. In contrast, despite being expressed under the regulatory influences of *G1HRD*, a transactivation-defective mutant, ΔNTD, did not efficiently rescue the defects of *p45*-null megakaryocytes, indicating that *G1HRD*-p45 rescue requires intact p45 activity. The second approach showed that *G1HRD*-p45 partially corrected the defects in *Gata1*-deficient megakaryocytes, indicating that p45 is one of the effectors regulated by GATA1 but that GATA1 requires additional effectors to direct the full differentiation process of megakaryocytes. These features of the GATA1-p45 regulatory axis are summarized in Fig. 8.

GATA1 is a prototypical lineage-restricted transcription factor that controls erythroid and megakaryocytic cell differentiation. The transcriptional regulatory cascade governed by GATA1 plays an essential role in promoting differentiation of these lineages. In addition to *p45*, genes encoding a number of transcription factors, including EKLF, GATA2, c-Myb, c-Myc, and PU.1, have been identified as downstream target genes of GATA1 (2, 6, 11, 18, 50). Of these transcription factors, GATA1 activates *p45* and *EKLF* gene expression while it represses expression of the others. Supplementation of transgene-derived p45 did not fully restore the gene expression and maturation arrest with proliferative ten-

TABLE 4. Blood count measurement for compound mutant mice in the *Gata1*$^{\Delta neo\Delta HS/Y}$ background[a]

Genotype (n[b])	Blood count[c]			
	RBC ($10^4/\mu l$)	Hb (g/dl)	Htc (%)	Plt ($10^4/\mu l$)
Gata1$^{X/Y}$ (9)	924 ± 52	15.1 ± 0.6	45.4 ± 2.0	87.7 ± 12.7
Gata1$^{X/Y}$:Tg (9)	894 ± 83	13.2 ± 0.6	40.1 ± 1.8	88.5 ± 6.3
Gata1$^{\Delta neo\Delta HS/Y}$ (3)	740 ± 32	12.9 ± 0.3	40.0 ± 1.4	12.4 ± 3.0
Gata1$^{\Delta neo\Delta HS/Y}$:Tg (3)	750 ± 85	12.2 ± 1.0	37.2 ± 2.1	24.9 ± 21.6

[a] *Gata1*$^{X/Y}$, *Gata1*$^{X/Y}$:Tg, *Gata1*$^{\Delta neo\Delta HS/Y}$, and *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg mice were examined at 2 months of age. Results from line 207 are shown as representative. Values are expressed as means ± standard deviations.
[b] n, no. of mice.
[c] For abbreviations, see footnote *c* of Table 2.

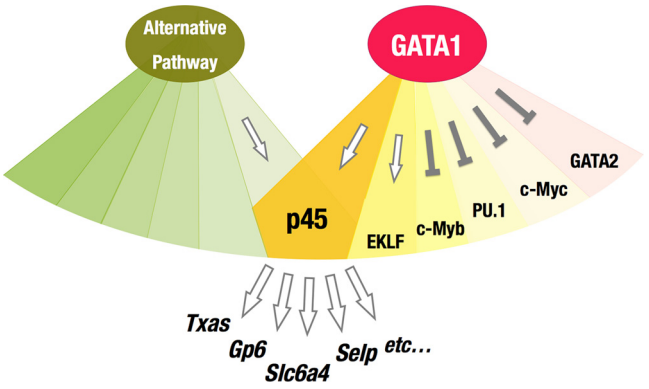

FIG. 8. Schematic model of a transcriptional regulation hierarchy surrounding p45. p45 is regulated by GATA1-dependent and -independent mechanisms. GATA1 activates or represses several key transcription factors, including p45. Among these hierarchical relations, the GATA1-p45 regulatory axis is demonstrated in this study. Upon transgenic expression, p45 directly or indirectly activates downstream targets in megakaryocytes, including Txas, Slc6a4, Gp6, and Selp, even in the absence of GATA1.

dency of GATA1-deficient megakaryocytes, indicating that regulatory pathways other than p45 are also indispensable for GATA1 to regulate the megakaryopoiesis program.

Our analyses have revealed critical contributions of the GATA1-p45 regulatory axis to the suppression of fibrosis in the spleen (see Fig. 2G and 5F) and bone marrow (data not shown). We found extensive fibrosis in spleens and bone marrow from *p45*-null mice, but these mice were rescued from fibrosis by *G1HRD*-p45, suggesting that fibrosis is a consequence of p45 deficiency in GATA1-expressing hematopoietic cells, especially megakaryocytes. This is consistent with the observation that mice suffering from pathological accumulation of megakaryocytes in bone marrow and the spleen, including TPO-overexpressing mice (52), *Gata1*$^{\Delta neo\Delta HS}$ (or *Gata1*^{low}) mice (48), Bach1-overexpressing mice (46) and small Maf mutant mice (H. Motohashi, unpublished data), often develop myelofibrosis and spleen fibrosis. In this regard, it has been reported that an osteosclerotic phenotype also is present in the *p45*-null mice, and spleen cells from *p45*-null mice are able to transfer osteosclerosis as well as megakaryocytosis adoptively to wild-type mice (14). These results support the contention that megakaryocytes in which the GATA1-p45 regulatory axis is operative are integral to bone and connective tissue homeostasis.

It is interesting to note that p45 was able to increase expression of some downstream effector genes in the absence of GATA1. Because *Txas* is a well-established direct target of p45 (7) and *Selp* and *Slc6a4* are newly identified target genes of p45 (R. Fujita, H. Motohashi, and M. Yamamoto, unpublished observation), the increased expression of these genes in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg megakaryocytes seems to be interpreted as p45 directly activating transcription of these genes in the absence of GATA1. However, in the case of *Slc6a4*, we surmise that additional factors regulated by GATA1 or GATA1 itself might cooperate with p45, because expression of *Slc6a4* was lower in the *Gata1*$^{\Delta neo\Delta HS/Y}$ background despite similar p45 expression levels (Fig. 3 and Fig. 7A).

Another interpretation of the restored expression of platelet genes in *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg megakaryocytes is an indirect effect through altered megakaryocyte differentiation. Considering that expression levels of platelet genes are elevated in the course of megakaryocyte maturation (26) and that the megakaryocyte fraction obtained from *Gata1*$^{\Delta neo\Delta HS/Y}$:Tg mice contained a higher frequency of mature megakaryocytes than that from *Gata1*$^{\Delta neo\Delta HS/Y}$ mice (Fig. 7B and C), the increased expression of the platelet genes in the former might reflect a difference in maturation stages between the two populations.

Three lines of evidence suggest that the *p45* gene may be finely tuned by transcription factors other than GATA1. First, substantial amounts of the 1a transcript were still detected in GATA1-deficient megakaryocytes (Fig. 1E). While the 1b promoter was heavily dependent on GATA1 and did not seem to be activated by other GATA factors, like GATA2, the 1a promoter was partially dependent on GATA1 and/or might be activated by other GATA factors. We surmise that the 1a promoter may be primarily utilized at progenitor stages, before GATA1 is expressed. Second, GATA1 and GATA2 are reported to play redundant roles in cooperation with FOG1 (4), and knockout mouse analysis revealed an indispensable role for FOG1 in early megakaryopoiesis (47). Because p45 contributes to the commitment and proliferation of megakaryocytic cells (9, 17) and GATA2 is expressed in immature megakaryocytes, GATA2 may also be involved in the regulation of *p45* gene expression at early stages of megakaryopoiesis. Indeed, other GATA1 target genes, such as *EKLF* and *PU.1*, are also under the control of GATA2 (6, 18). Third, deletion of SCL reduced p45 expression, and conversely, SCL augmented transcriptional activation of the *p45* 1b promoter by GATA1 (19). Thus, p45 may also be under the influence of SCL.

In summary, our results demonstrate that *G1HRD*-p45 rescues the defective megakaryopoiesis of *p45*-null mice, indicating that GATA1-dependent regulation of the *p45* gene substantially contributes to p45 function in megakaryopoiesis. The results also show that p45 supports a limited portion of GATA1 function in megakaryopoiesis, indicating that GATA1 needs to regulate other downstream effectors in addition to p45.

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