these c-CBL mutants strongly inhibit the E3 ligase activity of wild-type c-CBL, indicating that linker-RING finger mutants act in a dominant negative manner against wild-type c-CBL (10). This finding is expected because a simple loss-of-function would not explain the dominant effect of c-CBL mutant on transforming activity in NIH3T3 cells expressing wild-type c-CBL. Interestingly, this inhibitory effect does not seem to depend on dimerization with the wild-type c-CBL, but on intact binding to phosphorylated tyrosine kinases, because a G306E mutation abolishes oncogenic capacity of these c-CBL mutants.[10] Thus, when overexpressed in EGFR-transduced NIH3T3 cells, mutant c-CBL inhibits ubiquitinylation of EGFR, leading to prolonged activation of the receptor after EGF stimulation. Similarly, transduction of c-CBL mutants into hematopoietic cell lines results in prolonged activation of c-Kit, FLT3, and Jak2 kinases after stimulation with either their ligands or interleukin 3 (IL-3; Fig. 2A, lower panel; refs. 10, 55). Murine hematopoietic progenitors transduced with tumor-derived *c-CBL* mutants show increased cell survival in the presence of stem cell factor, similar to those from *c-CBL* null mice (10). Unexpectedly, however, the effect of these c-CBL mutants becomes much more prominent in the *c-CBL* null background, in which these c-CBL mutants induce exaggerated survival or even proliferative responses to stem cell factor. Moreover, the augmented proliferative and/or survival responses of mutant c-CBL–transduced cells are also found for a broader spectrum of cytokines, including thrombopoietin, IL-3, and FLT3 ligand (10). These effects of *c-CBL* mutants found in the *c-CBL* null background are not explained by either a simple loss of c-CBL functions or inhibition of wild-type c-CBL, but should be interpreted as true gain of function. Of particular interest, the gain of function of mutant c-CBL is lost in large part by the presence of either wild-type *c-CBL* allele or cotransduction of wild-type *c-CBL*. The gain of function becomes apparent in the *c-CBL* null background, explaining the observation that *c-CBL* mutations are found in a homozygous state with loss of the wild-type c-CBL in most cases (7–10).

Currently, the exact mechanism of the gain of function of c-CBL mutants is unclear. A possible mechanism is inhibition of CBL homologs (Fig. 2B, red arrow) and/or CBL-intrinsic positive regulatory machinery (Fig. 2B, blue arrow). Because the hypersensitive response to cytokines in mutant *c-CBL*–transduced cells is markedly diminished by wild-type c-CBL, it is mediated by inhibition of "CBL-like" activity still present in *c-CBL* null cells, most likely CBL-b. Mutant c-CBL also inhibits E3 ubiquitin ligase activity of CBL-b, which is expressed in hematopoietic progenitor cells (10). *c-CBL/CBL-b* double knockout T cells show exaggerated proliferative response to anti-CD3 stimulation and prolonged T-cell receptor signaling, as compared with *c-CBL* or *CBL-b* single knockout T cells (57). According to this model, two mutant *c-CBL* alleles could functionally titrate out two wild-type *CBL-b* alleles, whereas one mutant *c-CBL* allele might not be sufficient to overcome one wild-type *c-CBL* plus two wild-type *CBL-b* alleles (Fig. 2C).

Another possible mechanism of the gain of function of mutated c-CBL is related to its function as a multi-adaptor, which is implicated in positive regulatory functions in signal transduction (Fig. 2B, blue arrow). As an adaptor protein, kinase-bound c-CBL recruits a number of molecules involved in signal transductions and cytoskeletal regulations. For examples, upon either IL-4 or granulocyte colony-stimulating factor stimulation, c-CBL is tyrosine-phosphorylated and binds to the p85 subunit of phosphoinositide 3 kinase (PI3K) to transmit mitogenic and/or survival signals (58, 59). Similarly, CBL was shown to regulate integrin-mediated cell adhesion, spreading, and migration in a PI3K-dependent manner (60, 61). CBL promotes activation of MAP kinases after stimulation of Met tyrosine kinase through binding to Crk (62). c-CBL is one of the downstream substrates and/or effectors of Src kinase signaling, necessary for bone resorption and osteoclast migration (63). It is also involved in cytoskeletal regulation via activation of Rac1 or Cdc42, and *R*-RAS (64). In the face of loss of negative regulatory functions due to compromised E3 ubiquitin ligase activity, the intrinsic role in positive signaling of c-CBL protein could be unmasked as gain of function (Fig. 2B). This model could explain the observation that c-CBL mutations were much more frequent than CBL-b mutations in MDS-MPN, because both proteins clearly have different functionalities, as evident from the different phenotypes of their knockout mice (51, 52, 65).

## Clinical-Translational Advances

Gene mutations in signal transduction pathways are a common feature of MPN. Deregulated kinase activity caused by *bcr-abl* and mutated *JAK2* is a hallmark of chronic myelocytic leukemia and classical myeloproliferative disorders, including polycythemia vera, essential thrombocythemia, and primary myelofibrosis (66). Genes for RTKs, such as PDGFRs (PDGFRA/B) and fibroblast growth factor receptors (FGFR) are also recurrent targets of gene fusions in hypereosinophilic syndrome (PDGFRA) and subsets of CMML (FGFR; ref. 67). Finally, gene mutations commonly involving RAS pathway genes, including *NF-1*, *RAS*, and *PTPN11*, occur in more than 70% of CMML cases, responsible for their hypersensitivity to granulocyte-macrophage colony-stimulating factor (15, 67). The recent finding of frequent *c-CBL* mutations in the MDS-MPD subgroup revealed a novel mechanism for excessive cell signaling through deregulated kinase activity in MPN, especially MDS-MPN subtypes, and also provided an insight into the therapeutics of *c-CBL*–mutated myeloid neoplasms.

Because *c-CBL* mutations induce excessive tyrosine kinase signaling, use of tyrosine kinase inhibitors could be

[10] Unpublished data.

a logical approach to the control of *c-CBL*–mutated neoplasms. However, the broad spectrum of c-CBL–regulated tyrosine kinases may preclude the efficacy of selective kinase inhibitors, whereas the use of pan-kinase inhibitors would increase a risk of the development of unacceptable adverse effects. Otherwise, identification of functionally relevant kinases regulated by mutated c-CBL would enable efficient targeting of such inhibition. Alternatively, the downstream signaling pathways, including JAK/STAT, PI3K, as well as RAS/extracellular signal-regulated kinase (ERK) signalings, are also potential therapeutic targets for inhibition with low molecular-weight compounds.

Given the gain-of-function nature of c-CBL mutants, inhibition of these mutant proteins would be a more reasonable approach, regardless of the exact mechanism of the gain-of function. Because the oncogenic action of mutant c-CBL proteins depends on their intact binding to target kinases, inhibition of this binding would be a potential approach, especially when the inhibition could be specifically directed to mutant c-CBL, but be saved for CBL-b. Recently, piceatannol, a naturally occurring phenol stilbenenoid, was shown to induce loss of the CBL family of proteins including mutant CBL (70Z mutant; ref. 68). Piceatannol was initially isolated as an antileukemic agent from a domesticated oilseed and was shown to inhibit a broad spectrum of tyrosine kinases including Sky, Src, Lck, and FAK, as well as some serine-threonine kinases (69–72). It also induces selective loss of CBL-associated proteins; levels of PDGFRβ, c-Abl, and EGFR are reduced by piceatannol treatment, whereas those of c-Src, Lyn, Syk, and Grb2 are unaffected (68). The molecular mechanism that underlies piceatannol-induced CBL loss is still unclear. It does not depend on proteasome, lysosome, and caspase activation, but rather on reactive oxygen species, which seems to be distinct from the mechanism of inhibition of kinase activities (68). Although piceatannol shows a broad spectrum of biological activity as an anti-inflammatory, antihistamine, and general antitumor agent *in vitro* (73–75), because of its broad biochemical actions, it has not been determined if, or to what extent, the biological activities of piceatannol depend on piceatannol-induced loss of CBL proteins. Although loss of both c-CBL and CBL-b is likely to result in increased tyrosine kinase activity, it also induces CBL-associated molecules and inhibits activity of a number of kinases, actually showing general antitumor activity. Unfortunately, no information is currently available about the antitumor effect of piceatannol on c-CBL–mutated leukemia. In c-CBL–mutated leukemic cells, loss of mutant c-CBL may further augment antitumor activity of this agent.

## Conclusion

*c-CBL* mutations are tightly associated with myeloproliferative myeloid neoplasms, especially the MDS-MPD subtype. c-CBL seems to act as a tumor suppressor, but when mutated, it is converted to an oncogenic protein. Although the oncogenic potential of c-CBL mutants is thought to be related to a type of gain of function, the molecular basis of this gain of function has not been fully understood. Undoubtedly, the effect of these mutations on the E3 ubiquitin ligase activity is essential for the gain of function. What complicates the mechanism is the fact that c-CBL has dual functionalities; it can behave as a multi-adaptor signal transducer, while also terminating signals by ubiquitinylating activated tyrosine kinases. Clearly, to understand the exact oncogenic mechanism of c-CBL mutants and to develop effective therapeutics, further *in vivo* and *in vitro* analyses are required.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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bjh short report

# Spectrum of molecular defects in juvenile myelomonocytic leukaemia includes *ASXL1* mutations

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## Summary

Mutations in *NF1, PTPN11, NRAS, KRAS* and *CBL* have been reported to play a pathogenetic role in juvenile myelomonocytic leukaemia (JMML), a rare myelodyplastic/myeloproliferative neoplasm occurring in children. Recently, mutations in *ASXL1* were identified in chronic myelomonocytic leukaemia and other myeloid malignancies. We sequenced exon 12 of *ASLX1* in 49 JMML patients, and found 2 novel heterozygous (nonsense and frameshift) mutations, one occurring as a sole lesion, the other was in conjunction with a *PTPN11* mutation. *ASXL1* cooperates with *KDM1A* in transcriptional repression and thereby *ASXL1* mutations may synergize with or mimic other JMML-related mutations.

**Keywords:** juvenile myelomonocytic leukaemia, *ASXL1*, chronic myelomonocytic leukaemia, *PTPN11*, RAS signalling.

The molecular pathogenesis of juvenile myelomonocytic leukaemia (JMML) includes a number of recurrent mutations and chromosomal aberrations, all resulting in a similar clinical phenotype and the characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Koike & Matsuda, 2008). Constitutional mutations of *NF1*, a GTPase-activating protein that negatively regulates RAS, are associated with the characteristic neurofibromatosis type 1 (NF-1) features and JMML. During leukaemic evolution, heterozygous *NF1* alleles are duplicated through somatic uniparental disomy (UPD) of 17q in 2/3 of mutant cases, while the remaining 1/3 of affected children show compound-heterozygous inactivating *NF1* mutations (Steinemann *et al*, 2010). Other mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, have been reported in 10–20% and 35% of patients with JMML, respectively. Mutations of *PTPN11*, encoding tyrosine phosphatase SHP-2, lead to elevated levels of Ras-GTP, the active form of Ras. Recently, we and others have described ring finger domain mutations of *CBL*, an ubiquitin ligase involved in inactivation of activated phosphothyrosine kinase receptor (TRK)-mediated signals. The presence of *CBL* mutations also explains the laboratory findings of GM-CSF hypersensititvity. Similar to *NF1*, some heterozygous *CBL* mutations can also be constitutional (Loh *et al*, 2009; Muramatsu *et al*, 2009).

In some phenotypic features JMML may resemble chronic myelomonocytic leukaemia (CMML), but cytogenetic and mutational spectra differ between the conditions. While *PTPN11*, *NRAS* and *KRAS* mutations are not a common finding in CMML, *CBL* mutations have also been found in both CMML and JMML. Based on the identification of

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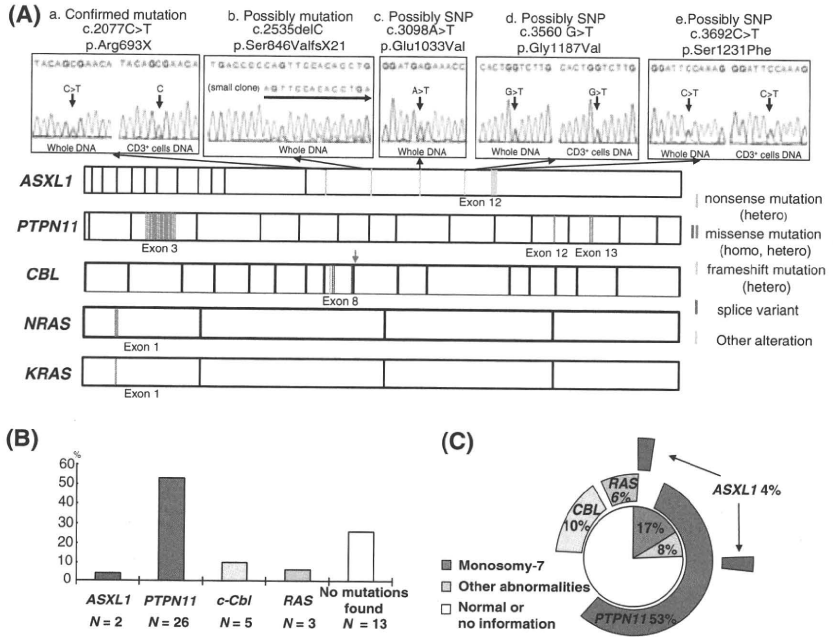


**Fig 1.** Mutational spectrum detected in JMML. High-density Affymetrix (250K) single nucleotide polymorphism array (SNP-A) karyotyping was performed on 49 patients with JMML and chromosomal lesions annotated. *PTPN11* (exon 2, 3, 4, 7, 8, 12 and 13), *NRAS*, *KRAS* (codons 12, 13 and 61), *TET2* (exon 3-11), *CBL* family members (exon 8 and 9 of *CBL*, exon 9 and 10 of *CBLB*, exon 7 and 8 of *CBLC*), *IDH1* (exon 4) and *ASXL1* (exon 12) were sequenced. For confirmation of somatic nature of observed mutations, immunoselected CD3+ cells were used as a germ line control. (A) Topography of mutations in *ASXL1*, *PTPN1*, *CBL*, *NRAS* and *KRAS* in JMML. Phenograms of nucleotide sequence alterations of *ASXL1* are shown. (B) Prevalence of each mutation in JMML. Percentage of each gene mutated cases in 49 JMML are shown. (C) Overlap between karyotype abnormalities and each mutation in JMML. Cytogenetic aberrations were identified by metaphase cytogenetics. Two cases with *ASXL1* mutations also harboured *PTPN11* mutation or monosomy-7, respectively.

frequent loss of heterozygosity (LOH) of 4q24 and an index microdeletion, mutations in *TET2* have been found in 40–50% of patients with CMML (Jankowska *et al*, 2009). Laboratory findings suggest that *TET2* mediates the hydroxylation of methylated cytosine residues in CpG islands and may thereby be a determinant of epigenetic instability (Tahiliani *et al*, 2009). However, unlike *CBL* mutations, *TET2* mutations have not been found in JMML and as a consequence, cases without a pathogenic mutation still constitute approximately 30% of patients with JMML (Muramatsu *et al*, 2009). While chromosomal abnormalities, particularly monosomy-7, are present in some of these cases, other pathogenetic mutations are likely and may involve genes within and outside of regions affected by LOH.

Recently, novel mutations involving isocitrate dehydrogenase 1 (*IDH1*) and the homologous gene *IDH2* have been identified in myeloproliferative neoplasms (MPN). *IDH1* and *IDH2* mutations are reported in 10–20% of de novo acute myeloid leukaemia (AML) (Mardis *et al*, 2009; Ward *et al*, 2010), and also in patients with secondary AML (sAML) that evolved from MPN (Green & Beer, 2010). These mutations are thought to lead to increased production of a pathological metabolite, 2-hydroxyglutarate (2HG), which contributes to malignant progression by yet an unknown mechanism.

In CMML, additional sex comb-like 1 (*ASXL1*) mutations have been found in approximately 40% of patients. This mutation has also been detected in a smaller proportion (10%) of patients with myelodysplastic syndrome (MDS), MPN and AML (primary AML 5%, secondary AML 50%) (Carbuccia *et al*, 2009; Gelsi-Boyer *et al*, 2009). *ASXL1* mutations are restricted to exon 12. ASXL1 cooperates with KDM1A (LSD1) in transcriptional repression, presumably by removing H3K4 methylation, an active histone mark, but not a repressive H3K9 methylation mark, recognized by HP1 (Lee *et al*, 2010). Based on evidence that some of the mutations are shared in related classes of myeloid malignancies, in particular CMML and JMML, we hypothesized that mutations in *IDH1*, *IDH2*, and *ASXL1* could also be present in children with JMML.

## Results and discussion

We studied 49 children with JMML. Written informed consent for sample collection was obtained from the patients' parents according to established institutional protocols. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria (Niemeyer *et al*, 1998) and excluded patients with Noonan syndrome.

By conventional metaphase cytogenetics, chromosomal aberrations are found in only 25% of JMML patients. Using single nucleotide polymorphism arrays (SNP-A) as a



 

**Table I.** Clinical and molecular data in 49 JMML patients.

| Case no. | Sex | Age (months) | Hb (g/l) | HbF (%) | WBC ($\times 10^9$/l) | Monocyte (%) | Platelets ($\times 10^9$/l) | Myeloblast (%) | SCT | Survival (month) | Karyotype | SNP-A | *ASXL1* | *PTPN11* | *CBL* | *RAS* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 41 | 97 | 34·5 | 48·5 | 10 | 49·0 | 2 | – | 3, Dead | 46,XY,inv(4)(p14p16) | – | WT | MT | WT | WT |
| 2 | M | 65 | 49 | 32·4 | 15·3 | 17 | 26·0 | 17 | – | 4, Dead | 46,XY | 19p13.3 loss | WT | MT | WT | WT |
| 3 | F | 66 | 78 | 3 | 32·2 | 46 | 58·0 | 2 | + | 6, Dead | 45,XX,-7 | monosomy-7, 12p13.2 loss | MT | WT | WT | WT |
| 4 | F | 6 | 87 | 22·4 | 100 | 14 | 49·0 | 4 | + | 216, Alive | 46,XX | – | WT | WT | WT | WT |
| 5 | M | 58 | 89 | 1 | 11·8 | 14 | 13·0 | 25 | – | 10, Dead | 45,XY,-7 | monosomy-7, trisomy 21 | WT | WT | WT | WT |
| 6 | F | 47 | 78 | 17·7 | 85·5 | 37 | 21·0 | 7 | + | 10, Dead | 46,XX | – | WT | WT | WT | WT |
| 7 | M | 49 | 114 | 11·3 | 16·8 | 7 | 36·0 | 3 | – | 19, Dead | 46,XY | – | WT | MT | WT | WT |
| 8 | M | 27 | 94 | 46·1 | 72·9 | 10 | 25·0 | 4 | + | 8, Dead | 48,XY,+X,+13 / 45,X,-Y | – | WT | MT | WT | WT |
| 9 | M | 29 | 106 | 14·9 | 10·9 | 26 | 8·9 | 6 | + | 3, Dead | 45,XY,-7 | monosomy-7 | WT | MT | WT | WT |
| 10 | M | 61 | 120 | 55·4 | 12·2 | 9 | 8·0 | 1 | + | 116, Alive | 46,XY | – | WT | MT | WT | WT |
| 11 | M | 53 | 85 | 24·7 | 15·1 | 6 | 29·4 | 5 | + | 48, Alive | 46,XY | – | WT | WT | WT | WT |
| 12 | M | 36 | 109 | 43·9 | 19·4 | 16 | 42·0 | 3 | + | 84, Alive | 46,XY | – | WT | WT | WT | WT |
| 13 | M | 34 | 69 | 28·4 | 15·7 | 20 | 1·4 | 35 | + | 9, Dead | 46,XY | – | WT | MT | WT | WT |
| 14 | M | 56 | 120 | 9 | 58·5 | 26 | 102·0 | 2 | + | 6, Dead | 45,XY,-7 | monosomy-7, 1p31.3 gain | WT | MT | WT | WT |
| 15 | F | 36 | 64 | 30·5 | 23·9 | 30 | 29·0 | 6 | + | 6, Dead | 46,XX | 17q11.2 loss, 6q21q25.3 loss | WT | WT | WT | WT |
| 16 | M | 8 | 72 | NA | 36 | 18 | 85·0 | 2 | + | 10, Dead | 46,XY | 7p21.1 gain, 11q13.3q25 UPD | WT | WT | MT | WT |
| 17 | M | 36 | 105 | 23·8 | 563 | NA | 59·0 | NA | + | 16, Dead | 47,XY,+8 | trisomy 8, 7q11.22 gain | WT | MT | WT | WT |
| 18 | M | 24 | 84 | 35·8 | 12·6 | 18 | 38·0 | 3 | + | 63, Alive | 46,XY | – | WT | MT | WT | WT |
| 19 | F | 1 | 85 | NA | 55·4 | 25 | 49·0 | 10 | – | 11, Dead | 46,XX | – | WT | WT | WT | WT |
| 20 | F | 19 | 114 | 10·2 | 23·3 | 9 | 17·0 | 8 | – | 38, Alive | 46,XX | 5q23.1 loss | WT | WT | WT | WT |
| 21 | M | 24 | 80 | NA | 49·8 | 6 | 8·0 | 19 | + | 19, Dead | 45,XY,del(6)(q?),-20 | – | WT | MT | WT | WT |
| 22 | M | 35 | 109 | 49·3 | 14·7 | 7 | 123·0 | NA | + | 22, Alive | 45,XY,-7 | 18q12.3 gain | WT | MT | WT | WT |
| 23 | M | 12 | 89 | 2 | 50·1 | 9 | 320·0 | 3 | – | 21, Alive | 46,XY | – | WT | WT | MT | WT |
| 24 | F | 48 | 108 | 30·5 | 20·1 | 13 | 156·0 | 9 | + | 10, Dead | 46,XX | – | WT | MT | WT | WT |
| 25 | M | 11 | 86 | 1·1 | 16·8 | 22 | 65·0 | 0 | – | 84, Alive | 46,XY | – | WT | WT | WT | MT |
| 26 | F | 15 | 104 | 4·4 | 24·1 | 20 | 134·0 | 0 | – | 20, Alive | 46,XX | – | WT | WT | WT | MT |
| 27 | F | 15 | 69 | NA | 29·5 | 17 | 28·0 | 0 | NA | NA | 46, XX | 11q12.1q25 UPD | WT | WT | MT | WT |
| 28 | F | 16 | 111 | 20·8 | 56·2 | 8 | 175·0 | 1 | + | 22, Alive | 46, XX | – | WT | MT | WT | WT |
| 29 | M | 39 | 95 | 19 | 22·6 | 5 | 93·0 | 0 | + | 28, Alive | 46, XY | 10p11.23 gain | MT | MT | WT | WT |
| 30 | M | 2 | 111 | 25·1 | 79·3 | 14 | 132·0 | 5 | + | 33, Alive | 46, XY | – | WT | WT | WT | MT |
| 31 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | – | WT | MT | WT | WT |
| 32 | M | 20 | 116 | 56·1 | 33 | 16 | 53·0 | 1 | + | 24, Alive | 46, XY | – | WT | MT | WT | WT |
| 33 | F | 45 | 87 | 21·9 | 81·5 | 14 | 23·0 | 5 | NA | NA | 46, XX | – | WT | WT | WT | WT |
| 34 | F | 18 | NA | NA | NA | NA | NA | NA | + | 35, Alive | 46, XX | – | WT | WT | WT | WT |
| 35 | F | 11 | 118 | 2·7 | 14·2 | 43 | 31·0 | 0 | + | 35, Alive | 45, XX, -7 | monosomy-7 | WT | WT | WT | WT |
| 36 | M | 28 | NA | NA | NA | NA | NA | NA | + | 36, Alive | 45, XY, -7 | monosomy-7, 8p21.2 loss | WT | MT | WT | WT |
| 37 | F | 29 | 89 | 55 | 16 | 18 | 23·0 | 11 | + | 4, Dead | 45, XX, -7 | monosomy-7 | WT | MT | WT | WT |
| 38 | F | 13 | 92 | 3·5 | 20·8 | 14 | 65·0 | 0 | + | 21, Alive | 46, XX | 11q23.3q25 UPD | WT | WT | MT | WT |
| 39 | M | 7 | 64 | NA | NA | NA | NA | NA | NA | NA | NA | 1q44 gain | WT | MT | WT | WT |

 

**Table I.** (Continued).

| Case no. | Sex | Age (months) | Hb (g/l) | HbF (%) | WBC ($\times 10^9$/l) | Monocyte (%) | Platelets ($\times 10^9$/l) | Myeloblast (%) | SCT | Survival (month) | Karyotype | SNP-A | *ASXL1* | *PTPN11* | *CBL* | *RAS* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 40 | M | 18 | 83 | 15 | 51 | 6 | 48·0 | 1 | + | 18, Alive | 46, XY | 5q31.3 loss | WT | MT | WT | WT |
| 41 | M | 2 | 76 | 37·4 | 59·5 | 17 | 59·0 | 2 | – | 1, Dead | 46, XY | – | WT | MT | WT | WT |
| 42 | M | 2 | 102 | NA | 10·5 | 18 | 71·0 | 6 | + | 5, Dead | 46, XY | – | WT | MT | WT | WT |
| 43 | F | 10 | 92 | 7·6 | 43·1 | 15 | 84·0 | 2 | – | 1, Dead | 46, XX | 11q23.3q25 UPD | WT | WT | MT | WT |
| 44 | M | 6 | 137 | NA | 23·8 | 21 | 16·0 | NA | – | 7, Dead | 46,XY | 1q25.3 loss | WT | WT | WT | WT |
| 45 | M | 41 | 99 | 32 | 49·9 | 8 | 19·0 | 1 | + | 14, Alive | 46, XY | – | WT | MT | WT | WT |
| 46 | M | 1 | 114 | 54 | 126·2 | 13 | 116·0 | 1 | + | 12, Alive | 46, XY | 2p22.1 loss | WT | WT | WT | WT |
| 47 | M | 75 | 100 | 62 | 41 | 49 | 105·0 | 1 | + | 12, Alive | 46, XY | 15q26.3 gain | WT | MT | WT | WT |
| 48 | M | 22 | 99 | 21 | 35·6 | 22 | 132·0 | 2 | + | 9, Alive | 46, XY | 17q11.2q25.3 UPD | WT | WT | WT | WT |
| 49 | M | 65 | 113 | 28 | 31·9 | 9 | 75·0 | 10 | – | 1, Alive | 46, XY | 17q11.2 loss | WT | MT | WT | WT |

NA, not available; SCT, stem cell transplantation; SNP-A, single nucleotide polymorphism arrays; WT, wild type; MT, mutant type.

karyotyping tool, we found chromosomal abnormalities in 49% of cases; UPD11q23, UPD17q or monosomy-7/7q- were present in 24% of cases (Muramatsu *et al*, 2009).

NF1 features, consistent with the presence of *NF1* mutations, were present in additional 2 patients. Recurrent del(7) in JMML could also be indicative of the presence of putative hemizygous mutations affecting specific genes on this chromosome. Mutations in genes involved in RAS pathway, including *KRAS*, *NRAS* and *PTPN11* were found in a significant proportion of JMML patients (59%). In contrast, these mutations were less frequently encountered in patients with the adult phenotypic counterpart of JMML, CMML. Based on the identification of *CBL* mutations in around 15% of CMML and other MDS/MPN cases (Makishima *et al*, 2009), we sequenced cases of JMML and have identified *CBL* mutations in 5/49 JMML cases. Both homozygous (1/49; due to UPD involving 11q23) and heterozygous (4/49) *CBL* mutations were found. However, unlike in CMML (Jankowska *et al*, 2009), *TET2* mutations were not detected in our JMML cohort, consistent with the total absence of LOH4q. Similarly, while advanced cases of CMML and sAML due to MPD may contain exon 4 *IDH1/2* mutations, they were totally absent in 49 JMML patients analysed in this study.

When exon 12 of *ASXL1* was sequenced in our cohort of JMML patients, we found 2 heterozygous mutations (4%): a nonsense (c.2077C>T p.Arg693X) and a frameshift (c.2535delC p.Ser846ValfsX21) mutation (Fig 1A). That these mutations prevent proper transcription into RNA suggests they represent somatic and not germline events. $CD3^+$ cells were used to confirm that the frameshift change was absent in the germline DNA; the non-sense mutation could not be confirmed. Neither mutation has been previously reported. LOH20q11 corresponding to *ASXL1* locus was not detected in any of the cases studied, indicating that the corresponding mutations were heterozygous. We have also identified a number of polymorphisms present in both tumour cells and $CD3^+$ cells (Fig 1A). The significance of these polymorphisms remains unclear but some have been observed in healthy controls.

Clinical analysis of patients with *ASXL1* mutations did not highlight any specific shared phenotypic features (Table I). Both cases presented with chromosomal abnormalities (none involving the *ASXL1* locus). In one case, monosomy-7 was detected in both metaphase cytogenetics and SNP-A. By SNP-A karyotyping, additional 12p13.2 loss was found in this case, while 10p11.23 gain was found in the second case. Patients affected by *ASXL1* mutations had a low myeloblast count in the marrow (2%, 0%) but only one displayed a significant monocytosis. Both patients underwent stem cell transplantation (SCT): patient 1 died 6 months after diagnosis, while the second patient is well 28 months after diagnosis.

*ASXL1* mutations detected in JMML induce truncation of the protein downstream of the ASXH domain and a consequent loss of the PDH domain, suggested to be functionally important in the tumour suppressor function of A*SXL1*. While a loss of function mouse model of *ASXL1* showed that it is

required for normal haematopoiesis, a MDS or AML phenotype was not observed (Fishe *et al*, 2010). These finding may also suggest that *ASXL1* mutations may contribute to the pathogenesis of haematologic malignancies through gain-of-function and consequently murine knock-in models may be needed for investigation of their consequences. In contrast to *PTPN*, *NRAS*, *KRAS* and *CBL* mutations, which were mutually exclusive, one patient with an *ASXL1* mutation had also a *PTPN11* mutation. Consequently, it is possible that *ASXL1* mutations play a cooperative role, for example, in disease progression. Because ASXL1 function is related to the regulation of epigenetic inactivation patterns, theoretically, mutations of this gene may affect expression of genes involved in RAS signalling networks or *CBL*, which can increase activity of RAS signalling thought to be responsible for GM-CSF sensitivity of JMML progenitor cells. Of note is that this characteristic feature was present in all 49 JMML cases, including 30% of cases in which no mutations were found.

## Conflict of interest

The authors declare no conflict of interest.

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# Analysis of mutations and recombination activity in RAG-deficient patients

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**KEYWORDS**
RAG deficiency;
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V(D)J recombination

**Abstract** Mutations in the recombination activating genes (*RAG1* or *RAG2*) can lead to a variety of immunodeficiencies. Herein, we report 5 cases of RAG deficiency from 5 families: 3 of Omenn syndrome, 1 of severe combined immunodeficiency, and 1 of combined immunodeficiency with oligoclonal TCR$\gamma\delta^+$ T cells, autoimmunity and cytomegalovirus infection. The genetic defects were heterogeneous and included 6 novel *RAG* mutations. All missense mutations except for Met443Ile in *RAG2* were located in active core regions of *RAG1* or *RAG2*. V(D)J recombination activity of each mutant was variable, ranging from half of the wild type activity to none, however, a significant decrease in average recombination activity was demonstrated in each patient. The reduced recombination activity of Met443Ile in *RAG2* may suggest a crucial role of the non-core region of RAG2 in V(D)J recombination. These findings suggest that functional evaluation together with molecular analysis contributes to our broader understanding of RAG deficiency.


## 1. Introduction

V(D)J recombination mediated by the recombination activating genes (RAG) 1 and RAG2 leads to the generation of diverse antigen receptors [1]. A complete lack of RAG activity causes severe combined immunodeficiency (SCID) with the

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absence of mature T and B cells, but the presence of natural killer (NK) cells ($T^-B^-$ SCID) [2], whereas partial loss results in variant syndromes, such as Omenn syndrome (OS) [3] or combined immunodeficiency (CID) presenting with oligoclonal TCR$\gamma\delta^+$ T cells, autoimmunity and cytomegalovirus (CMV) infection (CID with $\gamma\delta$/CMV) [4,5]. OS is characterized by early-onset generalized erythroderma, lymphadenopathy, hepatosplenomegaly, protracted diarrhea, failure to thrive, eosinophilia, hypogammaglobulinemia, elevated serum IgE levels, the absence of B cells, and the presence of activated and oligoclonal T cells [6]. In contrast to $T^-B^-$ SCID and OS, patients affected with CID with $\gamma\delta$/CMV exhibit autoimmune cytopenias, B cells, normal immunogulobulin levels, oligoclonal TCR$\gamma\delta^+$ T cells, and disseminated CMV infections [4,5]. Very recently, another distinct clinical syndrome caused by hypomorphic *RAG* mutations has been described. Schuetz et al. [7] reported 3 patients with late age of onset of illness characterized by hypogammaglobulinemia, diminished numbers of T and B cells, and the formation of granulomas in the skin, mucous membranes and internal organs. De Ravin et al. [8] described an adolescent patient presenting with destructive midline granulomatous disease who also exhibited autoimmunity, relatively normal numbers of T and B cells, and a diverse T-cell receptor (TCR) repertoire.

Herein, we report the identification of 8 *RAG* mutations including 6 novel mutations in a group of patients presenting with a variety of clinical phenotypes, and discuss the functional significance of these mutations by using the V(D)J recombination assay.

## 2. Materials and methods

### 2.1. Patients

We studied five patients with RAG deficiency from five families. Table 1 presents the immunological features of the patients. All patients except for patient 5 were born to non-consanguineous Japanese parents. The clinical and immunological data of patient 1 and patient 3 have been reported elsewhere [9]. Patient 2 was a 1-month-old boy who presented with generalized erythroderma, hepatosplenomegaly and *Pseudomonas aeruginosa* sepsis. Laboratory studies revealed hypereosinophilia, hypogammaglobulinemia, lack of B cells, and oligoclonal expansion of activated TCR$\alpha\beta^+$ T-cells. These findings were consistent with typical features of OS. Patient 4 was a 2-year-old girl who presented with prolonged diarrhea, bronchopneumonia, liver dysfunction and CMV infections. CMV was detected in her stool and sputum. Laboratory analysis revealed lymphopenia with normal immunoglobulin levels, an increased percentage of TCR$\gamma\delta^+$ T cells (61.7% of $CD3^+$), and multiple autoantibodies including anti-nuclear, anti-DNA, and antiparietal cell antibodies and Coombs test. In addition, IgG antibody against CMV was detected (20.7; normal, <2.0). Her elder sister suffered from autoimmune hemolytic anemia and immune mediated thrombocytopenia, and died of fatal interstitial pneumonia of adenovirus at age of 1 year. Patient 5 was the fourth child born to non-consanguineous parents of Indian origin. All of her 3 siblings were affected with immunodeficiency and died within the first year of life. Patient 5 showed lymphopenia, very low numbers of autologous T and B cells, preserved numbers of NK cells, and the presence of maternal $CD4^+$ T cell engraftment. At the age of 2 months, she remained asymptomatic except for oral thrush and microcephaly.

**Table 1** Immunological features of the patients at diagnosis.

| Patient | 1[a] | 2 | 3[a] | 4 | 5 |
|---|---|---|---|---|---|
| Diagnosis | OS | OS | Atypical OS | CID with $\gamma\delta$/CMV | Atypical SCID with MFT |
| Age at onset (month) | 0 | 0 | 7 | 8 | 0 |
| WBC | 26,900 | 19,000 | 2800 | 3900 | 3280 |
| Lymphocytes (/mm$^3$) | 8339 | 5700 | 1300 | 546 | 459 |
| $CD3^+$ (%) | 84.8 | 41.3 | 20.0 | 53.9 | 7.8 |
| $CD4^+$ (%) | 56.7 | 16.6 | 17.3 | 9.9 | 7.4 |
| $CD8^+$ (%) | 27.0 | 37.8 | 1.3 | 35.4 | 0.1 |
| $CD19^+$ or $20^+$ (%) | 0.0 | 0.2 | 0.1 | 11.6 | 0.1 |
| IgG (mg/dl) | 461 | 220 | 328 | 678 | 1475 |
| IgA (mg/dl) | <4 | <1 | 62 | 63 | 114 |
| IgM (mg/dl) | <4 | <2 | 31 | 65 | 147 |
| IgE (IU/ml) | 7 | <2 | 16 | NA | NA |

OS, Omenn syndrome; CID, combined immunodeficiency; $\gamma\delta$, TCR$\gamma\delta^+$ T cells; CMV, cytomegalovirus; SCID, severe combined immunodeficiency; MFT, maternal T-cell engraftment; WBC, white blood cells; NA, not available.

[a] Data of patient 1 and patient 3 have been reported previously [9].

Approval for this study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

### 2.2. Mutation analysis of RAG1 and RAG2

DNA was extracted from blood samples using standard methods. The *RAG1* and *RAG2* genes were amplified in several segments from genomic DNA using specific primers, as previously described [10,11]. Sequencing was performed on purified polymerase chain reaction (PCR) products using the ABI Prism BigDye Terminator Cycle sequencing kit on an ABI 3100 automated sequencer (Applied Biosystems, Foster, CA).

### 2.3. V(D)J recombination assay

*In vivo* V(D)J recombination assay was performed by using the recombination substrate pJH200 as described previously with modifications [3,12]. The complete open reading frames of human *RAG1* and *RAG2*, and the active core regions of mouse *RAG1* (aa 330–1042) and *RAG2* (aa 1–388) were subcloned into the mammalian expression vector pEF-BOS [13]. PCR products carrying the patients' mutations were also subcloned into the vector. Cotransfections of full-length human *RAG1*, the mouse *RAG2* active core, and pJH200, or of full-length human *RAG2*, the mouse *RAG1* active core, and pJH200 into 293T cells were performed using 1 μg of each plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Cells were harvested after 48-hours of culture, and the recombined products of signal joints were analyzed for recombination frequency by PCR using primers RA-CR2 and RA-14 [14]. After 30 cycles, the amplified products were visualized by ethidium bromide staining, and the intensity of each band was quantified using Image J software (NIH, Bethesda, MD).

### 2.4. Analysis of IgE production and somatic hypermutation (SHM) in variable regions of IgM

Peripheral blood mononuclear cells were isolated and incubated with 500 ng/ml of anti-CD40 (Diaclone, Besançon, France) and 100 U/ml of recombinant interleukin-4 (IL-4; R&D Systems, Minneapolis, MN) for 12 days. IgE production in culture supernatants was determined by enzyme-linked immunosorbent assay as previously described [15,16]. The frequency and characteristics of SHM in the $V_H$3-23 region of IgM were studied in purified $CD19^+$ $CD27^+$ B cells as previously described [15,16].

## 3. Results

### 3.1. RAG mutations

As shown in Table 2, we found 2 missense and 1 nonsense mutations in *RAG2* and 4 missense and 1 nonsense mutations in *RAG1*. Two distinct novel *RAG2* mutations, R73H and Q278X, were demonstrated in patient 1. Patient 2 was found to be homozygous for a novel M443I mutation in *RAG2*. Patient 3 was a compound heterozygote bearing R142X and R396H mutations in *RAG1*. The latter mutation has been repeatedly reported in OS patients [17]. Patient 4 was a compound heterozygote bearing R474C and L732P mutations in *RAG1*. These missense mutations are novel, although similar missense mutations, R474S, R474H and L732F, have been reported in patients with RAG deficiency [17–19]. Patient 5 carried a homozygotic novel E770K mutation in *RAG1*. All missense mutations but one (M443I in *RAG2*) were located in the active core regions of *RAG1* or *RAG2*, and all patients had at least one missense mutation. None of these mutations were found in 100 alleles of healthy controls.

**Table 2** *RAG* mutations and recombination activity.

| Patient | Gene | Nucleotide mutation | Effect | Relative recombination activity (%) [a] |
|---|---|---|---|---|
| 1 | RAG2 | 1419 G>A | R73H | 59.3±4.7 |
| | | 2033 C>T | Q278X | 0.4±0.3 |
| 2 | RAG2 | 2530 G>T [b] | M443I | 8.7±1.2 |
| 3 | RAG1 | 536 C>T | R142X | 51.2±9.2 |
| | | 1299 G>A | R396H | 1.0±0.5 |
| 4 | RAG1 | 1532 C>T | R474C | 47.2±7.9 |
| | | 2307 T>C | L732P | 0.5±0.4 |
| 5 | RAG1 | 2420 G>A [b] | E770K | 15.6±9.1 |
| Control | RAG2 | wild type | – | 100 |
| | RAG1 | wild type | – | 100 |

[a] Data are expressed as the percentage of activity as compared with that of the wild type protein, and represent the mean±standard deviation of three independent experiments.
[b] Homozygous mutation.

### 3.2. Recombination activity of RAG mutants

To elucidate the pathogenic significance of these novel mutations, we performed V(D)J recombination assay using the artificial extrachromosomal rearrangement substrate (Table 2). As expected, the recombined products were amplified from 293T cells transfected with both wild type RAG1 and RAG2, and no products were obtained from 293T cells transfected with either RAG1 or RAG2 (Fig. 1). Although the relative recombination activity of each mutant was variable, ranging from about half of the wild type activity to none, a significant decrease in average recombination activity was demonstrated in each patient (Fig. 1 and Table 2). The effects of the patients' missense mutations were also evaluated by the web-based analysis tools including Mutation@A Glance (http://rapid.rcai.riken.jp/mutation/) [20] and MutationTaster (http://www.mutationtaster.org/) [21]. Mutation@A Glance predicted all the mutation except for the E770K in *RAG1* to be deleterious on the basis of the SIFT program [22], whereas MutationTaster predicted all the missense mutations to be disease-causing.

### 3.3. B cell analysis of patient 4

The percentages of $IgD^-$ $CD27^+$ and $IgD^+$ $CD27^+$ cells within $CD19^+$ B cells from patient 4 were found comparable to controls (Fig. 2A) [23]. After stimulation with anti-CD40 and IL-4, B cells from patient 4 produced levels of IgE equivalent to normal, indicating their capability of undergoing class

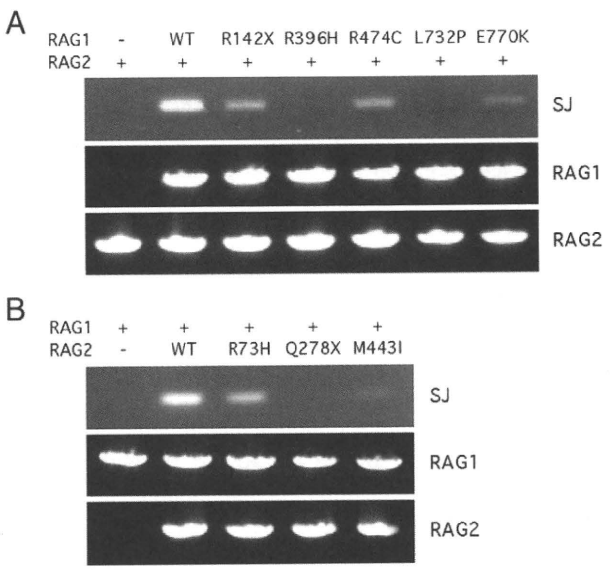
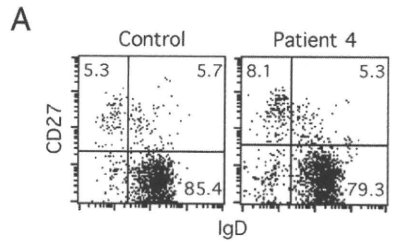
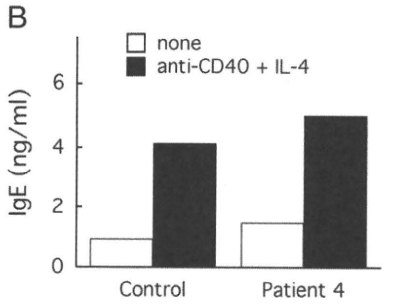



**Figure 1** V(D)J recombination assay. V(D)J recombination activity was assessed by using the recombination substrate pJH200 in 293T cells that were cotransfected with mutant RAG1 and wild type RAG2 (A), or with wild type RAG1 and mutant RAG2 (B). Recombined products (signal joints, SJ) were analyzed by PCR (top). The presence of RAG1 and RAG2 was verified by vector specific PCR (middle and bottom).




C

| % | Patient 4 | Controls |
|---|---|---|
| SHM | 4.8 | 2.6-6.3 |
| GC target | 56.0 | 62-66 |
| GC transition | 71.4 | 57-63 |
| AT transition | 50.0 | 33-67 |

To / From

| % | A | C | G | T | Total |
|---|---|---|---|---|---|
| A | | 5 | 13 | 10 | 28 |
| C | 2 | | 6 | 11 | 19 |
| G | 29 | 6 | | 2 | 37 |
| T | 2 | 9 | 5 | | 16 |

Patient 4

| % | A | C | G | T | Total |
|---|---|---|---|---|---|
| A | | 6.2 | 12.9 | 4.1 | 23.2 |
| C | 2.6 | | 6.0 | 11.4 | 20.0 |
| G | 26.1 | 13.2 | | 4.3 | 43.6 |
| T | 4.8 | 5.5 | 3.0 | | 13.2 |

Control

**Figure 2** B cell analysis of patient 4. (A) B cell subpopulations. Peripheral bloods were stained with FITC-labeled anti-IgD, PE-labeled anti-CD27, and APC-labeled anti-CD19 monoclonal antibodies. The dot plot of immunofluorescence profiles of IgD and CD27 expression within $CD19^+$ B cells is shown. The number indicates the percentage of cells in each quadrant. (B) IgE production. After stimulation of peripheral blood mononuclear cells with anti-CD40 and IL-4 for 12 days, concentrations of IgE in the culture medium were quantified. (C) The frequency and pattern of somatic hypermutation in the $V_H3$-23 region of the IgM in memory B cells. RT-PCR products amplified from purified $CD19^+$ $CD27^+$ B cells by using $V_H3$-23 and $C\mu$ primers were subcloned and sequenced. Nucleotide changes were evaluated and shown as percentages.

switch recombination and IgE synthesis *in vitro* (Fig. 2B). In addition, the frequency and nucleotide substitution patterns of SHM were similar to those of healthy individuals (Fig. 2C).

## 4. Discussion

RAG deficiency has been considered to display a range of phenotype from classical $T^-B^-$ SCID (complete RAG deficiency) to OS (partial RAG deficiency), depending on residual V(D)J recombination activity [24]. Atypical SCID/OS or leaky SCID may be also diagnosed in patients who show incomplete clinical and immunological characteristics and do not fulfill the criteria for SCID or OS [17]. However, it has recently been recognized that the clinical spectrum of RAG deficiency is much broader and includes CID with γδ/CMV [4,5], and CID with granulomatous inflammation [7], or destructive midline granulomatous disease [8]. In the present study, we studied 5 cases of RAG deficiency including 3 of OS, 1 of CID with γδ/CMV, and 1 of SCID with maternal T-cell engraftment, and identified 6 novel and 2 recurrent *RAG* mutations in these patients.

Hypomorphic RAG mutations leading to immunodeficiency have been shown to have up to 30% of wild type RAG activity by V(D)J recombination assay [7]. Although the R73H mutation in *RAG2* from patient 1, the R142X mutation in *RAG1* from patient 3, and the R474C mutation in *RAG1* from patient 4 exhibited around half of the wild type activity, all of these patients also had mutations with extremely low levels of recombination activity on the other allele, resulting in a substantial decrease in the average recombination activity due to a tetrameric complex formation of RAG1 and RAG2 during V(D)J recombination [1]. Similar results were obtained from an investigation of a RAG-deficient patient with destructive granulomatous disease who carried a W522C mutation with half of the recombination activity and a L541CfsX30 mutation with no recombination activity in *RAG1* [8]. It therefore seems reasonable that the clinical phenotype of partial RAG deficiency in patients 1, 3 and 4 is a consequence of these combinations of the mutations.

Biochemical studies have identified the core regions of RAG1 and RAG2 that are the minimal regions necessary for recombination of exogenous plasmid substrates *in vivo* and for DNA cleavage *in vitro* [1]. The M443I missense mutation demonstrated in patient 2 was located in the noncanonical plant homeodomain (PHD) of the non-core region of RAG2. Recent evidence indicates the importance of the non-core regions of RAG1 and RAG2 in V(D)J recombination and lymphocyte development [25]. The PHD of RAG2 has been shown to play crucial roles for chromatin and phosphoinositide binding, regulation of protein turnover, and cellular localization of RAG2 [26]. Additionally, the PHD of RAG2 is known to recognize histone H3 that has been trimethylated at the lysine at position 4 by interacting with 4 essential amino acids, Y415, M443, Y445, and W453 [27]. To date, 8 mutations of the non-core region in *RAG2* (W416L, K440N, W453R, A456T, C446W, N474S, C478Y, and H481P) have been reported in patients with $T^-B^-$ SCID or OS [28]. A significant decrease in recombination activity of the M443I mutation from our patient further supports the important role of PHD of RAG2 in regulating V(D)J recombination.

Although the R142X nonsense mutation found in the N-terminal domain of RAG1 in patient 3 should have resulted in a complete loss of function, it remained partially functional for recombination unlike the Q278X mutation in RAG2 in our assay. On the other hand, the same R142X mutation has been described in a typical OS patient who also had a nonfunctional frameshift mutation in the core region of RAG1 on the other allele, thus suggesting that the residual V(D)J recombination activity exists

with the R142X mutation [29]. One explanation for these findings is alternative usage of methionine as a translation start site, which has been reported in OS patients with N-terminal RAG1 frameshift mutations [30,31]. A translation start prediction program NetStart 1.0 also indicated that methionines at codon 183 and 202, which were the first and second methionines found after the R142X mutations, could be alternative translation start sites with scores comparable to the conventional initiator codon 1 (http://www.cbs.dtu.dk/services/NetStart/) [32]. Therefore, it is possible that an N-terminal truncated and partially functional RAG1 protein generated by alternative usage of methionine led to the OS phenotype in our patient.

The clinical features of patient 4 were consistent with CID with γδ/CMV. Despite decreased recombination activity, patient 4 exhibited normal immunogulobulin levels and a normal percentage of peripheral B cells. These findings were in contrast to SCID and OS, but were in agreement with previously described cases of this disease [4,5]. Moreover, our B cell analysis of patient 4 revealed normal maturation, normal production of IgE after stimulation with anti-CD40 and interleukin-4, and normal somatic hypermutation in $CD27^+$ B cells. Taken together, our case provided additional data of the genetic and immunological features of this unique disease.

*RAG* mutations found in patients with typical $T^-B^-$ SCID have been usually shown to abrogate recombination activity almost completely [2,33]. The residual V(D)J recombination activity resulting from the E770K mutation in RAG1 was associated with the SCID phenotype in patient 5. Despite trends towards more severe mutations, such as nonsense and frameshift mutations in SCID patients, missense mutations can lead to the SCID phenotype [33]. It is also known that the same mutations may cause different clinical phenotypes, presenting as either $T^-B^-$ SCID or OS [18], and as either $T^-B^-$ SCID or CID with γδ/CMV even within one family [34,35]. These findings suggest that that residual V(D)J recombination activity may not be solely responsible for the disease development. Further studies will be necessary to assess additional factors that influence the clinical phenotype of RAG deficiency.

In summary, our studies demonstrated the pathogenic significance of the 8 RAG mutations including 6 novel mutations from 5 patients with RAG deficiency. The characterization of the genetic defects and functional abnormalities in RAG-deficient patients will help define the role of RAG in V(D)J recombination and may lead to a better understanding of the variable phenotypic expression in RAG deficiency.

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# PU.1-mediated upregulation of *CSF1R* is crucial for leukemia stem cell potential induced by MOZ-TIF2

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**Leukemias and other cancers possess self-renewing stem cells that help to maintain the cancer[1,2]. Cancer stem cell eradication is thought to be crucial for successful anticancer therapy. Using an acute myeloid leukemia (AML) model induced by the leukemia-associated monocytic leukemia zinc finger (MOZ)-TIF2 fusion protein, we show here that AML can be cured by the ablation of leukemia stem cells. The MOZ fusion proteins MOZ-TIF2 and MOZ-CBP interacted with the transcription factor PU.1 to stimulate the expression of macrophage colony–stimulating factor receptor (CSF1R, also known as M-CSFR, c-FMS or CD115). Studies using PU.1-deficient mice showed that PU.1 is essential for the ability of MOZ-TIF2 to establish and maintain AML stem cells. Cells expressing high amounts of CSF1R (CSF1R$^{high}$ cells), but not those expressing low amounts of CSF1R (CSF1R$^{low}$ cells), showed potent leukemia-initiating activity. Using transgenic mice expressing a drug-inducible suicide gene controlled by the *CSF1R* promoter, we cured AML by ablation of CSF1R$^{high}$ cells. Moreover, induction of AML was suppressed in CSF1R-deficient mice and CSF1R inhibitors slowed the progression of MOZ-TIF2–induced leukemia. Thus, in this subtype of AML, leukemia stem cells are contained within the CSF1R$^{high}$ cell population, and we suggest that targeting of PU.1-mediated upregulation of CSF1R expression might be a useful therapeutic approach.**

Chromosomal translocations that involve the *MOZ* gene[3] (official gene symbol *Myst3*) are typically associated with acute myelomonocytic leukemia and predict a poor prognosis[4]. Whereas MOZ is essential for the self-renewal of hematopoietic stem cells[5,6], MOZ fusion proteins enable the transformation of non–self-renewing myeloid progenitors into leukemia stem cells[7]. We previously generated a mouse model for AML by introducing c-Kit$^+$ mouse myeloid stem/progenitor cells infected with a retrovirus encoding MOZ-TIF2 and EGFP into lethally irradiated mice[8].

To identify leukemia-initiating cells (LICs), we investigated the bone marrow cells of these mice for various cell surface markers by FACS analysis. CSF1R$^{high}$ and CSF1R$^{low}$ cells were present in the bone marrow (**Fig. 1a**) and expressed equivalent amounts of MOZ-TIF2 protein (**Fig. 1b**). To determine the LIC activity of these cell populations, we isolated CSF1R$^{high}$ and CSF1R$^{low}$ cells by cell sorting and transplanted limited numbers (10 to $1 \times 10^4$ cells) into irradiated mice. One hundred CSF1R$^{high}$ cells were sufficient to induce AML in all transplanted mice (**Fig. 1c**). Conversely, no mice developed AML after $1 \times 10^3$ CSF1R$^{low}$ cells were transplanted per mouse, and only half of the mice developed AML with delayed onset when $1 \times 10^4$ CSF1R$^{low}$ cells were transplanted (**Fig. 1d**). Thus, the CSF1R$^{high}$ cells showed a >100-fold stronger LIC activity than CSF1R$^{low}$ cells.

FACS analysis indicated that the CSF1R$^{high}$ cell population had the phenotype of both granulocyte-macrophage progenitors (GMPs, Kit$^+$Sca-1$^-$CD16/CD32$^+$) and differentiated monocytes (Mac-1$^{low}$Gr-1$^+$) (**Supplementary Fig. 1a**). Comparison of the CSF1R$^{high}$ and CSF1R$^{low}$ cell populations indicated that Mac-1 expression was lower in CSF1R$^{high}$ than in CSF1R$^{low}$ cells (**Fig. 1e**). However, we did not observe significant differences between the CSF1R$^{high}$ and CSF1R$^{low}$ cell populations with respect to their cell morphology (**Fig. 1f**), colony-forming ability in methylcellulose medium (**Fig. 1g**), cell cycle distribution (**Supplementary Fig. 1b**) or homeobox A9 (HoxA9) expression (**Supplementary Fig. 1c**). To investigate whether downstream pathways of CSF1R signaling were activated, we measured phosphorylation levels of signal transducer and activator of traranscription-5 (STAT5) and extracellular signal–regulated kinase (ERK) in CSF1R$^{high}$ and CSF1R$^{low}$ cells. STAT5 was highly phosphorylated in the CSF1R$^{high}$ cell population but not in the CSF1R$^{low}$ population, whereas ERK was equivalently phosphorylated in the two cell populations (**Fig. 1h**).

Side population cells, which are present in some types of normal and malignant stem cell populations, were present in the bone marrow of MOZ-TIF2–induced AML mice (**Supplementary Fig. 2a**). Whereas most side population cells were CSF1R$^{high}$, the non–side population fraction contained both CSFR1$^{high}$ and CSF1R$^{low}$ cells (**Supplementary Fig. 2b**). LICs were approximately tenfold more enriched in the side population fraction than in the non–side population fraction (**Supplementary Fig. 2c,d**). Because the side population fraction was very small (~0.12% of total bone marrow cells), the fraction of LICs in the side population fraction was also small (~1% of all LICs), and most LICs were present in the non–side population fraction (~99%).

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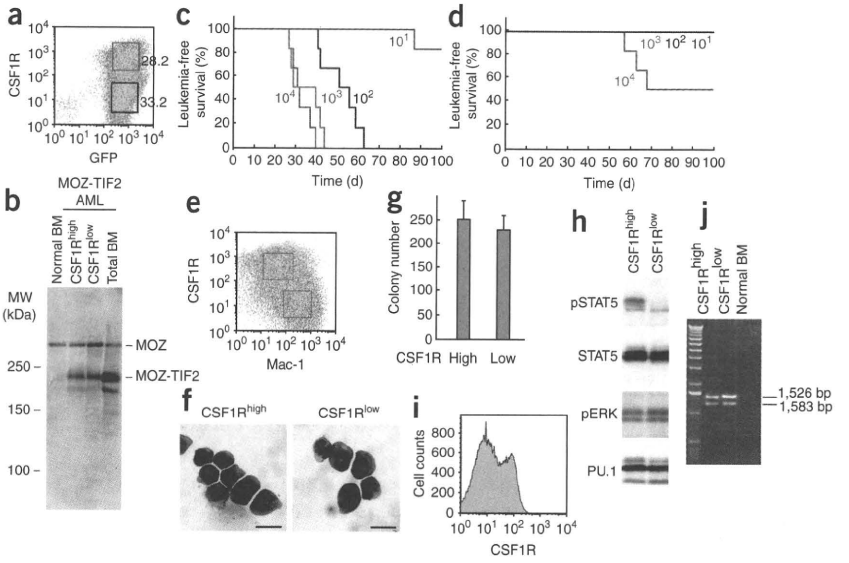

**Figure 1** CSF1R$^{high}$ cells show potent leukemia-initiating activity. (**a**) FACS analysis of bone marrow cells from mice with MOZ-TIF2–induced AML for expression of GFP and CSF1R. The red and black boxes signify CSF1R$^{high}$ and CSF1R$^{low}$ cell fractions, respectively. (**b**) Immunoblot analysis of MOZ-TIF2 expression in CSF1R$^{high}$ and CSF1R$^{low}$ cell populations (sorted by flow cytometry) with a MOZ-specific antibody. MW, molecular weight; BM, bone marrow. (**c**,**d**) Leukemia-free survival after the indicated numbers of flow-sorted CSF1R$^{high}$ (**c**) and CSF1R$^{low}$ (**d**) cells were transplanted into sublethally irradiated mice. $n = 6$, $P = 0.0001$ ($1 \times 10^4$, $1 \times 10^3$ and $1 \times 10^2$) and 0.3173 ($1 \times 10^1$) (CSF1R$^{high}$ versus CSF1R$^{low}$ cells). (**e**) FACS analysis of Mac-1 and CSF1R expression in bone marrow cells from mice with MOZ-TIF2–induced AML. The red and blue boxes signify CSF1R$^{high}$ and CSF1R$^{low}$ cell fractions, respectively. (**f**–**h**) CSF1R$^{high}$ and CSF1R$^{low}$ cells were sorted and analyzed for morphology by staining with May-Giemsa (**f**), colony-forming activity in methylcellulose medium (**g**) and levels of total and phosphorylated STAT5, phosphorylated ERK and PU.1 (**h**). Scale bars represent 10 μm in **f**. The error bars represent s.d. in **g**. (**i**) FACS analysis of CSF1R expression in bone marrow cells from an individual with AML with a t(8;16) translocation; the cells were cultured for 3 d in 10 ng ml$^{-1}$ human M-CSF. (**j**) RT-PCR analysis of MOZ-CBP transcripts in CSF1R$^{high}$ and CSF1R$^{low}$ cells of the individual with t(8;16) AML. The results are representative of 25 (**a**,**e**), four (**b**), three (**c**,**d**,**f**–**h**) and two (**i**,**j**) independent experiments.



To determine whether a high level of CSF1R expression also occurs in human AML cells with *MOZ* translocations, we investigated CSF1R expression in bone marrow cells from a subject with AML harboring a t(8;16) translocation, yielding a MOZ-CREB–binding protein (CBP, encoded by the *Crebbp* gene) fusion[9]. FACS analysis indicated that both CSF1R$^{high}$ and CSF1R$^{low}$ cells were present among the bone marrow cells with this translocation (**Fig. 1i**). We detected *MOZ-CBP* fusion transcripts in both the CSF1R$^{high}$ and CSF1R$^{low}$ cell populations (**Fig. 1j**).

These results suggest that leukemia stem cells in this subtype of AML express a high amount of CSF1R, indicating that leukemia might be cured by inducing apoptosis of CSF1R$^{high}$ cells. To test this idea, we used transgenic mice expressing a drug-inducible FK506-binding protein (FKBP)-Fas suicide gene and EGFP under the control of the *CSF1R* promoter[10] (**Fig. 2a**). The suicide gene products are inactive monomers under normal conditions but can be activated by injection of the AP20187 dimerizer, inducing apoptosis of cells expressing high amounts of CSF1R[10]. We infected c-Kit$^+$ bone marrow cells of transgenic mice with the MOZ-TIF2 retrovirus and transplanted them into lethally irradiated wild-type mice. These mice developed AML ~2 months after transplantation. In the bone marrow of these mice, we observed morphologically indistinguishable CSF1R$^{high}$ and CSF1R$^{low}$ cells. As expected, endogenous CSF1R expression was proportional to EGFP and FKBP-Fas expression (**Fig. 2b** and **Supplementary Fig. 3a**).

Next, we transplanted the bone marrow cells of these AML mice ($1 \times 10^5$ cells per mouse) into secondary sublethally irradiated recipient mice. Seven days after transplantation, we injected the mice with

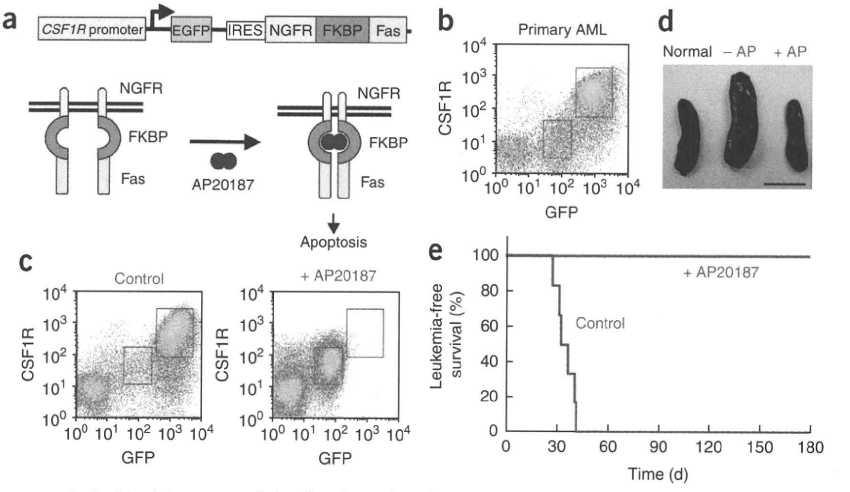

**Figure 2** Cure of AML by ablation of CSF1R$^{high}$ cells. (**a**) Top, structure of the *CSF1R* promoter-EGFP-NGFR-FKBP-Fas suicide construct. Bottom, schematic showing the activation of the NGFR-FKBP-Fas fusion protein: in transgenic mice carrying this suicide construct, ablation of cells expressing high levels of CSF1R can be induced by exposure to the AP20187 dimerizer. (**b**) FACS analysis of GFP and CSF1R expression in bone marrow cells of mice with AML 2 months after the transplantation of MSCV-MOZ-TIF2-IRES-GFP–transfected bone marrow cells derived from transgenic mice into lethally irradiated C57BL/6 mice. The red boxes signify CSF1R$^{high}$ and CSF1R$^{low}$ cell fractions. (**c**–**e**) Bone marrow cells ($1 \times 10^5$) of primary transplanted mice with AML, generated as in **b**, were transplanted into sublethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary transplanted mice was started by intravenous injection 3 weeks after transplantation. Expression of GFP and CSF1R in bone marrow cells (**c**) and spleen sizes (**d**) were analyzed 4 weeks after transplantation. Scale bars, 1 cm. (**e**) Leukemia-free survival of the untreated ($n = 6$) and AP20187-treated ($n = 6$) secondary transplanted mice. $P < 0.0001$. The results are representative of five (**b**), four (**c**) and three (**d**,**e**) independent experiments.

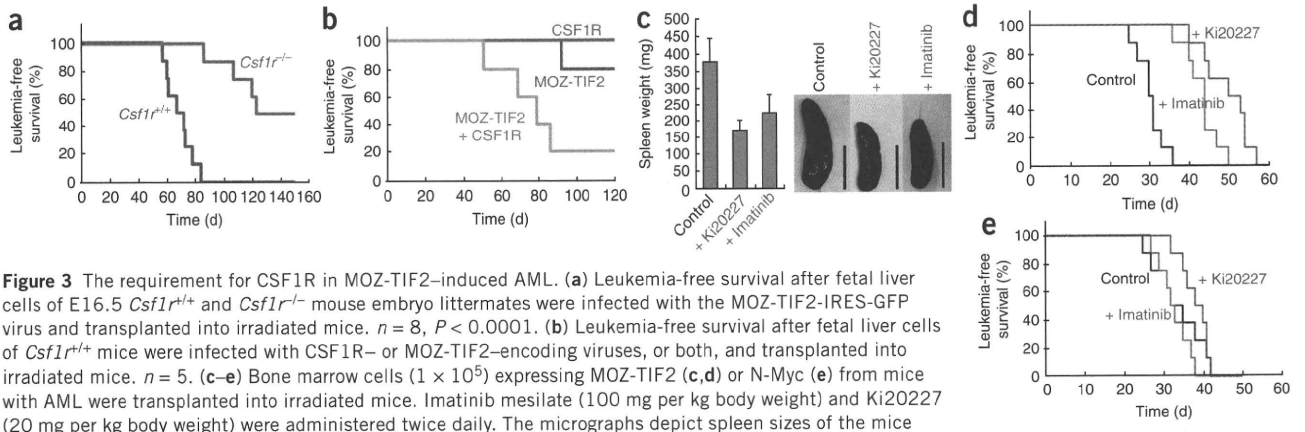


**Figure 3** The requirement for CSF1R in MOZ-TIF2–induced AML. (**a**) Leukemia-free survival after fetal liver cells of E16.5 *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ mouse embryo littermates were infected with the MOZ-TIF2-IRES-GFP virus and transplanted into irradiated mice. $n = 8$, $P < 0.0001$. (**b**) Leukemia-free survival after fetal liver cells of *Csf1r*$^{+/+}$ mice were infected with CSF1R– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. $n = 5$. (**c–e**) Bone marrow cells ($1 \times 10^5$) expressing MOZ-TIF2 (**c**,**d**) or N-Myc (**e**) from mice with AML were transplanted into irradiated mice. Imatinib mesilate (100 mg per kg body weight) and Ki20227 (20 mg per kg body weight) were administered twice daily. The micrographs depict spleen sizes of the mice transplanted with MOZ-TIF2–expressing cells, analyzed three weeks after transplantation (**c**). Scale bars, 1 cm. (**d**,**e**) Leukemia-free survival of the control and drug-treated mice was analyzed. In **d**, $n = 8$, $P < 0.0001$ (control versus + Ki20227 and control versus + imatinib). In **e**, $n = 8$, $P = 0.3825$ (control v.s. + Ki20227) and 0.4051 (control versus + imatinib).



AP20187 or a control solvent, as previously described[10]. We observed an increase in the number of CSF1R$^{high}$ cells (**Fig. 2c**) and splenomegaly (**Fig. 2d**) in the control-treated mice 3 weeks after transplantation. However, we detected neither CSF1R$^{high}$ cells nor splenomegaly in the AP20187-treated mice after a 1-week course of treatment (**Fig. 2c,d**). Although we observed CSF1R$^{low}$ cells in the bone marrow and peripheral blood after the 1-week treatment course, we did not detect these cells after three months of treatment (**Fig. 2c** and **Supplementary Fig. 3b**). All control-treated mice developed AML 4–6 weeks after transplantation, but none of the AP20187-treated mice died of AML within 6 months of transplantation (**Fig. 2e**). These results indicate that ablation of the CSF1R$^{high}$ cells was sufficient to cure MOZ-TIF2–induced AML, and that a high level of CSF1R expression is a key contributor to leukemia stem cell potential.

As it has been reported that N-Myc overexpression rapidly causes AML in mice[11], we next tested the specificity of the requirement for CSF1R$^{high}$ cells in AML progression. We transfected the bone marrow cells of suicide gene–expressing transgenic mice with a retrovirus encoding N-Myc and EGFP, and transplanted the cells into lethally irradiated recipient mice, which developed AML. In these mice, GFP$^+$ leukemia cells were Mac1$^+$Gr1$^+$CSF1R$^-$ blast cells (**Supplementary Fig. 4a,b**), and treatment with AP20187 did not affect AML induction (**Supplementary Fig. 4c**). These results indicate a specific role of CSFR expression in MOZ-TIF2–induced AML.

To investigate the role of CSF1R in the development of MOZ-TIF2–induced AML, we infected wild-type and *Csf1r*$^{-/-}$ (ref. 12) mouse fetal liver cells of embryonic day 16.5 (E16.5) littermate embryos with the MOZ-TIF2 virus and transplanted them into lethally irradiated mice. All mice transplanted with wild-type cells developed AML within 3 months (**Fig. 3a**). In contrast, AML induction was initially suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, but half of the mice developed AML after a longer latency period (**Fig. 3a**). The suppression of AML was rescued by co-infection with the retrovirus encoding CSF1R (**Fig. 3b**). STAT5, which was highly phosphorylated in CSF1R$^{high}$ cells but not in CSF1R$^{low}$ cells (**Fig. 1h**), was phosphorylated in the bone marrow of recipient mice transplanted with *Csf1r*$^{+/+}$ cells but not with *Csf1r*$^{-/-}$ cells (**Supplementary Fig. 5**). To test the specificity of the requirement of CSF1R for AML induction by MOZ-TIF2, we transfected *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ fetal liver cells with the retrovirus encoding N-Myc and transplanted them into irradiated recipient mice. All of the mice transplanted with either *Csf1r*$^{+/+}$ or *Csf1r*$^{-/-}$ cells expressing N-Myc developed AML (**Supplementary Fig. 4d**). These results indicate that CSF1R has a key role in AML induction by MOZ-TIF2, but not by N-Myc.

The above results suggest that signaling through CSF1R might be a therapeutic target for kinase inhibitors in leukemogenesis induced by MOZ fusions. To test this, we used the CSF1R-specific inhibitor Ki20227 (ref. 13) and the tyrosine kinase inhibitor imatinib mesylate (STI571), which inhibits CSF1R1[14–16]. Oral administration of Ki20227 or imatinib inhibited MOZ-TIF2–induced splenomegaly (**Fig. 3c**) and slowed MOZ-TIF2–induced AML onset (**Fig. 3d**). However, the drugs did not affect the progress of N-Myc–induced AML (**Fig. 3e**).

Next, we investigated the molecular mechanism of CSF1R expression in the leukemia cells. Monocyte-specific expression of CSF1R is reportedly regulated by transcription factors such as AML1, PU.1 and CCAAT/enhancer-binding proteins (C/EBPs)[17]. We previously found that MOZ interacts with AML1 and PU.1, but not with C/EBPα or C/EBPε, to stimulate transcription of their target genes[5,18]. Deletion analysis indicated that PU.1 interacted with the N-terminal and central regions of MOZ (**Fig. 4a** and **Supplementary Fig. 6**), and that the acidic amino acid–rich region (DE region) of PU.1 was required for its high-affinity interaction with MOZ (**Fig. 4a** and **Supplementary Fig. 7a–d**). Although binding of PU.1 to N-terminal MOZ (amino acids 1–513) was inhibited by several deletions in the PU.1 protein (**Supplementary Fig. 7c**), binding to full-length MOZ was not completely inhibited by these deletions (**Supplementary Fig. 7b**), suggesting that there may be other PU.1-binding sites in MOZ, its associated proteins or both. A pull-down assay with *Escherichia coli*–produced GST-PU.1 or GST-AML1 and *in vitro*–produced N-terminal MOZ indicated a direct interaction between both PU.1 and MOZ and between AML1 and MOZ (**Supplementary Fig. 8**). However, we cannot rule out a possibility that other factors may facilitate interactions between PU.1 or AML1 and MOZ *in vivo*.

To investigate transcriptional regulation of CSF1R, we performed reporter analysis with a *CSF1R* promoter–luciferase construct and found that MOZ, MOZ-TIF2 and MOZ-CBP could all activate the *CSF1R* promoter in the presence of PU.1 but not in the presence of AML1 (**Fig. 4b**). Moreover, MOZ, MOZ-TIF2 and MOZ-CBP did not activate a *CSF1R* promoter mutant lacking PU.1-binding sites (**Fig. 4c**). These results suggest that MOZ and MOZ fusion

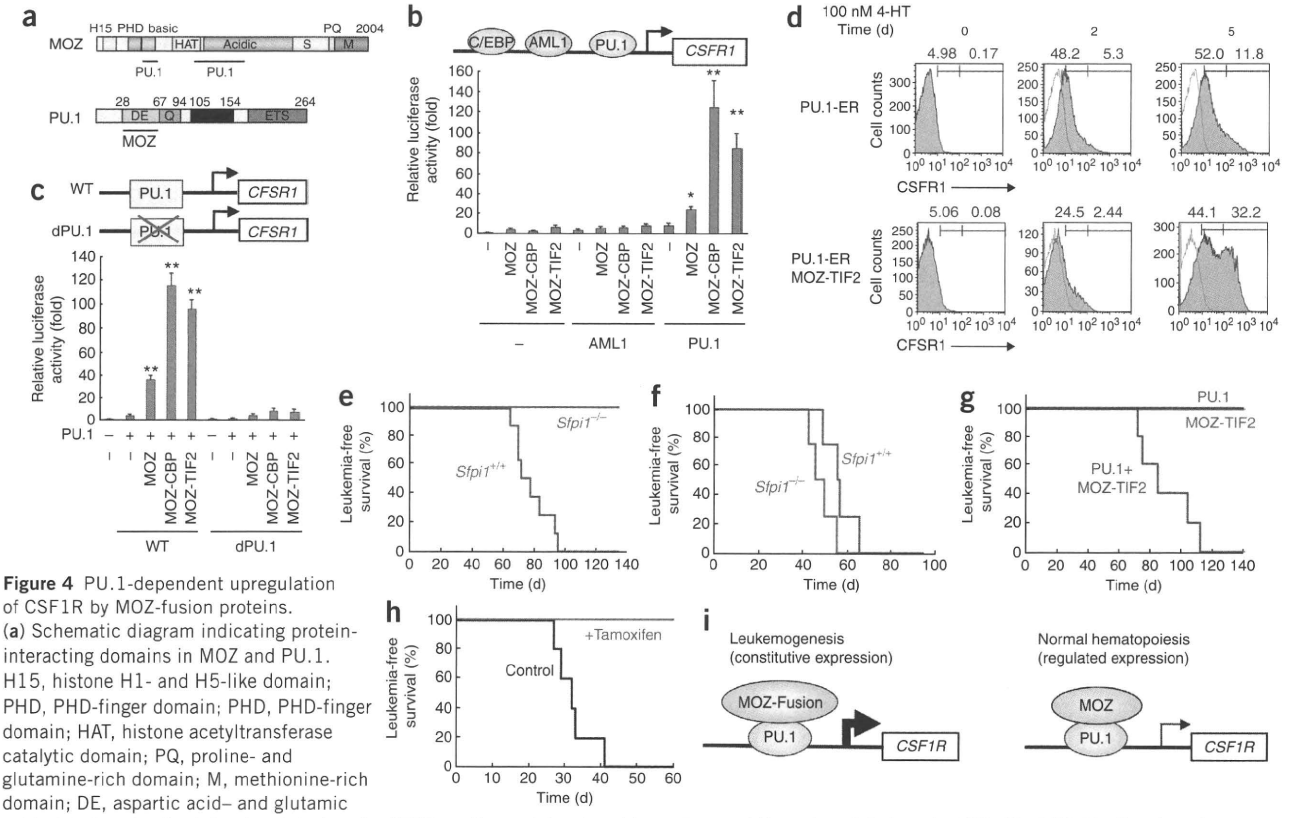


**Figure 4** PU.1-dependent upregulation of CSF1R by MOZ-fusion proteins. (**a**) Schematic diagram indicating protein-interacting domains in MOZ and PU.1. H15, histone H1- and H5-like domain; PHD, PHD-finger domain; PHD, PHD-finger domain; HAT, histone acetyltransferase catalytic domain; PQ, proline- and glutamine-rich domain; M, methionine-rich domain; DE, aspartic acid– and glutamic acid–rich domain; Q, glutamine-rich domain; PEST, proline-, glutamic acid–, serine- and threonine-rich domain; ETS, Ets DNA-binding domain. (**b**) Effects of MOZ, MOZ-CBP and MOZ-TIF2 on AML1- and PU.1-mediated transcription of the *CSF1R* promoter. Osteocarcinoma SaOS2 cells were transfected with the CSF1R-luciferase construct and the indicated effector constructs encoding AML1 or PU.1 together with MOZ, MOZ-CBP or MOZ-TIF2. Luciferase activity was analyzed 24 h after transfection. Error bars represent s.d. $*P < 0.01$ and $**P < 0.005$ (comparison to PU.1 only). The results are representative of six independent experiments in which three samples were tested for each group in each experiment. (**c**) PU.1-dependent activation of CSF1R promoter. SaOS2 cells were transfected with the wild-type (WT) *CSF1R*-luciferase construct or its mutant lacking the PU.1-binding site (dPU.1), together with the indicated effectors. Error bars represent s.d. $*P < 0.01$ and $**P < 0.005$ (comparison to PU.1 only). The results are representative of three independent experiments in which three samples were tested for each group in each experiment. (**d**) FACS analysis of CSF1R expression in PUER cells infected with MSCV-GFP (top) or MSCV-MOZ-TIF2-IRES-GFP (bottom) retroviruses and exposed to 100 nM 4-HT for 0, 2 or 5 d. Population (%) of $CSF1R^{high}$ and $CSF1R^{low}$ cells were indicated. The results are representative of three independent experiments. The horizontal lines and the numbers above the graphs indicate $CSF1R^{high}$ (right) and $CSF1R^{low}$ (left) cell fractions and their populations (%), respectively. (**e**,**f**) Leukemia-free survival after fetal liver cells of E12.5 $Sfpi1^{+/+}$ and $Sfpi1^{-/-}$ mouse embryo littermates were infected with either MOZ-TIF2– (**e**) or N-Myc– (**f**) encoding viruses and transplanted into irradiated mice. (**g**) Leukemia-free survival after fetal liver cells of $Sfpi1^{-/-}$ mice were infected with PU.1– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. In **e**, $n = 8$, $P < 0.0001$; in **f**, $n = 4$, $P = 0.0943$; in **g**, $n = 5$, $P = 0.0001$ (PU.1 + MOZ-TIF2 versus either PU.1 or MOZ-TIF2). (**h**) Fetal liver cells of E14.5 $Sfpi1^{flox/flox}$ ER-Cre mice were infected with the MOZ-TIF2–encoding virus and transplanted into irradiated mice, which developed AML. The bone marrow cells of these mice were then transplanted into sublethally irradiated wild-type mice. Tamoxifen or solvent (control) was administered to the secondary transplanted mice every 2 d by intravenous injection starting 17 d after transplantation, when $GFP^+$ cells were detected in peripheral blood. Leukemia-free survival of the secondary transplanted mice is shown. $n = 5$, $P = 0.0018$. (**i**) Model for transcriptional regulation by normal and fusion MOZ proteins. MOZ fusion proteins stimulate constitutive CSF1R expression to induce leukemia (left). Normal MOZ protein controls CSF1R expression by binding to PU.1 to regulate normal hematopoiesis (right).

proteins activate *CSF1R* transcription in a PU.1-dependent manner. It was recently reported that although chromatin reorganization of *Csf1r* requires prior PU.1 expression together with AML1 binding, stable transcription factor complexes and active chromatin can be maintained at the *Csf1r* locus without AML1 once the full hematopoietic program has been established[19]. This might explain why we found that AML1 was not required for MOZ-TIF2–mediated activation of *Csf1r*. Deletion analysis indicated that the DE-rich, Q-rich and ETS DNA-binding domains of PU.1, as well as the histone H1 and H5-like (H15) and the central PU.1-binding domains of MOZ and MOZ fusion proteins, are required for the activation of *CSF1R* transcription (**Supplementary Figs. 7e** and **9**). A truncated version of MOZ (1–1518) lacking the C-terminal region failed to activate transcription, indicating that the transcriptional activity of MOZ-TIF2 and MOZ-CBP, which do not contain that C-terminal region, requires the TIF2 or CBP portion of the fusion protein.

To test the requirement of PU.1 for the expression of endogenous *CSF1R*, we used PU.1-deficient ($Sfpi1^{-/-}$) myeloid progenitors expressing the PU.1–estrogen receptor fusion protein (PUER). Upon restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT), PUER cells can differentiate into macrophages[20]. We infected PUER cells with the MOZ-TIF2 retrovirus or control retrovirus, sorted them for GFP expression and cultured the $GFP^+$ cells in the presence of 4-HT. The results of FACS (**Fig. 4d**) and quantitative RT-PCR (**Supplementary Fig. 10**) analyses indicated that CSF1R expression was induced after exposure to 4-HT, and that MOZ-TIF2 enhanced

the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected CSF1R$^{high}$ and CSF1R$^{low}$ cells in the population of PUER cells expressing MOZ-TIF2, but only CSF1R$^{low}$ cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and *Sfpi1*$^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with *Sfpi1*$^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with *Sfpi1*$^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or *Sfpi1*$^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice (*Sfpi1*$^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in *MOZ*$^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both CSF1R$^{high}$ and CSF1R$^{low}$ cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected *Csf1r*$^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.I. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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