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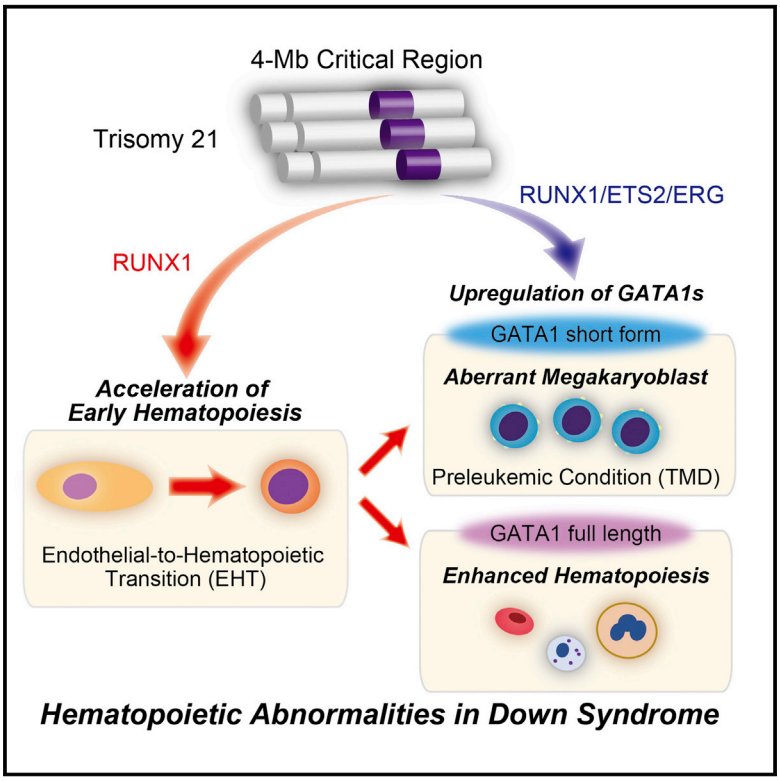
Systematic Cellular Disease Models Reveal Synergistic Interaction of Trisomy 21 and GATA1 Mutations in Hematopoietic Abnormalities

Graphical Abstract



Highlights

- We established iPSC-based cellular disease models of TMD in Down syndrome
- Trisomy 21 promotes expansion of early hematopoietic progenitors
- GATA1s is upregulated by trisomy 21, leading to aberrant megakaryopoiesis
- RUNX1, ETS2, and ERG within a 4-Mb region are critical for these phenotypes

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In Brief

To investigate how trisomy 21 and GATA1 mutations perturb hematopoiesis in Down syndrome, Banno et al. establish systematic disease models using human iPSCs and genome/chromosome-editing technologies and demonstrate interaction between trisomy 21 and GATA1 mutations. The authors thus identify RUNX1/ETS2/ERG as critical regulators in the hematopoietic disorder of Down syndrome.

Banno et al., 2016, Cell Reports *15*, 1228–1241
May 10, 2016
http://dx.doi.org/10.1016/j.celrep.2016.04.031

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Systematic Cellular Disease Models Reveal Synergistic Interaction of Trisomy 21 and GATA1 Mutations in Hematopoietic Abnormalities

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http://dx.doi.org/10.1016/j.celrep.2016.04.031

SUMMARY

Chromosomal aneuploidy and specific gene mutations are recognized early hallmarks of many oncogenic processes. However, the net effect of these abnormalities has generally not been explored. We focused on transient myeloproliferative disorder (TMD) in Down syndrome, which is characteristically associated with somatic mutations in *GATA1*. To better understand functional interplay between trisomy 21 and *GATA1* mutations in hematopoiesis, we constructed cellular disease models using human induced pluripotent stem cells (iPSCs) and genome-editing technologies. Comparative analysis of these engineered iPSCs demonstrated that trisomy 21 perturbed hematopoietic development through the enhanced production of early hematopoietic progenitors and the upregulation of mutated GATA1, resulting in the accelerated production of aberrantly differentiated cells. These effects were mediated by dosage alterations of *RUNX1*, *ETS2*, and *ERG*, which are located in a critical 4-Mb region of chromosome 21. Our study provides insight into the genetic synergy that contributes to multi-step leukemogenesis.

INTRODUCTION

Down syndrome (DS; trisomy 21) is the most common chromosomal aneuploidy, caused by the presence of three copies of human chromosome 21 (HSA21). Among various medical symptoms, DS patients have an increased likelihood of certain hematopoietic abnormalities, including transient myeloproliferative disorder (TMD) and leukemia (Roy et al., 2009). TMD involves the clonal proliferation of immature megakaryoblasts, which typically occurs in ~10% of infants with DS. In addition, 20%–30% of TMD survivors progress to acute myeloid leukemia (ML-DS; myeloid leukemia associated with Down syndrome, also called DS-AMKL, M7) within 4 years (Pine et al., 2007). Both DS-TMD and ML-DS cells harbor somatic mutations in *GATA1*, a hematopoietic transcription factor located on the X chromosome, which result in loss of the full-length protein product and the exclusive production of a short GATA1 variant (GATA1s) (Hitzler and Zipursky, 2005). Except for some rare hereditary diseases (Hollanda et al., 2006; Sankaran et al., 2012), somatically acquired GATA1s-producing mutations are found solely in cells that are trisomic for chromosome 21. These two factors (i.e., a single acquired *GATA1* mutation and constitutive trisomy 21) are now considered necessary and sufficient to cause TMD (Yoshida et al., 2013). Thus, the distinctive features of DS, TMD, and ML-DS provide ideal models to study the multistep process of leukemia caused by aneuploidy and specific gene mutations.

Here, we established systematic and strategically designed cellular disease models by combining patient-derived human induced pluripotent stem cells (hiPSCs), genome-editing technologies, and chromosome engineering techniques. 20 types (and more than 40 clones) of hiPSCs were generated and subjected to hematopoietic differentiation to explore the synergistic interaction between *GATA1* mutation and gene-dosage effects of trisomy 21 in hematopoiesis (Figures 1 and 6A). Constitutive trisomy 21 not only accelerates the production of hematopoietic

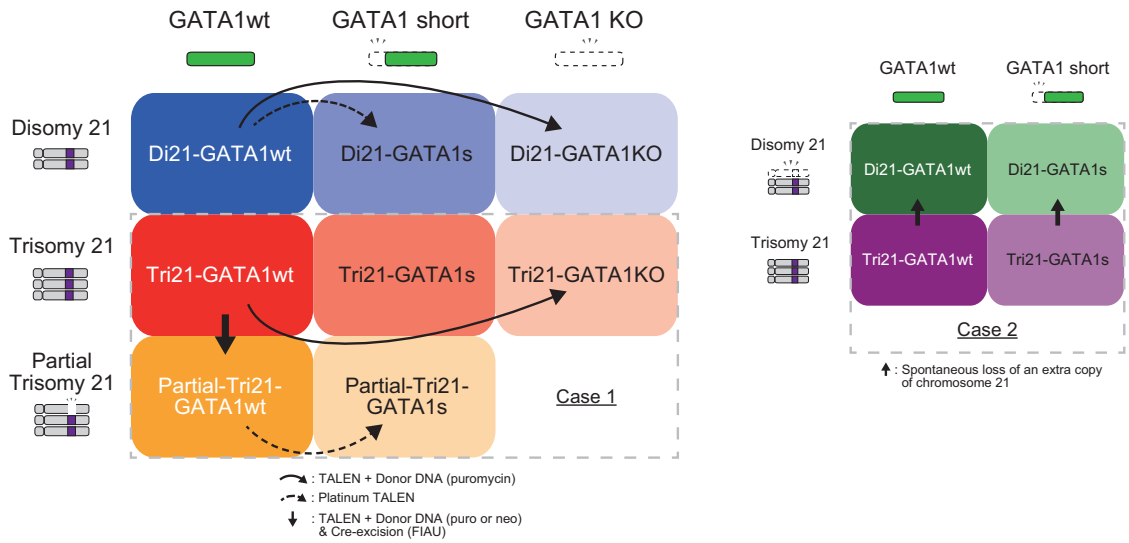


Figure 1. Human iPSC-Based Modeling of Hematopoietic Abnormalities in DS

Schematic overview depicting the strategy to generate twelve types of genome-edited iPSC lines for in vitro disease modeling. (Left, case 1) First, Di21-GATA1wt, Tri21-GATA1wt, and Tri21-GATA1s iPSC lines were directly generated from human blood samples using Sendai virus (SeV). Second, GATA1KO lines were generated from Di21- and Tri21-GATA1wt iPSC lines using a TALEN and donor DNA (Di21- and Tri21-GATA1KO, respectively; arrow). Third, a 17-bp deletion was introduced into the Di21-GATA1wt iPSC line using a Platinum TALEN (Di21-GATA1s; dotted arrow). Fourth, a 4-Mb region was deleted from a single chromosome 21 in the Tri21-GATA1wt iPSC line (Partial-Tri21-GATA1wt; bold arrow). Finally, the Partial-Tri21-GATA1s iPSC line was generated using a Platinum TALEN (dotted arrow). (Right, case 2) Additional Tri21-GATA1wt and -GATA1s iPSC lines (case2-Tri21-GATA1wt and -GATA1s) were generated from another DS patient (case 2), and isogenic disomy iPSC lines were obtained by spontaneous loss of chromosome 21 (case2-Di21-GATA1wt and -GATA1s; upward bold arrow).

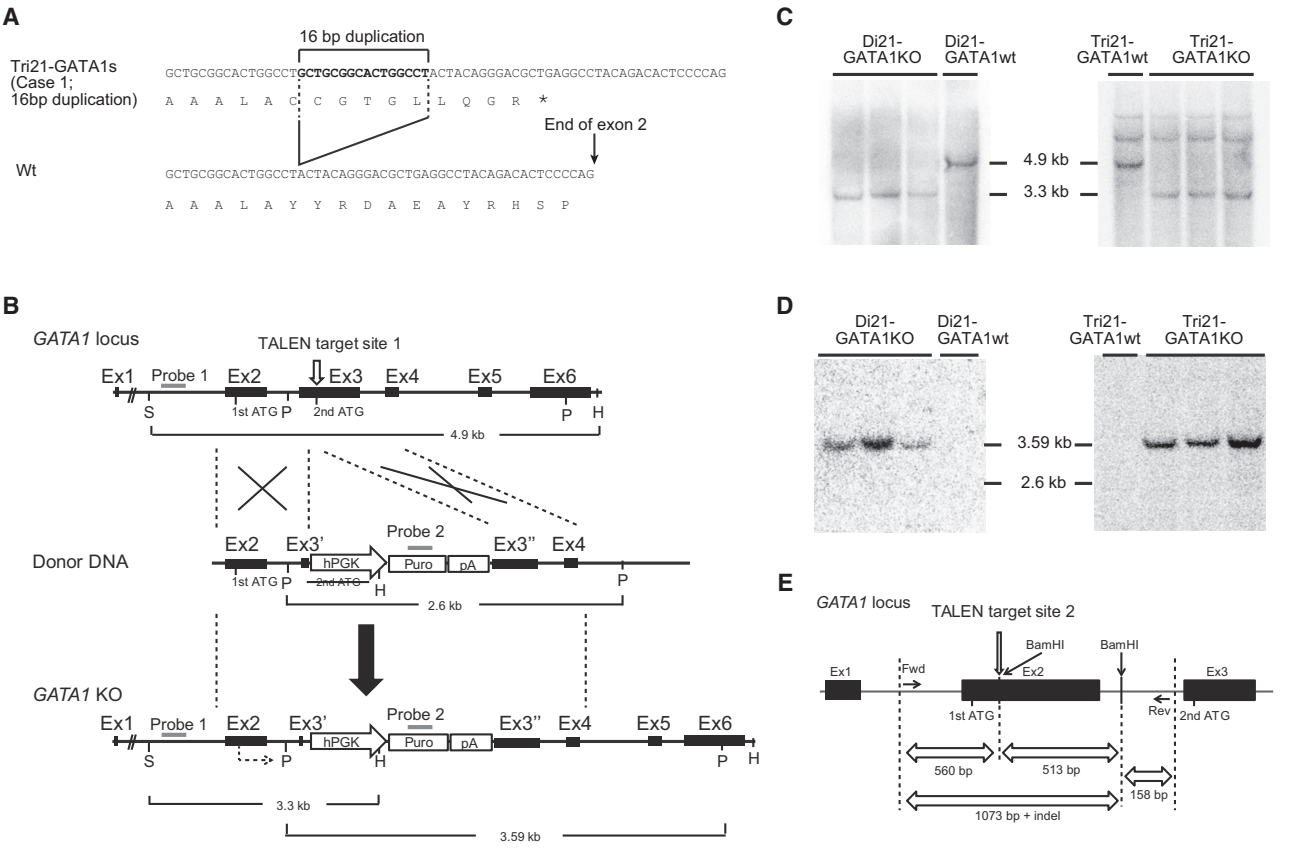
progenitors, which leads to enhanced multilineage differentiation, but also upregulates GATA1s expression, giving rise to the excessive generation of abnormal megakaryoblasts. We succeeded in isolating a 4-Mb region critical for hematopoietic defects in DS and identified three genes, *RUNX1*, *ETS2*, and *ERG*, as key molecules involved in an interconnected regulatory network. The results demonstrate synergistic effects of aneuploidy and a specific gene mutation on the leukemogenesis.

RESULTS

Generation of Systematic Cellular Disease Models Incorporating Trisomy 21 and *GATA1* Mutations

Our first aim was to establish an iPSC-based in vitro model system that would enable us to investigate the interaction between *GATA1* mutations and trisomy 21 in TMD. For this purpose, multiple iPSC lines based on the chromosome 21 copy number (disomy, Di; or trisomy, Tri) and variations due to *GATA1* mutation (no mutation, GATA1wt; short-form mutation, GATA1s; and knockout mutation, GATA1KO) were designed (Figure 1). We identified two newborn male patients with DS-TMD, who typically presented with constitutive trisomy 21, high white blood cell counts, hepatomegaly, and pericardial/pleural/ascitic effusions (cases 1 and 2; Table S1). Introduction of reprogramming factors into peripheral blood cells derived from case 1, which included cell types with and without *GATA1* mutations, resulted in the establishment of two different isogenic iPSC lines: one with normal *GATA1* (Tri21-GATA1wt) and the other with the GATA1s-producing mutation (Tri21-GATA1s). All iPSC clones in the Tri21-GATA1s line from case 1 shared the same 16-bp duplication in exon 2 of *GATA1*, resulting in a premature stop codon in the N-terminal activation domain (Figure 2A). No acquired mutations were found in the protein-coding sequences of *RAD21*, *CTCF*, or *EZH2*, which are representative mutational targets in ML-DS (Yoshida et al., 2013). A control iPSC line with a normal karyotype and wild-type *GATA1* were also generated from cord blood mononuclear cells obtained from a euploid male subject (Di21-GATA1wt). To generate iPSC lines carrying a targeted *GATA1* gene mutation, the most active pair of transcription activator-like effector nucleases (TALENs), which recognize unique sequences in the proximity of the second ATG in exon 3 of *GATA1* (Figures 2B and S2A), and a targeting donor construct were introduced into the Di21- and Tri21-GATA1wt iPSC lines. This procedure resulted in the generation of Di21-GATA1KO and Tri21-GATA1KO iPSC lines, respectively (Figures 2C and 2D).

To generate the Di21-GATA1s iPSC line, we introduced a highly active Platinum TALEN and performed one round of sib-selection (Figures 2G, S2B, S2C, S2E, and S2F see also Figure S5I, method 1) (Sakuma et al., 2013). Di21- and Tri21-GATA1s lines expressed only short-form products accompanied by unstable transcripts leading to nonsense-mediated decay (NMD), confirming the absence of full-length products (Figure 2H). Interestingly, GATA1s expression was evidently higher in Tri21-GATA1s cells than in Di21-GATA1s cells, indicating the direct influence of trisomy 21 on GATA1s expression (Figures 2H and S2H; see also Figures 4I and S7). In contrast, neither GATA1 nor GATA1s transcripts were detected in Di21- and Tri21-GATA1KO lines. All iPSC clones exhibited a typical morphology and normal euploidy or trisomy 21 karyotypes



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(Figures S2D and S2G). The assessments of pluripotency and the differentiation ability of iPSC lines are described in Figure S1.

Trisomy 21 Promotes Early Hematopoietic Commitment and Multilineage Differentiation: Comparison between Di21- and Tri21-GATA1wt iPSCs

Several reports have demonstrated that trisomy 21 augments fetal hematopoiesis (Chou et al., 2012; Maclean et al., 2012). To confirm these findings and further examine the effect of trisomy 21 in our models, Di21- and Tri21-GATA1wt iPSCs were differentiated into a hematopoietic lineage by generating embryoid bodies (EBs) using published protocols with some modifications (Figure 3A) (Grigoriadis et al., 2010; Ng et al., 2008). qRT-PCR analysis of stage-specific β-like globins in day 10 EBs showed predominant γ-globin expression, suggesting that our differentiation protocol reflected fetal hematopoiesis (Figure 3B). We first examined the early hematopoietic developmental stage, in which hematopoietic stem cells (HSCs) emerge from a specialized subset of vascular endothelial cells (hemogenic endothelium). Previous reports have shown that the human pluripotent stem cell-derived KDR^+CD31^+ cell population appears at the beginning of the hematopoietic development and has the potential to generate blast colonies that display both hematopoietic and endothelial cells (Choi et al., 2012). There was no significant difference in the KDR^+CD31^+ cell population between Di21- and Tri21-GATA1wt iPSC-derived EBs (Figure 3C). However, the proportion of $CD43^+$ cells in the $CD34^+CD38^-Lin^-$ fraction, representing putative hematopoietic progenitors, was significantly increased in the Tri21-GATA1wt line compared with that in Di21-GATA1wt line (Figures 3D, S3A, and S3B) (Vodyanik et al., 2006). This result suggests that trisomy of chromosome 21 accelerates the early stage of hematopoiesis, leading to the increased production of committed hematopoietic progenitors. Reflecting this enhanced commitment to a hematopoietic lineage, Tri21-GATA1wt iPSCs gave rise to erythroid-lineage cells (CD235/glycophorin A) at a rate more than double that for Di21-GATA1wt cells (Figures 3E and S3C). A similar increase in the $CD33^+$ fraction, which reflects myeloid cells and their progenitors, was detected in trisomic cells (Figure 3F). This pattern was even more apparent in a methylcellulose colony-forming assay with Tri21-GATA1wt iPSCs generating more than 5-fold the numbers of multilineage colonies of CFU-G/GM/M, -E, and -GEMM cells (Figures 3G and S3D). Moreover, the megakaryocyte colony-forming assay showed that trisomy 21 iPSC lines generated over 5-fold more megakaryocyte colonies than disomic iPSCs (Figures 3H and S3E). These results are consistent with the increased number of hematopoietic progenitor cells in Tri21-GATA1wt iPSC lines.

Full-Length GATA1 Is Indispensable for Erythropoiesis: Comparison among GATA1wt, GATA1s, and GATA1KO iPSCs

The acquisition of a somatic short-form mutation in *GATA1* is the second step of leukemogenesis in DS, which impairs the production of mature erythrocytes during erythropoiesis (Byrska-Bishop et al., 2015). To further address whether this effect is due to the presence of the short-form protein or the absence of the full-length form, we examined the erythropoiesis of the GATA1wt, GATA1s, and GATA1KO iPSCs. In contrast to the clear production of $CD235^+$ erythroid cells by GATA1wt-containing iPSC lines, clones lacking the full-length form of GATA1 (i.e., GATA1s and GATA1KO iPSC lines) exhibited severely impaired erythrocytes, suggesting that the normal function of full-length GATA1 is indispensable for erythrocyte development, regardless of the number of copies of chromosome 21 (Figure 3E).

Short-Form GATA1 Mutation Causes Aberrant Differentiation of Megakaryocytes

A clinical characteristic of TMD is the abnormal proliferation of megakaryoblasts (Roy et al., 2009). We thus examined the functional effects of GATA1s and GATA1KO on megakaryopoiesis. A two-step protocol, in which EB formation was followed by coculture with MS5 (murine stromal) or AFT024 (murine fetal liver stromal) cells, generated highly consistent results in terms of the later differentiation stages (Figure 4A). In this protocol, on day 15, the numbers of $CD34^+$ cells were significantly increased in trisomic lines compared with those in disomic ones, which is

Figure 2. Gene Editing of *GATA1* by TALEN-Mediated HDR and NHEJ in Human iPSCs

(A) *GATA1* sequence data from the Tri21-GATA1s iPSC line (case 1; 16-bp duplication). The asterisk indicates the premature stop codon.

(B) Schematic representation of the *GATA1*-targeting strategy by homology-directed repair (HDR). Top: structure of the endogenous *GATA1* gene. Middle: the targeting vector containing the puromycin-resistance gene and ~800-bp homology arms. Bottom: schematic of the targeted *GATA1* locus. Exons (black boxes), restriction enzyme sites (S, SpeI; H, HincII; and P, PvuII), TALEN target site 1 (vertical white arrow), and the probe for Southern blot analysis (gray bars) are indicated.

(C and D) Southern blot analysis of iPSC lines before (Di21- and Tri21-GATA1wt) and after (Di21- and Tri21-GATA1KO) targeting using a 5′ probe (probe 1; C) and internal probe (probe 2; D). Expected fragment sizes: probe 1 (SpeI/HincII digest) wild-type allele, 4.9 kb; targeted allele, 3.3 kb. Probe 2 (PvuII digest) wild-type allele, no band; targeted allele, 3.59 kb; random integration, 2.6 kb.

(E) Schematic representation of the *GATA1*-modification strategy by non-homologous end-joining (NHEJ). Exons (black boxes), restriction enzyme sites (BamHI), TALEN target site 2 (vertical white arrow), and PCR primer sites are indicated.

(F) PCR mutation analysis of the genomic *GATA1* locus in TALEN-injected Di21-GATA1wt iPSCs (+/−BamHI digest). Expected fragment sizes: Di21-GATA1wt (−BamHI) 1,231 bp, (+BamHI) 560/513/158 bp; Di21-GATA1s (+BamHI) 1,073 (+indel)/158 bp. A 17-bp deletion by NHEJ resulted in production of 1,056/158 bp.

(G) *GATA1* sequence data for the Di21-GATA1s iPSC line (17-bp deletion). Microhomologies flanking the double-strand break are underlined.

(H) Representative photomicrographs showing RT-PCR products for GATA1 and β-actin (ACTB) mRNAs in each human iPSC line ($CD34^+CD38^-$ cells isolated from day 8 EBs; left). Fragment sizes: Di21- and Tri21-GATA1wt, 359/120 bp; Di21- and Tri21-GATA1KO, no band; Di21-GATA1s (17-bp deletion), 342/120 bp; Tri21-GATA1s (16-bp duplication), 375/120 bp. A schematic of each mRNA with the PCR primers, corresponding amplicon lengths, and resulting protein products is shown on the right. NMD, nonsense-mediated decay. Note that DNA fragments in each band were extracted from the gel and sequenced to confirm the expected gene editing.

See also Figures S1 and S2.

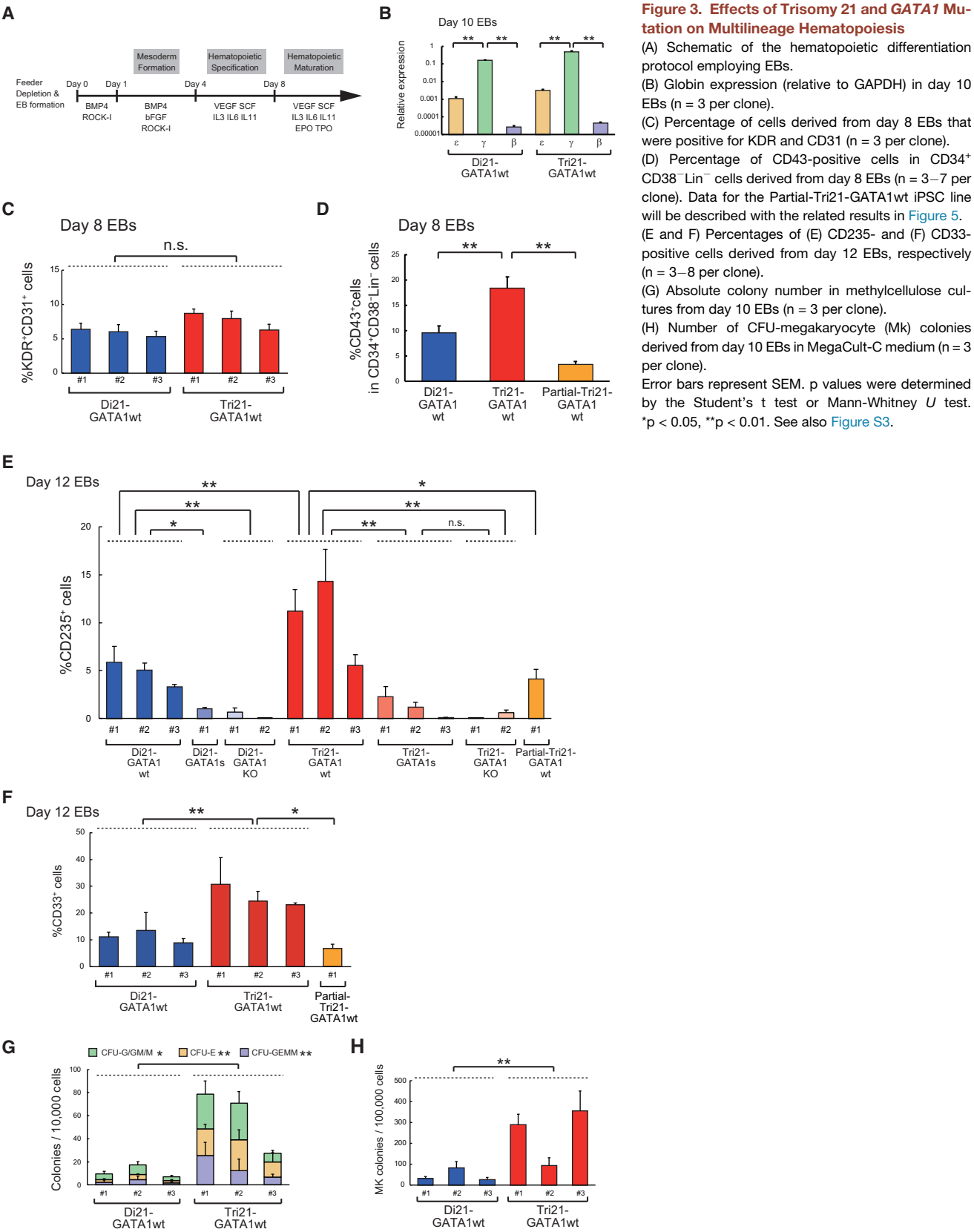


Figure 3. Effects of Trisomy 21 and *GATA1* Mutation on Multilineage Hematopoiesis

(A) Schematic of the hematopoietic differentiation protocol employing EBs.

(B) Globin expression (relative to GAPDH) in day 10 EBs (n = 3 per clone).

(C) Percentage of cells derived from day 8 EBs that were positive for KDR and CD31 (n = 3 per clone).

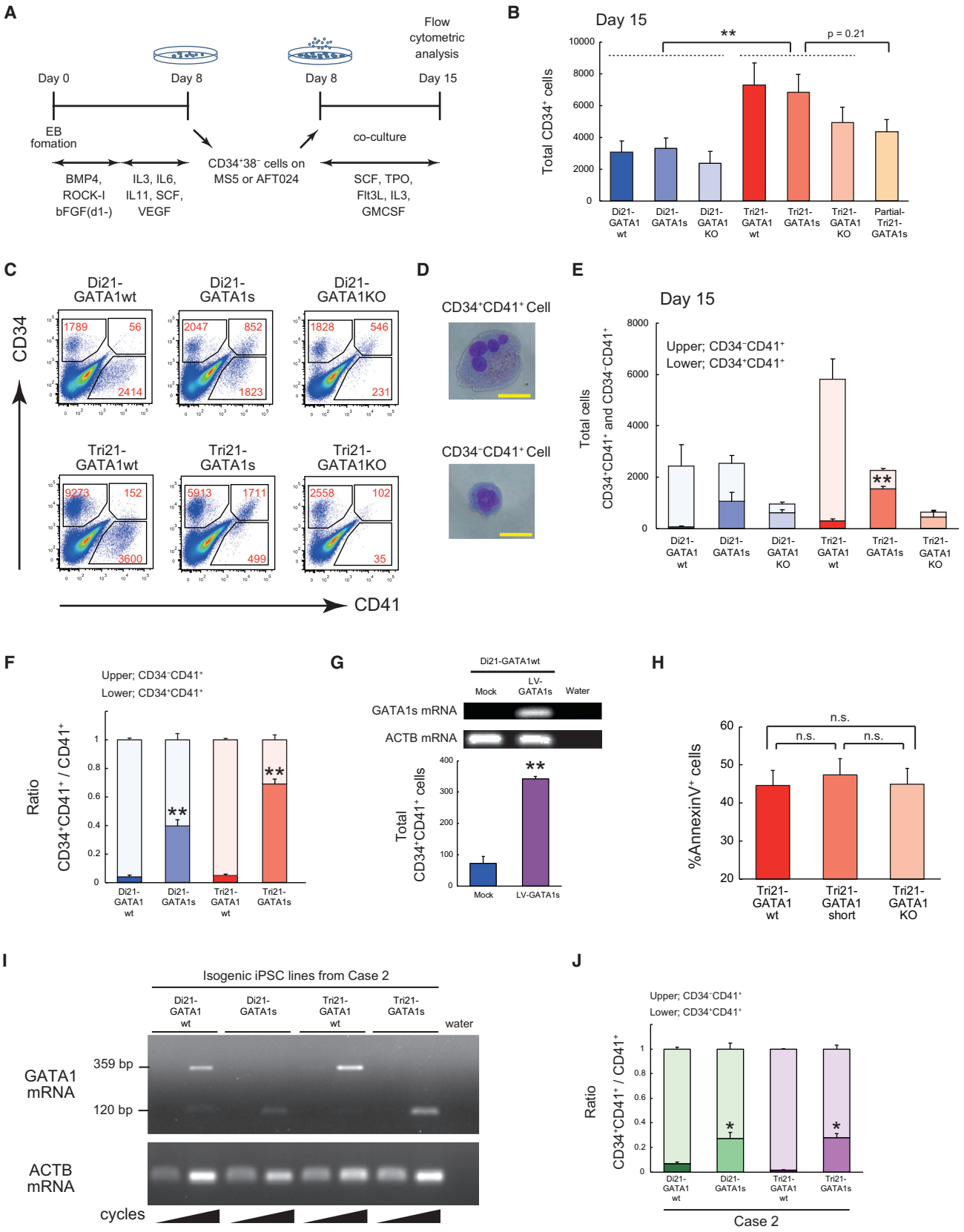
(D) Percentage of CD43-positive cells in $CD34^+$ $CD38^-Lin^-$ cells derived from day 8 EBs (n = 3–7 per clone). Data for the Partial-Tri21-GATA1wt iPSC line will be described with the related results in Figure 5.

(E and F) Percentages of (E) CD235- and (F) CD33-positive cells derived from day 12 EBs, respectively (n = 3–8 per clone).

(G) Absolute colony number in methylcellulose cultures from day 10 EBs (n = 3 per clone).

(H) Number of CFU-megakaryocyte (Mk) colonies derived from day 10 EBs in MegaCult-C medium (n = 3 per clone).

Error bars represent SEM. p values were determined by the Student's t test or Mann-Whitney *U* test. *$p < 0.05$, **$p < 0.01$. See also Figure S3.



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consistent with the enhanced early hematopoiesis in trisomy 21 (Figure 4B).

The development of hematopoietic progenitors into the megakaryocytic lineage is characterized by the expression patterns of CD34 and CD41, which are defined by the cell types or differentiation protocols (Pick et al., 2013; Schipper et al., 2003). Di21- and Tri21-GATA1wt iPSC lines produced sufficient numbers of $CD34^{-}CD41^{+}$ cells, which were generally larger than $CD34^{+}CD41^{-}$ hematopoietic progenitors and had one or a few nuclear lobes, representing promegakaryocytes or immature megakaryocytes. Consistent with the results from the megakaryocyte colony-forming assay, Tri21-GATA1wt iPSCs produced more $CD34^{-}CD41^{+}$ cells than Di21-GATA1wt iPSCs (Figure 4C–4E). In contrast, both Tri21- and Di21-GATA1s iPSC lines generated a significantly larger population of $CD34^{+}CD41^{+}$ double-positive cells, which was remarkably small in the presence of the full-length GATA1 form. This cell fraction was occupied by the dysplastic form of megakaryocytes with multiple separate nuclei, which are characteristically found in the bone marrow of patients with myelodysplastic syndrome (Figures 4C–4F and S4A) (Bhasin et al., 2013). $CD34^{+}CD41^{+}$ cells were similarly generated in Di21- and Tri21-GATA1KO iPSC lines to some extent, but the cell population was smaller than in the Di21- and Tri21-GATA1s iPSC lines. A previous report demonstrated the dominant action of GATA1s on the megakaryopoiesis in murine fetal liver cells (Li et al., 2005). On the other hand, loss of the full-length form of GATA1 has been reported to cause the excessive proliferation of megakaryocytes (Muntean and Crispino, 2005). To study whether this aberrant megakaryopoiesis is caused by the direct action of GATA1s, the loss of function of the full-length form of GATA1, or both, we forced GATA1s expression in the Di21-GATA1wt iPSC line by lentiviral transduction. This forced expression of GATA1s led to a significant increase in the production of $CD34^{+}CD41^{+}$ cells in the Di21-GATA1wt iPSC line, but the cell number was low compared with the number in the Di21-GATA1s iPSC line (Figures 4G and S4B). This suggests the dominant accelerating effect of GATA1s and the possible suppressive effect of full-length GATA1 on the generation of $CD34^{+}CD41^{+}$ cells. There were no significant differences in annexin V-positive cell numbers between trisomic iPSC lines (Figure 4H). These results indicate that mutant GATA1s perturbs proper megakaryocyte differentiation and that the production of aberrant megakaryoblasts can be further accelerated on a trisomy 21 background.

Isogenic hiPSC Lines Derived from Another Patient Reproduce the Effect of GATA1s on Megakaryocyte Differentiation

In our established iPSC lines, the genome-edited disomic or trisomic iPSC clones shared a genetic background, except for the *GATA1* gene. However, because Di21- and Tri21-GATA1wt iPSCs were derived from different individuals (non-DS and DS) and not genetically matched, it is difficult to attribute the observed phenotype solely to the chromosomal abnormality. Therefore, we generated additional trisomic and isogenic disomic iPSC lines from a different male DS-TMD patient (case 2) (see Figure 1, right; spontaneous loss). Two iPSC lines (case2-Tri21-GATA1wt and -GATA1s) were simultaneously generated from cord blood samples from case 2. Over several passages, some trisomic cells spontaneously lost one copy of chromosome 21, giving rise to disomic cells (case2-Di21-GATA1wt and -GATA1s), which were isogenic except for chromosome 21 (Figure S4C). Case2-Di21- and case2-Tri21-GATA1s iPSC lines shared a *GATA1* point mutation in the 5′ splice site of intron 2, resulting in the loss of normal expression of the full-length transcript (Figures 4I and S4D). During megakaryocyte differentiation, both case2-Di21- and case2-Tri21-GATA1s iPSCs appeared to produce aberrant $CD34^{+}CD41^{+}$ megakaryoblasts (Figure 4J), which was consistent with the data obtained from case 1. Taken together, these findings demonstrate that the expression of GATA1s and the absence of the full-length form lead to the suppression of normal megakaryocyte maturation and increased production of aberrant megakaryoblastic cells.

Generation of Partial Trisomy 21 iPSCs by Targeted Deletion of a 4-Mb Region on Chromosome 21

During gene expression analysis of hematopoietically differentiated trisomy 21 iPSCs, we noticed that a group of genes on chromosome 21 showed higher than expected expression (1.5-fold) and formed a cluster in the restricted region (an ~4-Mb segment between *RUNX1* and *ETS2*) (Figure S5A). Thus, we determined whether this region is critical for the hematopoietic defects in DS by generating a partial trisomy iPSC line in which this 4-Mb

Figure 4. Synergistic Interaction between Trisomy 21 and *GATA1* Mutation during Megakaryocyte Differentiation

(A) Schematic of the two-step differentiation protocol using a co-culture system with MS5 or AFT024 cells.

(B) Average number of total $CD34^{+}$ cells per well on day 15 (n = 3 per line). Histogram bar colors correspond to the iPSC lines in Figure 1. Data for the Partial-Tri21-GATA1s iPSC line are described with the related results in Figure 5.

(C) Representative flow cytometric analysis showing $CD34^{+}CD41^{-}$, $CD34^{+}CD41^{+}$, and $CD34^{-}CD41^{+}$ cells on day 15. Total cell counts in each gate are indicated.

(D) Representative microphotographs of May-Grünwald-Giemsa-stained cytospins of $CD34^{+}CD41^{+}$ and $CD34^{-}CD41^{+}$ cells on day 15. Scale bars represent 25 μm.

(E) Absolute number of $CD34^{+}CD41^{+}$ cells (dark color; lower column) and $CD34^{-}CD41^{+}$ cells (light color; upper column) per well on day 15 (n = 3 per line).

(F) Ratio of $CD34^{+}CD41^{+}$ cells to the total $CD41^{+}$ cell number on day 15.

(G) Upper: semiquantitative RT-PCR analysis of GATA1s and β-actin mRNA in lentivirus-transduced iPSCs. Lower: average number of total $CD34^{+}CD41^{+}$ cells per well on day 15 (n = 3 per line).

(H) Percentage of annexin V^{+} cells from $CD34^{+}CD38^{-}$ progenitors (n = 3 per line).

(I) Semiquantitative RT-PCR analysis of GATA1 and β-actin mRNA in $CD34^{+}CD38^{-}$ progenitors isolated from day 8 EBs formed by case2-Di21- and case2-Tri21-iPSC lines. A total of 30- and 35-PCR cycles were performed. Expected PCR fragment sizes: GATA1wt, 359 bp; GATA1s, 120 bp.

(J) Ratio of $CD34^{+}CD41^{+}$ cells to the total $CD41^{+}$ cell number on day 15 (n = 3 per line).

Error bars represent SEM. p values were determined by the Student's t test to compare two independent groups, or Dunnett's or Steel's test to compare three independent groups. *$p < 0.05$, **$p < 0.01$. See also Figure S4.

region was deleted from a single copy of chromosome 21, resulting in reversion to disomy in this deleted region alone. If these partial trisomy 21 iPSCs lost their pathological features, the phenotype could be attributed to this 4-Mb region. Two *loxP*-flanked cassettes were introduced sequentially into single alleles of the *RUNX1* and *ETS2* genes located on the same chromosome in Tri21-GATA1wt iPSCs. The resultant double-targeted clones were subjected to Cre recombinase-mediated excision (Figures 5A and S5B–S5G). Deletion of the 4-Mb region was confirmed by comparative genomic hybridization (CGH) analysis, DNA fluorescent in situ hybridization, and gene expression analysis (Figures 5B, 5F, and S5H) (hereafter referred to as Partial-Tri21-GATA1wt iPSCs).

The 4-Mb Region on Chromosome 21 Is Critical for Hematopoiesis in DS

To assess the role of this 4-Mb region in the hematopoietic abnormalities of DS, we differentiated the Partial-Tri21-GATA1wt iPSC line into hematopoietic lineages. Notably, the proportion of $CD43^+$ cells in the $CD34^+CD38^-Lin^-$ fraction was decreased dramatically in Partial-Tri21-GATA1wt iPSCs compared with that in the Tri21-GATA1wt line (Figure 3D). Similarly, the number of both $CD235^+$ and $CD33^+$ cells produced by Partial-Tri21-GATA1wt iPSCs was significantly lower than that produced by Tri21-GATA1wt, which returned to the normal level observed in the Di21-GATA1wt iPSC line (Figures 3E and 3F). These findings suggest that the accelerating effect on the production of early hematopoietic precursors and subsequent multilineage hematopoiesis in trisomy 21 requires the extra copy of this 4-Mb region.

Trisomy of the 4-Mb Region on Chromosome 21 Perturbs Megakaryocyte Development via GATA1s Upregulation

We introduced the short-form mutation of *GATA1* into Partial-Tri21-GATA1wt iPSCs using a Platinum TALEN (Figures S2C and S5I, method 2). Importantly, the generation of aberrant $CD34^+CD41^+$ megakaryoblastic cells was markedly decreased in the Partial-Tri21-GATA1s iPSC line compared with that in the Tri21-GATA1s iPSCs line, indicating that trisomy of this 4-Mb region plays a critical role in abnormal megakaryocyte differentiation (Figures 5C and S5J).

GATA1s expression in $CD34^+CD38^-$ cells on day 8 EBs was increased significantly in trisomy 21 cells compared with that in disomy cells at in both transcriptional (Figure 5E) and protein levels (Figure S2H). This upregulation of GATA1s was similarly observed in the case2-Tri21-GATA1s iPSC line compared with that in the case2-Di21-GATA1s iPSC line (Figures 4I and S5K). Because the latter was derived from the former by the spontaneous loss of an extra copy of chromosome 21, the *GATA1* loci of these two isogenic iPSC lines have the same mutation, ruling out the possibility that the upregulation of the short form was due to different types of mutation in *GATA1* (Kanezaki et al., 2010). Importantly, GATA1s expression was markedly decreased in Partial-Tri21-GATA1s iPSCs (Figures 5D, 5E, and S2H), with the cell numbers of $CD34^+CD41^+$ double-positive megakaryoblasts appearing to correlate with GATA1s expression levels (Figure 5C). These results strongly suggest that trisomy of the 4-Mb region causes megakaryoblast hyperproliferation via GATA1s upregulation in a dose-dependent manner.

To verify this hypothesis, we forced the expression of GATA1s in the Partial-Tri21-GATA1s iPSC line by lentiviral transduction. As expected, this enhanced GATA1s expression resulted in a significant increase in the production of abnormal $CD34^+$ $CD41^+$ megakaryoblasts, which returned to a level similar to that observed in the Tri21-GATA1s iPSC line (Figure 5C). The fraction of mature $CD34^-CD41^+$ megakaryocytes was increased slightly, but was far from the level observed in the Di21-GATA1s iPSC line (Figure S5J).

RUNX1, ETS2, and ERG are Critical Regulators of Hematopoietic Abnormalities in Trisomy 21

The 4-Mb region on HSA21 contains 20 protein-coding genes and several noncoding RNA genes (Table S2). To identify key molecules with copy-number alterations that are specifically associated with hematopoietic abnormalities, we further prepared several genome-edited trisomy 21 iPSC lines, in which candidate genes were targeted only in a single chromosome, with or without GATA1s mutation. Because our data suggested that trisomy 21 had a significant effect on the generation of hematopoietic progenitors, we focused on RUNX1, an essential regulator in this process, and its cofactors ETS2 and ERG. The resultant alleles are referred to as $RUNX1^{+/+/m}$ ($R^{+/+/m}$), $ETS2^{+/+/m}$ ($Et^{+/+/m}$), and $ERG^{+/+/m}$ ($Er^{+/+/m}$) (Figures 6A and S6A). Note that trisomy of HSA21 was conserved in these cells, and the dosages of the target genes were restored to normal (i.e., two copies). The loss of one copy of the *RUNX1* gene ($R^{+/+/m}$-Tri21-GATA1wt and -Tri21-GATA1s) was sufficient to cancel the accelerating effect on early hematopoietic specification ($CD43^+$ cells) and later differentiation ($CD235^+$ and $CD33^+$ cells) stages caused by trisomy 21 (Figures 6B, S6B, and S6C). Double targeting of *RUNX1* and *ETS2*, or *RUNX1* and *ERG* in a single chromosome ($R^{+/+/m}Et^{+/+/m}$-Tri21-GATA1wt and -GATA1s, and $R^{+/+/m}Er^{+/+/m}$-Tri21-GATA1s), also eliminated the accelerating effect on early hematopoiesis, whereas single targeting of *ETS2* ($Et^{+/+/m}$-Tri21-GATA1wt and -GATA1s) or *ERG* ($Er^{+/+/m}$-Tri21-GATA1s) was insufficient to fully cancel out this effect. This result indicated that an extra copy of the *RUNX1* gene is indispensable for the accelerated hematopoiesis caused by trisomy 21. RUNX1 has three major isoforms: RUNX1a, RUNX1b, and RUNX1c. The expression of the RUNX1b/c isoforms was higher than that of RUNX1a in our day 8 EBs (data not shown), which is consistent with a previous study (Real et al., 2013). Lentiviral transduction of RUNX1c into Di21- and Partial-Tri21-GATA1wt iPSCs increased the expression of RUNX1 to levels similar to that observed in Tri21-GATA1wt iPSCs (Figure 6C). This enforced RUNX1 expression caused no significant change in $CD43^+$ cell production, whereas a slight increase was observed in Partial-Tri21-GATA1wt iPSCs. This suggests that the dosage effects of the additional genes may be required for hematopoietic enhancement.

In contrast, single targeting of *RUNX1* in Tri21-GATA1s iPSCs ($R^{+/+/m}$-Tri21-GATA1s) was not sufficient to decrease the $CD34^+CD41^+$ double-positive cell population during megakaryocyte differentiation (Figure 6D). *ERG* alone ($Er^{+/+/m}$-Tri21-GATA1s) or double targeting of *RUNX1* and *ETS2*

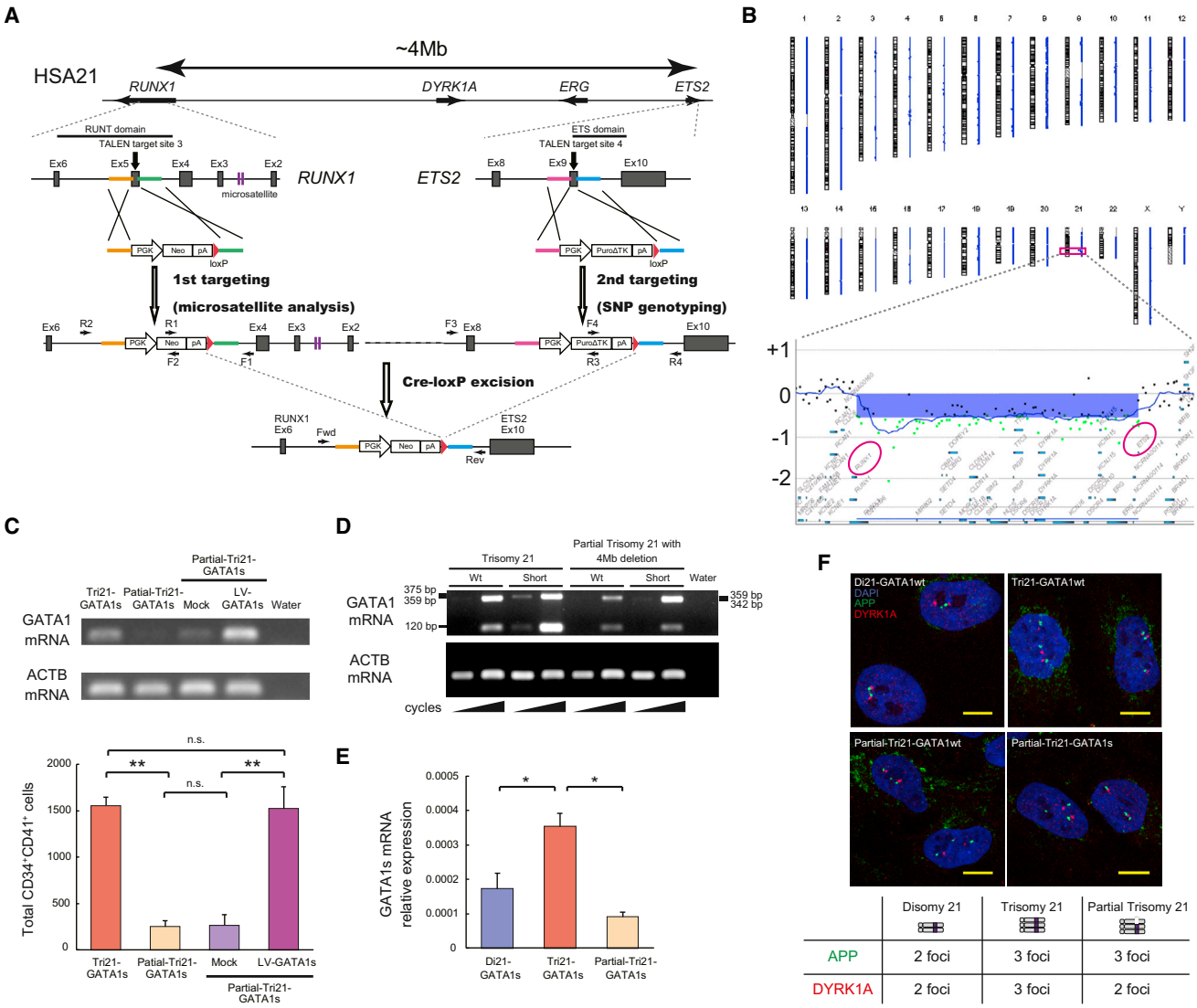

Figure 5. Trisomy of the 4-Mb Region on Chromosome 21 Perturbs Megakaryocyte Development via GATA1s Upregulation

(A) Schematic overview depicting the targeting strategy for the Partial-Tri21-GATA1wt iPSC line. Enlarged schemas indicate each gene locus, including exons (gray boxes), TALEN target sites (arrows), microsatellite sequences (purple stripe), homology arms (*RUNX1*, orange and green bars; *ETS2*, pink and blue bars), and functionally important domains (*RUNX1*, RUNT domain; *ETS2*, ETS domain) (black bars). Below are schemas of each donor plasmid including homology arms, human PGK promoters, drug-resistance genes (Neo or PuroΔTK), poly(A) sequences, *loxP* sequences (red triangles), and the primers (black arrows) used in junctional PCR analysis. PuroΔTK, fusion protein of puromycin and a truncated version of herpes simplex virus type 1 thymidine kinase.

(B) Array CGH profile of the Partial-Tri21-GATA1wt iPSC line compared with Tri21-GATA1wt iPSCs. Top: complete genomic view (vertical orientation) created by Agilent Genomic Workbench. Bottom: expanded chromosome view (horizontal view) of the 4-Mb region. x and y axes indicate the genomic location and intensity log2 ratio, respectively. The 4-Mb region located between *RUNX1* and *ETS2* is indicated by a blue-translucent rectangle.

(C) Top: semiquantitative RT-PCR analysis of GATA1s expression in sorted $CD34^+CD38^-$ progenitors derived from lentivirus-transduced iPSCs on day 8. Bottom: average $CD34^+CD41^+$ cell number per well on day 15 (n = 3 per line).

(D) Representative photomicrographs showing semiquantitative RT-PCR products for GATA1 and β-actin mRNAs in each iPSC line. The short-form product was detected as a 120-bp band. Expected fragment sizes: Tri21-GATA1wt, 359/120 bp; Tri21-GATA1s, 375/120 bp; Partial-Tri21-GATA1wt, 359/120 bp; Partial-Tri21-GATA1s, 342/120 bp (see also Figures 2H and 4I).

(E) Relative expression levels of GATA1s to β-actin in $CD34^+CD38^-$ cells (day 8) derived from Di21-, Tri21-, and Partial-Tri21-GATA1s EBs (n = 3 per line).

(F) Fluorescence in situ hybridization of chromosome 21-specific probes for each hiPSC line. The *APP* gene, which is located outside the 4-Mb region, was labeled with a green probe, while the *DYRK1A* gene, which is located inside and was deleted in partial trisomy 21 iPSCs, was labeled with a red probe. DAPI-stained nuclei are in blue. Expected number of *APP* and *DYRK1A* foci in each iPSC line is shown below. Scale bars represents 10 μm.

Error bars represent SEM. Student's t tests were used to compare Tri21-GATA1s iPSC line and the other lines. Paired t tests were used to compare between mock- and GATA1s-transduced iPSCs. *$p < 0.05$, **$p < 0.01$. See also Figure S5.

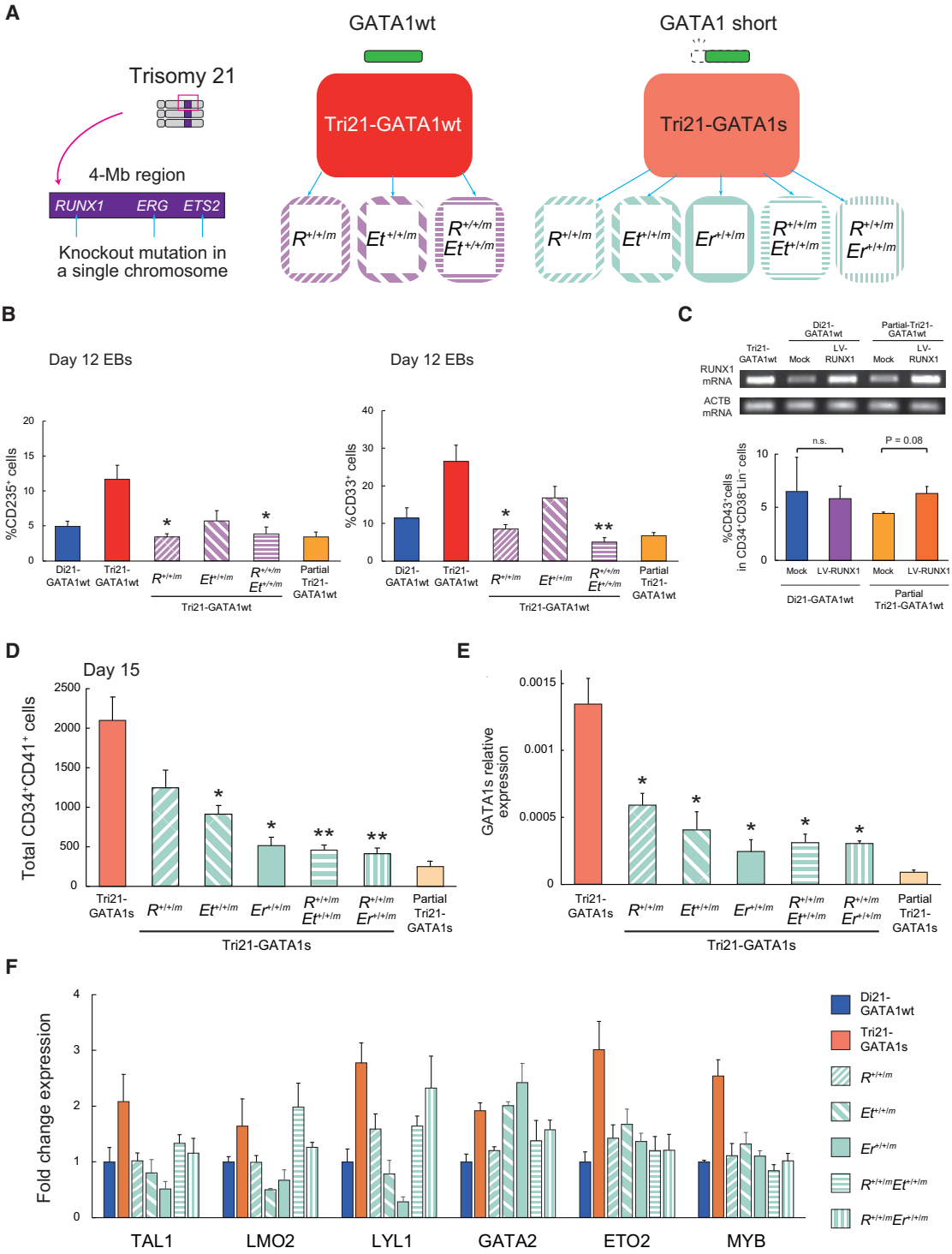

Figure 6. RUNX1, ETS2, and ERG Are Critical Regulators of Hematopoietic Abnormalities in Trisomy 21

(A) Schematic illustration of an additional eight types of iPSC lines. One or two candidate genes in the 4-Mb region were disrupted in a single chromosome in the Tri21-GATA1wt or -GATA1s iPSC line (*RUNX1*, $R^{+/+/m}$; *ETS2*, $Et^{+/+/m}$; *ERG*, $Er^{+/+/m}$; *RUNX1* and *ETS2*, $R^{+/+/m}Et^{+/+/m}$; and *RUNX1* and *ERG*, $R^{+/+/m}Er^{+/+/m}$).

(B) Percentage of CD235- and CD33-positive cells derived from day 12 EBs (n = 4–16 per line).

(C) Top: semiquantitative RT-PCR analysis of RUNX1 expression in sorted $CD34^+CD38^-Lin^-$ cells derived from lentivirus-transduced iPSCs on day 8. Bottom: percentage of CD43-positive cells among $CD34^+CD38^-Lin^-$ cells derived from day 8 EBs (n = 3 per line).

(D) Average number of total $CD34^+CD41^+$ cells per well on day 15 (n = 3–5 per line).

(legend continued on next page)

($R^{+/+/m}Et^{+/+/m}$-Tri21-GATA1s) as well as *RUNX1* and *ERG* ($R^{+/+/m}Er^{+/+/m}$-Tri21-GATA1s) significantly decreased the generation of $CD34^+CD41^+$ megakaryoblasts. Importantly, this reduction was accompanied by decreased mRNA expression of GATA1s (Figure 6E). Taken together, the increased gene dosage of *RUNX1*, *ETS2*, and *ERG* perturbs megakaryocyte differentiation synergistically via GATA1s upregulation.

Hematopoiesis is tightly controlled by numerous key transcription factors that form a densely interconnected auto-regulatory network (Sive and Göttgens, 2014; Wilson et al., 2011). The expression levels of RUNX1, ETS2, and ERG in Tri21-GATA1wt iPSCs were increased by ~1.8-fold compared with those in Di21-GATA1wt iPSCs, which were slightly higher than the expected change in gene dosage (i.e., 1.5-fold; data not shown). The upregulation of these three genes was further enhanced up to 2- to 3-fold by GATA1s in Tri21-GATA1s iPSCs (Figure S5A). It is known that GATA1, TAL1, and LYL1 are involved in a complex combinatorial interaction network in hematopoietic stem/progenitor cells, together with RUNX1, ETS2, and ERG (Wilson et al., 2010). Intriguingly, mRNA expression levels of these transcriptional factors at the early developmental stage were markedly upregulated in Tri21-GATA1s iPSCs compared with those in Di21-GATA1wt iPSCs (Figure 6F). The level of expression of TAL1, a key regulator of hematopoietic gene expression that acts as an oncogene when expressed aberrantly, was approximately doubled in trisomy cells and decreased by reductions in the dosage of *RUNX1*, *ETS2*, or *ERG*. The expression of both LMO2, a scaffolding protein involved in the TAL1 complex (Ryan et al., 2008), and LYL1, a paralog of TAL1 (Curtis et al., 2012), showed similar increases in trisomic cells and decreases by gene modification to some extent, suggesting the presence of an interconnected circuit involving these factors. In addition, ETO2, the transcriptional corepressor, and MYB, an essential regulator of definitive hematopoiesis (Mucenski et al., 1991), were upregulated ~3-fold in trisomy 21 and returned to normal levels in *RUNX1*-, *ETS2*-, and *ERG*-targeted trisomy cells. These findings imply that these molecules are distinct targets of a dysregulated network leading to aberrant hematopoiesis.

DISCUSSION

The pathological phenotypes of DS are likely to result from extra copies of dosage-sensitive genes located in HSA21. Genotype/phenotype studies in partial trisomy 21 patients suggest that there are critical segment(s) responsible for DS pathogenesis (commonly referred to as the Down syndrome critical region) (Korbel et al., 2009; Lyle et al., 2009). However, no studies have successfully determined the minimal range of the region(s) and proved causal genotype/phenotype relationships experimentally. Our cellular disease models enabled us to identify a 4-Mb segment as a strong candidate for the pathogenesis of hematopoietic abnormalities.

Further differentiation experiments using these iPSC models demonstrated that the hematopoietic defects in DS progress via two steps: (1) constitutive trisomy 21 causes RUNX1-mediated but GATA1-independent acceleration of early hematopoiesis, and (2) the acquired GATA1s mutation leads to RUNX1/ETS2/ERG-mediated and GATA1s dose-dependent hyperproliferation of aberrant megakaryoblasts in TMD.

Normal definitive hematopoiesis is maintained by the two copies of chromosome 21 and the full-length form of GATA1 (Figure 7, left). In the first step, a one-copy gain of the *RUNX1* gene to three copies plays a critical role (Figure 7, middle). RUNX1 is essential for the emergence of HSCs from the hemogenic endothelium through a process called "endothelial-to-hematopoietic transition" (EHT), and its deficiency leads to a significant decrease in the numbers of hematopoietic precursors (Chen et al., 2009; Lancrin et al., 2009). Our results demonstrated a remarkably sensitive response of the generation of $CD43^+$ cells to only a 1.5-fold increase in the *RUNX1* copy number, indicating that trisomy 21 may exert its effect on early hematopoiesis through the acceleration of this EHT process (Figures 3D, S6B, and S6C). The RUNX1-mediated acceleration of early hematopoiesis expanded the HSC pool and contributed to subsequent increases in the numbers of differentiated hematopoietic cells in the presence of the GATA1 full-length form. Our lentiviral transduction experiments showed that the enforced expression of RUNX1c induced no change in Di21-GATA1wt iPSCs and a modest, but not significant, enhancement in Partial-Tri21-GATA1wt iPSCs in terms of hematopoietic commitment. This suggests the existence of other dosage-sensitive genes both inside and outside the 4-Mb region, in addition to the essential function of RUNX1. Coexpression experiments of ETS2, ERG, DYRK1A, or BACH1, which are also implicated in hematopoiesis, should be performed (Chou et al., 2012; Malinge et al., 2012).

In the second step, an acquired somatic GATA1s mutation perturbs a transcriptional network involved in the regulation of fetal HSCs, in close synergy with trisomy 21 (Figure 7, right). Pathogenic crosstalk between constitutive trisomy 21 and the specific GATA1s mutation is most typically observed in megakaryocyte development. A recent study showed the combinatorial effects of trisomy 21 and GATA1s on erythrocyte and megakaryocyte differentiation and successfully elucidated the functional roles of the GATA1 N terminus in erythropoiesis (Byrska-Bishop et al., 2015). Our current study confirms most of these results and further shifts the focus to the underlying mechanism of the synergistic interaction between trisomy 21 and GATA1s. Notably, we found that GATA1s expression is significantly upregulated by trisomy 21, leading to further excessive production of aberrant megakaryocytes.

(E) Relative expression levels of GATA1s in $CD34^+CD38^-Lin^-$cells derived from GATA1s iPSC lines. Red, Tri21-GATA1s iPSC line; light blue, Tri21-GATA1s iPSC lines in which one or two genes were disrupted in a single chromosome (day 8; n = 3).

(F) Fold changes of the expression of hematopoietic transcription factors in $CD34^+CD38^-Lin^-$cells derived from gene-edited Tri21-GATA1s iPSC lines compared with the Di21-GATA1wt iPSC line (day 8; n = 3).

Error bars represent SEM. Student's t tests or Mann–Whitney *U* tests were used to compare Tri21-GATA1wt or -GATA1s lines and the others. Paired t tests were used to compare between mock- and RUNX1-transduced iPSCs.*$p < 0.05$, **$p < 0.01$. See also Figure S6.

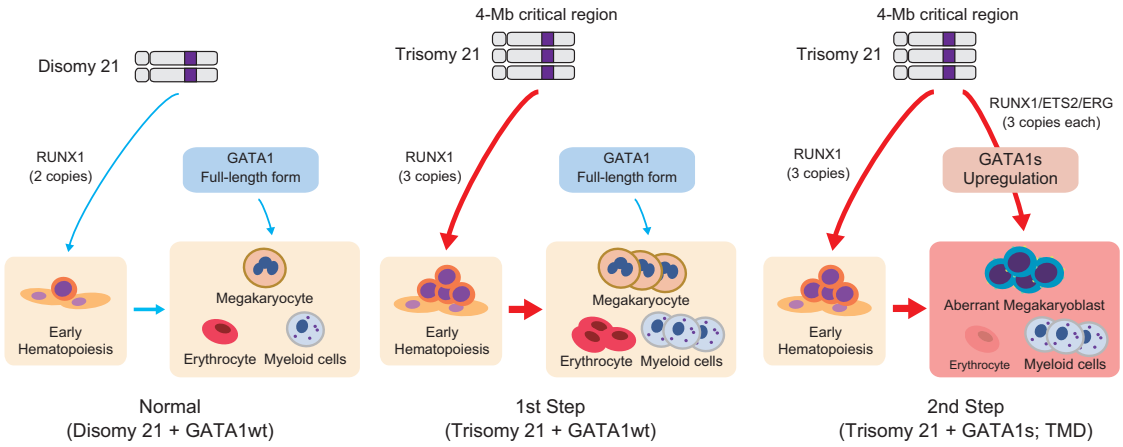

Figure 7. Synergistic Interactions between Trisomy 21 and GATA1 Mutation during Hematopoiesis

Normal hematopoiesis is maintained in the presence of disomy 21 and the GATA1 full-length form. Moderate expression of the two copies of the *RUNX1* gene is essential for early hematopoiesis. The GATA1 full-length form supports normal differentiation of megakaryocyte and erythrocyte lineages (disomy 21 + GATA1wt, left). Constitutive trisomy 21 (the first step) involving a 1.5-fold increase in the *RUNX1* gene dosage promotes early hematopoiesis, resulting in an expanded hematopoietic progenitor pool and increased numbers of differentiated cells of multiple lineages (trisomy 21 + GATA1wt, middle). The GATA1 full-length form is indispensable for normal differentiation of erythrocyte and megakaryocyte lineages, and the absence of the full-length form severely impairs erythropoiesis and megakaryopoiesis. In addition, the GATA1 short-form mutation (the second step) causes exclusive generation of aberrant megakaryoblasts. Trisomy 21 accelerates abnormal megakaryocyte differentiation through not only enhancement of early hematopoiesis but also upregulation of GATA1s expression, which is mediated by dosage alterations in *RUNX1*, *ETS2*, and *ERG* (trisomy 21 + GATA1s, right).

The GATA1 protein forms a pentameric complex by interacting with TAL1, LMO2, LDB1, and E2A, which constitutes a core transcriptional circuit through direct/indirect binding to RUNX1, ETS2, and ERG (Rainis et al., 2005; Taoudi et al., 2011). In leukemic cells, these transcription factors form a positive feed-forward loop (Sanda et al., 2012), and even a subtle increase in the gene dosage of some network members on HSA21 (i.e., *RUNX1*, *ETS2*, and *ERG*) can upregulate the entire network and robustly activate the key target molecules RUNX1 and GATA1s by up to nearly 3-fold. Increased expression of GATA1s enhances the generation of aberrant megakaryocytes in a dose-dependent manner, and the expansion of the $CD43^+$ progenitor pool, which is stimulated by RUNX1, further accelerates this abnormal differentiation. Intriguingly, the proportion of $CD43^+$ progenitor cells (as well as the generation of subsequent $CD235^+$ cells and $CD33^+$ cells) was even smaller in the Partial-Tri21-GATA1wt iPSC line than in the Di21-GATA1wt iPSC line (Figures 6B and S6B). Similarly, the double-positive $CD34^+$ $CD41^+$ cell population was smaller in the Partial-Tri21-GATA1s iPSC line than in the Di21-GATA1s iPSC line (Figure S5J), which was accompanied by lower GATA1s expression (Figure 5E). These observations indicate that there may be an additional key molecule that functions as a suppressor of the hematopoietic transcriptional network outside the 4-Mb region.

In addition to TMD and ML-DS, neonatal DS patients may have a variety of hematological abnormalities such as neutrophilia and polycythemia (Choi, 2008; Henry et al., 2007). The data obtained in the present study are consistent with these DS symptoms and provide an explanation for the clinical findings by suggesting possible causative factors in HSA21 and cross-regulatory interactions between aneuploidy and specific gene mutations. Our results suggest that many pathological features observed in DS are caused not by the simple summation of individual gene-dosage effects but by the complex orchestration of critical regulators. Our collection and engineering of DS-derived iPSC lines will provide an unprecedented experimental platform for detailed comparative analyses of this multi-step and multi-factorial disease process and may contribute to the development of new therapies.

EXPERIMENTAL PROCEDURES

Full details are provided in the Supplemental Experimental Procedures.

Generation of Human iPSCs

Induced pluripotent stem cells (iPSCs) were generated using a Sendai virus (SeV) vector encoding tetracistronic factors (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) with the miR-302 target sequence (SeVp [KOSM302L]) as described previously (Nishimura et al., 2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.031.

AUTHOR CONTRIBUTIONS

K.B. and Y.K. conceived and designed the experiments and wrote the manuscript. K.B. conducted most of the experiments. S.O. constructed TALENs and generated partial trisomy iPSC lines. K.H., N. Nawa, and N. Nakagawa assisted with iPSC culture and differentiation experiments. K.N., N.O., and M.N. generated the Sendai virus. T.S. and Takashi Yamamoto generated Platinum TALENs. T.T. and E.I. performed biochemical analysis. Toshiyuki Yamamoto performed the CGH array analysis. C.K. and J.T. provided vectors and supervised the experimental work. C.K. helped with writing of the manuscript. H.T., H.A., K.W., and Y.K. collected clinical data to characterize patients for iPSCs

establishment. K.O. and Y.K. supervised the entire project. All authors reviewed and commented on the manuscript.

ACKNOWLEDGMENTS

We thank S. Nishikawa at JT Biohistory Research Hall for encouragement and advice regarding this project as well as M. Tokunaga at Osaka University and K. Horie at Nara Medical University for helpful discussions and advice. We thank A. Nishida for providing technical assistance and Y. Hashii, T. Okuda, and T. Yokota (Osaka University), M. Furue-Kusuda (National Institute of Biomedical Innovation), and T. Tamaoki (Hyogo College of Medicine) for assistance with maintenance, differentiation, and analysis of iPSCs. This work was supported by Precursory Research for Embryonic Science and Technology (PRESTO) of the Japan Science and Technology Agency, a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#25461546 to Y.K.), a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows from JSPS (#A2640750 to S.O.), and the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development (AMED). This work was also supported in part by the Japan Foundation for Pediatric Research and the Takeda Science Foundation.

Received: August 15, 2015
Revised: December 22, 2015
Accepted: April 4, 2016
Published: April 28, 2016

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