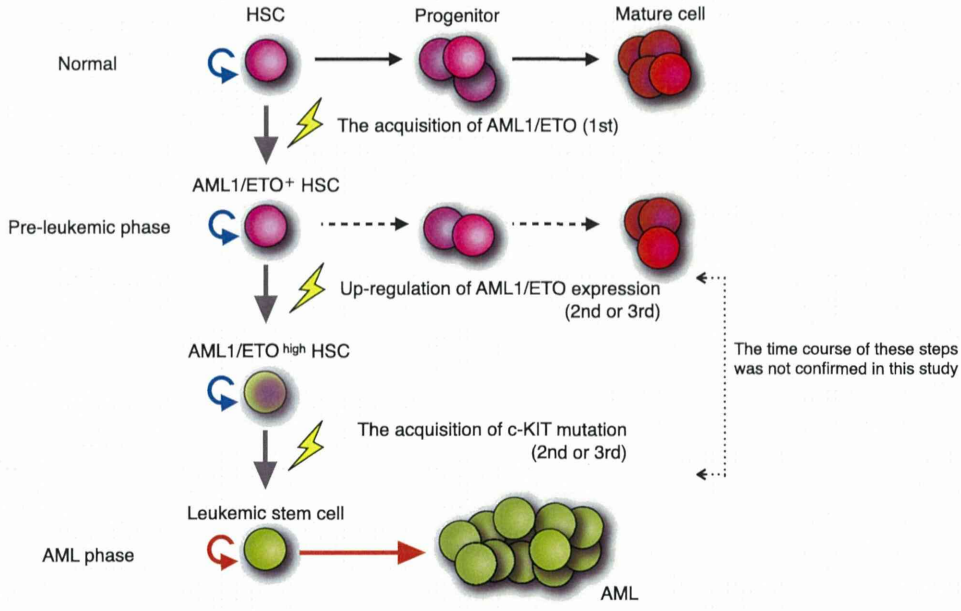

Figure 5. The proposed multistep developmental scheme of human t(8;21) AML with *c-KIT* mutations. A normal HSC acquires t(8;21) and forms a reservoir of preleukemic *AML1/ETO*$^+$ HSCs as the first step. These preleukemic *AML1/ETO*$^+$ HSCs upregulate *AML1/ETO* transcripts (second step), and the acquisition of a *c-KIT* mutation (3rd step) finally transforms preleukemic *AML1/ETO*^{high} HSCs into LSCs. The detailed mechanism of *AML1/ETO* upregulation at the second step remains unknown. The time course of the second and third steps was not confirmed in this study.

abnormalities cooperatively transform normal HSCs into LSCs [1,2]. Our intensive analysis of human t(8;21) AML with *c-KIT* mutation revealed that, at diagnosis, all single LSCs had both *AML1/ETO* and *c-KIT* mutants (Table 2). During remission, a small fraction (~1%) of HSCs or their myeloerythroid colonies had *AML1/ETO*, and such single *AML1/ETO*$^+$ HSCs always had wild-type *c-KIT*. Hematopoietic stem cells with only the *c-KIT* mutation were never observed (Tables 2 and 3).

Furthermore, the breakpoint of *AML1/ETO* was identical at diagnosis and in remission in all three patients analyzed (Fig. 1C), indicating that *AML1/ETO*$^+$ cells at diagnosis and those in remission originated from the same preleukemic clone. In addition, we found a patient who had N822K at diagnosis but newly obtained D816Y at relapse (Patient 14, Table 1), suggesting that acquisition of *c-KIT* mutation is a subsequent event. Thus, our sequential analysis provides definitive evidence that HSCs first acquire *AML/ETO* fusion (Class II) and then *c-KIT* mutation (Class I) for transformation into LSCs. The proposed developmental model for t(8;21) AML is schematized in Figure 5.

Because all three types of *c-KIT* mutations found in t(8;21) AML in this study constitutively transduce an active c-KIT signaling [36], the enhanced c-KIT signals may play a critical role in leukemic transformation. Our patients had D816V, D816Y, and N822K *c-KIT* mutants, and microarray analysis showed that *c-KIT* mutations at least caused upregulation of *NFKB1A*, *BCL2*, and *MCL1*, whose signals may promote proliferation/survival of leukemic cells [32,33,35]. A variety of *c-KIT* mutations have been found in several other malignant diseases [36]. Consistently, in a mouse model, the enforced *c-KIT* mutant signaling induced a myeloproliferative neoplasm-like disease [9]. Notably, D816V and D816Y are frequently found in mast cell leukemia as well as in t(8;21) AML [36]. Thus, it is possible that active *c-KIT* signaling itself does not decide the phenotype of leukemia, but it may contribute toward development of AML in the presence of CBF mutations such as *AML1/ETO*.

It is also important to note that the significant upregulation of *AML1/ETO* transcripts may also be a key event in leukemic transformation. Because AML1/ETO knock-in mice did not present any leukemia phenotype [17], AML1/ETO may not play a role in the inhibition of CBF function. In contrast, in LSCs, *AML1/ETO* mRNA levels were elevated up to one hundredfold at diagnosis, and this high level of *AML1/ETO* may be effective in inhibiting myeloid differentiation. Our data suggest that c-KIT signaling is independent of *AML1/ETO* upregulation (Fig. 4B). Collectively, an unknown, presumably epigenetic mechanism that elevates *AML1/ETO* mRNA levels may also be critical for the development of t(8;21) AML. This event may precede the acquisition of *c-KIT* mutations, since no *AML1/ETO*$^+$ HSCs in remission had mutated *c-KIT* (Table 2).

The reason the ordered acquisition of Class II and Class I mutation is consistently observed in t(8;21) AML patients remains unclear. To achieve leukemic hematopoiesis, the single cell with the first oncogenic hit needs to have a clonal advantage for self-renewal compared with normal HSCs. Class I mutations, when their expression is enforced, are capable of conferring cytokine-independent growth activity to cell lines [9,37–39]. In mouse models, HSCs with a high level of BCR-ABL (Class I) enforced by retroviruses

showed myeloproliferation, whereas, in healthy human adults, a low level of BCR-ABL transcripts is sometimes detectable [40–42]. This suggests that the acquisition of BCR-ABL in HSCs cannot directly provide clonal advantages against normal HSCs. Previous studies reported that mice having HSCs with *FLT3-ITD* showed expansion of hematopoietic progenitors resulting in myeloproliferation [7,43]; however, the HSC compartment declines because *FLT3-ITD* signals perturb the self-renewal of HSCs [44]. Therefore, in the light of de novo development of human AML, if a single HSC achieves *FLT3-ITD*, the *FLT3-ITD*$^+$ HSCs may not be able to outgrow normal HSCs. It is possible that HSCs with a *c-KIT* mutation alone cannot exhibit the advantage of self-renewal against normal HSCs to become a dominant clone because *c-KIT* and *FLT3* use similar signal transduction pathways [29,30].

In contrast, *AML1/ETO*$^+$ HSCs can persist for over 10 years, maintaining their clones at the level of a few percent of normal HSCs [19,20]. This evidence indicates that HSCs achieved through *AML1/ETO* do not have advantages in self-renewal but can coexist with normal HSCs for a long period. This is probably because the expression level of *AML1/ETO* in t(8;21)$^+$ HSCs is low, and it does not significantly block hematopoietic differentiation. It is assumed that the long-term coexistence of normal and t(8;21)$^+$ HSCs allows the latter to acquire second or third oncogenic hits, including Class I mutations and some unknown abnormalities that can cause upregulation of *AML1/ETO*. It is critical to test whether this hypothesis can be applied to AML with other Class II mutations.

Collectively, the results of our intensive analysis of de novo t(8;21) human AML suggest that there are at least three independent leukemogenic steps in this type of leukemia (Fig. 5). First, the normal HSC acquires t(8;21), which generates a low level of *AML1/ETO*. Second, the long-term existence of such AML1/ETO$^+$ HSCs allows them to obtain additional epigenetic or genetic abnormalities that upregulate *AML1/ETO*. Finally, the acquisition of Class I mutations, such as *c-KIT* mutants, transforms *AML1/ETO*$^+$ preleukemic HSCs into AML LSCs. Thus, the original description of Class I and Class II mutations [4,45,46] is very useful in the understanding of the leukemogenesis of AML. In future studies, intensive tracking of mutational processes during clinical courses is critical to understand the step-wise leukemogenesis involved in de novo human AML.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Supplementary Table E1. Primers for the genomic PCR analysis of *c-KIT* mutation

	Forward primers (5′—3′)	Reverse primers (5′—3′)	°C	Length (bp)
c-KIT exon 1 external	GAGAGCTGGAACGTGGACC	GAGGGCCACCTGGAGTCT	60	267
c-KIT exon 2 external	GGGACCAAATGTGACCCTCA	AAAGCACCAGCACAAATGGG	60	764
c-KIT exon 3 external	GCCATTTGGGCCACTAGTCA	ACAGCTAGCTCCCTGATTGAC	60	499
c-KIT exon 4 external	ACAGATAGGTTAGCACCATGCTT	TGTGGAGGTATGAAAGGGGGA	60	442
c-KIT exon 5 external	GAAGTGTTCCAATGACAGACTTGT	GTGCTTTCATTGCAAGAGGCT	60	507
c-KIT exon 6 external	TGTGACAGTGATTCTACAAGAGCA	GCAGCCCCAGACTTTCTTCT	60	494
c-KIT exon 7 external	CTGTCAGGTTGCCTTTGTGC	ACCACCAAACCACGAAGTCT	60	512
c-KIT exon 8 external	TTGAACTTGCTCCCTCAGGC	CCTGCAGGCTAGAAATTGCAT	60	505
c-KIT exon 9 external	ATCTGACTCCGAAGCCTCCT	TGACAGTATGGTGTGATGCATGT	60	535
c-KIT exon 10 and 11 external	GGGGTCAGTTTGGGACTGAG	ACCTATCAAAAGGGGCGCAA	60	546
c-KIT exon 12 and 13 external	ATGTCTCTGGACAACATTG	ACTAGGGTATGTCCTGGGCT	60	632
c-KIT exon 14 external	TGTTACTCCACATAAGGCTGCT	ATCAGCCTTGATTGCAAACCC	60	414
c-KIT exon 15 external	TGGGCATGGACCCCAATATC	ATGCTCTGACCCCAAACACC	60	475
c-KIT exon 16 external	ATTCGCCATCCCTCTCCTCT	TCCAAAGAGACAGCAGTTGGA	60	537
c-KIT exon 17 external	TTATGTGAACATCATTCAAGGCGT	TGTTTCCTTCACATGCCCCA	60	543
c-KIT exon 18 and 19 external	CACATTTCAGCAACAGCAGCA	GACCAGTGTGTCATAAAGA	60	555
c-KIT exon 20 external	TGTCCAGTTGCATAGCCCTG	CACCCTGAAAGCCTGAGGAG	60	293
c-KIT exon 21 external	TGGGTTTTGGCCACAAAGTTC	AAGACAGGATTGCAGTGGGG	60	403
c-KIT exon 1 internal	GGAACGTGGACCAGAGCTCGGAT	GGATGCACCCTGGCGGGTACCA	60	193
c-KIT exon 2 internal	AGCAGGGCAGCTTTGTCCTA	CAGTCCTCCAGCTGGGGCCA	60	600
c-KIT exon 3 internal	GTGTTTTCAGTGTCTGTGAC	GATCAACGAGAAGAGAAGTC	56	416
c-KIT exon 4 internal	TGTACACATTTGAGGAGAAA	CTGACAGACGCACTAGTCG	56	330
c-KIT exon 5 internal	TGGAGAAGTTAATTGCTGCT	ACTGTCTAATAATTTCTTC	53	336
c-KIT exon 6 internal	TTGTAATTCCAAGATGAGG	GATCTGATAAGCCCACTG	56	338
c-KIT exon 7 internal	TATGTGTGTGCGTGTTTATG	CAAGTTGAGTCCTTGCAGCTG	60	374
c-KIT exon 8 internal	CTCAGGAAGGTTGTAGGG	TAGAGAAGTCATTCAGTAA	56	426
c-KIT exon 9 internal	TTCCTAGAGTAAGCCAGGG	AATCATGACTGATATGGT	56	299
c-KIT exon 10 internal	GATCCCATCCTGCCAAAGTT	ATTGTCTCAGTCATTAGAGCAC	56	206
c-KIT exon 11 internal	CAGGTAACCATTTATTTGT	TCATTGTTTCAGGTGGAAC	56	327
c-KIT exon 12 internal	CACCAGCACCATCACCACTT	AGCAAAAAGCACAACTGGC	60	209
c-KIT exon 13 internal	TGGTACTGCATGCGCTTGACA	AAAGGCAGCTTGGACACGGCT	60	206
c-KIT exon 14 internal	GTCTGATCCACTGAAGCTG	ACCCCATGAACTGCCTGTC	56	319
c-KIT exon 15 internal	AACTTTACATGACTTTCCTC	ACCCACTTGCAACCCTAACT	56	386
c-KIT exon 16 internal	GAAGTGATCTGCCTGCAAG	GGCTCTAAAATGCTCTGTTCT	56	350
c-KIT exon 17 internal	GTTTTCACTCTTTACAAGT	ATGTGTGATATCCCTAGACAGGAT	56	301
c-KIT exon 18 internal	TGAGCTTCTGAATTAACAT	CCTTCCTTGATCATCTTGT	56	331
c-KIT exon 19 internal	GATCCTTGCCAAAGACAAC	TGAAAACCCTCAACATCTGGGT	56	292
c-KIT exon 20 internal	TACTGAAGTTGCTGGATGC	GGACACACCTGGAACTGGG	56	244
c-KIT exon 21 internal	AGTATGCCTTTTGTTGCTAT	TCATTCCTGGAGGGGTG	56	232

Supplementary Table E2. Patients' specific primers for *AML1/ETO* mutation

	Forward primers (5′—3′)	Reverse primers (5′—3′)
Patient 8 external	TGAGTCTTGAGGGCTGGTCT	GGAGCTTGAAAGAAACCTGCC
Patient 8 internal	GCCCTGCTAGCTCAGTCAAT	CCTGCCAAGAGTTTGTTGGT
Patient 11 external	TCTAGGATTGGGTCAGGGCA	AGCAGTCTACTGACATGGGCT
Patient 11 internal	GATTGGGTCAGGGCATGTGA	GCCTTTCCACAGGTCTTCTCA
Patient 23 external	AAGGGGCTTCAGGAAGAGTC	AAGCTGAGCCGACCACTTTT
Patient 23 internal	GGGCTTCAGGAAGAGTCACA	GTGCTTTTAACCCCCTGGGA

Supplementary Table 3. Primers for the single cell quantitative PCR analysis

	Forward primers (5′—3′)	Reverse primers (5′—3′)	Probe (FAM-MGB)
AML1 qPCR external	CAAACCCACCGCAAGTCGCC	GGCTGACCCTCATGGCTGTGC	-
AML1 qPCR internal	CCGCAAGTCGCCACCTACCA	GCCGCAGCTGCTCCAGTTCA	CATCGGCAGAAACT
AML1/ETO qPCR external	CAAACCCACCGCAAGTCGCC	TTGGAGGAGTCAGCCTAGATTGCGT	-
AML1/ETO qPCR internal	CCGCAAGTCGCCACCTACCA	CCGCAAGTCGCCACCTACCA	CTCGAAATCGTACTGAGAAG

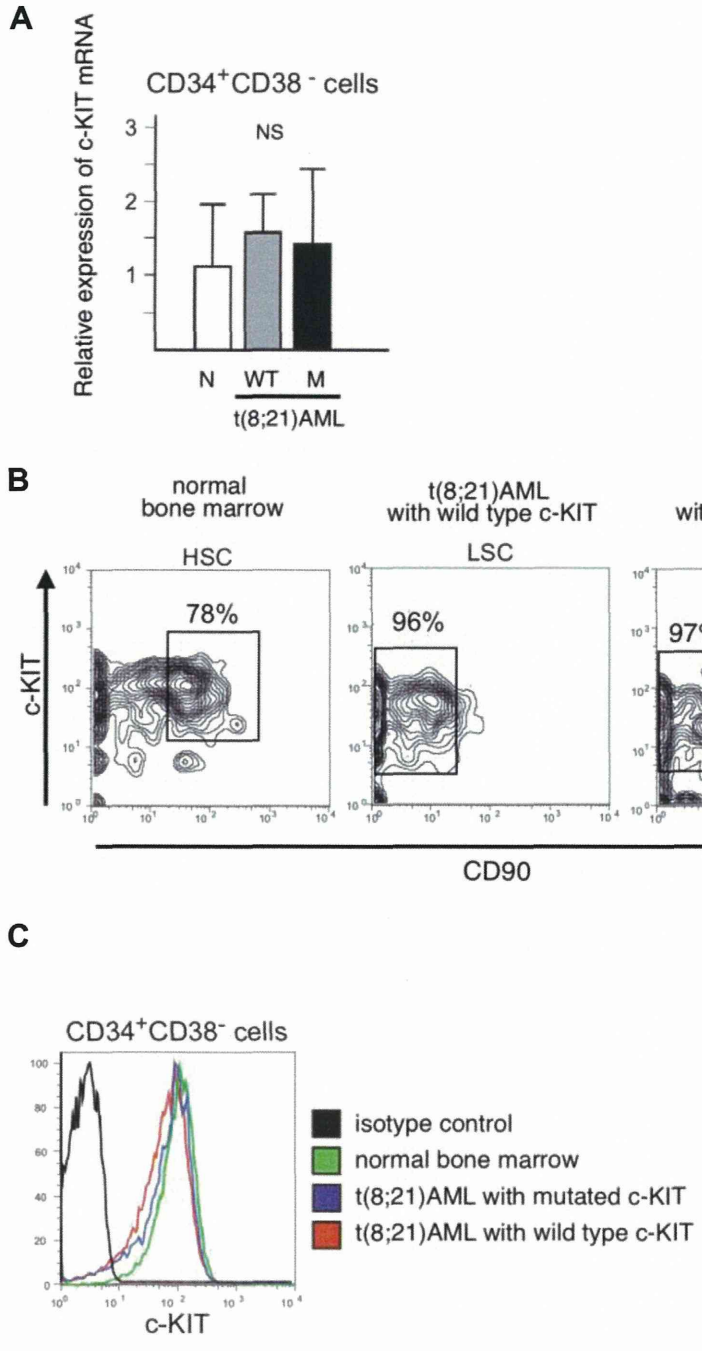
Supplementary Table E4. Genes upregulated or downregulated by over twofold in patients with mutant *c-KIT*

Gene	Fold change	Regulation
NEU4	16.14177	Up
IL8	8.642307	Up
ATF3	5.3436446	Up
NR4A2	4.6731033	Up
PDE4B	4.560626	Up
SIK1	4.2487206	Up
CSDE1	4.216702	Up
NR4A2	4.1884294	Up
KLF6	4.1034	Up
RGS2	4.0624347	Up
DKFZp451A211	3.9222076	Up
TIPARP	3.7905805	Up
JUN	3.77206	Up
CD83	3.6409142	Up
KLHL15	3.4783096	Up
C13orf15	3.394554	Up
AXUD1	3.3869207	Up
GAS7	3.2704463	Up
CD83	3.2681978	Up
LOC644936	3.187798	Up
XAGE1B	3.1597843	Up
TNFAIP3	3.0895545	Up
SGK	3.0253735	Up
XAGE1A	2.968178	Up
CDKN2D	2.8945742	Up
NLRP3	2.882177	Up
TSC22D3	2.8238995	Up
NLRP3	2.815252	Up
IL8	2.8040483	Up
PTGS2	2.7757351	Up
PDE4B	2.7746418	Up
KLF6	2.774551	Up
TSC22D3	2.7563179	Up
MYADM	2.727143	Up
PSCDBP	2.7197104	Up
CD69	2.719084	Up
BCL11A	2.7186139	Up
CYTIP	2.6962366	Up
PMAIP1	2.6845686	Up
IFIT2	2.6844542	Up
F11R	2.6836522	Up
RGPD5	2.6785529	Up
SPTBN1	2.6567411	Up
HIF1A	2.625503	Up
OSM	2.6132405	Up
TPPP3	2.613133	Up
BCL2	2.573377	Up
RIPK2	2.551046	Up
IDS	2.5435395	Up
DNAJB14	2.5313227	Up
IRS2	2.5246117	Up
PIGA	2.502675	Up
PABPC4L	2.4957426	Up
CDKN1A	2.4884536	Up
FTH1	2.4739304	Up
ARL4A	2.4711127	Up
SIK1	2.4488606	Up
SLC31A2	2.4468434	Up
LBR	2.4299662	Up
PER1	2.428982	Up
RNF10	2.4289358	Up
CXCR4	2.4257038	Up
HIST2H2BE	2.4105597	Up
TREM1	2.407748	Up
DUSP5	2.403791	Up
STK17B	2.397199	Up
KLF11	2.3865235	Up
DAD1L	2.3744328	Up
HIST1H4A	2.3475258	Up
HIST1H1C	2.3427014	Up
LMNA	2.3376257	Up
MCL1	2.3375716	Up
LOC100132418	2.3364425	Up
LOC651309	2.3218596	Up
KLF2	2.3194962	Up
RANBP9	2.3180208	Up
HIST1H3D	2.3122156	Up
LOC652226	2.2992957	Up
TRA2A	2.289847	Up
COPS2	2.2791734	Up
ARL4A	2.27622	Up
ERN1	2.2705226	Up
FTHL11	2.2671752	Up
LDLR	2.266	Up
GALR2	2.2607083	Up
LMNA	2.25953	Up
ITGA9	2.2548873	Up
CDC42SE1	2.2428436	Up
KLF6	2.2388575	Up
SKP1	2.229393	Up
C1orf55	2.1997943	Up
RAB17	2.1989782	Up
MXD1	2.1932738	Up
UBE2D3	2.1830506	Up
LOC654126	2.177946	Up
RMND5A	2.1719003	Up
FTHL2	2.1643596	Up
CD69	2.1608243	Up
DNAJB6	2.1585906	Up
SLC25A24	2.1563485	Up
ANXA2P1	2.1527424	Up
WHAMM	2.1476474	Up
FTHL11	2.1474578	Up
FLJ33590	2.130018	Up
SAP30	2.1245534	Up
FAM26F	2.1223474	Up
KLF11	2.1153169	Up
LOC654126	2.0836039	Up
EAF1	2.083288	Up
LOC100129905	2.0823586	Up
LOC285074	2.0809927	Up
NFKBIA	2.0617783	Up
LOC644422	2.0600662	Up
BCL2	2.0487194	Up
ETNK1	2.0485618	Up
SDHALP1	2.0467134	Up
FTHL3	2.044776	Up
UBE2L3	2.0408573	Up
FAM65B	2.0376937	Up
ITCH	2.0362887	Up
FEM1B	2.0333986	Up

Supplementary Table E4. (*continued*)

Gene	Fold change	Regulation
FEM1C	2.0307894	Up
WWP2	2.0043664	Up
PIAS2	2.0022597	Up
F13A1	2.0020864	Up
VCAM1	−3.6228995	Down
CSTF3	−3.2687597	Down
MAGED1	−3.1033447	Down
CD247	−2.8345284	Down
MFGE8	−2.8191347	Down
MGC39900	−2.6340804	Down
SLC40A1	−2.6316283	Down
IRF5	−2.626895	Down
C2orf44	−2.5530567	Down
KLHL3	−2.537466	Down
MYC	−2.533614	Down
POLQ	−2.519416	Down
RSPO1	−2.4792924	Down
C1S	−2.4603832	Down
CD247	−2.4599783	Down
ZNF616	−2.4583354	Down
RNASE3	−2.4497318	Down
GPT2	−2.4312274	Down
STARD8	−2.4143083	Down
SPIRE1	−2.4086332	Down
GYG2	−2.362726	Down
C2orf40	−2.3493814	Down
FAM43A	−2.339291	Down
RARRES2	−2.3389773	Down
ZNF135	−2.337041	Down
TNS3	−2.3365765	Down
ANGPT1	−2.335653	Down
TCTEX1D1	−2.329428	Down
LOC284757	−2.3185284	Down
ZNF526	−2.3017328	Down
TRAPPC5	−2.2595809	Down
CCL23	−2.2565548	Down
C6orf125	−2.2436416	Down
GINS2	−2.2227316	Down
CENPM	−2.2061203	Down
WDR40A	−2.2041402	Down
TRIM6	−2.193719	Down
CCDC34	−2.1790016	Down
IFI27L2	−2.174044	Down
MGC16121	−2.171578	Down
C9orf40	−2.1645977	Down
ZNF84	−2.159642	Down
FANCE	−2.1517787	Down
ANKRD35	−2.1510656	Down
CYP46A1	−2.146884	Down
SLC44A1	−2.1467984	Down
PTGR1	−2.1382608	Down
CKS1B	−2.1347759	Down
NDUFB7	−2.1328485	Down
VANGL2	−2.1275373	Down
SLMO1	−2.1170223	Down
TRO	−2.1113238	Down
HRASLS3	−2.0928133	Down
SALL4	−2.0898502	Down
ASPM	−2.0893974	Down
ZNF232	−2.06298	Down
TM4SF1	−2.0553544	Down
LOC554206	−2.0506268	Down
SH3BP4	−2.0495594	Down
DPM3	−2.032855	Down
CENTG3	−2.0327954	Down
N6AMT1	−2.0305235	Down
CDCA5	−2.0081842	Down
NLGN2	−2.0042732	Down
RCOR2	−2.0032747	Down
WDR54	−2.0021079	Down
TRO	−2.0018616	Down
PRRT3	−2.0001419	Down

(*continued*)



Supplementary Figure E1. Analyses of $CD34^+CD38^-$ fraction of normal, diagnostic and remission bone marrow. (**A**) The expression level of c-KIT in normal HSCs, t(8;21) AML with wild-type *c-KIT* LSCs, and t(8;21) AML with mutated *c-KIT* LSCs in a quantitative PCR analysis. Each bar shows the fold difference of the level of c-KIT mRNA in comparison to normal HSCs. There was no significant difference of c-KIT mRNA expression levels among these cells. (**B**) Phenotype of normal HSCs, t(8;21) AML with wild-type *c-KIT* LSCs, and t(8;21) AML with mutated *c-KIT* LSCs by five-color flow cytometer. The phenotype of normal HSCs was $CD34^+CD38^-CD90^+c\text{-}KIT^+$. LSCs displayed the $CD34^+CD38^-CD90^-c\text{-}KIT^+$ phenotype, irrespective of additional *c-KIT* mutation. Representative data (Patients 1 and 3) are shown. (**C**) The expression level of c-KIT did not differ among HSCs and LSCs with or without *c-KIT* mutant.