Single-cell transcriptomics reveal synergistic and antagonistic effects of T21 and GATA1s on hematopoiesis

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Abstract

Trisomy 21 (T21), or Down syndrome (DS), is associated with baseline macrocytic erythrocytosis, thrombocytopenia, and neutrophilia, and transient abnormal myelopoiesis (TAM) and myeloid leukemia of DS (ML-DS). TAM and ML-DS blasts both arise from an aberrant megakaryocyte-erythroid progenitor and exclusively express GATA1s, the truncated isoform of *GATA1*, while germline *GATA1s* mutations in a non-T21 context lead to congenital cytopenias without a leukemic predisposition. This suggests that T21 and GATA1s perturb hematopoiesis independently and synergistically, but this interaction has been challenging to study in part due to limited human cell and murine models. To dissect the developmental impacts of GATA1s on hematopoiesis in euploid and T21 cells, we performed a single-cell RNA-sequencing timecourse on hematopoietic progenitors (HPCs) derived from isogenic human induced pluripotent stem cells differing only by chromosome 21 and/or *GATA1* status. These HPCs were surprisingly heterogeneous and displayed spontaneous lineage skew apparently dictated by T21 and/or GATA1s. In euploid cells, GATA1s nearly eliminated erythropoiesis, impaired MK maturation, and promoted an immature myelopoiesis, while in T21 cells, GATA1s appeared to compete with the enhanced erythropoiesis and suppressed megakaryopoiesis driven by T21 to give rise to immature erythrocytes, MKs, and myeloid cells. T21 and GATA1s both disrupted temporal regulation of lineage-specific transcriptional programs and specifically perturbed cell cycle genes. These findings in an isogenic system can thus be attributed specifically to T21 and GATA1s and suggest that these genetic changes together enhance HPC proliferation at the expense of maturation, consistent with a pro-leukemic phenotype.

Introduction

Trisomy 21 (T21), or Down syndrome (DS), is associated with baseline macrocytic erythrocytosis, thrombocytopenia, and neutrophilia particularly in the newborn period,[1–4] and transient abnormal myelopoiesis (TAM) and myeloid leukemia of DS (ML-DS).[5,6] TAM and ML-DS blasts are characterized by somatic mutations in the transcription factor *GATA1* (GATA-binding protein 1), which is essential for normal erythropoiesis, megakaryopoiesis, and myelopoiesis;[7,8] the mutations result in exclusive expression of the amino (N) terminus-truncated isoform GATA1s[9–11] with impaired transactivation activity.[9,12–15] Both myeloproliferative disorders are attributed to an aberrant megakaryocytic-erythroid progenitor (MEP),[16,17] and the blasts typically carry a megakaryocyte (MK)-like surface marker signature.

The mechanisms by which T21 and GATA1s exert their hematopoietic phenotypes remain incompletely understood. In individuals without T21, germline *GATA1s* mutations lead to congenital cytopenias but are not associated with a leukemic predisposition,[18,19] while co-occurring *GATA1s* mutations and acquired T21 in leukemic clones give rise to a ML-DS-like phenotype.[20–22] Previous studies have shown that the extra copy of chromosome 21 (HSA21) itself perturbs histone modifications throughout the genome and that the addition of a *GATA1s* mutation further perturbs acetylation specifically at genes involved in hematopoiesis and cell-cycle regulation,[23,24] suggesting that genome-wide gene dysregulation underlies the hematopoietic and other end-organ manifestations of DS. These findings emphasize the unique genetic interactions between T21 and *GATA1*.

Prior murine DS models have only partially recapitulated human disease: HSA21 orthologs map to three different murine chromosomes,[25] DS mice tend to exhibit macrocytic anemia,[26,27] and crosses between DS and *Gata1s* mice do not develop leukemia.[26,28,29] Studies of primary human samples have been limited by the individual variation in genetic backgrounds that overwhelm the transcriptional changes resulting from T21 and/or GATA1s,[30,31] requiring isogenic or large-scale approaches.[24,32] These challenges underscore the value of human model systems in which HSA21 or GATA1

can be manipulated in isolation.

Human T21 induced pluripotent stem cell (iPSC) models accurately reflect DS hematopoietic phenotypes.[33,34] T21 increases erythropoiesis, while GATA1s severely impairs erythroid development but enhances MK and myeloid proliferation in both euploid and T21 backgrounds. However, bulk assessments obscure the heterogeneity of hematopoietic progenitor cell (HPC) populations,[35,36] including those derived from iPSCs.[34,37,38] Thus, to further dissect the developmental impacts of GATA1s on hematopoiesis in the euploid and T21 contexts, we performed single-cell RNA sequencing (scRNA-seq) of multipotent and late HPCs derived from isogenic human iPSCs differing only by HSA21 copy number and/or GATA1 status. Our studies reveal individual and synergistic effects of T21 and GATA1s on hematopoietic development that appear to have distinct kinetics and are lineage-dependent. In euploid cells, GATA1s nearly eliminated erythropoiesis, impaired MK maturation, and promoted myelopoiesis, while in T21 cells, GATA1s appeared to compete with the enhanced erythropoiesis and suppressed megakaryopoiesis driven by T21 to give rise to immature erythrocytes, MKs, and myeloid cells, consistent with a pro-leukemic phenotype.

Methods

Generation of isogenic iPSCs

Under a protocol approved by the Institutional Review Board (IRB) at the Children's Hospital of Philadelphia (CHOP), peripheral blood was collected from an individual who presented with TAM and then in remission. Isolated PBMCs were reprogrammed with Sendai virus expressing the Yamanaka factors as previously described[39,40] to yield isogenic T21/GATA1s and T21/wild-type GATA1 (wtGATA1) iPSC clones.[41] The isogenic euploid/GATA1s clone was isolated from a T21/GATA1s clone that spontaneously lost its redundant copy of HSA21.[41,42] The CRISPR/Cas9 nuclease system and gRNAs (Integrated DNA Technologies) targeting the individual's known *GATA1* mutation were used to generate the isogenic euploid/wtGATA1 clone from the euploid/GATA1s clone (**Figure 1A**). Successful editing was confirmed by TOPO cloning. All iPSC lines were characterized by CNV (copy number variation) analysis, karyotype, *GATA1* Sanger

sequencing (GENEWIZ, Inc.), and Western blot for GATA1 (**Supplemental Figure 1**). Sequences of gRNAs, oligonucleotides for homologous recombination, and PCR and sequencing primers (Integrated DNA Technologies) are shown in **Supplemental Table 1**.

Western blot

Cells were lysed in Pierce RIPA Buffer (ThermoFisher) with 1% Halt Protease Inhibitor and Phosphatase Inhibitor Cocktails (ThermoScientific). The protein contents of extracted samples were normalized using the Pierce BCA Protein Assay Kit (ThermoFisher) according to the manufacturer's instructions, fractionated on Bolt 4-12% Bis-Tris gradient gels (Invitrogen), and transferred to 0.2 µm PVDF membranes (GE Healthcare). SuperSignal™ West Femto Maximum Sensitivity Substrate was used for imaging. Primary and secondary antibodies are shown in **Supplemental Table 2**.

Hematopoietic differentiation of iPSCs

iPSCs were cultured and differentiated into hematopoietic cells by embryoid body (EB) formation using modifications on a protocol previously described by our laboratory.[33,34,41,43] On the initial day of differentiation (Day -2), iPSCs were incubated with Accutase (ThermoFisher Scientific) at 37°C x 2 minutes, then washed with phosphate-buffered saline (PBS) x 2 to remove the mouse embryonic fibroblast feeder layer. The iPSCs were resuspended in human embryonic stem media with 10 ng/mL of bFGF (beta fibroblast growth factor, Invitrogen) (HES-10) and ROCK inhibitor (10 µM final concentration, Cayman Chemical) as aggregates of 5-10 cells each at a density of 1.5-2 x 10^6 cells/2mL per well in 6-well ultra-low attachment plates (Corning).

Cells were maintained at 37 °C, 5% CO_2, and 5% O_2 on a rotary shaker at 100 rotations per minute. On Days -1 and 0, EBs were isolated by gravity x 20 minutes, then resuspended in HES-10 (Day -1) or StemPro-34 (Invitrogen) with glutamine (1%, Gibco), ascorbic acid (50 ng/mL, Sigma-Aldrich), monothioglycerol (3 µL/mL, Sigma-Aldrich), BMP-4 (bone morphogenic protein 4, 25 ng/mL, Invitrogen) and VEGF (vascular endothelial growth factor, 10 ng/mL, Invitrogen) (Day 0) and redistributed into the 6-well

plates. EBs were subsequently cultured in multi-cytokine differentiation media to induce mesoderm and hematoendothelial specification as previously described.[33,34]

Isolation of hematopoietic progenitors

On Day 7 of differentiation, HPCs were collected from the supernatant and disaggregated EBs. EBs were isolated by centrifugation at 600 rpm x 1 minute, then chemically disaggregated in collagenase B (Sigma-Aldrich) at 37 °C x 60 minutes and then 0.25% trypsin-EDTA (Gibco) at room temperature x 2 minutes followed by trypsin inactivation with 50% fetal bovine serum (FBS, GeminiBio) in IMDM (Iscove's Modified Dulbecco's Medium, Corning). EBs were then mechanically disaggregated by passing through a 20G needle/5mL syringe and filtered through a 40 µm cell strainer to remove stromal debris to isolate the HPCs. These were combined with HPCs isolated from the supernatant by additional centrifugation at 1200 rpm x 5 minutes and incubated with fluorescently-conjugated antibodies against CD41a and CD235a (**Supplemental Table 3**) in FACS buffer (0.1% bovine serum albumin (BSA), 0.0002% NaN_3, 0.005 M ethylenediaminetetraacetic acid (EDTA) in PBS) at 4 °C x 20 minutes. $CD41^+/CD235^+$ cells were sorted on a FACSAria Fusion (BD Biosciences) or MoFlo Astrios EQ (Beckman Coulter) (**Figure 1B**). On Days 9 and 11 of differentiation, late HPCs were collected from the supernatant only with sorting for live cells (**Figure 1B**).

Flow cytometry

Flow cytometric analysis was performed on a FACSCanto II (BD Biosciences) as previously described,[34] using conjugated antibodies for surface-marker staining (**Supplemental Table 3**). Data were analyzed using FlowJo Software (BD Biosciences).

Single-cell RNA-sequencing

Sorted HPCs were resuspended in 0.04% BSA in PBS at 1000 cells/µL. Samples were processed at the Center for Applied Genomics and Single Cell Technology Core at CHOP. 2×10^4 cells per sample were loaded onto gel beads-in-emulsion wells (10x Genomics), and cDNA libraries were prepared from extracted RNA using the Chromium Single Cell Platform (10x Genomics). cDNA quality and integrity were measured by TapeStation High

Sensitivity DNA analysis (Agilent). cDNA sequencing was performed by the High Throughput Sequencing Core (CHOP) using a standard 3' assay with the NovaSeq 6000 platform and S1 Reagent Kit v1.5 (Illumina).

Single-cell profiling data analysis

Raw base calls (BCL) files were demultiplexed into FASTQ files using CellRanger (v7.1.0)[44] *mkfastq*, then processed with *counts* to generate feature-barcode counts matrices aligned to the 10x Genomics Human reference GRCh38 (GENCODE v32/Ensembl98). Contaminating ambient RNA (SoupX v1.6.2),[45] doublets (DoubletFinder v2.0.3),[46] and cells with mitochondrial RNA (Seurat v4.4.0)[46] (**Supplemental Table 3**) were removed. High-quality data representing all differentiation time points were merged and individual sample objects normalized (*sctransform*) with Seurat. Samples were integrated using the top 6000 highly variable genes with the first 20 estimated principal components and clustered using the Louvain algorithm (resolution 0.4).

Cell clusters were manually annotated based on calculated module scores for cell lineage-specific expression of selected key hematopoietic marker genes (**Supplemental Table 5**) and previously published hematopoietic gene sets[34,47,48] (Seurat *AddModuleScore*). Differential gene expression was performed (Seurat *FindMarkers*) for cells with the same identity within each time point, comparing integrated GATA1s and wtGATA1 samples. Genes detected in ≥25% of the cells in the two samples, with a log-fold change ($\log_2$FC) >0.25 and adjusted *p*-value <0.05, were considered significantly differentially expressed. Gene set enrichment analysis (GSEA) was performed on differentially expressed genes (DEGs) using the fgsea (v1.30.0) R package[47,48] on MsigDB Hallmark (v7.5.1)[47,49] gene sets to identify enriched pathways (adjusted *p*-value <0.05). To simplify interpretation and assess the effects of GATA1s in each chromosomal context, comparisons were restricted to within euploid or T21 genotypes.

For RNA velocity analysis, sample-specific aligned bam files from CellRanger were quantified for spliced and unspliced abundances (velocyto v0.17.17),[50] and cell velocities

and latent times estimated with scVelo (v0.3.1).[51] Pre-processed data from Seurat and velocyto-derived expression matrices were merged, and the resulting dataset was filtered and normalized using the top 6000 highly variable genes with a minimum of 20 counts per gene in both spliced and unspliced matrices. Each cell's first- and second-order moments across its nearest neighbors were computed with the default parameters and velocities estimated in the dynamical mode.

Cell differentiation trajectories were inferred using the CytoTRACE[52] kernel of CellRank (v2.0.2).[53] CytoTRACE scores and pseudotimes were computed on the previously-generated scVelo count matrices. Transition probabilities were calculated, and we set the first four predicted macrostates, which neatly matched annotated cell lineages, as the target terminal states. Fate probabilities for cells to transition to the terminal states were estimated and driver genes determined.

Genes that promote stalled cell lineage maturation in T21/GATA1s were identified by fitting a regression model testing the effect of GATA1s on the ploidy conditions while treating the estimated latent times as a continuous covariate (i.e. GATA1*T21 + latent time) (MAST v1.30.0).[54] Genes upregulated in the MAST analysis (interaction coefficient > 0, false discovery rate <0.05) and also identified as lineage drivers by CellRank CytoTRACE (correlation coefficient >0, *q*-value <0.05) were considered putative genes involved in stalled lineage maturation. GSEA was performed on the resulting subset of driver genes for each target terminal state.

Statistical analysis

Continuous variables were assessed using the Wilcoxon rank-sum test and categorical data with the χ^2 test. A 2-tailed or adjusted *p*-value <0.05 was considered statistically significant.

Study approval

All patients or legal guardians provided written informed consent to participate in this study. The IRBs at CHOP and the University of Pennsylvania approved the study

protocols.

Results

HPCs demonstrate lineage bias in multi-cytokine media. Isogenic iPSCs differing only by HSA21 and/or GATA1 status were generated from an individual with DS (**Figure 1A**). Flow cytometry of HPCs generated from hematopoietic differentiation of these iPSCs (**Figure 1B**) showed that on Day 7, >80% of HPCs from each genotype (euploid/wtGATA1, euploid/GATA1s, T21/wtGATA1, T21/GATA1s) were $CD41^+/CD235^+$, indicating multipotency, but also had distinct profiles that suggested early biases towards the erythroid ($CD235^+$) or megakaryocytic ($CD41^+$) lineages (**Figure 1C, left**). HPCs exhibited further lineage biases on Days 9 and 11 despite continued exposure to multi-cytokine media (**Figure 1C, middle and right**). Notably, euploid/GATA1s HPCs progressively lost CD235 expression across the 3 timepoints, consistent with the clinical human phenotype of anemia associated with exclusive expression of GATA1s, but a subpopulation of T21/GATA1s HPCs surprisingly retained CD235 expression throughout.

scRNA-seq of flow-purified Days 7, 9, and 11 HPCs identified HPCs, HPCs with a megakaryocytic or an erythroid bias, MKs, erythroid cells, and myeloid cells (**Figure 2A-B**). On Day 7, T21 was associated with increased lineage bias, with smaller multipotent HPC populations compared to euploid that was not affected by GATA1s (**Table 1**). This underscores the heterogeneity of even purified $CD41^+/CD235^+$ Day 7 progenitors that can only be detected by single-cell approaches. Consistent with the flow cytometry, the scRNA-seq revealed the evolution of lineage-committed subpopulations over time (**Figure 2C-D**).

GATA1s impairs erythroid lineage commitment in euploid cells but results in an incomplete erythroid block in T21 cells. On Day 7, 21.9% of the euploid/wtGATA1 cells were erythroid-biased HPCs or erythroid compared to only 12.0% of the euploid/GATA1s (p <0.0001) (**Figure 2D; Table 1**), consistent with the anemia observed in individuals with germline *GATA1s* mutations. T21 was associated with an erythroid drive, consistent with the DS phenotype, but surprisingly T21/GATA1s cells exhibited the strongest erythroid

skew at this stage (T21/wtGATA1: 25.4% vs. T21/GATA1s: 31.7%, p <0.0001) along with a greater proportion of committed, rather than erythroid-biased, cells (**Figure 2C-E; Table 1**).

The suppressive effect of GATA1s on erythropoiesis became more apparent by Day 11. Committed erythroid cells were the most abundant in the euploid/wtGATA1 sample (30.3%) but negligible in the euploid/GATA1s sample (0.5%) (**Figure 2C-D, F; Table 1**). The early erythroid drive in T21/GATA1s cells was no longer as strong, with 17.9% total erythroid-biased HPCs or erythroid cells compared to 33.9% of T21/wtGATA1 (p <0.0001) (**Figure 2D, F; Table 1**). A greater proportion of these T21/GATA1s cells were erythroid-biased rather than erythroid (**Figure 2F**), further suggesting impaired, but not eliminated, erythropoiesis as a result of the competing effects of T21 and GATA1s.

GATA1s enhances megakaryopoiesis in euploid and T21 cells at distinct developmental timepoints. On Day 7, MK-biased HPCs and MKs together represented the largest subgroup of cells for all four genotypes (**Figure 2C-D; Table 1**). GATA1s enhanced megakaryocytic lineage skew in euploid cells (euploid/wtGATA1: 56.1% vs. euploid/GATA1s: 67.9%, p <0.0001) and furthermore appeared to promote early commitment, with a greater proportion of these cells representing MKs rather than MK-biased HPCs (euploid/wtGATA1 5.0% vs. euploid/GATA1s 9.5%, p <0.0001) (**Figure 2G**). However, these trends were reversed by Day 11. Out of the four genotypes, only euploid/wtGATA1 cells developed a distinct population of committed MKs (**Figure 2C**). The euploid/GATA1s sample contained fewer megakaryocytic cells overall (euploid/wtGATA1: 46.1% vs. euploid/GATA1s: 35.9%, p <0.0001) but disproportionately more MK-biased HPCs (**Figure 2H**).

GATA1s did not appear to strongly impact megakaryocytic lineage commitment in Day 7 T21 cells (**Figure 2D, G; Table 1**). Although T21 was associated with decreased megakaryocytic lineage skew on Day 11 (euploid/wtGATA1: 46.1% vs. T21/wtGATA1: 31.1%, p <0.0001), GATA1s restored an incomplete megakaryocytic drive: 42.6% of T21/GATA1s cells were identified in the megakaryocytic lineage, but they were primarily

MK-biased HPCs rather than committed MKs (**Figure 2H**). This suggests that the effects of T21 and GATA1s compete in not only erythropoiesis but also megakaryopoiesis to give rise to cells with impaired lineage commitment, reminiscent of the proliferative, aberrant MEP that underlies TAM.

GATA1s drives myelopoiesis in both euploid and T21 cells. On Day 7, a small minority of all genotypes were myeloid (**Figure 2C-D; Table 1**). The myeloid drive associated with GATA1s became more apparent over time, particularly in euploid cells; by Day 11, 55.1% of euploid/GATA1s cells were myeloid compared to 9.3% of euploid/wtGATA1 cells (p <0.0001) (**Figure 2D; Table 1**). At Day 11, T21 was associated with increased myelopoiesis compared to euploid (T21/wtGATA1: 30.8%, T21/GATA1s: 35.8% vs. euploid/wtGATA1: 9.3%; p <0.0001 for both) (**Figure 2C-D; Table 1**). In T21/GATA1s cells, the increased myelopoiesis of GATA1s appeared to be blunted by the T21-driven increases in erythropoiesis and megakaryopoiesis.

T21 and GATA1s independently and synergistically perturb gene regulation. GATA1s was associated with gene dysregulation in both euploid and T21 cells, although the number of DEGs varied with lineage and timepoint (**Figure 3A-B**). On Day 7, GATA1s in T21 cells overall resulted in more DEGs compared to euploid, except in HPCs and MKs (**Figure 3A**). Notably, the number of DEGs identified between euploid/wtGATA1 vs. T21/wtGATA1 also tended to be higher than that identified between euploid/wtGATA1 vs. euploid/GATA1s, suggesting that T21 and GATA1s independently and synergistically perturb gene regulation. By Day 11, GATA1s resulted in more DEGs in euploid HPCs, MK-biased HPCs, and MKs but more DEGs in T21 erythroid-biased HPCs, erythroid cells, and myeloid cells (**Figure 3B**). Of note, the erythroid analyses were extremely limited on Day 11 as there were almost no euploid/GATA1s erythroid cells (**Table 1**).

The mean $\log_2$FC of the DEGs tended to be highest between T21/wtGATA1 vs. T21/GATA1s cells and lowest between euploid/wtGATA1 vs. T21/wtGATA1. Genes associated with the cell cycle, p53 network, histones, NF-κB network, globins, and platelet development and function had the greatest $\log_2$FC, particularly in euploid/wtGATA1 vs.

T21/wtGATA1 and T21/wtGATA1 vs. T21/GATA1s cells (**Figure 3C-D)**. Overall, many DEGs were identified recurrently **(Supplemental File 1**), and the timepoint- and lineage-specific differences again suggest individual and synergistic effects of T21 and GATA1s on hematopoiesis.

GATA1s in both euploid and T21 cells exerts distinct effects on the cell cycle depending on lineage and timepoint. GSEA analysis of DEGs identified in Day 7 euploid/wtGATA1 vs. euploid/GATA1s cells revealed enriched pathways in HPCs, HPCs – MK bias 1, erythroid-biased HPCs, erythroid cells, and myeloid cells. MYC targets, G2M checkpoint genes, and E2F targets were most significantly enriched in wtGATA1 cells (**Figure 3E**), but by Day 11 these pathways were enriched in MK-lineage cells with GATA1s (**Figure 3F**); this may reflect that GATA1s predominantly affects megakaryopoiesis at later stages to promote proliferation at the expense of terminal maturation.[55–64] On Day 11, heme metabolism was enriched in all wtGATA1 lineages, consistent with the severe block in erythropoiesis observed in euploid/GATA1s cells.

GSEA analysis of DEGs identified in Day 7 T21/wtGATA1 vs. T21/GATA1s cells revealed significantly enriched pathways in HPCs, HPCs – MK bias 1 and 2, MKs, and myeloid cells. MYC targets, G2M checkpoint genes, and E2F targets were again identified, this time in GATA1s rather than wtGATA1 cells, while conversely apoptosis was identified in wtGATA1 cells (**Figure 3G**). On Day 11, MYC targets were primarily enriched in wtGATA1 cells, but MYC targets, G2M checkpoint genes, and E2F targets were again enriched in GATA1s MKs (**Figure 3H**). Taken together, these findings suggest that the effect of GATA1s on cell cycle control is mediated by HSA21 context and stage of cell differentiation. Notably, both euploid and T21 MKs with GATA1s were enriched for MYC targets, G2M checkpoint genes, and E2F targets on Day 11, suggesting that GATA1s enhances aberrant, proliferative megakaryopoiesis regardless of HSA21 background.

T21 and GATA1s alter the kinetics of hematopoietic lineage maturation. Trajectory analysis of the integrated Days 7, 9, and 11 cells revealed the most mature cells in the periphery of the UMAP (**Figure 4A**). T21 was associated with impaired maturation of MKs

and myeloid cells but enhanced maturation of erythroid cells (**Figure 4B**). GATA1s was broadly associated with delayed maturation of all lineages in both chromosomal contexts and tended to have a greater impact with T21. This was particularly notable in the erythroid lineage, in which GATA1s skewed T21 cells from the most to the least mature of all genotypes.

Four terminal states—MK, erythroid, and myeloid 1 and 2—were inferred (**Figure 4C**), and these terminal states were validated using known marker genes (**Figure 4D**). It was not possible with the given data to distinguish the biological significance of the two terminal myeloid states. Latent time analysis of lineage-specific driver genes identified in this validation revealed the distinct dynamic transcriptional programs associated with each terminal state (**Figure 4E**).

GATA1s perturbs lineage maturation through dysregulation of transcriptional programs. Genes that may drive the stalled maturation of T21/GATA1s were identified from DEGs that were associated with wtGATA1 vs. GATA1s and also identified as lineage drivers.

Analysis of the driver genes for MK development contained 4 subsets of gene expression patterns (**Figure 5A**). A subset of closely segregated DEGs had expression levels that varied by genotype and appeared to have no relationship with pseudotime (**Figure 5A, blue**). In euploid/wtGATA1 cells, the expression level of the 2 largest gene subsets increased with pseudotime (**Figure 5A, purple/green**); a small subset of genes was highly expressed throughout maturation (**Figure 5A, yellow**). In euploid/GATA1s cells, these genes had globally lower and less uniformly controlled expression intensities (**Figure 5A, purple/green/yellow**). Both T21/wtGATA1 and T21/GATA1s cells failed to upregulate gene expression with pseudotime (**Figure 5A, purple/green**), and a subset of genes were inappropriately downregulated in T21/wtGATA1 cells and never expressed in T21/GATA1s cells (**Figure 5A, yellow**). Overall, T21/GATA1s was associated with the lowest expression intensities. Taken together, this is consistent with our findings that T21/GATA1s MKs exhibit a maturation block compared to T21/wtGATA1, which in turn are less mature than euploid/wtGATA1 (**Figure 4C**). These MK driver genes were

enriched most notably for cell cycle pathways (MYC targets, G2M checkpoint, E2F targets, mitotic spindle); heme metabolism, which may reflect aberrant expression of an erythroid program; and mTORC1, which is required megakaryopoiesis and specifically for maturation of neonatal MKs, and upregulates *GATA1*[65,66] (**Figure 5B**).

Erythroid driver genes were progressively upregulated in euploid/wtGATA1 cells (**Figure 5C**). These genes were less uniformly regulated in euploid/GATA1s, with failure to upregulate some genes throughout development and premature upregulation of others. In contrast, in T21/wtGATA1 and T21/GATA1s cells, gene expression levels did not significantly change with pseudotime, and T21/GATA1s cells had the lowest expression intensity overall. These differentially regulated genes were again enriched for cell cycle pathways and processes essential to erythroid differentiation including mTORC1 signaling,[67–69] oxidative phosphorylation,[68] and heme metabolism (**Figure 5D**).

Finally, the two myeloid terminal states displayed distinct patterns of gene regulation. Although the number of genes driving maturation towards myeloid1 was limited, they again tended to be progressively upregulated in euploid/wtGATA1 cells (**Figure 5E, purple**). These genes were not as strongly upregulated in euploid/GATA1s cells and only moderately upregulated in cells with T21; a separate subset of genes was inappropriately upregulated throughout in T21/GATA1s (**Figure 5E, blue**). The myeloid2 driver genes segregated into three broad subsets: in euploid/wtGATA1 cells, one subset had expression apparently unrelated to pseudotime (**Figure 5F, green**), and two subsets increased in expression intensity with pseudotime (**Figure 5F, purple/blue**). Globally, the genes were insufficiently upregulated in euploid/GATA1s and T21/wtGATA1 cells. In contrast, two subsets of genes had inappropriately high expression levels in T21/GATA1s cells (**Figure 5F, green/blue**). The driver genes for both terminal myeloid states were enriched for E2F targets, G2M checkpoint genes and MYC targets (**Figure 5G-H**).

In all three lineages, both T21 and GATA1s were associated with disruption of coordinated transcriptional programs, and GATA1s had distinct effects depending on euploid vs. T21 context. The recurrent enrichment of cell cycle genes suggests that the

impaired maturation of T21/GATA1s cells is driven in part by an uncoupling between lineage development and proliferation, consistent with a myeloproliferative disease phenotype.

Discussion

Using a novel, isogenic human iPSC system, we dissect the effects of GATA1s on hematopoiesis in euploid and T21 cells as the basis of GATA1-related cytopenias vs. TAM. Although T21 and GATA1s have been previously studied in iPSC-derived hematopoietic cells,[33,34,60,70–73] our investigation of the perturbations in lineage commitment and maturation at the single-cell level reveal how HSA21 copy number mediates the consequences of GATA1s. In euploid cells, GATA1s nearly eliminates erythropoiesis, slows MK maturation, and has a variable effect on myeloid development, while in T21 cells, GATA1s competes with the erythroid drive of T21 and delays maturation of all 3 lineages. This is consistent with germline euploid *GATA1s* mutations typically causing anemia, dysplastic MKs, and occasionally neutropenia,[19,74–76] while T21/GATA1s perturbs an MEP and underlies both TAM and ML-DS. We demonstrate that the heterogeneity of HPCs is driven by genotype and is dynamic throughout development. Although our findings on the early erythroid bias of T21/GATA1s cells appears to contradict the phenotype that T21/GATA1s HPCs fail to differentiate into erythroid cells,[34] these findings instead suggest that the effects of T21 and GATA1s are cell stage-specific.

Our analyses suggest that temporal dysregulation of transcriptional programs underlies the hematopoietic perturbations, consistent with GATA1's role as a transcription factor and the impaired transactivation activity of GATA1s. The recurrent enrichment of MYC targets, E2F targets, and G2M checkpoint genes suggests a specific role for GATA1 in the cell cycle, proliferation, and maturation, particularly in MKs for which all 3 pathways were uniquely enriched on Day 11. Impaired *Myc* downregulation has been found in *Gata1s* murine MKs,[14] and conversely, sustained *c-MYC* overexpression in euploid iPSC-derived platelets has been associated with decreased GATA1 levels and impaired platelet function.[77] The N-terminus of GATA1 is required to block the interaction between E2F and its targets and thus control cell cycle progression, proliferation, and terminal

differentiation of HPCs into erythrocytes, MKs, and eosinophils.[60,78–80] The enrichment in G2M checkpoint genes we observed may reflect this disruption of E2F activity by GATA1s. Although our focus here was how GATA1s perturbs euploid and T21 hematopoiesis, we also found specific enrichment of these three pathways in T21/wtGATA1 MKs compared to euploid/wtGATA1 (data not shown). This suggests that HSA21 copy number modulates GATA1's interactions with MYC and E2F and remains an active area of study.

A limitation of our differentiation protocol is that it recapitulates primitive hematopoiesis, which represents the first wave of hematopoiesis in the extraembryonic yolk sac, while TAM is a disease of definitive fetal hematopoiesis. However, iPSC-derived T21/GATA1s HPCs that more closely recapitulate definitive hematopoiesis exhibited a similar incomplete suppression of erythropoiesis and a block in terminal MK maturation.[72] Our findings are also consistent with prior T21 murine models. The bone marrow and spleen of adult DS mice contained increased numbers of erythroid cells[26] and small, immature MKs,[26,28] similar to the erythroid drive and delayed MK maturation in our T21/wtGATA1 cells. Tc1Gata1s fetal livers contained increased erythroid progenitors although the mice were anemic,[26] reminiscent of the early erythroid drive and subsequent developmental block in our T21/GATA1s cells. Ts65Dn fetal liver cells with ectopic expression of GATA1s formed hyperproliferative, immature colonies on methylcellulose assays and were less mature *in vitro*,[14] echoing our prior and current findings of increased proliferative capacity[34,60] and impaired maturation of T21/GATA1s MKs.

While the hematopoietic phenotypes associated with GATA1s and DS have both been studied extensively, isolating the underlying mechanisms has been challenging in part due to stage- and lineage-specific effects that may not be captured in prior human models. iPSCs provide a source of human HPCs that are otherwise difficult to obtain, and our single-cell approach attempts to answer these questions by examining the heterogeneous evolution of isogenic HPCs. Given the extensive number of known GATA1 targets and the genome-wide driver genes identified here, our findings here are likely just one, albeit key, component of the perturbations in hematopoiesis. Future studies focusing on later waves of hematopoiesis may provide additional insight on these diseases.

Author Contributions

KT designed research studies, performed experiments, acquired data, interpreted the data, and wrote the manuscript. EW and DS analyzed data and edited the manuscript. SSK and AG performed experiments. DLF and CST interpreted the data and edited the manuscript. STC designed research studies, interpreted the data, and wrote the manuscript.

Acknowledgements

The authors thank members of the CHOP Human Pluripotent Stem Cell Core Laboratory, Flow Cytometry Core, Center for Applied Genomics, Single Cell Technology Core, and High Throughput Sequencing Core for their expertise. This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute T32 HL0007150-41 (KT) and R01 HL151260-01 (STC), American Society of Hematology Research Training Award for Fellows (KT), Doris Duke Charitable Foundation Physician Scientist Fellowship (KT), and Mizuno Fund in Hematology (KT). Figures were compiled and edited in BioRender.

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Table 1. Frequencies of cells assigned to hematopoietic lineages in integrated analysis of Days 7-11 of differentiation, by genotype. Boxed sections highlight key findings.

		HPCs	HPCs – Ery bias	Ery	HPCs – MK bias 1	HPCs – MK bias 2	MK	Myeloid
Day 7	Euploid/wtGATA1	2517 (19.4%)	1320 (10.2%)	1515 (11.7%)	2289 (17.7%)	4615 (35.6%)	362 (2.8%)	336 (2.6%)
	Euploid/GATA1s	1544 (17.0%)	709 (7.8%)	381 (4.2%)	2722 (30.0%)	2843 (31.4%)	582 (6.4%)	278 (3.1%)
	T21/wtGATA1	1209 (10.9%)	1458 (13.1%)	1367 (12.3%)	2490 (22.4%)	4014 (36.1%)	306 (2.7%)	284 (2.6%)
	T21/GATA1s	1322 (10.4%)	1774 (14.0%)	2243 (17.7%)	1776 (14.0%)	4708 (37.2%)	369 (2.9%)	463 (3.7%)
Day 9	Euploid/wtGATA1	591 (5.1%)	970 (8.3%)	635 (5.4%)	1801 (15.4%)	2747 (23.5%)	2747 (23.5%)	2193 (18.8%)
	Euploid/GATA1s	789 (5.6%)	285 (2.0%)	21 (0.1%)	2742 (19.3%)	2915 (20.6%)	1736 (12.2%)	5691 (40.1%)
	T21/wtGATA1	389 (3.0%)	1930 (15.1%)	1206 (9.4%)	2147 (16.8%)	3474 (27.2%)	2159 (16.9%)	1475 (11.5%)
	T21/GATA1s	2854 (18.9%)	1963 (13.0%)	1467 (9.7%)	1636 (10.8%)	4291 (28.4%)	1001 (6.6%)	1921 (12.7%)
Day 11	Euploid/wtGATA1	625 (4.1%)	1562 (10.2%)	4625 (30.3%)	1784 (11.7%)	2081 (13.6%)	3176 (20.8%)	1422 (9.3%)
	Euploid/GATA1s	554 (5.7%)	270 (2.8%)	48 (0.5%)	963 (9.9%)	1093 (11.3%)	1430 (14.7%)	5340 (55.1%)
	T21/wtGATA1	500 (4.2%)	1336 (11.1%)	2736 (22.8%)	1179 (9.8%)	1268 (10.6%)	1289 (10.7%)	3704 (30.8%)
	T21/GATA1s	457 (3.7%)	928 (7.6%)	1255 (10.3%)	1558 (12.8%)	2131 (17.5%)	1500 (12.3%)	4364 (35.8%)

Ery = erythroid; HPC = hematopoietic progenitor cell; MK = megakaryocyte

Supplemental Table 1. CRISPR/Cas9 targeting guide RNAs, oligonucleotides for homologous recombination, and PCR and sequencing primers.

	Sequence
GATA1 Exon 2 gRNA (5'-3')	TTAATCCCCAGAGGCTCCATGGG
GATA1 Exon 2 correction oligonucleotide (3'-5')	TGGGGGAGGGGCTCTGAGGTCCCCAGGGACCCCAGG CCAGGGAA**T**TCCATGGAGCCTCTGGGGATTAACCTGC GAGGACAGAAGGGGTCCTCAGACAC
GATA1 Exon 2 PCR and sequencing primers	F: GAAGGAGAAATATGGAGACTGAGGTGATGG R: GAGATATCCTCACAGTGGTATTCTGACCTA

Supplemental Table 2. Antibodies for Western blot.

Antibody	Primary/Secondary	Manufacturer	Catalog #	Dilution
GATA1	Primary	Cell Signaling Technology	4589	1:1000
GAPDH	Primary	Abcam	ab181602	1:10000
Rabbit IgG (HRP-conjugated)	Secondary	Cell Signaling Technology	7074	1:10000

Supplemental Table 3. Antibodies for flow cytometry.

Antibody	Fluorophore	Manufacturer	Catalog #	Dilution
CD18	FITC	Fisher Scientific	555923	1:20
CD235a	APC	BD Biosciences	551336	1:5000
CD34	APC	BioLegend	561209	1:100
CD41a	PE/Cy7	BioLegend	303718	1:800
CD42a	FITC	BD Biosciences	558818	1:20
CD45	APC	BD Biosciences	55485	1:20

Supplemental Table 4. Mitochondrial RNA content of each sample.

Sample		Mitochondrial RNA content
Day 7	Euploid/wtGATA1	25%
	Euploid/GATA1s	20%
	T21/wtGATA1	15%
	T21/GATA1s	15%
Day 9	Euploid/wtGATA1	15%
	Euploid/GATA1s	15%
	T21/wtGATA1	15%
	T21/GATA1s	25%
Day 11	Euploid/wtGATA1	15%
	Euploid/GATA1s	15%
	T21/wtGATA1	15%
	T21/GATA1s	15%

Supplemental Table 5. Manually selected hematopoietic genes for cluster annotation.

Gene	Associated lineage	Gene	Associated lineage	Gene	Associated lineage
ACHE	Ery	*CNGB1*	MK	*GATA3*	HPC
ACLY	MK	*COPA*	MK	*GFI1*	MK
ACSL1	Myeloid	*CREB1*	MK	*GFI1B*	MK
ACTB	Housekeeping	*CSF1R*	Myeloid	*GLO1*	Ery
ADD1	Ery	*CSF2RA*	Myeloid	*GP1BA*	MK
ADD2	Ery	*CSF3R*	Myeloid	*GP9*	MK
ALAS2	Ery	*CSTA*	Myeloid	*GPD2*	MK
ALB	MK	*CST3*	Ery	*GPI*	MK
ALDH1A1	Ery	*CTSE*	Ery	*GSR*	Ery
ANGPT1	HPC	*CXCL8*	Myeloid	*GTF2H4*	MK
ANK1	MK	*CYB5A*	Ery	*GYPA*	Ery
ARHGAP6	MK	*CYB5R3*	Ery	*HBA1*	Ery
ARHGAP15	HPC	*CYBB*	Myeloid	*HBA2*	Ery
ATP2B1	Ery	*DAAM1*	MK	*HBB*	Ery
ATP2B4	Ery	*DAAM2*	MK	*HBE1*	Ery
AZU1	Myeloid	*DCTN1*	MK	*HBG1*	Ery
BACH1	Ery	*DDI1*	MK	*HBZ*	Ery
BBS1	Ery	*DIAPH1*	MK	*HDHD1*	Ery
BCL11A	Ery	*DMTN*	Ery	*HK1*	MK
BIN2	MK	*DYNC1H1*	MK	*HMBS*	Ery
BLVRB	Ery	*DYRK1A*	MK	*HMHA1*	MK
BMI1	Ery	*EGR2*	Myeloid	*HOXA10*	HPC
CASS4	MK	*EHD1*	MK	*HOXA9*	HPC
CAT	Ery	*ENPP1*	MK	*HSP90AA1*	MK
CCDC181	MK	*EPB41*	Ery	*HSP90B1*	MK
CCL3	Myeloid	*EPB42*	Ery	*IFI16*	Myeloid
CCL4	Myeloid	*EPOR*	Ery	*IKZF1*	Myeloid
CCT3	MK	*ERG*	MK	*IKZF2*	Myeloid
CD34	HPC	*ETS2*	MK	*IL8*	Myeloid
CD44	Ery	*EVI1*	HPC	*INF2*	Myeloid
CD47	Ery	*F10*	MK	*IQGAP2*	HPC
CD58	Ery	*F5*	MK	*IRAK3*	Myeloid
CD74	Myeloid	*FGA*	MK	*IRF1*	Myeloid
CEBPA	Myeloid	*FGB*	MK	*IRF7*	Myeloid
CEBPB	Myeloid	*FLI1*	HPC/MK	*ITGA1*	MK
CEBPG	Myeloid	*FLNA*	MK	*ITGA4*	HPC
CFAP36	MK	*FOXO3*	HPC	*ITGA2B*	MK
CFH	Myeloid	*GABPA*	HPC	*ITGB3*	MK
CLC	Myeloid	*GAPDH*	Housekeeping	*JAK3*	Myeloid
CLTC	MK	*GATA1*	Ery	*KIF16B*	MK
CREM	MK	*GATA2*	HPC	*KIT*	Ery

Gene	Associated lineage	Gene	Associated lineage	Gene	Associated lineage
KLF1	Ery	*PRDX6*	Ery	*TRIM10*	Myeloid
LANCL1	Ery	*PRKAR2B*	MK	*TSTA3*	Ery
LDB1	Ery	*PRG2*	Myeloid	*UBE2O*	MK
LGMN	Myeloid	*PRG3*	Myeloid	*UBR4*	MK
LTBP1	MK	*PRTN3*	Myeloid	*UGGT1*	MK
LTF	MK	*PSPC1*	MK	*UGGT2*	MK
LYL1	Myeloid	*PYGB*	MK	*UNC13D*	MK
MED12L	MK	*PYGL*	MK	*UTRN*	MK
MEIS1	HPC	*RBM47*	Myeloid	*VCL*	MK
MMRN1	MK	*RNF220*	HPC	*VCP*	MK
MPL	Myeloid	*RCAN1*	Myeloid	*VDR*	Myeloid
MPO	Myeloid	*RUNX1*	HPC/MK	*VIM*	Myeloid
MPP1	Ery	*S100A8*	Myeloid	*VPS13A*	MK
MRC1	Myeloid	*S100A9*	Myeloid	*VWF*	MK
MS4A3	Myeloid	*SAMHD1*	Myeloid	*WDFY3*	MK
MT1E	Ery	*SDHA*	Housekeeping	*WDR1*	MK
MYB	HPC	*SELP*	MK	*WDR44*	MK
MYC	HPC	*SKP1*	Ery	*WNT10A*	HPC
MYO18A	MK	*SLC12A1*	MK	*WNT8B*	MK
NAB2	Myeloid	*SLC4A1*	Ery	*YARS*	MK
NBEAL1	MK	*SMAD1*	HPC	*ZC3H12C*	Myeloid
NBEAL2	MK	*SMC3*	MK	*ZFPM1*	MK
NCAM1	Myeloid	*SPINK2*	HPC	*ZNF559*	MK
NCF1	MK	*SON*	HPC	*ZSCAN12*	MK
NEXN	MK	*SOX17*	HPC		
NFE2	HPC	*SOX4*	HPC		
NFX1	HPC	*SOX6*	Ery		
NRP1	Myeloid	*SP1*	Myeloid		
P4HB	MK	*SPTA1*	MK		
PBX1	HPC	*SPTB*	MK		
PCMT1	Ery	*SPTBN2*	MK		
PDE4D	Myeloid	*SPTBN4*	MK		
PDIA4	MK	*STAT2*	Myeloid		
PDZD3	MK	*STAT3*	Myeloid		
PF4	MK	*STIP1*	MK		
PF4V1	MK	*STOM*	Ery		
PGK1	Ery	*SYTL4*	MK		
PIEZO2	MK	*TAF1B*	MK		
PLSCR1	Ery	*TAL1*	HPC		
PPBP	MK	*TFEC*	HPC		
PPIA	HPC	*TMC1*	MK		
PRDX2	Ery	*TPP2*	MK		

Figure Legends

Figure 1. Characterization of early hematopoietic progenitors differentiated from isogenic iPSCs. A) Generation of isogenic iPSCs differing by HSA21 and/or GATA1 status. B) Isolation and characterization of D7, D9, and D11 hematopoietic progenitors. C) Flow cytometry plots of D7, D9, and D11 hematopoietic progenitors differing only by HSA21 and/or GATA1 status. $CD41^+/CD235^+$ indicates multipotency, $CD41^+/CD235^-$ suggests megakaryocytic skew, $CD41^-/CD235^+$ suggests erythroid skew, and $CD41^-/CD235^-$ suggests myeloid skew.

Figure 2. scRNA-seq characterization of HPCs generated from isogenic iPSCs differing by HSA21 and/or GATA1 status. A) Annotated integrated UMAP of euploid/wtGATA1, euploid/GATA1s, T21/wtGATA1, and T21/GATA1s HPCs collected on Day 7, Day 9, and Day 11. B) Relative expression of canonical hematopoietic genes, organized by lineage/cluster. C) Individual annotated UMAPs for each genotype and timepoint. D) Relative proportions of cells in each lineage per genotype and timepoint. E) Percentage of erythroid-biased HPCs and committed erythroid cells within each genotype on Day 7. F) Percentage of erythroid-biased HPCs and committed erythroid cells within each genotype on Day 11. G) Percentage of MK-biased HPCs and committed MKs within each genotype on Day 7. H) Percentage of MK-biased HPCs and committed MKs within each genotype on Day 11. *p*-values reflect comparisons of relative proportions of biased HPCs vs. committed cells; ****$p < 0.0001$, ***$p = 0.0007$, **$p = 0.016$.
HPC = hematopoietic progenitor cell; MK = megakaryocyte.

Figure 3. Differentially expressed genes and regulated pathways in HPCs generated from isogenic iPSCs differing by HSA21 and/or GATA1 status. A) Number of DEGs between wtGATA1 vs. GATA1s D7 cells, organized by HSA21 status and lineage. B) Number of DEGs between wtGATA1 vs. GATA1s D11 cells, organized by HSA21 status and lineage. C) Magnitude of $\log_2$FC of DEGs between wtGATA1 vs. GATA1s D7 cells, organized by HSA21 status and lineage. D) Magnitude of $\log_2$FC of DEGs between wtGATA1 vs. GATA1s D11 cells, organized by HSA21 status and lineage. E) GSEA analysis of DEGs identified in D7 euploid cells, organized by lineage. F) GSEA analysis of DEGs identified

in D11 euploid cells, organized by lineage. G) GSEA analysis of DEGs identified in D7 T21 cells, organized by lineage. H) GSEA analysis of DEGs identified in D11 T21 cells, organized by lineage.
HPC = hematopoietic progenitor cell; MK = megakaryocyte; Ery = erythroid; NES = normalized enrichment score.

Figure 4. Trajectory analysis of hematopoietic cells generated from isogenic iPSCs differing by HSA21 and/or GATA1 status. A) Projection of latent time indicating degree of differentiation and lineage maturation, with 0 indicating least mature and 1 indicating most mature. B) Inferred mean trajectories for MK, erythroid, and myeloid cells across all timepoints, separated by genotype. C) Terminal states inferred by latent time analysis. D) Sample plot of gene expression, correlated with fate probabilities, to identify driver genes for all trajectories. Upper left quadrant identifies driver genes for erythroid development and lower right quadrant identifies driver genes for MK development. E) Heatmaps of pseudotemporal gene expression across all samples for the 4 lineage-specific terminal states identified in (C).
HPC = hematopoietic progenitor cell; MK = megakaryocyte.

Figure 5. Analysis of driver genes associated with stalled lineage maturation of cells with T21 and/or GATA1s. A) Heatmaps showing developmental expression along pseudotime for DEGs identified between euploid vs. T21 and wtGATA1 vs. GATA1s for A) MKs C) erythroid cells E) myeloid 1 cells and F) myeloid 2 cells. Enrichment analysis of these DEGs for B) MKs D) erythroid cells G) myeloid 1 cells and H) myeloid 2 cells.
MK = megakaryocyte; NES = normalized enrichment score.

Supplemental Figure 1. Characterization of isogenic iPSCs derived from single individual with TAM. A) CNV analysis of iPSCs. B) Karyotypes of iPSCs indicating 46,XY or 47,XY+21. C) Sanger sequencing of iPSCs showing the patient-specific c.4_5insG mutation in iPSC lines with GATA1s. Additional c.6G>A mutation in euploid/wtGATA1 line is silent mutation introduced during CRISPR/Cas9 gene targeting. D) Western blot showing expression of GATA1 and GATA1s in iPSCs.

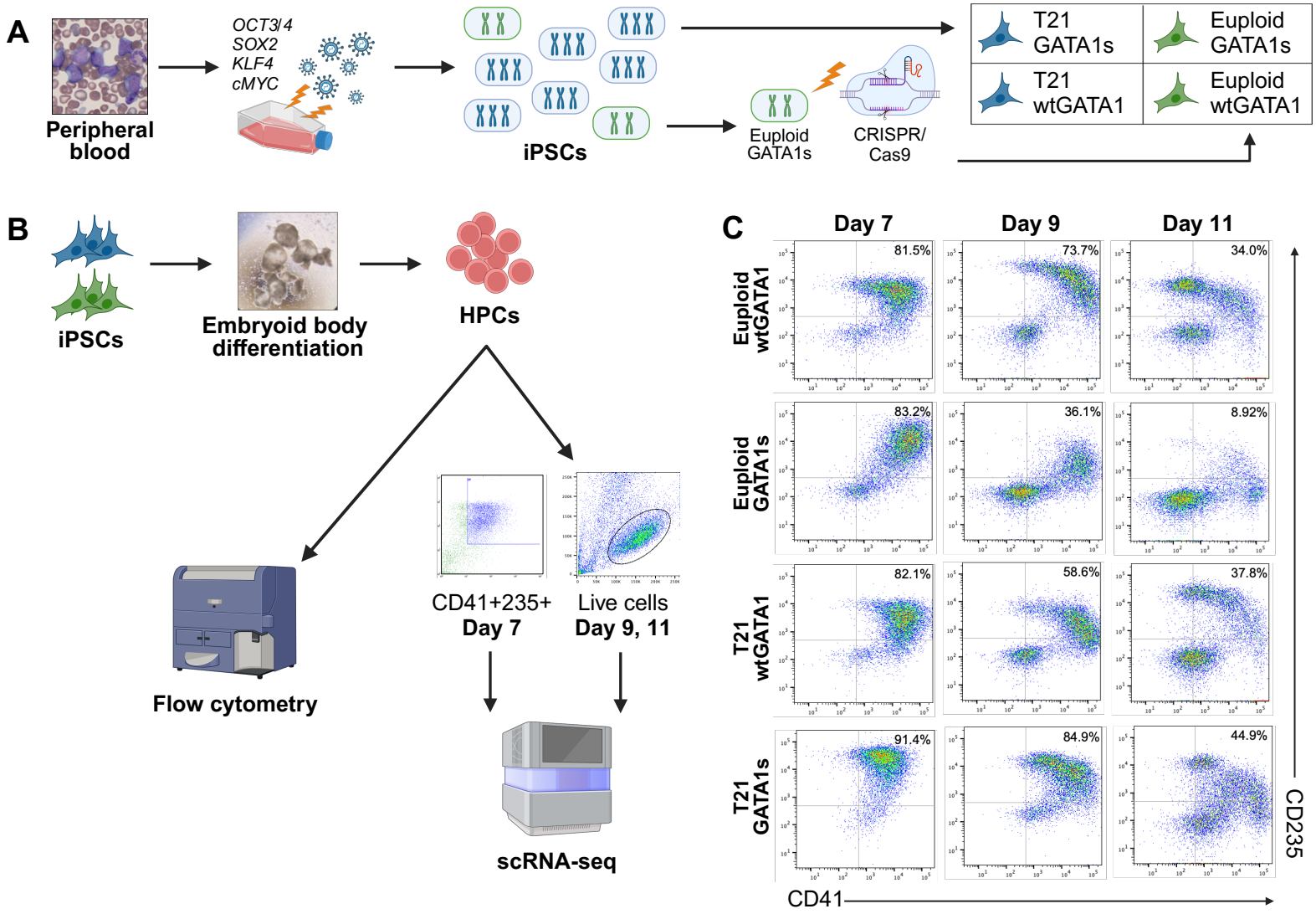

A
Peripheral blood
OCT3/4
SOX2
KLF4
cMYC
iPSCs
Euploid GATA1s
CRISPR/ Cas9
T21 GATA1s
Euploid GATA1s
T21 wtGATA1
Euploid wtGATA1
B
iPSCs
Embryoid body differentiation
HPCs
Flow cytometry
CD41+235+ Day 7
Live cells Day 9, 11
scRNA-seq
C
Day 7
Day 9
Day 11
Euploid wtGATA1
Euploid GATA1s
T21 wtGATA1
T21 GATA1s
81.5%
73.7%
34.0%
83.2%
36.1%
8.92%
82.1%
58.6%
37.8%
91.4%
84.9%
44.9%
CD41
CD235

Figure 1

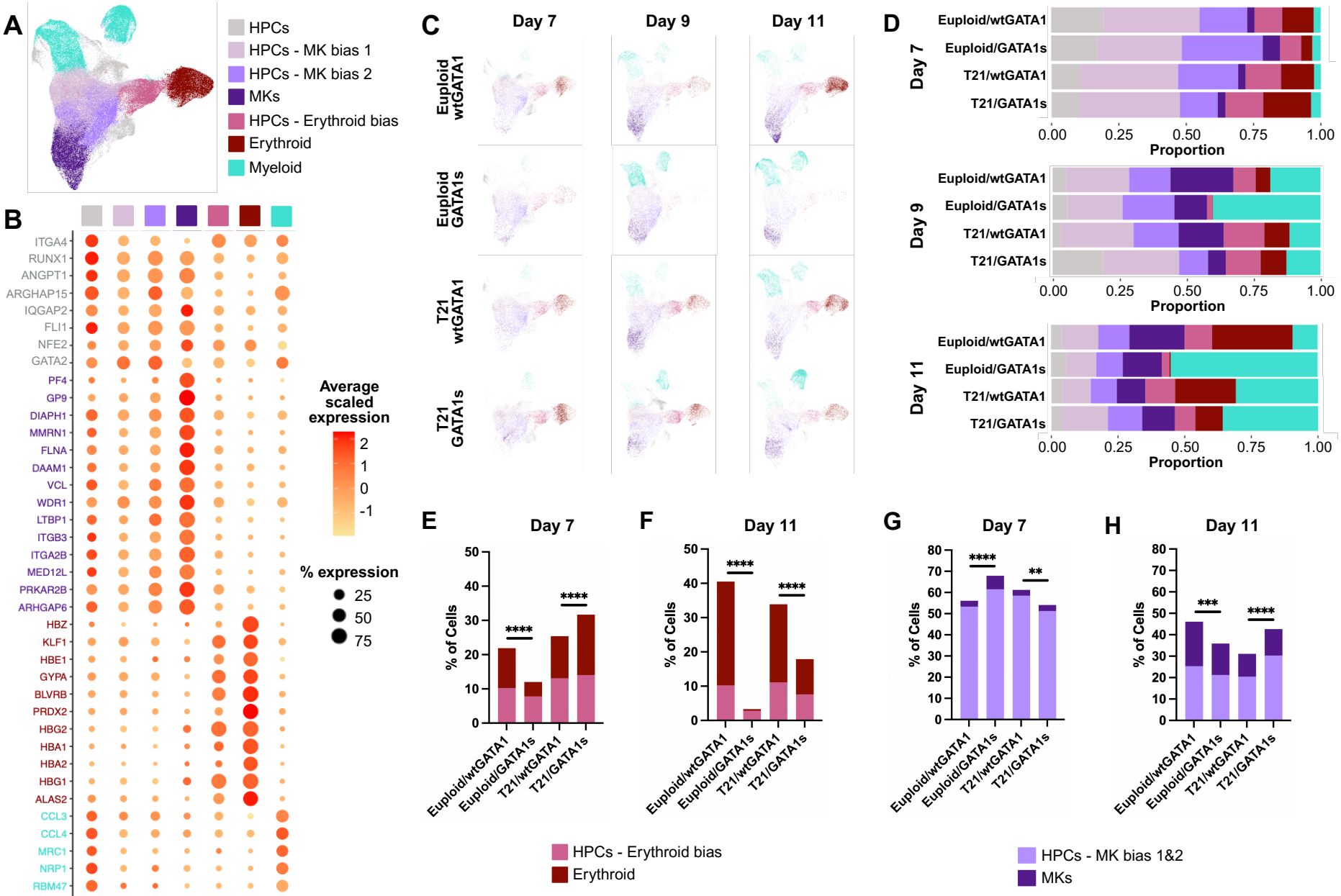

A
HPCs
HPCs - MK bias 1
HPCs - MK bias 2
MKs
HPCs - Erythroid bias
Erythroid
Myeloid
B
ITGA4
RUNX1
ANGPT1
ARGHAP15
IQGAP2
FLI1
NFE2
GATA2
PF4
GP9
DIAPH1
MMRN1
FLNA
DAAM1
VCL
WDR1
LTBP1
ITGB3
ITGA2B
MED12L
PRKAR2B
ARHGAP6
HBZ
KLF1
HBE1
GYPA
BLVRB
PRDX2
HBG2
HBA1
HBA2
HBG1
ALAS2
CCL3
CCL4
MRC1
NRP1
RBM47
SAMHD1
CD74
CST3
Average scaled expression
2
1
0
-1
% expression
25
50
75
C
Day 7
Day 9
Day 11
Euploid wtGATA1
Euploid GATA1s
T21 wtGATA1
T21 GATA1s
D
Day 7
Day 9
Day 11
Euploid/wtGATA1
Euploid/GATA1s
T21/wtGATA1
T21/GATA1s
0.00
0.25
0.50
0.75
1.00
Proportion
E
Day 7
F
Day 11
G
Day 7
H
Day 11
% of Cells
****
**
***
Euploid/wtGATA1
Euploid/GATA1s
T21/wtGATA1
T21/GATA1s
HPCs - Erythroid bias
Erythroid
HPCs - MK bias 1&2
MKs

Figure 2

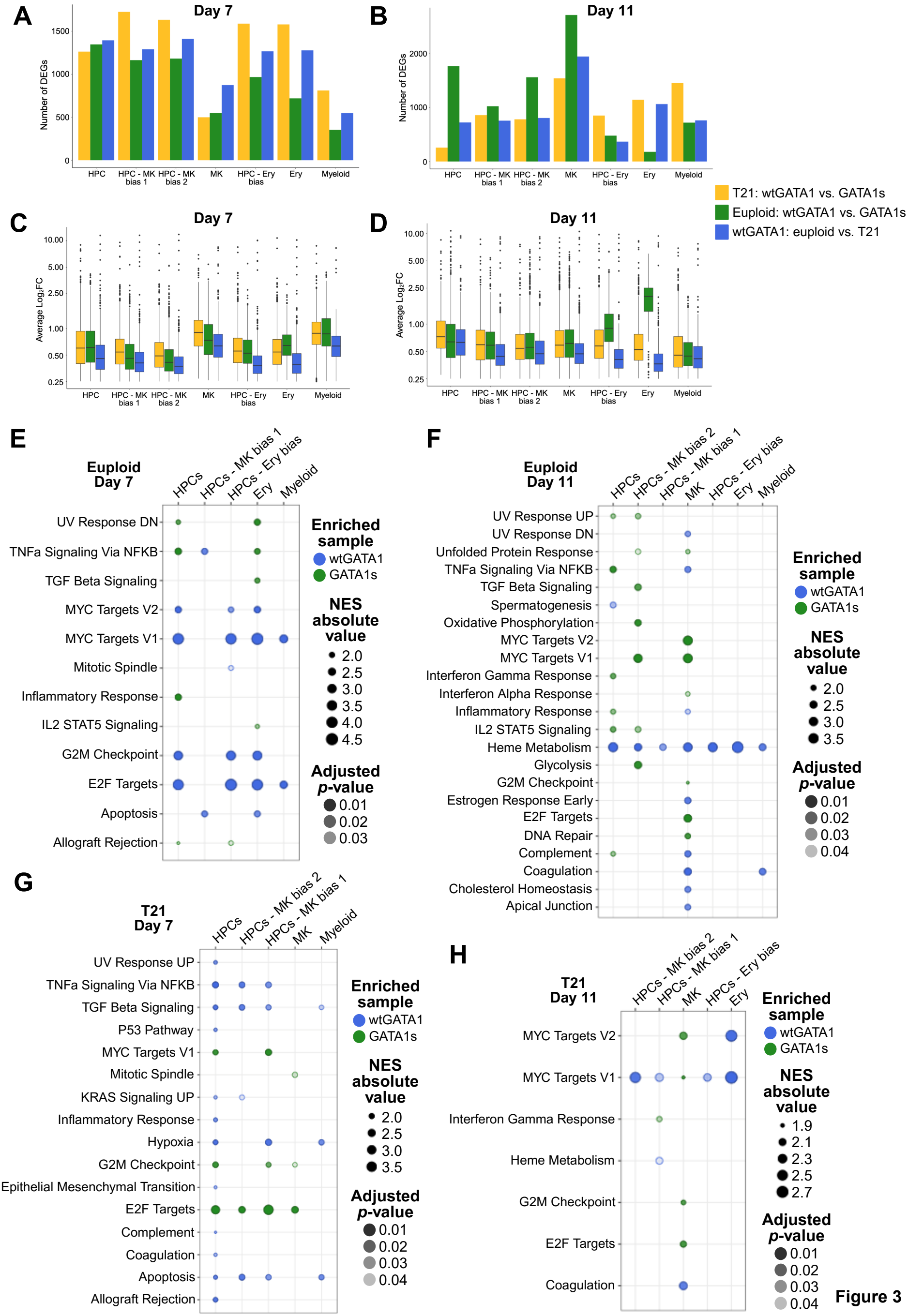

A
Day 7
Number of DEGs
HPC
HPC - MK bias 1
HPC - MK bias 2
MK
HPC - Ery bias
Ery
Myeloid
B
Day 11
T21: wtGATA1 vs. GATA1s
Euploid: wtGATA1 vs. GATA1s
wtGATA1: euploid vs. T21
C
Average Log2FC
D
E
Euploid Day 7
HPCs
HPCs - MK bias 1
HPCs - Ery bias
UV Response DN
TNFa Signaling Via NFKB
TGF Beta Signaling
MYC Targets V2
MYC Targets V1
Mitotic Spindle
Inflammatory Response
IL2 STAT5 Signaling
G2M Checkpoint
E2F Targets
Apoptosis
Allograft Rejection
Enriched sample
wtGATA1
GATA1s
NES absolute value
Adjusted p-value
F
Euploid Day 11
HPCs - MK bias 2
UV Response UP
Unfolded Protein Response
Spermatogenesis
Oxidative Phosphorylation
Interferon Gamma Response
Interferon Alpha Response
Heme Metabolism
Glycolysis
Estrogen Response Early
DNA Repair
Complement
Coagulation
Cholesterol Homeostasis
Apical Junction
G
T21 Day 7
P53 Pathway
KRAS Signaling UP
Hypoxia
Epithelial Mesenchymal Transition
H
T21 Day 11

Figure 3

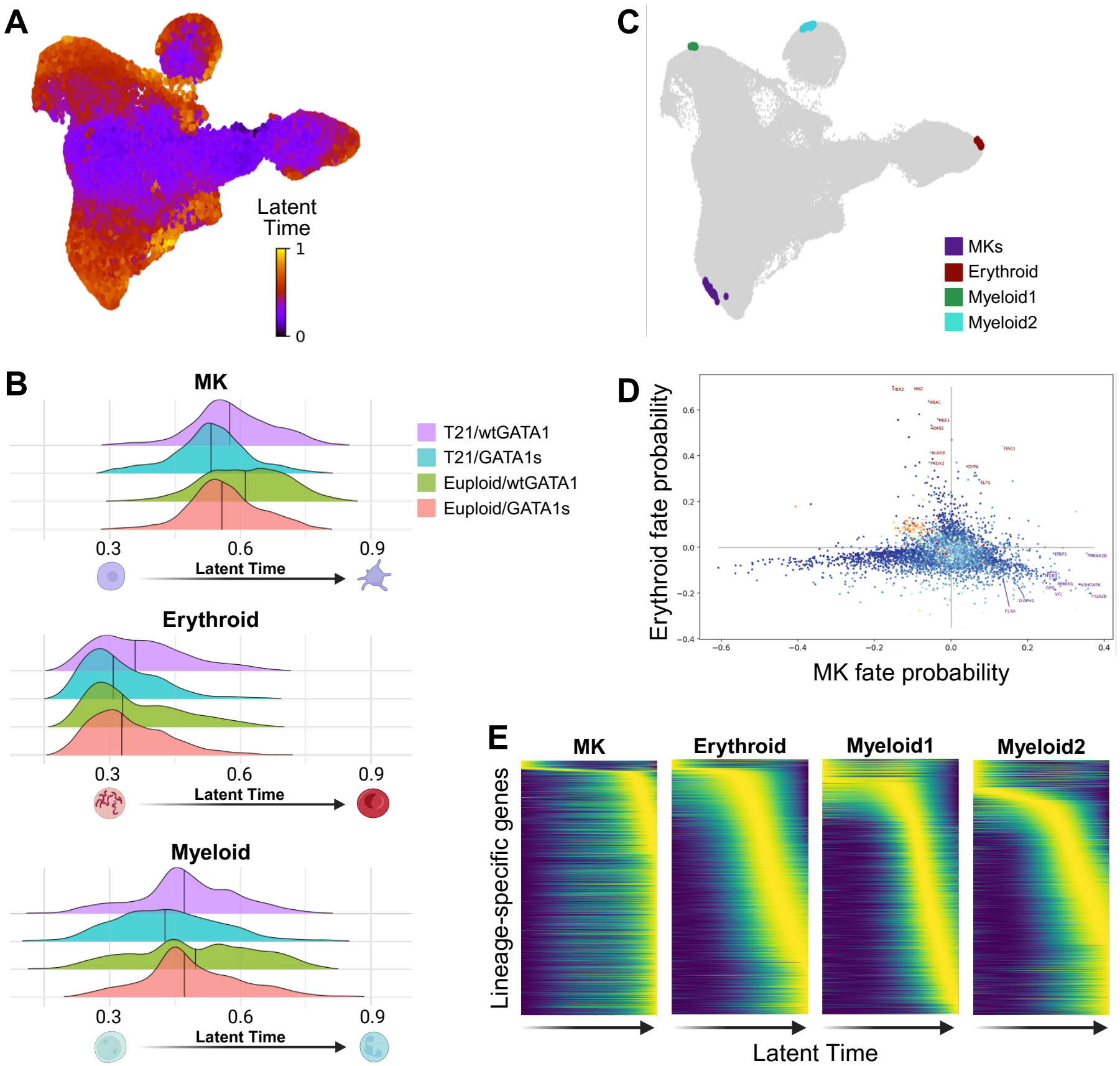

A
Latent Time
1
0
C
MKs
Erythroid
Myeloid1
Myeloid2
B
MK
T21/wtGATA1
T21/GATA1s
Euploid/wtGATA1
Euploid/GATA1s
0.3
0.6
0.9
Latent Time
Erythroid
0.3
0.6
0.9
Latent Time
Myeloid
0.3
0.6
0.9
Latent Time
D
Erythroid fate probability
MK fate probability
E
MK
Erythroid
Myeloid1
Myeloid2
Lineage-specific genes
Latent Time

Figure 4

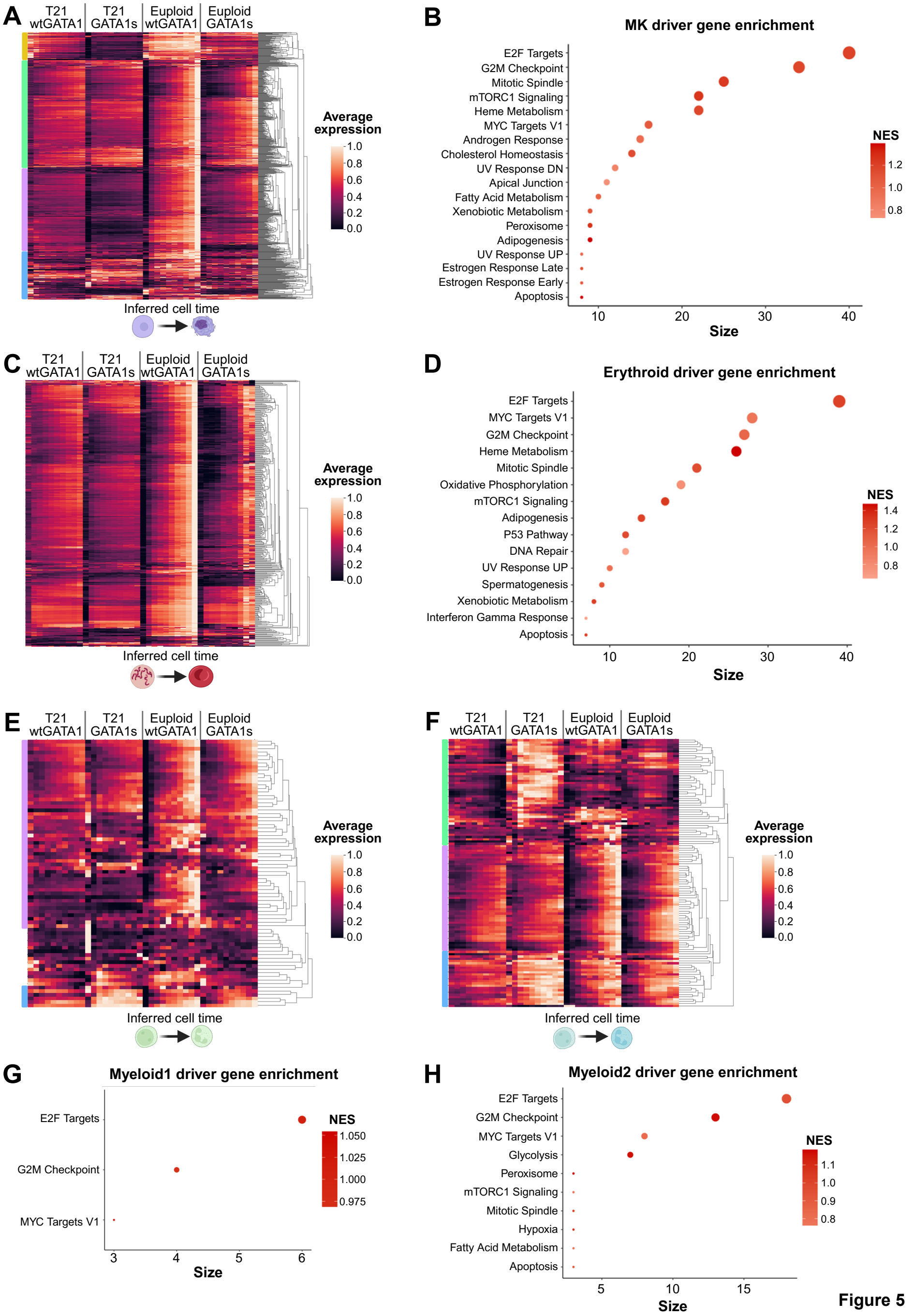
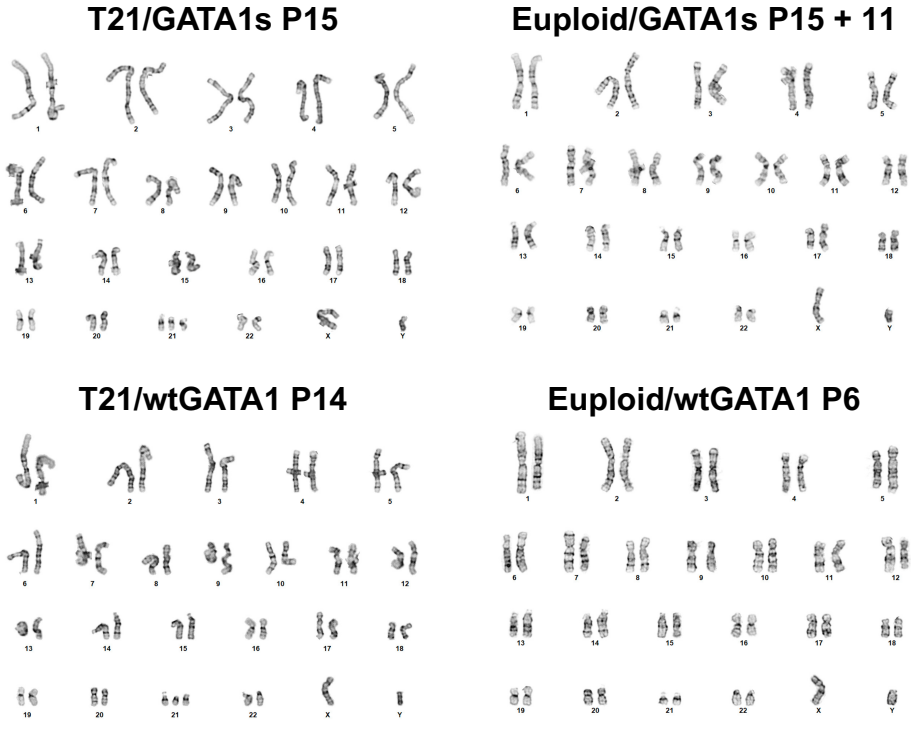

A
T21 wtGATA1
T21 GATA1s
Euploid wtGATA1
Euploid GATA1s
Average expression
1.0
0.8
0.6
0.4
0.2
0.0
Inferred cell time
B
MK driver gene enrichment
E2F Targets
G2M Checkpoint
Mitotic Spindle
mTORC1 Signaling
Heme Metabolism
MYC Targets V1
Androgen Response
Cholesterol Homeostasis
UV Response DN
Apical Junction
Fatty Acid Metabolism
Xenobiotic Metabolism
Peroxisome
Adipogenesis
UV Response UP
Estrogen Response Late
Estrogen Response Early
Apoptosis
10
20
30
40
Size
NES
1.2
1.0
0.8
C
T21 wtGATA1
T21 GATA1s
Euploid wtGATA1
Euploid GATA1s
Average expression
1.0
0.8
0.6
0.4
0.2
0.0
Inferred cell time
D
Erythroid driver gene enrichment
E2F Targets
MYC Targets V1
G2M Checkpoint
Heme Metabolism
Mitotic Spindle
Oxidative Phosphorylation
mTORC1 Signaling
Adipogenesis
P53 Pathway
DNA Repair
UV Response UP
Spermatogenesis
Xenobiotic Metabolism
Interferon Gamma Response
Apoptosis
10
20
30
40
Size
NES
1.4
1.2
1.0
0.8
E
T21 wtGATA1
T21 GATA1s
Euploid wtGATA1
Euploid GATA1s
Average expression
1.0
0.8
0.6
0.4
0.2
0.0
Inferred cell time
F
T21 wtGATA1
T21 GATA1s
Euploid wtGATA1
Euploid GATA1s
Average expression
1.0
0.8
0.6
0.4
0.2
0.0
Inferred cell time
G
Myeloid1 driver gene enrichment
E2F Targets
G2M Checkpoint
MYC Targets V1
3
4
5
6
Size
NES
1.050
1.025
1.000
0.975
H
Myeloid2 driver gene enrichment
E2F Targets
G2M Checkpoint
MYC Targets V1
Glycolysis
Peroxisome
mTORC1 Signaling
Mitotic Spindle
Hypoxia
Fatty Acid Metabolism
Apoptosis
5
10
15
Size
NES
1.1
1.0
0.9
0.8

Figure 5

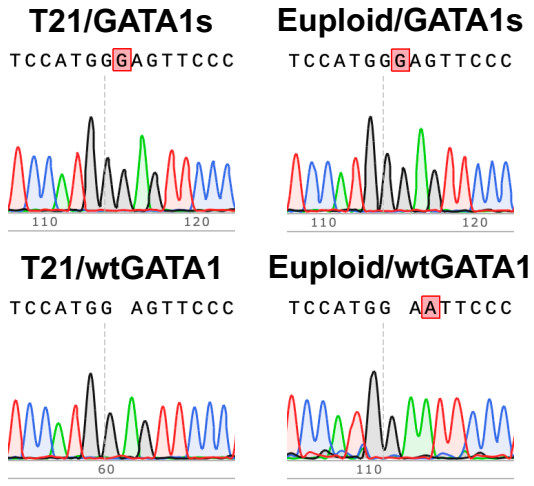
A

	Passage	CNV Result
T21/GATA1s	7	0.1 Mb duplication 3q26 0.1 Mb deletion 8q24 32 Mb duplication 21q11
T21/wtGATA1	12	0.1 Mb duplication 3q26 0.1 Mb deletion 8q24 32 Mb duplication 21q11
Euploid/GATA1s	15 + 2	0.1 Mb duplication 3q26 0.1 Mb deletion 8q24
Euploid/wtGATA1	5	0.1 Mb duplication 3q26 0.1 Mb deletion 8q24

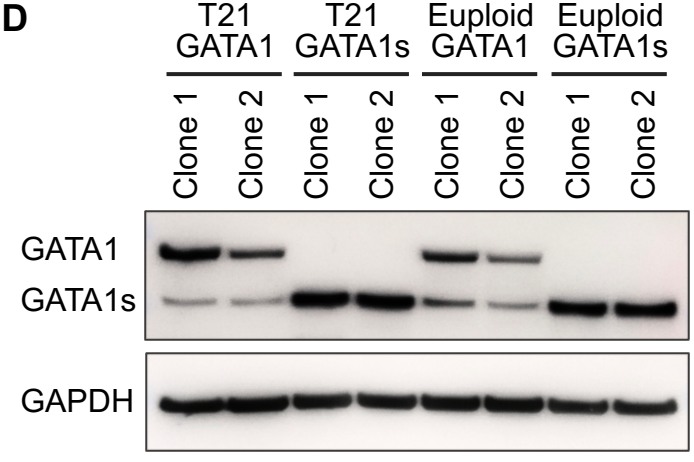
B



C



D



Supplemental Figure 1