Expression levels of GATA1s isoform affect megakaryocyte differentiation and leukemic predisposition in mice

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Supplementary information: Materials and Methods

Increase of megakaryocytes in Δ NTR-H and Δ NTR-M embryos



(A) Dot plot depicting the frequencies of CD41+CD61+ megakaryocytes in live cells from the fetal liver at E18.5. (B) Hematoxylin and eosin-stained sections of E12.5 Δ NTR-M and Δ NTR-H livers exhibit accumulation of large cells with a diameter exceeding 10 µm (indicated by yellow allowheads).

Histopathological analyses of liver sections of leukemic ANTR-M mice



Histopathological analyses on liver sections from two ΔNTR-M mice exhibiting splenomegaly and a control wild-type mouse. Hematoxylin-Eosin staining (left panels) and silver staining (right panels) are shown. d/o: days-old.



Peripheral blood smear of a leukemic ANTR-M mouse

A lower magnification image (upper panel) and an enlarged image (lower panel) of the inset are presented. In these images, multiple blast cells with or without bleb formation are observed, which are indicated by red and black arrows, respectively. Additionally, numerous smudge cells are present, suggesting a hematopoietic neoplastic disease.

Leukemic cells in ANTR-M mouse show autonomous proliferation in recipient nude mice.



(A-C) A leukemia-developing Δ NTR-M mouse of 76 days-old (d/o) used for the transplantation analysis. Results include the macroscopic appearance (A), May-Grünwald–Giemsa staining of a peripheral blood smear (B), and flow cytometry analysis of the spleen (C). (C) Cells are visualized on FSC versus SSC (left panel), and the abnormal cells gated in the red polygon are c-Kit-positive and CD41-dull (right panel). (D) Kaplan-Meier graph depicting the outcome of five nude mice transplanted with 1 x 10^6 splenic cells from the leukemia developing Δ NTR-M mouse shown in (A). (E) Representative images from macroscopic analysis of two nude mice at 45 days (Case 1; left) and 52 days (Case 2; right) after transplantation. The spleen, liver, and body weights of mice in Case 1 are 1.97 g, 3.33 g and 22.4 g, respectively, while those in Case 2 are 0.98 g, 1.79 g and 13.5 g, respectively. (F) Flow cytometry analysis of spleen mononuclear cells from the Case 1 nude mouse (left) and that from Case 2 nude mouse (right). Cells within the abnormal fractions, marked by small black polygons in both left panels, exhibited characteristics of c-Kit⁺CD41^{dull}, as seen in both right panels. (G) Blast cells in the peripheral blood smear sample (left panel) and cytospin samples from bone marrow (middle panel) and spleen (right panel). These samples were collected from the Case 1 leukemic nude mouse. Note that these leukemic cells in the nude mice exhibit similar characteristics to those observed in the donor \triangle NTR-M mouse.

Integration sites of transgenes in the Δ NT-H and Δ NT-M lines.



(A) Schematic representation of the G1HRD-G1 and G1HRD- Δ NT transgene constructs. Locations of primers used to identify the transgenes are indicated with blue arrows. (B, C) Schematic diagrams of the transgene-integrated sites on chromosome 3 in the Δ NT-H line of mice (B) and on chromosome 13 in the Δ NT-M line of mice (C). Notably, multiple copies (more than three [6]) of transgenes were integrated into the genomes of Δ NT-H and Δ NT-M transgenic mice. The orientations of the transgenes at both ends are depicted. Genomic position data is referenced from the GRCm38 mouse assembly. Locations of primers used to identify the transgenic and wild-type alleles are denoted with blue arrows. A reverse primer, specific for endogenous chromosome 13 allele, was designed within the sequence that was replaced by transgene in Δ NT-M line. We have determined that the transgene in the Δ NT-H mice is integrated within the body of the *Ndst4* gene, which may represent a relatively open chromatin structure. In contrast, the transgene in Δ NT-M mice is in an intergenic region approximately 100 Mb away from the neighboring genes, suggesting a probable closed structure. We surmise that the difference in the *Gata1s* expression levels between the two transgenic mouse lines is attributable largely to the insertion site of the transgene. Supplementary Table 1. List of mice used for experiment shown in Figure 1. *: Mice that underwent necropsy

No.	ID	Age (day)	Genotype	Outcome
1	710	180	G1R	Surviving
2	957	180	G1R	Surviving
3	306	180	G1R	Surviving
4	308	180	G1R	Surviving
5	309	180	G1R	Surviving
6	310	180	G1R	Surviving
7	481	180	G1R	Surviving
8	487	180	G1R	Surviving
9	488	180	G1R	Surviving
10	491	180	G1R	Surviving
11	432	180	G1R	Surviving
12	183	180	G1R	Surviving
13	059	180	G1R	Surviving
14	738	180	G1R	Surviving
15	966	180	G1R	Surviving
16	298	77	∆NTR-H	Unknown
17	441	180	∆NTR-H	Surviving
18	496	180	ΔNTR-H	Surviving
19	501	180	∆NTR-H	Surviving
20	433	180	∆NTR-H	Surviving
21	434	180	∆NTR-H	Surviving
22	435	180	∆NTR-H	Surviving
23	436	180	∆NTR-H	Surviving
24	350	180	∆NTR-H	Surviving
25	222	180	∆NTR-H	Surviving
26	187	180	∆NTR-H	Surviving
27	724	180	∆NTR-H	Surviving
28	727	180	∆NTR-H	Surviving
29	188	180	∆NTR-H	Surviving
30	191	180	∆NTR-H	Surviving
31	195	180	∆NTR-H	Surviving
32	448	180	∆NTR-H	Surviving
33	351	180	∆NTR-H	Surviving
34	619	180	∆NTR-H	Surviving
35	029	180	∆NTR-H	Surviving
36	030	180	∆NTR-H	Surviving
37	484	180	∆NTR-H	Surviving
38	779	180	∆NTR-H	Surviving
39	223	180	∆NTR-H	Surviving
40	083	180	∆NTR-H	Surviving
41	670	180	∆NTR-H	Surviving
42	672	180	ANTR-H	Surviving

No.	ID	Age (day)	Background	
43	673	180	∆NTR-H	
44	674	180	∆NTR-H	
45	031	180	∆NTR-H	
46	061	180	∆NTR-H	
47	005	180	∆NTR-H	
48	808	180	∆NTR-H	
49	470	180	∆NTR-H	
50	482	180	∆NTR-H	
51	337	180	∆NTR-H	
52	653	180	∆NTR-H	
53	174	180	∆NTR-H	
54	176	180	∆NTR-H	
55	165	180	∆NTR-H	
56	166	180	∆NTR-H	
57	167	180	∆NTR-H	

Supplementary	Table 1	(Continued)
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44		674	180	ΔNTR-H	Surviving
45		031	180	ΔNTR-H	Surviving
46		061	180	ΔNTR-H	Surviving
47		005	180	ΔNTR-H	Surviving
48		808	180	ΔNTR-H	Surviving
49		470	180	ΔNTR-H	Surviving
50		482	180	ΔNTR-H	Surviving
51		337	180	ΔNTR-H	Surviving
52		653	180	ΔNTR-H	Surviving
53		174	180	ΔNTR-H	Surviving
54		176	180	ΔNTR-H	Surviving
55		165	180	ΔNTR-H	Surviving
56		166	180	ΔNTR-H	Surviving
57		167	180	ΔNTR-H	Surviving
58		198	180	ΔNTR-H	Surviving
59		104	180	ΔNTR-H	Surviving
60		101	180	∆NTR-H	Surviving
61		102	180	∆NTR-H	Surviving
62		194	180	ΔNTR-H	Surviving
63		957	180	∆NTR-H	Surviving
64		491	180	ΔNTR-H	Surviving
65		329	180	∆NTR-H	Surviving
66		782	180	ΔNTR-H	Surviving
67		964	180	ΔNTR-H	Surviving
68		684	180	ΔNTR-H	Surviving
69		017	33	Δ NTR-M	Unknown
70		199	33	Δ NTR-M	Unknown
71		044	35	Δ NTR-M	Unknown
72		414	35	Δ NTR-M	Unknown
73	*	592	38	Δ NTR-M	Leukemia
74		842	41	$\Delta NTR-M$	Unknown
75		864	41	Δ NTR-M	Unknown
76		808	45	$\Delta NTR-M$	Unknown
77		966	45	Δ NTR-M	Unknown
78	*	548	45	$\Delta NTR-M$	Leukemia
79	*	723	45	Δ NTR-M	Leukemia
80	*	387	50	Δ NTR-M	Leukemia
81		728	52	$\Delta NTR-M$	Unknown
82		757	52	Δ NTR-M	Unknown
83	*	724	53	$\Delta NTR-M$	Leukemia
84	*	756	53	ΔNTR-M	Leukemia

Outcome Surviving

85 693 60 ANTR-M Unk 86 721 60 ANTR-M Unk 87 130 67 ANTR-M Unk 87 130 67 ANTR-M Unk 88 209 67 ANTR-M Unk 89 384 88 ANTR-M Unk 90 * 071 93 ANTR-M Leul 91 * 155 93 ANTR-M Leul 92 * 162 120 ANTR-M Leul 93 * 209 120 ANTR-M Leul 94 * 337 143 ANTR-M Leul 95 * 516 143 ANTR-M Leul 96 * 494 146 ANTR-M Leul 97 * 516 146 ANTR-M Leul 98 663 149 ANTR-M Leul 100 * 094 150 ANTR-M Leul <tr< th=""><th>ID</th><th>ID Age (day)</th><th>Background</th><th>Outcome</th></tr<>	ID	ID Age (day)	Background	Outcome
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110 004 180 ΔNTR-MH Surv 111 009 180 ΔNTR-MH Surv 112 599 180 ΔNTR-MH Surv 113 427 180 ΔNTR-MH Surv 114 256 180 ΔNTR-MH Surv 115 470 180 ΔNTR-MH Surv 116 373 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MH Lead 121 431 138 ΔNTR-MM Lead 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	471	471 180	∆NTR-MH	Surviving
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112 599 180 ANTR-MH Surv 113 427 180 ANTR-MH Surv 114 256 180 ANTR-MH Surv 115 470 180 ANTR-MH Surv 116 373 180 ANTR-MH Surv 117 148 180 ANTR-MH Surv 118 205 180 ANTR-MH Surv 119 088 180 ANTR-MH Surv 120 * 406 132 ANTR-MM Leul 121 431 138 ANTR-MM Surv 123 355 180 ANTR-MM Surv 124 071 180 ANTR-MM Surv	009	009 180	ΔNTR-MH	Surviving
113 427 180 ΔNTR-MH Surv 114 256 180 ΔNTR-MH Surv 115 470 180 ΔNTR-MH Surv 116 373 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leul 121 431 138 ΔNTR-MM Unk 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	599	599 180	∆NTR-MH	Surviving
114 256 180 ΔNTR-MH Surv 115 470 180 ΔNTR-MH Surv 116 373 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leul 121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	427	427 180	ΔNTR-MH	Surviving
115 470 180 ΔNTR-MH Surv 116 373 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 117 148 180 ΔNTR-MH Surv 118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leul 121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	256	256 180	∆NTR-MH	Surviving
116 373 180 ANTR-MH Surv 117 148 180 ANTR-MH Surv 118 205 180 ANTR-MH Surv 119 088 180 ANTR-MH Surv 120 * 406 132 ANTR-MM Leul 121 431 138 ANTR-MM Unk 122 657 180 ANTR-MM Surv 123 355 180 ANTR-MM Surv 124 071 180 ANTR-MM Surv	470	470 180	ΔNTR-MH	Surviving
117 148 180 ΔNTR-MH Surv 118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leuk 121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	373	373 180	∆NTR-MH	Surviving
118 205 180 ΔNTR-MH Surv 119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leul 121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	148	148 180	ΔNTR-MH	Surviving
119 088 180 ΔNTR-MH Surv 120 * 406 132 ΔNTR-MM Leul 121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv	205	205 180	∆NTR-MH	Surviving
120 * 406 132 ANTR-MM Leul 121 431 138 ANTR-MM Unk 122 657 180 ANTR-MM Surv 123 355 180 ANTR-MM Surv 124 071 180 ANTR-MM Surv 125 920 100 ANTR-MM Surv	088	088 180	∆NTR-MH	Surviving
121 431 138 ΔNTR-MM Unk 122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv 125 920 100 ΔΝΤR-MM Surv	* 406	406 132	ΔNTR-MM	Leukemia
122 657 180 ΔNTR-MM Surv 123 355 180 ΔNTR-MM Surv 124 071 180 ΔNTR-MM Surv 125 020 100 ΔΝΤR-MM Surv	431	431 138	ΔNTR-MM	Unknown
123 355 180 ΔΝΤR-MM Surv 124 071 180 ΔΝTR-MM Surv 125 020 100 ΔΝΤR-MM Surv	657	657 180	ΔNTR-MM	Surviving
124 071 180 △NTR-MM Surv	355	355 180	ΔNTR-MM	Surviving
	071	071 180	ΔNTR-MM	Surviving
125 020 180 ANTR-MM Surv	020	020 180	ΔNTR-MM	Surviving
126 151 180 <u>ANTR-MM</u> Surv	151	151 180	∆NTR-MM	Surviving

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		Primer sequence (5'-3')
Gata 1.05 allele	F	AAGTATCCATCATGGCTGATGC
<i>Guiu1.05</i> allele	R	TAGCCAACGCTATGTCCTGATA
V chromosomo	F	CCTATTGCATGGACTGCAGCTTATG
	R	GACTAGACATGTCTTAACATCTGTCC
G1HRD-G1 and G1HRD-ΔNT	F	AGATGAATGGTCAGAACCGG
transgene	R	AGGCATAAGATGGCTGACAG
		GGAGACAGGATCTTCTGTAGC
An I-M transgene	R	GGGTGATTGGGTTACCTTACTC
ANT H transcope	F	CCAGAGGATAGAGAGCAATATC
An I-ri uansgene	R	CAAACCACAACTAGAATGCAG
Endogenous allele at the Δ NT-M	F	GGAGACAGGATCTTCTGTAGC
transgene integration site		GTAGTCCTACCTGAGGGACCAG

Supplementary Table 2. Primer sequences used for mouse genotyping.

Supplementary Table 3. Primer sequences used for Identification of transgene insertion sites.

Primer name	Primer sequence (5'-3')
G1HRD outer	GAGTACTCACATGAATCTGAGATGTC
G1HRD nested	GAGTATTCTGATCTGTCTACTGAGTGC
∆NT3'UTR outer	GAGACAGGATCTTCTGTAGCTATGTAG
∆NT3'UTR nested	TTTGGACAAACCACAACTAGAATGCAG

		Primer sequence (5'-3')
Catal	F	CAGAACCGGCCTCTCATCC
Galai	R	TAGTGCATTGGGTGCCTGC
C and dle	F	GTCGTGGAGTCTACTGGTGTCTT
Gapan	R	GAGATGATGACCCTTTTGGC

Supplementary Table 4. Primer sequences used for qPCR.

Supplementary methods

Mice

Experimental procedures involving animals are approved by the Institutional Animal Experiment Committee of the Tohoku University. Experiments were carried out in compliance with the Regulation for Animal Experiments in Tohoku University (2015MdA-001, 2018MdA-058, 2018MdA-245, 2019MdA-289). Mice were continuously monitored for survival and were humanely euthanized upon reaching predetermined endpoints. Mice having enlarged spleen over the midline of body at necropsy were defined as those developing leukemia. Hematopoietic indices of embryos were measured by collecting blood after decapitation with surgical scissors and using a Celltac- α autohemocytometer (Nihon Koden). The *Gata1.05* allele was identified through genomic polymerase chain reaction (PCR) using a pair of primers specific to the neomycin resistance gene [6]. Gatal transgenes were genotyped via PCR, using a primer set designed to target the region flanking exon 4 and exon 6 of Gata1 cDNA (Supplementary Fig. 5A). Δ NTR-MH mice were produced by crossing *Gata1.05/X* mice carrying Δ NT-H or Δ NT-M transgene with Δ NT-M or Δ NT-H transgenic male mice, respectively. Specific primer sets were used to identify Δ NT-H and Δ NT-M transgenes (Supplementary Fig. 5B, C). Δ NTR-MM mice were generated by crossing Gata1.05/X mice carrying the Δ NT-M transgene with Δ NT-M transgenic male mice. To distinguish Δ NTR-MM from Δ NTR-M, we used a primer set designed to recognize the endogenous allele at the site where the Δ NT-M transgene was incorporated (Supplementary Fig. 5). The primer sequences used for mouse genotyping are shown in Supplementary Table 2.

Identification of insertion sites of transgenes

Transgene insertion sites were determined using the Universal Genomic Walker Kit from

Clontech Laboratories, following the manufacturer's instruction. In brief, we amplified fragments containing genome-transgene boundary sequences from the transgenic mouse genome through nested PCR. This was achieved using transgene-specific primers in conjunction with adaptor primers provided by the manufacturer. The sequences of transgene-specific primers are shown in Supplementary Table 3. DNA fragments were purified from agarose gel and cloned into a pGEM-T vector (Promega). Subsequently, sequencing of the cloned fragments was carried out using T7 and SP6 primers, employing BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). After excluding fragment sequences originating from the fusion of two transgenes, sequence data were analyzed through the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) and the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp/index-e.html) websites. Schematic illustrations depicting the genomic regions surrounding the transgenes are shown in Supplementary Fig. 5.

Flow cytometry analysis

Mononuclear cells collected from fetal liver and spleen were suspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Fetal liver cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (eBioscience) and phycoerythrin (PE)-conjugated anti-CD61 (eBioscience) antibodies. Spleen cells were labeled with FITC-conjugated anti-CD41, PE-conjugated anti-CD71 (BioLegend) and allophycocyanin conjugated anti-cKit (BD Biosciences) antibodies. After two washes with PBS, the cells were resuspended in PBS containing 2% FBS and analyzed using FACS CaliburTM (Becton Dickinson Biosciences). Dead cells were excluded by propidium iodide.

RNA extraction and quantitative real-time PCR

CD41-positive cells were isolated using biotin anti-CD41 antibody (eBioscience) and

streptavidin-conjugated beads (Invitrogen). Total RNA from the CD41-positive cells was isolated using ISOGEN (NIPPON GENE). Subsequently, first-strand cDNA was synthesized using ReverTra Ace (TOYOBO). Quantitative real-time PCR was conducted utilizing the ABI PRISM 7300 sequence detector system and StepOnePlus Real-Time PCR System (Applied Biosystems) along with THUNDERBIRD SYBR qPCR Mix (TOYOBO). The data were normalized to the *Gapdh* mRNA level. The primer sequences employed for the expression analysis are provided in Supplementary Table 4.

Proplatelet formation assay

Mononuclear cells were suspended in CATCH-medium (doi: 10.1083/jcb.69.1.159) and overlaid on a discontinuous BSA gradient (2%/3%/4%/16% in CATCH-medium). After 1 hour, the upper half of the gradient was combined and washed with CATCH-medium. Subsequently, cells were resuspended in 2-ml IMDM-medium containing Nutridoma-SP (Behringer-Mannheim). After a 24 hour-incubation at 37°C, the frequencies of megakaryocytes forming proplatelets were assessed.

Histological and cytological analysis and microscope

Cytospin samples of bone marrow and spleen cells were prepared using a cytocentrifuge (Thermo Fisher Scientific). Peripheral blood smear samples and cytospin samples were stained with May–Grünwald staining solution (Merck) and Giemsa staining solution (Merck). Formalinfixed tissue sections were stained with Hematoxylin-eosin and Watanabe's silver impregnation method. Images of mice were monitored by µTOUGH-8000 (Olympus). Images of tissues were captured using a BX51 microscope (Olympus) and images of cells expressing GFP were captured using MZFLIII stereomicroscopes (Leica), and were photographed using a DP73 CCD-camera (Olympus).

Statistical analysis

All statistical analyses were done using JMP software (SAS Institute Inc.). Survival rates were analyzed using the Kaplan-Meier method. The data was analyzed using a Mann-Whitney U test. Mortality of various mouse groups were compared using the Log-rank test and Generalized Wilcoxson test.