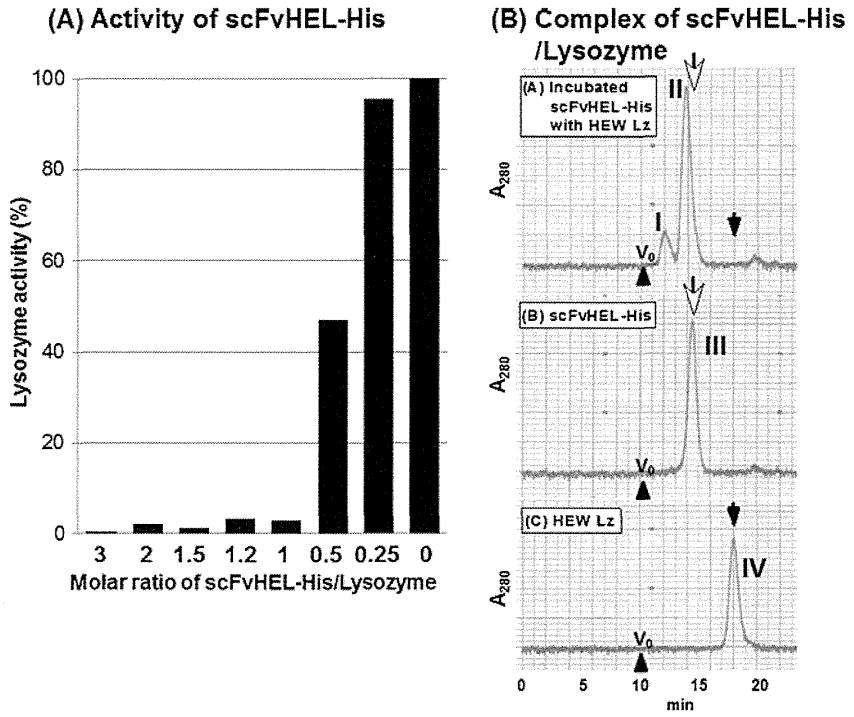

Fig. 5 Antibody activity of scFvHEL-His and detection of complex of scFvHEL-His/hen egg white lysozyme with Superdex 75 SEC analysis. **a** Antibody activity of scFvHEL-His. Hen egg white lysozyme (0.043 nmol) was incubated with various molar ratios (3–0) of scFvHEL-His for 1 h at 25 °C, and then lysozyme activity was measured. Results shown here are average data of three experiments and deviation is less than 5 %. **b** Hen egg white lysozyme (HEW Lz) and scFvHEL-His (both 0.344 nmol) was incubated for 1 h at 25 °C, and subjected to Superdex 75 SEC analysis. *Upper panel* (*A*) shows SEC of complex. Peaks I and II represent dimer and monomer of complex. *White arrow* shows position of scFvHEL-His monomer and *black arrow* shows position of lysozyme. *Middle panel* (*B*) and *lower panel* (*C*) show control experiments: peak III in (*B*) represents monomer position of scFvHEL-His and peak IV in (*C*) represents position of lysozyme

complex formed may be fairly compact and hence have only a slightly increased hydrodynamic size relative to the size of scFvHEL-His (white arrow). A small peak was observed at earlier elution position (peak I), which appeared to be dimers of scFvHEL-His/lysozyme complex.

The scFvFUL-His protein was also similarly purified from the BLA fusion (Fig. 3d, lane 2 and Fig.4a, lower panel). The monoclonal antibody, from which scFvFUL-His was derived, has been shown to stoichiometrically inhibit the fluorescence of traditional fluorophore, fluorescein (Kudou et al. 2011). Fluorescence of fluorescein was titrated with the purified scFvFUL-His. Figure 6 plots the fluorescence intensity of 0.6 μM fluorescein as a function of scFvFUL-His concentration. At the ratio of 0.5, the fluorescence intensity was about half, again suggesting nearly stoichiometric binding of scFvFUL-His to fluorescein. However, when the ratio was increased to 1, there were small but significant amounts of fluorescence. The ratio above 1.5 appeared to be required for greater fluorescence suppression, indicating that the binding affinity of scFvFUL-His is not as strong as for scFvHEL-His binding to lysozyme. A similar titration experiment was done with bovine serum albumin, showing no inhibition of fluorescein. Thus, it may be concluded that the observed suppression of fluorescein fluorescence by scFvFUL-His is due to its specific binding.

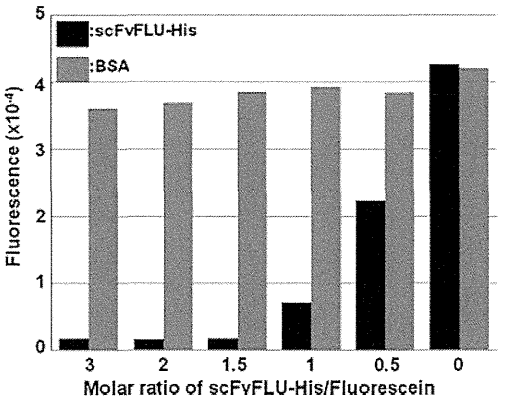

Fig. 6 Antibody activity of scFvFLU-His protein. Fluorescein (0.6 μM) was incubated with various molar ratios of scFvFLU-His for 1 h at 25 °C, and then the fluorescence intensity (*black bar*) was measured at excitation 480 nm and emission 515 nm. *Gray bar* shows control experiment using the same amount of bovine serum albumin (BSA) instead of scFvFLU-His. Results shown here are average data of two experiments and deviation is less than 10 %

Expression of scFv fusion protein with *Brevibacillus* secretory protein P45 as a partner protein

Expression of these scFv with another fusion partner, i.e., P45, in *Brevibacillus* was attempted in a similar manner to the BLA fusions. This P45 is an intrinsic 45-kDa (417 amino acid residues) abundant secretory protein of *Brevibacillus* bacterium, as supposed to a foreign protein of BLA (halophilic origin) and may enhance soluble expression of the fusion protein. Both P45-scFvHEL-His (Fig. 1, construct 4) and P45-scFvFLU-His (construct 5) were clearly expressed in culture supernatant, as indicated by the arrowhead (Fig. 7a, lane 2 and lane 3). Both proteins were purified by His-Trap columns. Figure 7b shows expression of P45-scFvHEL-His in culture supernatant of 200 ml flask culture (lane 1, dot) and purified fraction from the His-Trap column (lane 2). Highly homogeneous preparation of P45-scFvHEL-His was obtained, indicating both soluble expression and stability of this construct. Figure 7c shows purification of P45-scFvFLU-His construct. For this protein as well, highly homogeneous preparation (lane 2) was obtained from crude supernatant (lane 1). However, thrombin cleavage was rather difficult and inconsistent, in particular for scFvFLU fusion construct, most likely due to weak ability of P45 to enhance the solubility of the scFv proteins. Greater aggregation tendency of scFvFLU described above made it more aggregating with P45 fusion. Thus, the analysis of scFv proteins from the P45 constructs was not possible and was done as a fusion protein.

Antibody activity of whole fusion proteins

Expression of both BLA fusion and P45 fusion has resulted in soluble scFv proteins. If these scFv proteins were functional in the fusion form, they could be a useful reagent, in particular when cleavage of the fusion partner is difficult (for example, the P45 fusion). The effects of BLA- and P45-fusion constructs on lysozyme activity and fluorescein fluorescence were thus examined without cleaving the fusion partners. Figure 8a shows titration of lysozyme activity at fixed lysozyme concentration as a function of BLA-scFvHEL-His concentration. Similar to scFvHEL-His, the lysozyme activity was nearly stoichiometrically inhibited by this fusion protein, indicating that BLA does not interfere with binding of scFvHEL to the enzyme. BLA-scFvFLU-His did not inhibit lysozyme activity, indicating that the BLA portion of BLA-scFvHEL-His fusion protein has no influence on lysozyme activity (data not shown). Lysozyme activity was similarly titrated with P45-scFvHEL-His, showing gradual reduction of enzyme activity by this fusion construct (Fig. 8b). However, it appears that the degree of inhibition was slightly less for P45 construct than for the BLA construct and hence that P45 may sterically interfere with binding of scFvHEL to the enzyme, leading to slightly reduced binding strength. Alternatively, the P45-scFvHEL-His preparation may be heterogeneous due to aggregation tendency of P45, resulting in lower concentration of functional monomeric form. Nevertheless, it is evident that P45-scFvHEL-His does bind to lysozyme. As with BLA-scFvFLU-His protein, P45-scFvFLU-His

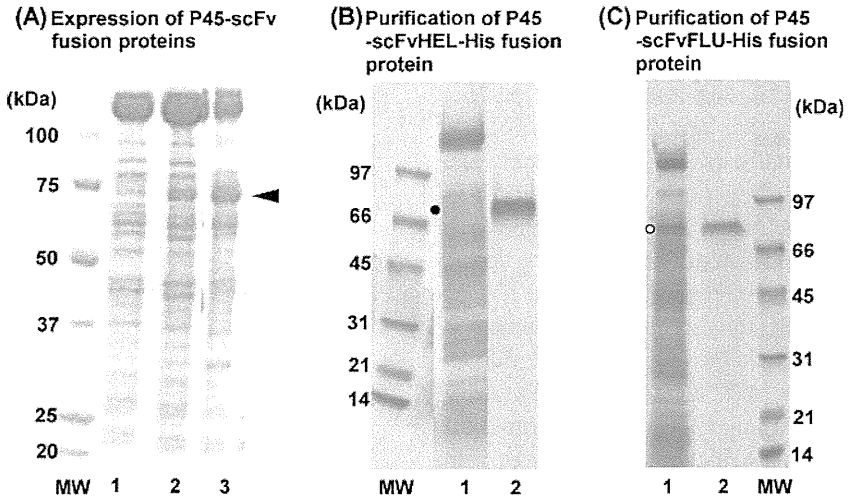

Fig. 7 Expression and purification of P45-scFv-His fusion proteins. **a** Expression of P45-scFvHEL-His (*lane 2*) and P45-scFvFLU-His (*lane 3*) in test tube culture (shown by *arrowhead*). *Lane 1* vector control without fusion gene. Each 4 μl of culture supernatant was applied. **b** Purification of P45-scFvHEL-His fusion protein from 200 ml batch culture. *Lane 1* culture supernatant. *Dot* shows P45-scFvHEL-His fusion protein; *lane 2* P45-scFvHEL-His fusion protein purified with His-Trap column. **c** Purification of P45-scFvFLU-His fusion protein from 200 ml batch culture. *Lane 1* culture supernatant. *White dot* shows P45-scFvFLU-His fusion protein; *lane 2* P45-scFvFLU-His fusion protein purified with His-Trap column

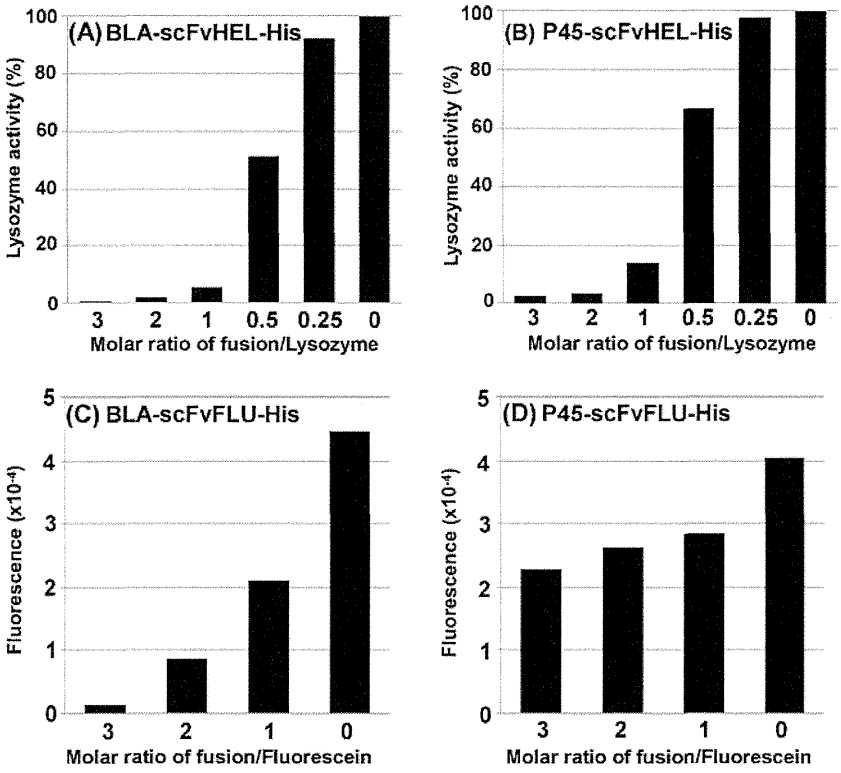

Fig. 8 Antibody activity of BLA- and P45-scFv fusion proteins. Whole BLA-scFv and P45-scFv fusion proteins (without protease digestion) were assayed using their antibody activities. Lysozyme (0.043 nmol) was incubated with various molar ratios of BLA-scFvHEL-His (**a**) or P45-scFvHEL-His (**b**) fusion protein for 1 h at 25 °C, and then lysozyme activity was measured. Fluorescein (0.6 μM) was incubated with various molar ratios of BLA-scFvFLU-His (**c**) or P45-scFvFLU-His (**d**) proteins for 1 h at 25 °C, and then fluorescence intensity was measured

protein had no effects on lysozyme activity, indicating that P45 portion as well did not affect lysozyme activity (not shown).

Titration of fluorescein was also done for BLA- and P45-fusion constructs. When fluorescein at a fixed concentration was titrated, the fluorescence gradually decreased with increasing BLA-scFvFLU-His concentration (Fig.8c, from right to left). However, there was significant fluorescence (about 50 %) at the molar ratio of 1. Nearly complete suppression of fluorescence required a molar ratio of 3, indicating that for this antigen, the BLA moiety does interfere with binding of scFvFLU to fluorescein. Alternatively, the BLA-scFvFLU-His preparation is heterogeneous due to aggregation tendency of scFvFLU, which caused reduced activity of the preparation used. As shown in Fig. 8d, only small reduction of fluorescence was observed by the addition of P45-scFvFLU-His. Even at the molar ratio of 3, the fluorescence intensity was still above 50 % of the original fluorescence (no P45-scFvFLU-His). This can be explained by reduced concentration of functional P45-scFvFLU-His due to aggregation-prone properties of this fusion protein, although possible steric hindrance of P45 portion on scFvFUL binding cannot be excluded.

Discussion

Antibody fragments, in particular scFv, are intensely investigated primarily as an anti-cancer drug (Beck et al. 2010; Demarest and Glaser 2008; Kontermann 2010). Strong interest is based on its small size, which allows more efficient penetration into solid tumors expressing antigen markers, to which the fragments bind. Cytotoxic agents are conjugated to the fragments that deliver the anti-cancer agents to the specific site (Beckman et al. 2007; Ottiger et al. 2009). However, production of antibody fragments, more specifically scFv, is not often straightforward, requiring laborious developmental work both in expression and purification. Refolding may be required when the fragments were expressed insoluble (Fujii et al. 2007; Fursova et al. 2009; Kurucz et al. 1995; Tsumoto et al. 1998). Here we were able to successfully express both scFv molecules, i.e., scFvHEL and scFvFLU, as a fusion protein in the culture media of *B. choshinensis*. Proteolytic cleavage of the fusions resulted in functional scFv proteins.

One of the fusion proteins used is halophilic BLA derived from *Chromohalobacter* sp. 560. We have shown before that BLA is extremely soluble even at high temperatures, at which it is fully unfolded (Arakawa et al. 2010; Tokunaga et al. 2004, 2006a). Such high solubility of heat-denatured structure is the key to its ability to support folding of the target protein, to which the BLA is fused (Tokunaga et al. 2010b). The scFv is an artificial molecule, in which two variable domains of antibody heavy and light chains are connected by an artificial linker, and hence may suffer both folding and aggregation problems during folding process, in particular when the folding is slow. The intermediate structures may aggregate during folding process in cellular

environments. High solubility of halophilic BLA may afford the solubility of the fusion protein, allowing a sufficient time for the fused scFv to fold into the native structure. P45 derived from *Brevibacillus* bacterium appears to function in a similar manner by maintaining the solubility of the fusion protein. Although the solubility and folding properties of P45 have not been extensively investigated, its solubilizing effects on scFv, in particular scFvFLU, appeared to be weaker than BLA.

Proteolytic cleavage of BLA fusion partner from two scFv was efficient, indicating no apparent aggregation. Repeated experiments showed more reproducible cleavages of BLA-scFvHEL fusion than BLA-scFvFLU fusion. When protein aggregates, proteolytic cleavage of peptide bonds will be normally compromised, thus suggesting greater aggregation tendency for BLA-scFvFLU fusion as described in the "Results" section. Both purified scFvHEL and scFvFLU stoichiometrically bound to their antigens and blocked the activity of the antigens. Due to more ideal solution property of scFvHEL, binding could be directly demonstrated by the SEC analysis of the complex formation between scFvHEL and lysozyme. The additional work on binding equilibrium and kinetics of the scFv proteins to the antigens will be necessary to be compared with each other and to commence further application of these preparations.

ScFv has been seen to suffer proteolytic cleavages during expression, although the exact sites and cleavage mechanism have not been determined. This often resulted in co-purification of cleaved scFv fragments. This problem does not appear to occur in the present fusion expression system, as no such fragments were observed in the final product, perhaps due to stabilization of scFv against proteolytic cleavage by the fusion partners.

Both scFv molecules were functional even before proteolytic cleavage, meaning that they were already folded. Thus, these scFv may have potential application as a diagnostic agent. Antigen binding was slightly reduced for BLA-scFvHEL-His and P45-scFvHEL-His, more so for the latter, perhaps due to steric hindrance by the BLA and P45: a larger size of P45 may have caused greater hindrance and thereby reduced binding. On the contrary, binding to fluorescein was greatly reduced by BLA and P45 fusion, more for P45. One possibility is steric hindrance as suggested for lysozyme. Alternatively, the reduced activity of the scFvFLU fusion proteins, in particular P45-scFvFLU, may be due to the strong aggregation tendency of scFvFLU protein as described earlier. It is possible that the preparations used for inhibition assay may be a mixture of various aggregated species. Since both BLA and scFvHEL are highly soluble, their fusion must be soluble and inhibits similarly to the scFvHEL. The observed slight reduction on lysozyme inhibition for the fusion may in fact be due to steric hindrance by BLA for binding of the scFvHEL to the lysozyme. Due to a lower solubility of P45 or scFvFLU, the fusions showed greater reduction in inhibition, more likely due to enhanced aggregation of the P45 fusion proteins. The fusion with both scFvFLU and P45 was the worst as expected from the aggregation tendency of scFvFLU and the weaker ability of P45 to enhance the solubility of the fusion proteins.

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nature genetics

The landscape of somatic mutations in Down syndrome–related myeloid disorders

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Transient abnormal myelopoiesis (TAM) is a myeloid proliferation resembling acute megakaryoblastic leukemia (AMKL), mostly affecting perinatal infants with Down syndrome. Although self-limiting in a majority of cases, TAM may evolve as non-self-limiting AMKL after spontaneous remission (DS-AMKL). Pathogenesis of these Down syndrome–related myeloid disorders is poorly understood, except for *GATA1* mutations found in most cases. Here we report genomic profiling of 41 TAM, 49 DS-AMKL and 19 non-DS-AMKL samples, including whole-genome and/or whole-exome sequencing of 15 TAM and 14 DS-AMKL samples. TAM appears to be caused by a single *GATA1* mutation and constitutive trisomy 21. Subsequent AMKL evolves from a pre-existing TAM clone through the acquisition of additional mutations, with major mutational targets including multiple cohesin components (53%), *CTCF* (20%), and *EZH2*, *KANSL1* and other epigenetic regulators (45%), as well as common signaling pathways, such as the JAK family kinases, *MPL*, *SH2B3* (*LNK*) and multiple RAS pathway genes (47%).

TAM represents a transient proliferation of immature megakaryoblasts that occurs in 5–10% of perinatal infants with Down syndrome[1,2]. Although morphologically indistinguishable from AMKL, TAM is self-limiting in the majority of cases and usually terminates spontaneously within 3–4 months of birth[1]. Hepatic infiltration of myeloid cells is a common finding and can be severe enough to be fatal, owing to hepatic failure, with liver fibrosis occurring in 5–16% of cases[2–4]. Moreover, even when spontaneous remission is achieved, approximately 20–30% of surviving infants develop DS-AMKL years after remission, although some DS-AMKL cases have no documented history of TAM[4]. In contrast to non–Down syndrome–related AMKL (non-DS-AMKL), which generally shows poor prognosis, individuals with DS-AMKL typically have a favorable prognosis. In molecular pathogenesis of these Down syndrome–related myeloid disorders, *GATA1* mutations are detected in virtually all affected infants, suggesting their central role in Down syndrome–related myeloid proliferation[5,6]. However, it is still open to question whether a *GATA1* mutation is sufficient for the development of TAM in individuals with Down syndrome, what is the cellular origin of the subsequent AMKL, whether additional gene mutations are required for progression to AMKL, and, if so, what are their gene targets, although several genes have been reported to be mutated in occasional cases with DS-AMKL, including *JAK1*, *JAK2* and *JAK3* (refs. 7–10), *TP53* (refs. 10,11), *FLT3* (ref. 8) and *MPL*[12]. We reasoned that identifying a comprehensive registry of gene mutations and tracking them at a clonal level using massively parallel sequencing would provide vital information for addressing these questions.

RESULTS

Genomic landscape of Down syndrome–related myeloid neoplasms

We performed whole-genome sequencing of 4 trios consisting of samples from TAM, AMKL and complete remission phases (**Supplementary Figs. 1** and **2** and **Supplementary Table 1**). In total,

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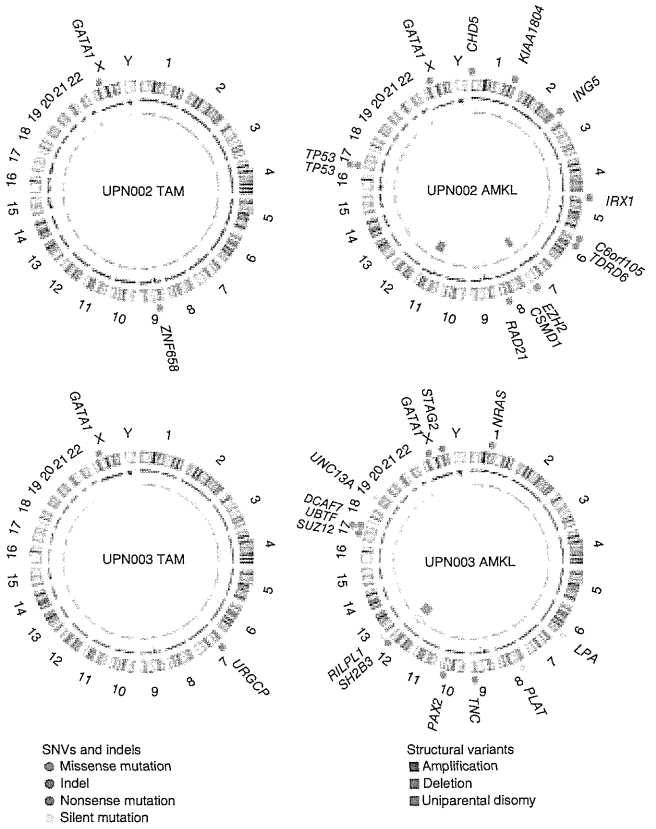

Figure 1 Representative Circos plots of paired TAM and DS-AMKL cases. Locations of somatic mutations, including of missense, frameshift, nonsense and silent mutations (colored circles), are indicated. Total (black) and allele-specific (red and green for alleles showing relatively larger and smaller copy numbers, respectively) genomic copy numbers, as well as somatic structural variants (colored bars), are indicated in the inner circle. Sample IDs are shown within each plot; plots were created with Circus[53].

we confirmed 411 single-nucleotide variants (SNVs) and 17 small nucleotide insertions and deletions (indels) by Sanger sequencing and/or deep resequencing (**Supplementary Fig. 1** and **Supplementary Table 2**). We detected only a few structural variants, including deletion, amplification and uniparental disomy, in the TAM and DS-AMKL genomes (**Fig. 1** and **Supplementary Fig. 3**). The mean number of validated somatic mutations in DS-AMKL samples (71 or 0.023 mutations/Mb) was twice the number observed in TAM samples (36 or 0.012 mutations/Mb) (**Supplementary Fig. 1a**). Mutation numbers in samples from both phases were substantially lower than in most other cancers (**Supplementary Fig. 4**), although differences in mutation rates could partly be affected by different definitions and algorithms for mutation calling. The spectrum of mutations was over-represented by C-to-T and G-to-A transitions in both TAM and DS-AMKL samples, resembling the mutational spectra in gastric and colorectal cancers[13] and in other blood cancers (**Supplementary Fig. 1b**)[14,15]. We unmasked the details of clonal evolution and expansion leading to AMKL through the use of deep sequencing of individual mutations detected by combined whole-genome and whole-exome sequencing (**Fig. 2** and **Supplementary Table 2**). Intratumoral heterogeneity was evident at initial diagnosis with TAM and in the AMKL phase in all cases (**Supplementary Fig. 5**). In UPN001, UPN002 and UPN004, AMKL evolved from one of the major subclones in the TAM phase with a shared *GATA1* mutation, as reported previously in relapsed acute myeloid leukemia (AML) in adults (**Fig. 2a,b,d**)[15]. In contrast, UPN003 showed a unique pattern of clonal evolution, in which AMKL originated from a minor subclone in the TAM phase that was totally unrelated to the predominant clone in terms of somatic mutations, with no mutation shared by both phases, and carried an independent *GATA1* mutation (**Fig. 2c**). In both scenarios, progression to AMKL seemed to be accompanied by many additional mutations, including common driver mutations that were absent in the original TAM population, indicating a multistep process of leukemogenesis.

Exome sequencing

We further investigated non-silent mutations by whole-exome sequencing of additional samples to generate a full registry of driver mutations that are relevant to the development of TAM and subsequent progression to AMKL (**Supplementary Fig. 6** and **Supplementary Table 1**). We detected *GATA1* mutations in all TAM and DS-AMKL cases, indicating sufficient sensitivity in our whole-exome analysis. In total, we confirmed 26 and 81 non-silent somatic mutations identified in the exome analysis of 15 TAM and 14 DS-AMKL samples, respectively, with 3 *GATA1* mutations common to both phases (**Supplementary Table 3**). The mean number of non-silent mutations was significantly higher in DS-AMKL samples (5.8; range of 1–11) than in TAM samples (1.7; range of 1–5) ($P = 0.0002$) (**Fig. 3a**). Of the 107 mutations, 84 were single-nucleotide substitutions that were mostly within coding sequences, except for 4 splice-site mutations. We also observed predominantly C-to-T and G-to-A transitions for non-silent substitutions (**Supplementary Fig. 7**). The remaining mutations were frameshift ($n = 21$) or non-frameshift ($n = 2$) indels, most frequently involving *GATA1* ($n = 13$). One individual with DS-AMKL (UPN004) had no SNVs or indels (**Fig. 3a**), but copy number analysis identified a large deletion at 16q involving the *CTCF* locus (**Supplementary Fig. 3**), suggesting that the alteration of *CTCF* could be a driver event in this case. Therefore, at least one additional genetic lesion other than *GATA1* mutation was detected in our whole-exome sequencing, despite the low frequency of leukemic cells appearing to show the morphology of immature megakaryoblasts (blast percentage) in many cases, which is a known characteristic of DS-AMKL samples[16,17]. Whole-exome sequencing results suggested the presence of intratumoral heterogeneity in the majority of DS-AMKL cases (**Fig. 3b**).

Spectrum of recurrent mutations in DS-AMKL

Recurrently affected genes are of primary interest in identifying driver mutations. Whereas *GATA1* was the only recurrent mutational target in TAM samples, an additional eight genes were recurrently mutated in the DS-AMKL samples, including *RAD21*, *STAG2*, *NRAS*, *CTCF*, *DCAF7*, *EZH2*, *KANSL1* and *TP53* (**Table 1**). These genes are expressed in a wide variety of hematopoietic compartments, including in both myeloid and lymphoid cells, except for *EZH2*, whose expression is largely confined to $CD34^+$ cells[18] (**Supplementary Fig. 8**). We also found that these genes were expressed in DS-AMKL cells at similar levels to common hematopoietic genes[19], although we did not observe significant difference in their expression levels in DS-AMKL and non-DS-AMKL cells (**Supplementary Fig. 9**).

We then performed targeted deep sequencing of these 8 genes in an extended set of 109 samples (including 29 samples in 25 discovery cases) consisting of 41 TAM, 49 DS-AMKL and 19 non-DS-AMKL samples (**Supplementary Tables 1** and **4**). We also included additional genes in targeted sequencing that were either functionally related to the above eight genes or were mutated only in single cases but had been previously reported to be mutated in DS-AMKL (*JAK3*) or other myeloid neoplasms (*SH2B3*, *SUZ12*, *SRSF2* and *WT1*), together with other common mutational targets in adult myeloid malignancies

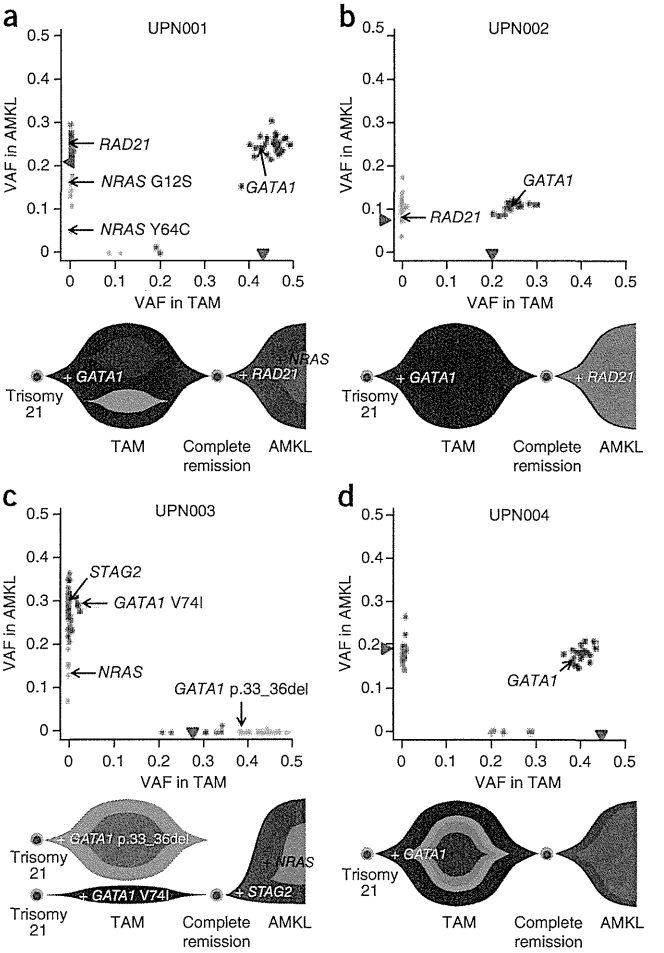

Figure 2 Clonal evolution of Down syndrome–related myeloid disorders. (**a**–**d**) Observed VAFs of validated mutations listed in **Supplementary Table 2** in both TAM and AMKL phases are shown in diagonal plots (top) for UPN001 (**a**), UPN002 (**b**), UPN003 (**c**) and UPN004 (**d**), where VAFs of genes on the X chromosome in male cases or in regions of uniparental disomy were halved. Half the value of the blast percentage, which corresponds to the allele frequency of a heterozygous mutation distributed in all tumor cells, is also shown by a red arrowhead, except for UPN003 AMKL, for which clinical data were not available. Driver mutations including in *GATA1*, *STAG2*, *RAD21* and *NRAS* are indicated by black arrows. Predicted chronological behaviors of different leukemia subclones are depicted below each diagonal plot. Distinct mutation clusters are indicated by color. In UPN001, UPN002 and UPN004, founding clones of TAM shown in blue became dominant in the AMKL samples, in which some subsequent subclones evolved through the serial acquisition of SNVs. In contrast, in UPN003, a subclone in the TAM phase (blue) and not the founding clone of TAM (aqua) became dominant in the AMKL sample. VAFs of some mutations were higher than for *GATA1* but seem to be actually equivalent to it given the error range of PCR-based deep sequencing.

(**Supplementary Fig. 10** and **Supplementary Tables 5** and **6**). We also analyzed by RT-PCR two recurrent fusion genes previously reported in non-DS-AMKL cases, *RBM15-MKL1* (*OTT-MAL*)[20,21] and *CBFA2T3-GLIS2* (refs. 22,23).

Mutations of cohesin and associated molecules

Major components of the cohesin complex, including *RAD21* and *STAG2*, were frequent targets of gene mutations in DS-AMKL (**Table 1**). Including an additional mutation in *NIPBL*, 8 of the 14 discovery DS-AMKL cases (57%) had a mutated cohesin or associated component (**Supplementary Table 3**). Cohesin is a multiprotein complex consisting of 4 core components, including the SMC1, SMC3, RAD21 and STAG proteins[24,25]. In concert with several functionally associated proteins, such as the NIPBL and ESCO proteins, cohesin is engaged in the cohesion of newly replicated sister chromatids by forming a ring-like structure[25], preventing their premature separation before late anaphase. Cohesin has also been implicated in post-replicative DNA repair and long-range regulation of gene expression[26–30]. Targeted deep sequencing confirmed recurrent mutations and deletions in all core cohesin components (*STAG2*, *RAD21*, *SMC3* and *SMC1A*) and in *NIPBL* in 26 of 49 DS-AMKL cases (53%) but in none of the 41 TAM cases, although 2 non-DS-AMKL cases (11%) had *STAG2* mutations (**Fig. 4a,b** and **Supplementary Tables 7** and **8**). Strikingly, all mutations and deletions in different cohesin components were completely mutually exclusive, suggesting that cohesin function was the common target of these mutations. All but one *STAG2* mutation (encoding a p.Arg370Gln substitution) was either a nonsense, frameshift or splice-site change (**Fig. 4a,b**, **Supplementary Figs. 11** and **12a**, and **Supplementary Table 7**). Similarly, 6 of 9 *RAD21* mutations were heterozygous nonsense or frameshift alterations. Four of the five mutations in *NIPBL*, *SMC1A* and *SMC3* were also nonsense or splice-site changes causing abnormal exon skipping (**Fig. 4a** and **Supplementary Table 7**). Thus, most of these mutations were thought to result in premature truncation, leading to loss of cohesin function. The leukemogenic mechanism of mutated cohesin components is still elusive; some studies have implicated aneuploidy caused by cohesin dysfunction in oncogenic actions[31]. However, DS-AMKL cases have been characterized by a largely normal karyotype[32]. We found no significant difference in the frequency of aneuploidy between cases with mutated and wild-type cohesin in the current DS-AMKL cohort. Many cases with mutated cohesin had completely normal karyotypes, except for constitutive trisomy 21, arguing against the hypothesis that aneuploidy has a major role in the pathogenesis of cohesin-mutated DS-AMKL (**Fig. 5a**).

CTCF mutations

Given the high frequency of cohesin mutations, new recurrent *CTCF* mutations were of particular interest because the functional interaction of cohesin and CTCF proteins has been of emerging interest in the long-range regulation of gene expression[26,30,33,34]. CTCF is a zinc-finger protein implicated in diverse regulatory functions, including transcriptional activation and/or repression, insulation, formation of chromatin barrier, imprinting and X-chromosome inactivation[35]. CTCF binds to target sequence elements and blocks the interaction of enhancers and promoters through DNA loop formation (insulator activity)[36], and several lines of evidence suggest that cohesin occupies CTCF-binding sites to contribute to the long-range regulation of gene expression by participating in the formation and stabilization of a repressive loop[26,37]. *CTCF* was mutated or deleted in ten DS-AMKL cases (20%), one TAM case (2%) and four non-DS-AMKL cases (21%), with seven mutations representing nonsense, frameshift or splice-site changes and an additional six alterations representing deletions resulting in the loss of protein function (**Fig. 4a,b**, **Supplementary Figs. 11** and **12b**, and **Supplementary Tables 7** and **8**). To our knowledge, this is the first report of frequent recurrent *CTCF* mutations in cancer, although rare mutations (occurring in approximately 2% of cases) have recently been reported in breast cancer sequencing[38].

Mutations in epigenetic regulators

EZH2, which encodes a catalytic subunit of the Polycomb repressive complex 2 (PRC2) that is responsible for di- and trimethylation of histone H3 lysine 27 (H3K27)[39], is another recurrent mutational target in DS-AMKL (**Table 1**). Inactivating mutations in *EZH2* have

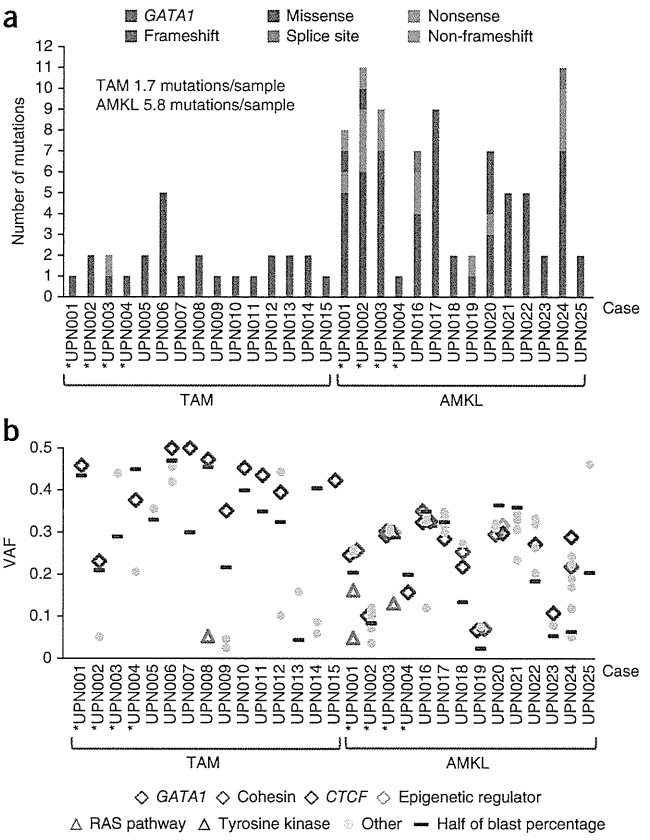

Figure 3 Somatic mutations detected by whole-exome sequencing of Down syndrome–related myeloid disorders. (**a**) Number of validated somatic mutations in 25 individuals with TAM and DS-AMKL identified by whole-exome sequencing. Paired samples are indicated by asterisks. The mutation rates per phase are given. (**b**) VAFs of individual mutations determined by deep sequencing, with VAFs adjusted for genomic copy numbers. Long indels of >3 bp were excluded from the analysis because their VAFs were difficult to accurately estimate. The VAF for each sample estimated on the basis of blast percentage is indicated by a purple horizontal bar.

been reported in up to 13% of myelodysplastic syndromes and related chronic myeloid neoplasms[40]. Although rarely mutated in adult AML[41], *EZH2* represents one of the most frequently mutated and deleted genes in childhood AMKL, as we identified mutations or deletions in 16 of 49 DS-AMKL cases (33%) and in 3 of 19 non-DS-AMKL cases (16%) (**Fig. 4a,b**, **Supplementary Fig. 12c** and **Supplementary Tables 7** and **8**). No other PRC2 components were mutated, except for *SUZ12*, which was mutated in a single DS-AMKL case (**Fig. 4a** and **Supplementary Table 7**). Although frequent mutations in other epigenetic regulators, including in *TET2*, *IDH1* or *IDH2*, *DNMT3A* and *ASXL1*, are cardinal features of myeloid neoplasms in adults, we rarely found these mutations in DS-AMKL and non-DS-AMKL cases, only identifying occasional *DNMT3A* ($n = 1$), *ASXL1* ($n = 1$) and *BCOR* ($n = 2$) mutations in DS-AMKL (**Fig. 4a**).

KANSL1 (encoding KAT8 regulatory NSL complex subunit 1; also known as MSL1V1 or NSL1) represents a new recurrent mutational target in human cancer (**Table 1**), although haploinsufficiency of *KANSL1* through germline deletions or mutations has been implicated in a congenital disease known as 17q21.31 microdeletion syndrome (MIM 610443)[42,43]. We found heterozygous mutations in *KANSL1* in three DS-AMKL and three non-DS-AMKL cases, and most of these mutations were nonsense or frameshifts, leading to loss of protein function (**Fig. 4a** and **Supplementary Table 7**). KANSL1 protein is necessary and sufficient for the activity of the KAT8 (MOF) histone acetyltransferase complex, which is engaged in the acetylation of histone H4 lysine 16 (H4K16), leading to transcriptional activation. Loss of acetylation of H4K16 has been reported to be a common hallmark of human cancer, and other histone acetyltransferases for H4K16 have been reported to form recurrent fusion partners in leukemia, including MOZ and MORF[44], suggesting a role for compromised H4K16 acetylation by *KANSL1* mutations in leukemogenesis. Of interest, KANSL1 is also responsible for the acetylation of the TP53 tumor suppressor that is important for TP53-dependent transcriptional activation[45]. KAT8 also interacts with a histone H3 lysine 4 (H3K4) methyltransferase, MLL, and the interaction of MLL and KAT8 complexes facilitates the cooperative recruitment of both complexes to gene promoters and enhances transcription initiation at target genes[45]. Thus, impaired TP53 function and/or deregulated expression of MLL gene targets could also contribute to leukemogenesis by *KANSL1* mutations.

Table 1 Recurrently mutated genes other than *GATA1* in DS-AMKL samples in whole-exome sequencing

Gene	Mutation type	RefSeq	Amino acid change	Nucleotide change	Sample (UPN) number
CTCF	Splice site	NM_006565	p.Gly318_splice	c.953–2A>G	016
CTCF	Frameshift	NM_006565	p.Asn314fs	c.940_941insAC	020
DCAF7	Missense	NM_005828	p.Leu340Phe	c.1018C>T	001
DCAF7	Missense	NM_005828	p.Leu340Phe	c.1018C>T	003
EZH2	Frameshift	NM_004456	p.710_716del	c.2129_2148delATCACAGGA TAGGTATTTTT	001
EZH2	Missense	NM_004456	p.Arg25Gln	c.74G>A	002
KANSL1	Frameshift	NM_001193466	p.Arg720fs	c.2159_2160insCG	020
KANSL1	Nonsense	NM_001193466	p.Arg462*	c.1384C>T	024
NRAS	Missense	NM_002524	p.Gly12Ser	c.34G>A	001
NRAS	Missense	NM_002524	p.Tyr64Cys	c.191A>G	001
NRAS	Missense	NM_002524	p.Gly12Ala	c.35G>C	003
RAD21	Nonsense	NM_006265	p.Arg139*	c.415A>T	001
RAD21	Frameshift	NM_006265	p.374_375del	c.1120_1124delTCTTT	002
RAD21	Missense	NM_006265	p.Leu611Arg	c.1832T>G	018
RAD21	Nonsense	NM_006265	p.Arg65*	c.193C>T	024
STAG2	Nonsense	NM_001042750	p.Arg604*	c.1810C>T	003
STAG2	Nonsense	NM_001042750	p.Arg216*	c.646C>T	019
STAG2	Frameshift	NM_001042750	p.Asn863fs	c.2588_2589insT	020
TP53	Nonsense	NM_000546	p.Glu68*	c.202G>T	002
TP53	Non-frameshift	NM_000546	p.157_162del	c.469_486delGTCCGCGCCA TGGCCATC	002

Other mutations in DS-AMKL

RAS pathway mutations are common in hematopoietic malignancies and other human cancers but have not to our knowledge been described in DS-AMKL. In the current cohort, we identified RAS pathway

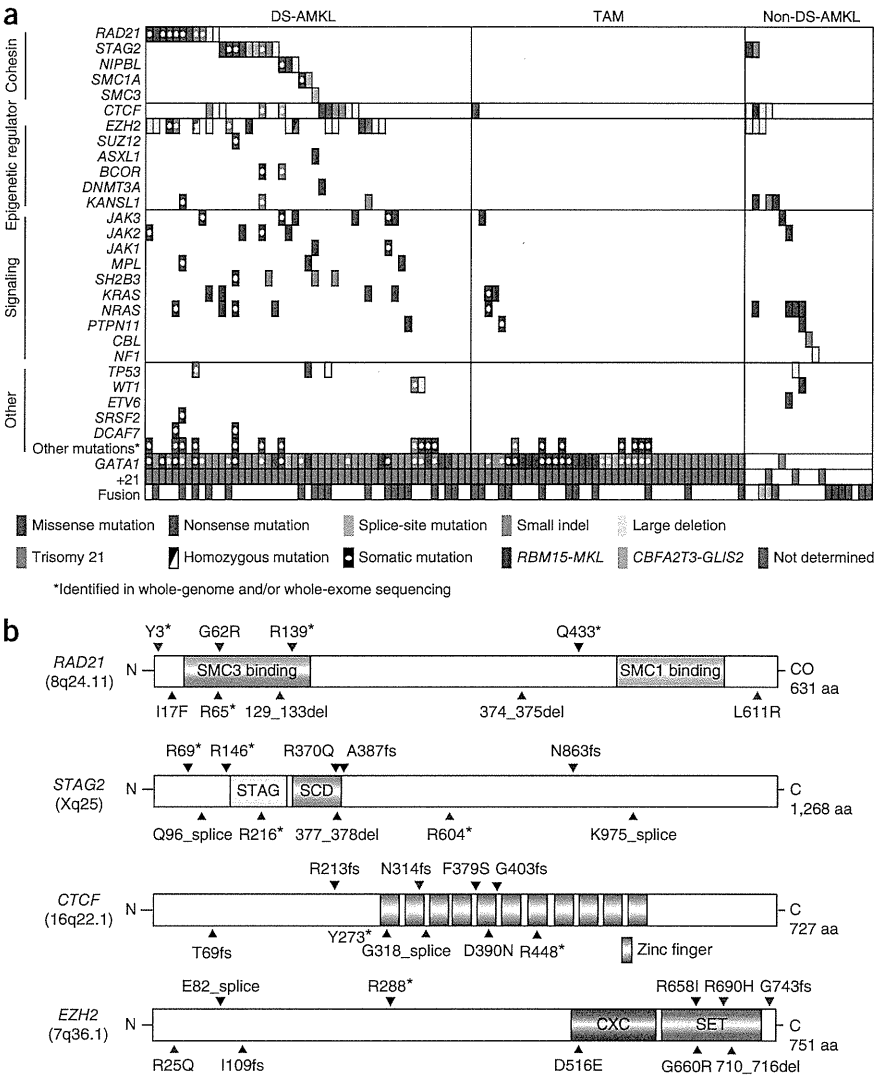

Figure 4 Driver mutations in Down syndrome–related myeloid disorders and non-DS-AMKL. (**a**) Driver mutations in 109 samples of 49 DS-AMKL, 41 TAM and 19 non-DS-AMKL cases. Types of mutations are distinguished by color. Each sample is also described in **Supplementary Table 12**. (**b**) Distribution of RAD21, STAG2, CTCF and EZH2 alterations. Alterations encoded by confirmed somatic mutations are indicated by red arrowheads.

mutations in the *NRAS*, *KRAS*, *PTPN11*, *NF1* and *CBL* genes in 8 DS-AMKL cases (16%) and 6 non-DS-AMKL cases (32%), but these mutations were rarely found in TAM cases (n = 3; 7%) (**Fig. 4a**). Tyrosine kinase and cytokine receptor mutations were also common in DS-AMKL. We found mutations in *JAK1*, *JAK2*, *JAK3*, *MPL* or *SH2B3* (*LNK*) in 17 DS-AMKL cases (35%) but rarely in TAM (n = 1) and non-DS-AMKL (n = 2) cases. We found no *FLT3* mutations in our cohort. The identified mutations were largely mutually exclusive. We found *JAK2* mutations in 4 DS-AMKL cases and 1 non-DS-AMKL case, including mutations encoding p.Val617Phe (n = 2), p.Leu611Ser (n = 1), p.Arg683Ser (n = 1) and p.Arg867Gln (n = 1); of these, *JAK2* mutations encoding p.Arg683Ser and p.Arg867Gln substitutions have been reported in acute lymphoblastic leukemia (ALL)[46,47] but not in myeloid malignancies[8,46]. Thus, we re-evaluated the diagnosis of AMKL in both UPN097 (p.Arg683Ser) and UPN023 (p.Arg867Gln), in whom the initial diagnosis of AMKL was strongly supported by typical surface marker expression of CD41, CD41b, CD117, CD13, CD33, CD34 and CD36 in UPN097 and of CD7, CD13, CD34, CD41a and CD42b in UPN023, together with characteristic cytomorphology. Similarly, the mutation encoding p.Leu611Ser was reported in both ALL[48] and polycythemia vera[49]. Thus, it seems that some *JAK2* mutations are involved in both myeloid and lymphoid leukemogenesis. As reported previously[10,11], *TP53* mutations were found in approximately 10% of DS-AMKL cases. Two identical somatic mutations found in the *DCAF7* gene (encoding p.Leu340Phe) might be interesting because the DCAF7 protein interacts with the DYRK1a kinase encoded within the Down syndrome critical region on chromosome 21 (ref. 50). DCAF7 has been shown to interact with DYRK1a through its N-terminal or C-terminal region, and the p.Leu340Phe substitution identified in our study was also located in the C-terminal domain. However, no additional mutation was detected in the extended cohort; therefore, the relevance of *DCAF7* remains to be determined.

Allelic burden of major recurrent mutations relative to *GATA1* mutations

We assessed intratumoral heterogeneity and the clonal origin of mutations by calculating the variant allele frequency (VAF) of each mutation relative to that of the *GATA1* mutation using deep sequencing. Mutations in cohesin components, *CTCF* and *EZH2* showed comparable VAFs to *GATA1* mutations (**Fig. 5b**), suggesting their role in the early stage of DS-AMKL development. In contrast, RAS pathway and other tyrosine kinases and cytokine receptor mutations showed significantly lower VAFs than corresponding *GATA1* mutations (P = 0.0001) (**Fig. 5b**), indicating that they are more likely to represent subclonal mutations, which were typically preceded by mutations in cohesin components, *CTCF* and *EZH2* and were involved in the evolution of multiple DS-AMKL subclones. Although RAS and JAK pathways activated by gene mutations represent potentially druggable targets and several promising compounds are currently available, this observation may largely preclude the efficient use of such compounds in eradicating founding DS-AMKL clones.

Distinct genetic features of Down syndrome– and non–Down syndrome–related AMKL

Despite their morphological similarities, both forms of AMKL in childhood are characterized by distinctive genetic features. According to the current study and a recent report of integrated analysis of non-DS-AMKL[22], *GATA1* mutations and trisomy 21 are less common in non-DS-AMKL than in DS-AMKL cases (**Fig. 4a** and **Supplementary Table 9**). In our series, DS-AMKL was characterized by high frequencies of mutations in the cohesin complex, *EZH2* and other epigenetic regulators, as well as in JAK family kinases, which were less

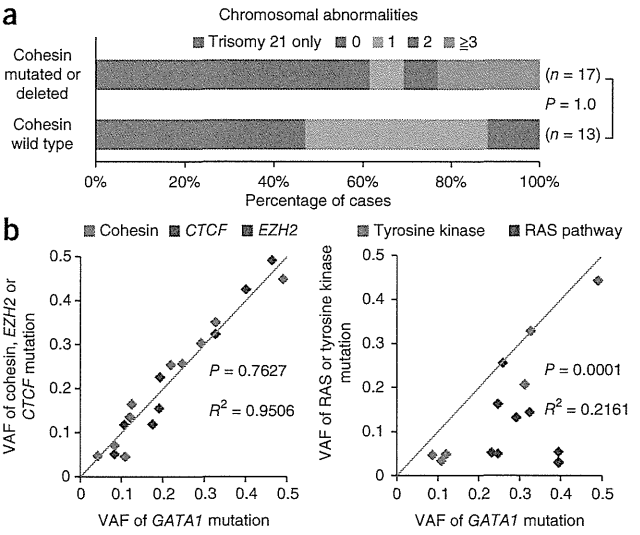

Figure 5 Relationship of cohesin mutations with karyotypes and comparison of mutation loads between major gene targets in DS-AMKL and *GATA1*. (**a**) The number of chromosomal abnormalities is compared between cases with and without cohesin mutations or deletions for DS-AMKL cases. Zero signifies chromosomal abnormalities without change in chromosome count, such as partial amplification or deletion of the chromosomal region or balanced translocation. (**b**) Diagonal plots of copy number–adjusted VAFs comparing coexisting *GATA1* and other pathway mutations, including cohesin, *CTCF*, *EZH2*, tyrosine kinase and the RAS pathway mutations, as indicated by color.

common mutational targets in non-DS-AMKL. Previous studies identified recurrent *CBFA2T3-GLIS2* and *RBM15-MKL* gene fusions in non-DS-AMKL, which were found in 27% and 15.2% of non-DS-AMKL cases, respectively[22,51], whereas these fusions were not detected in DS-AMKL cases in another report (n = 10 cases)[23]. Similarly, in the current cohort, RT-PCR analysis identified 2 *CBFA2T3-GLIS2* and 3 *RBM15-MKL* fusion genes in 19 non-DS-AMKL cases but not in TAM and DS-AMKL cases (**Fig. 4a** and **Supplementary Table 10**), illustrating the genetic differences between DS-AMKL and non-DS-AMKL. In addition, our RNA sequencing of the current cases (n = 17) (**Supplementary Table 11**) also showed no *CBFA2T3-GLIS2* and *RBM15-MKL* fusions.

DISCUSSION

Whole-genome and/or whole-exome analyses and follow-up targeted sequencing identified several new aspects of the pathogenesis of Down syndrome–related myeloid proliferation. First, the initial TAM phase was characterized by a paucity of somatic mutations. The mean number of non-silent mutations per sample (1.7; range of 1–5) was surprisingly small compared with that reported in other human cancers (**Supplementary Fig. 13**), in line with a recent report that identified 1.2 (range of 1–2) mutations per sample by whole-exome sequencing in 5 TAM samples[52]. In addition to reporting a low somatic mutation frequency in their initial TAM phase, Nikolaev *et al.*[52] also reported accumulation of somatic mutations (including single cases of *SMC3* and *EZH2* mutation) during progression from TAM to DS-AMKL. Excluding common *GATA1* mutations, we identified no other recurrent mutations, with only 0.7 non-silent mutations per case, indicating that TAM could be caused by a single acquired *GATA1* mutation in addition to constitutive trisomy 21.

Intratumoral heterogeneity was evident not only in the DS-AMKL phase but also at the initial diagnosis of TAM, and subsequent DS-AMKL originated from one of the multiple subclones present in the TAM phase, usually representing the progeny of the largest subpopulation. In most cases, the DS-AMKL clone was accompanied by newly acquired driver mutations not shared by the original TAM population, generating a unique landscape of gene mutations in DS-AMKL, which was characterized by high mutational frequencies in cohesin or *CTCF* (65%), other epigenetic regulators (45%), and RAS or signal-transducing molecules (47%) (**Fig. 4a**). Tumor recurrence or evolution has not to our knowledge been characterized by the distinct gene mutations in greater detail than in the present study. In total, 44 of the 49 DS-AMKL cases had additional mutations beyond those in *GATA1* (**Fig. 4a**), even though there was a clear limitation on capturing mutations using the targeted sequencing approach.

The very high frequency of cohesin (53%) and *EZH2* (33%) mutations and deletions in DS-AMKL but not in TAM or non-DS-AMKL cases was noteworthy because the reported mutation rates of cohesin and *EZH2* in adult AML and other human cancers remain approximately 10% (refs. 14,40,41), underscoring a major role for these mutations in the pathogenesis of DS-AMKL. The leukemogenic mechanism of mutated cohesin remains elusive, and frequent *CTCF* mutations also need further evaluation to characterize their possible cooperative role with cohesin mutations[26,30,33,34]. To our knowledge, *KANSL1* mutations have not been reported previously and represent a new recurrent mutational target in human cancer, although their functional impact on AMKL development remains unknown. Evaluation of the allelic burden of these mutations by deep sequencing disclosed a clonal hierarchy among different driver mutations in which clonal mutations in cohesin, *CTCF* and epigenetic regulators frequently preceded subclonal mutations in RAS and signal transduction molecules.

In conclusion, Down syndrome–related myeloid proliferation is shaped by multiple rounds of acquisition of new mutations and clonal selection, which are initiated by a *GATA1* mutation in the TAM phase and further driven by mutation in cohesin or *CTCF*, *EZH2* or other epigenetic regulators, and RAS or signal-transducing molecules, leading to AMKL. DS-AMKL and non-DS-AMKL showed similar phenotypes but had distinct genetic features, which may underlie their different clinical characteristics.

URLs. European Genome-phenome Archive (EGA), https://www.ebi.ac.uk/ega/; EBCall, https://github.com/friend1ws/EBCall; Catalogue of Somatic Mutations in Cancer (COSMIC), http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/; PubMed, http://www.ncbi.nlm.nih.gov/pubmed/; UCSC Genome Browser, http://genome.ucsc.edu/; Integrative Genomics Viewer, http://www.broadinstitute.org/igv/; DNACopy, http://biostatistics.oxfordjournals.org/content/5/4/557.full.pdf; Genomon-fusion (in Japanese), http://genomon.hgc.jp/rna/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequencing data have been deposited in the European Genome-phenome Archive (EGA) under accession EGAS00001000546.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.O., Y. Shiraishi, A.S.-O., K.C., H.T. and S.M. performed bioinformatics analyses of the resequencing data. M.S., A.S.-O., Y. Sato, A.H. and H.M. performed microarray experiments and analyses. R.K. and A.H. performed RT-PCR analyses. M.P., K. Terui, R.W., D.H., K.N., H.K., K. Tsukamoto, S.A., K. Kawakami, K. Kato, R.N., S.I., Y.H., S.K. and E.I. collected specimens and were involved in planning the project. K.Y., T.T., H.S., Y.N. and N.S. processed and analyzed genetic materials, prepared the library and performed sequencing. K.Y., T.T., Y.O., A.K. and S.O. generated figures and tables. E.I. and S.O. led the entire project. K.Y. and S.O. wrote the manuscript. All authors participated in discussions and interpretation of the data and results.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects and samples. Genomic DNA from 84 individuals with Down syndrome–related myeloid disorders (41 samples from the TAM phase and 49 from the AMKL phase) and 19 with non-DS-AMKL were analyzed by whole-genome and/or whole-exome and/or targeted deep sequencing. In six cases with Down syndrome–related myeloid disorders, samples were collected from both the TAM and AMKL phases. RNA sequencing was also performed for 12 of the 49 DS-AMKL cases and for 5 additional DS-AMKL cases. RNA samples were also available for RT-PCR analysis from 30 cases with TAM, 32 cases with DS-AMKL and 15 cases with non-DS-AMKL. Written informed consent was obtained from each subject's parents before sample collection (**Supplementary Note**). This study was approved by the Ethics Committees of the University of Tokyo according to the Helsinki convention. *GATA1* mutations were detected by Sanger sequencing of all TAM and DS-AMKL samples according to the previously described procedure[5]. Detailed information on subjects and samples is provided in **Supplementary Tables 1, 4, 11** and **12**. Tumor DNA was extracted from bone marrow– or peripheral blood–derived mononuclear cells at diagnosis. Genomic DNA samples from peripheral blood from subjects in remission or from nail tissues at diagnosis were used as germline controls. Genomic DNA was extracted using a QIAamp DNA Blood Mini kit and a QIAamp DNA Investigator kit (Qiagen). Total RNA was extracted using the RNeasy kit (Qiagen) with RNase-free DNase (Qiagen).

Whole-genome sequencing. DNA samples were processed for whole-exome sequencing using NEBNext DNA sample Prep Reagent (New England Biolabs) according to the modified Illumina protocol. Sequence data were generated on the Illumina HiSeq 2000 platform in 100-bp paired-end reads. Data processing and variant calling were performed as described previously[54]. All candidate variants were validated by deep sequencing.

Validation and quantitative measurements of the frequencies of mutant alleles by deep sequencing. Individual mutation sites were amplified by genomic PCR using primers tagged with NotI cleavage sites and subjected to high-throughput sequencing as described previously[55], except that target DNA was not pooled. Deep sequencing was performed using the MiSeq or HiSeq 2000 platform. Data processing was performed according to the previously described method with minor modifications[55]. Briefly, each read was aligned to a set of PCR-amplified target sequences using BLAT[56], and dichotomic variant alleles were differentially enumerated. For indels, individual reads were first aligned to each of the wild-type and indel sequences and then assigned to the one to which better alignment was obtained in terms of the number of matched bases. Each SNV and indel whose VAF in the tumor sample was equal to or greater than 2.0% and significantly higher than the frequency in the germline sample was adopted as a somatic mutation. The error size for estimated VAFs was evaluated by assuming binomial distributions in deep sequencing, which were confirmed by observed allele frequencies at heterozygous SNPs in normal DNA samples (**Supplementary Fig. 14a**), in which the variance (σ^2) ranged from $4.0–11.0 \times 10^{-4}$ (**Supplementary Fig. 14b**).

Clustering analysis of mutations. To identify the chronological behavior of the structure of the tumor subpopulation for the TAM and AMKL phases, somatic mutations detected in both phases by whole-genome sequencing were clustered according to their VAFs as measured by deep sequencing. Copy number–adjusted deep sequencing data, in which the VAFs of genes on the X chromosome in male cases or in regions of uniparental disomy were halved, were subjected to unsupervised clustering. Six mutations located in amplified or deleted genomic regions were excluded from the analysis. Long indels of >3 bp, except for those affecting key genes such as *GATA1* and *RAD21*, and mutations in repetitive regions were excluded from the analysis because their VAFs could tend to be underestimated.

All validated mutations were grouped into three categories according to the following criteria: (i) mutations found only in TAM (VAF in AMKL < 0.02), (ii) mutations found only in AMKL (VAF in TAM < 0.02) and (iii) mutations found in both TAM and AMKL (VAF in TAM > 0.02 and VAF in AMKL > 0.02). Clustering of mutations in each category was performed using Mclust, provided as an R package, on the basis of the VAFs of the mutations in the TAM and AMKL phases, where one-dimensional clustering of mutations in categories (i) and (ii) was performed on the basis of the homoscedastic model and two-dimensional clustering was performed for mutations in category (iii) on the basis of the ellipsoidal model. The most appropriate number of clusters was determined by using the Bayesian information criterion (BIC) score. Singleton points identified by this algorithm were regarded as outliers. Clonal subpopulations within tumors were also evaluated by kernel density analysis (**Supplementary Fig. 5**), where we drew kernel density estimate plots for the VAFs of validated variants using the density function in R.

Whole-exome sequencing and detection of somatic mutations. Exome capture was performed using SureSelect Human All Exon V3 or V4 (Agilent Technologies) or the TruSeq Exome Enrichment kit (Illumina). Enriched exome fragments were then subjected to massively parallel sequencing using the Genome Analyzer IIx or HiSeq 2000 platform (Illumina). Candidate somatic mutations were detected using our in-house pipeline EBCall (Empirical Bayesian mutation Calling; see URLs)[57]. All candidates were validated by Sanger sequencing or independent deep sequencing.

PCR-based targeted deep sequencing. Deep sequencing of *DCAF7*, *EED*, *JAK1*, *JAK3*, *KANSL1*, *SH2B3*, and *SUZ12* was performed using the primers tagged with NotI cleavage sites whose sequences are listed in **Supplementary Table 6**. Data processing and variant calling were performed as described previously[58]. All candidate variants were validated by Sanger sequencing or independent deep sequencing using non-amplified DNA.

Targeted deep sequencing. In total, 39 gene targets were exhaustively examined for mutations in all 109 cases using deep sequencing (**Supplementary Table 5**). Genomic DNA (1–1.5 μg) from bone marrow–derived mononuclear cells or peripheral blood was enriched for target exons using a SureSelect custom kit (Agilent Technologies) designed to capture all of the coding exons from the 39 target genes, and high-throughput sequencing was performed on the enriched targets using the HiSeq 2000 platform with a standard 100-bp paired-end read protocol. Sequencing reads were aligned to hg19 using Burrows-Wheeler Aligner (BWA) version 0.5.8 with default parameters. The allele frequencies of SNVs and indels were calculated at each genomic position by enumerating the relevant reads with SAMtools[59]. Initially, all variants showing VAF > 0.02 were extracted and annotated using ANNOVAR[60] for further consideration if they were found in >6 reads out of >10 total reads and appeared in both plus- and minus-strand reads. For the cases for which no germline DNA was available, relevant somatic mutations were called by eliminating the following entries, unless they were registered in the Catalogue of Somatic Mutations in Cancer (COSMIC) v60 (ref. 61) or reported as somatic mutations in PubMed: (i) synonymous variants and those having ambiguous (unknown) annotations, (ii) known SNPs in public and private databases, including dbSNP131, the 1000 Genomes Project as of 23 November 2010 and our in-house database, (iii) sequencing or mapping errors, (iv) all missense SNVs with allele frequencies of 0.45–0.55 and (v) variants localized to duplicated regions found in SegDups of the UCSC Genome Browser. To eliminate sequencing errors in category (iii), we excluded all variants found in 31 normal Japanese samples at, on average, allele frequency > 0.25. Mapping errors were removed by visual inspection with the Integrative Genomics Viewer browser[62]. All candidate variants were validated by Sanger sequencing or independent deep sequencing.

Calculation of copy numbers for target exons. Letting $d_j^{i,s}$ be the sequencing depth at the ith nucleotide of the jth exon in sample s, the standardized depth of the jth exon is calculated as

$$D_j^s = k_s \sum_i d_i^{j,s}$$

where k_s is determined to satisfy

$$k_0 = \sum_j D_j^s$$

for a fixed constant k_0 (for example, $k_0 = 1$). The correlation coefficient ($R = R^{s,t}$) between two vectors D_i^s and D_i^t was calculated, where D_i^s and D_i^t represent the depth for a given sample (sample s) and each of the 443

samples (sample t), analyzed for other projects, with completely normal copy numbers in array–comparative genomic hybridization (aCGH; $t = 1, 2, 3, \ldots, 443$), respectively, through which a total of m_0 (= 12) control samples showing the largest R values were selected (T_m; $m = 1, 2, 3, \ldots, m_0$) and used for copy number calculation. The copy number of the ith target exon of sample s (Cn_i^s) was calculated as

$$Cn_i^s = D_i^s / \hat{D}_i^s$$

where $\hat{D}_i^s$ was calculated by averaging m_0 samples by

$$\hat{D}_i^s = \sum_{m=1}^{m_0} D_i^{T_m} / m_0$$

Copy numbers were calculated for exons with mean depth of >500. Circular binary segmentation was also used to identify discrete copy number segments using DNACopy (see URLs); segmented copy number ($\widehat{Cn_i^s}$) was defined for the ith exon of sample s. The distribution of $\widehat{Cn_i^s}$ was calculated for all samples, and exons showing $|\widehat{Cn_i^s} - E(\widehat{Cn_i^s})| > 4$ s.d. were considered to have copy number losses or gains.

Screening for *CBFA2T3-GLIS2* and *RBM15-MKL1* fusion genes. *CBFA2T3-GLIS2* and *RBM15-MKL1* fusion genes were screened by RT-PCR[22,63]. Primer sequences are given in **Supplementary Table 13**. PCR amplification was performed by 40 cycles at 94 °C for 2 min, 60 °C for 30 s and 68 °C for 1 min, followed by denaturation at 94 °C for 2 min and extension at 68 °C for 7 min.

SNP array analyses. All tumor samples subjected to whole-exome sequencing were also analyzed for copy number alterations using SNP arrays (Affymetrix GeneChip Human Mapping 250K NspI Array or Genome-Wide Human SNP Array 6.0) as described previously[10,64,65].

RT-PCR analysis of *STAG2* and *CTCF* transcripts. To confirm abnormal splicing of *CTCF* in UPN016 and UPN071 and that of *STAG2* in UPN067, RT-PCR were performed using cDNA derived from each subject, with cDNA from CMK11-5 (DS-AMKL–derived cell line with no known mutations in both genes) used as a control (**Supplementary Fig. 11**). Primer sequences are given in **Supplementary Table 14**. Total RNA (1 μg) was subjected to reverse transcription using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Electrophoresis was performed using Experion (Bio-Rad).

RNA sequencing. Detailed information on samples is provided in **Supplementary Table 11**. Library preparation and sequencing were performed as described previously[54]. Fusion transcripts were detected using Genomon-fusion.

Gene expression analysis of recurrently mutated genes. Expression data for the recurrently mutated genes in whole-exome sequencing were retrieved from the BioGPS database[18] for normal hematopoietic cells, including whole bone marrow, $CD33^+$ myeloid cells, $CD34^+$ cells, $CD19^+$ B cells and $CD4^+$ T cells, and from published data[19] and our RNA sequencing data for DS-AMKL samples.

Statistical analysis. The number of non-silent mutations identified by whole-exome sequencing in TAM and DS-AMKL samples (**Fig. 2a**) and the number of chromosome abnormalities in DS-AMKL cases with and without cohesin mutations or deletions (**Fig. 5a**) were compared using the Mann-Whitney U test. The difference in VAF between two mutations (**Fig. 5b**) was tested by Wilcoxon signed-rank test.

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Corrigendum: The landscape of somatic mutations in Down syndrome–related myeloid disorders

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In the version of this article initially published, the discussion of cited reference 52 should also have noted that the work "reported accumulation of additional somatic mutations (including single cases of *SMC3* and *EZH2* mutation) during progression from TAM to DS-AMKL." The error has been corrected in the HTML and PDF versions of the article.

nature genetics

Somatic *SETBP1* mutations in myeloid malignancies

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Here we report whole-exome sequencing of individuals with various myeloid malignancies and identify recurrent somatic mutations in *SETBP1*, consistent with a recent report on atypical chronic myeloid leukemia (aCML)[1]. Closely positioned somatic *SETBP1* mutations encoding changes in Asp868, Ser869, Gly870, Ile871 and Asp880, which match germline mutations in Schinzel-Giedion syndrome (SGS)[2], were detected in 17% of secondary acute myeloid leukemias (sAML) and 15% of chronic myelomonocytic leukemia (CMML) cases. These results from deep sequencing demonstrate a higher mutational detection rate than reported with conventional sequencing methodology[3–5]. Mutant cases were associated with advanced age and monosomy 7/deletion 7q (–7/del(7q)) constituting poor prognostic factors. Analysis of serially collected samples indicated that *SETBP1* mutations were acquired during leukemic evolution. Transduction with mutant *Setbp1* led to the immortalization of mouse myeloid progenitors that showed enhanced proliferative capacity compared to cells transduced with wild-type *Setbp1*. Somatic mutations of *SETBP1* seem to cause gain of function, are associated with myeloid leukemic transformation and convey poor prognosis in myelodysplastic syndromes (MDS) and CMML.

During the past decade, substantial progress has been made in the understanding of the pathogenic gene mutations driving myeloid malignancies. Following the early identification of mutations in *RUNX1* (ref. 6), *JAK2* (ref. 7) and *RAS*[8,9], SNP array karyotyping led to the discovery of mutations in *CBL*[10], *TET2* (ref. 11) and *EZH2* (ref. 12). More recently, new sequencing technologies have enabled exhaustive screening of somatic mutations in myeloid malignancies, leading to the discovery of unexpected mutational targets, such as *DNMT3A*[13], *IDH1* (ref. 14) and spliceosomal genes[15–17]. Insights into the progression to sAML constitute an important goal of biomedical investigations, now augmented by the availability of next-generation sequencing technologies[18,19].

We performed whole-exome sequencing of 20 index cases with myeloid malignancies (**Supplementary Table 1**) and identified 38 non-silent somatic mutations that were subsequently confirmed by Sanger sequencing and targeted deep sequencing. We found that seven genes were recurrently mutated in multiple samples (**Supplementary Tables 2–4**). Of these, we identified a new recurrent somatic mutation in *SETBP1* (encoding a p.Asp868Asn alteration) in two cases with refractory anemia with excess blasts (RAEB) (**Fig. 1** and **Supplementary Tables 1–3** and **5**), which were confirmed using DNA from both tumor and $CD3^+$ T cells.

SETBP1 was initially identified as a 170-kDa nuclear protein that binds to SET[20,21] and is activated to support the recovery of granulopoiesis in chronic granulomatous disease[22]. Mutations in *SETBP1* are causative in SGS, a congenital disease characterized by a higher than normal prevalence of tumors, typically neuroepithelial neoplasia[23,24]. Notably, the mutations identified in our cohort exactly corresponded with the recurrent *de novo* germline mutations responsible for SGS, which prompted us to investigate *SETBP1* mutations in a large cohort of 727 cases with various myeloid malignancies (**Supplementary Table 6**).

SETBP1 mutations were found in 52 of 727 cases (7.2%). Consistent with recent reports[1,3–5,25,26], p.Asp868Asn (n = 28), p.Gly870Ser (n = 15) and p.Ile871Thr (n = 5) alterations were more frequent than p.Asp868Tyr, p.Ser869Asn, p.Asp880Asn and p.Asp880Glu alterations (n = 1 for each) (**Fig. 1** and **Supplementary Tables 1** and **7**). All these alterations were located in the SKI homology region, which is highly

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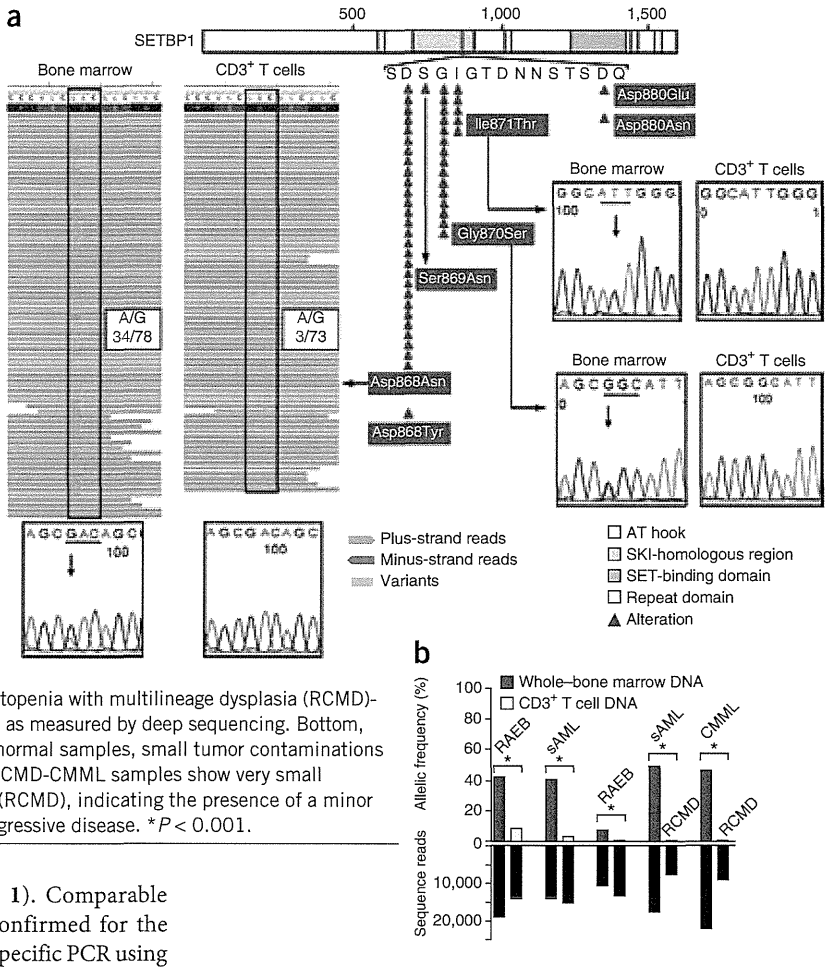

Figure 1 Somatic *SETBP1* mutations as detected by next-generation whole-exome sequencing and Sanger sequencing. (**a**) Distribution of *SETBP1* mutations detected in 52 of 727 myeloid neoplasms, all of which were located within the portion of the gene encoding the SKI-homologous domain. Bottom and right, representative mutations confirmed by Sanger sequencing (horizontal lines and vertical arrows indicate affected codons and nucleotides, respectively). Left, a somatic *SETBP1* mutation (encoding a p.Asp868Asn alteration) detected by whole-exome sequencing of paired tumor (bone marrow) and normal ($CD3^+$ T cell) DNA from a case with RAEB (whole exome 4), where red and blue bars indicate positive and negative strands, respectively. Mutated nucleotides (c.2602G>A) are shown in green. Black rectangles highlight the codon affected by mutation. A small amount of tumor cell contamination caused occasional mutant reads in the $CD3^+$ T cell sample, where the presence of multiple single-nucleotide variants (SNVs) of similar frequencies precluded the possibility of somatic mosaicism. (**b**) Top, allele frequencies in paired bone marrow and $CD3^+$ T cell samples in two RAEB cases and one sAML case and in paired refractory cytopenia with multilineage dysplasia (RCMD)-sAML and RCMD-CMML samples from the same individual as measured by deep sequencing. Bottom, depth of coverage of independent reads. In paired tumor-normal samples, small tumor contaminations were detected in $CD3^+$ T cells. Paired RCMD-sAML and RCMD-CMML samples show very small numbers of mutant reads in the initial MDS presentation (RCMD), indicating the presence of a minor *SETBP1*-mutated clone, which evolved later into more aggressive disease. $^*P < 0.001$.

conserved between species (**Supplementary Fig. 1**). Comparable expression of mutant and wild-type alleles was confirmed for the p.Asp868Asn and p.Gly870Ser alterations by allele-specific PCR using genomic DNA and cDNA (**Supplementary Fig. 2**). *SETBP1* mutations were significantly associated with advanced age ($P = 0.01$) and −7/del(7q) ($P = 0.01$) and were frequently found in sAML (19 of 113 cases; 16.8%; $P < 0.001$) and CMML (22 of 152 cases; 14.5%; $P = 0.002$), whereas they were less frequent in primary AML (1 of 145 cases; <1%; $P = 0.002$) (**Table 1** and **Supplementary Fig. 3a**). The lack of apparent segmental allelic imbalance involving the *SETBP1* locus (18q12.3) in SNP array karyotyping in all mutated cases (**Supplementary Fig. 4**), together with no more than 50% mutant allele frequencies in deep sequencing and allele-specific PCR, suggested the presence of heterozygous mutations (**Fig. 1b** and **Supplementary Fig. 2**). Medical history and physical findings did not support clinical diagnosis with SGS in any of these cases, and formal confirmation of the somatic origin of all types of mutation found was carried out using germline DNA from $CD3^+$ T cells and/or serial samples ($n = 21$).

Of the cases with *SETBP1* mutations, 12 had clinical material available to successfully analyze samples collected serially at multiple clinical time points. None of the 12 cases had *SETBP1* mutations at the time of initial presentation, indicating that the mutations were acquired only upon or during leukemic evolution (**Figs. 1** and **2**). Most of the *SETBP1* mutations (17 of 19) showed comparable or higher allele frequencies relative to other secondary events, suggesting a potential permissive role of *SETBP1* mutations (**Supplementary Fig. 5**). Such a secondary nature for *SETBP1* mutations was confirmed by mutational analysis of colonies derived from individual progenitor cells grown in methylcellulose culture (**Supplementary Fig. 6**).

To test potential associations with additional genetic defects, the frequencies of mutations in 13 common genes relevant to myeloid leukemogenesis were compared in the cases with *SETBP1* mutations and in cases with wild-type *SETBP1* (**Fig. 2c,d** and **Supplementary Table 8**). Only *CBL* mutations were significantly associated with *SETBP1* mutations ($P = 0.002$; **Supplementary Table 9**). Notably, mutations of *FLT3* and *NPM1* were not found in cases with *SETBP1* mutation. Coexisting *SETBP1* and *CBL* mutations were found in 12 cases, of which 6 were subjected to deep sequencing, and *CBL*-mutated clones were significantly smaller than *SETBP1*-mutated clones, suggesting that *CBL* mutations were acquired by a subclone with *SETBP1* mutation (**Supplementary Fig. 5**). The significant association of *CBL* and *SETBP1* mutations suggests their potential cooperation in leukemia progression. Although direct physical interaction between mutant Setbp1 and CBL proteins was not detected (**Supplementary Fig. 7**), it is possible that *CBL* mutations cooperate with *SETBP1* mutations indirectly by reducing the cytokine dependence of leukemia cells[10,27]. *SETBP1* mutations were also found in aCML[1] and juvenile chronic myelomonocytic leukemia[28], characterized by RAS pathway defects, including *CBL* mutations.

Analysis of the expression patterns of *SETBP1* mRNA in normal hematopoietic tissues showed relatively low levels of this transcript in myeloid and/or monocytic cells as well as in $CD34^+$ cells (**Supplementary Fig. 8**). In contrast, *SETBP1*-mutant cases showed significantly higher expression levels than samples with wild-type *SETBP1* ($P = 0.03$; **Supplementary Fig. 9**). When *SETBP1* expression was also evaluated using expression array data in the cases with different subtypes of myeloid neoplasm (**Supplementary Fig. 10**), *SETBP1* was found to be overexpressed in cases with non–core binding factor (CBF) primary AML, including MDS, whereas CBF leukemias showed normal levels of the corresponding mRNA. In particular, *SETBP1*

Table 1 Clinical characteristics of myeloid malignancies with or without *SETBP1* mutation

Characteristic	Wild-type *SETBP1*	Mutant *SETBP1*	*P*[a]
Number	675	52	
Age at study entry (years), mean ± s.d.	61 ± 15	67 ± 12	**0.01**[b]
Age range (years)	16–91	26–83	
Ancestry, number			0.27
Caucasian	222	29	
African American	10	0	
Asian	298	23	
Other	2	0	
Male sex, number	376	29	0.23
Increased (≥10%) bone marrow blasts, number	376	33	0.31
Diagnosis, number			
5q– syndrome	7	1	1.00
RCMD	52	2	1.00
RAEB	86	4	1.00
sAML	94	19	**<0.001**
CMML	130	22	**0.002**
CML BP	25	2	1.00
PMF	25	1	1.00
pAML	144	1	**0.002**
Cytogenetics, number			
Normal	208	17	1.00
–5,del(5q)	39	1	1.00
–7,del(7q)	72	15	**0.01**
–Y only	9	0	1.00
–20,del(20q)	18	1	1.00
+8	45	2	1.00
Complex (≥3)	69	2	1.00

CML BP, chronic myelogenous leukemia blast phase; PMF, primary myelofibrosis.
[a]A Fisher's exact test was used to determine *P* values, except where otherwise indicated. *P* values in multiple comparisons were evaluated by Bonferroni correction, and statistically significant *P* values are indicated with bold font. [b]A Wilcoxon test was used to calculate the *P* value.

expression was significantly higher in cases with loss of chromosome 7 ($P = 0.03$) and complex karyotype ($P < 0.001$) (**Supplementary Fig. 3**). Clustering analysis of gene expression profiles suggested that *SETBP1*-mutant cases had a similar expression pattern to that of cases with overexpression of wild-type *SETBP1*, including overexpression of *TCF4*, *BCL11A* and *DNTT* (**Supplementary Fig. 10** and **Supplementary Table 10**). Methylation array analysis showed that relative hypomethylation of the CpG site located in proximity to the *SETBP1* coding region was associated with higher expression and mutation of *SETBP1* (**Supplementary Fig. 11**). It remains unclear what factors drive the increase in *SETBP1* mRNA levels in these leukemias; however, these mechanisms may involve aberrant hypomethylation of the *SETBP1* promoter or activation of upstream regulators such as *MECOM*[22,29]).

Within the entire cohort, *SETBP1*-mutated cases were significantly associated with shorter overall survival time (hazards ratio (HR) = 2.27, 95% confidence interval (CI) = 1.56–3.21; $P < 0.001$), with this association especially prominent in the younger age group (<60 years; HR = 4.92, 95% CI = 2.32–9.46; $P < 0.001$). The presence of *SETBP1* mutations was also associated with compromised survival in the cohort with normal karyotype (HR = 3.13, 95% CI = 1.66–5.41; $P = 0.002$) (**Fig. 3**). Multivariate analysis confirmed that *SETBP1* mutation was an independent prognostic factor (HR = 2.90, 95% CI = 1.71–4.83; $P < 0.001$) together with male sex, advanced age and the presence of *ASXL1*, *CBL* and *DNMT3A* mutations. –7/del(7q) was associated with shorter length of survival in univariate analysis but did not remain an independent risk factor after multivariate analysis (**Supplementary Table 11**). The multivariate analysis in the subgroup of MDS and CMML cases (with white blood cell (WBC) counts of <12,000 cells/μl), in which the International Prognostic Scoring System (IPSS) score was applicable[30], also showed that *SETBP1* mutation was an independent prognostic factor (HR = 1.83, 95% CI = 1.04–3.12; $P = 0.04$), whereas the impact of the IPSS score

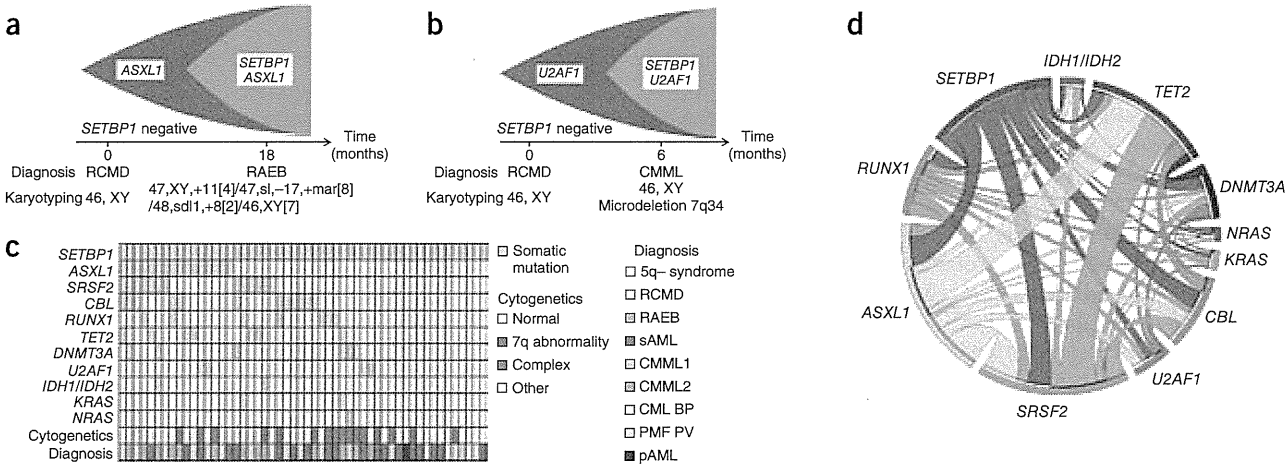

Figure 2 The relationship of *SETBP1* mutations with other common mutations. (**a**,**b**) Clonological profiles of gene mutations in two representative cases with MDS that transformed to RAEB (**a**) and CMML (**b**). Initially, hypocellular MDS (RCMD) was diagnosed on the basis of hypocellular bone marrow with normal karyotype in both cases. (**c**) Coexisting mutations in the *SETBP1*-mutated cohort are shown in a matrix: 36 of 52 cases (69%) were positive for other somatic concomitant mutations tested by Sanger sequencing. Sequenced genes are listed in **Supplementary Table 8**. CMML1 and CMML2 were discriminated by the number of blasts plus promonocytes in the peripheral blood and bone marrow. PV, polycythemia vera; pAML, primary AML. (**d**) Circos plots illustrating coexisting mutations in the selected 12 genes in the whole cohort. No mutations that occurred in a mutually exclusive manner were observed.

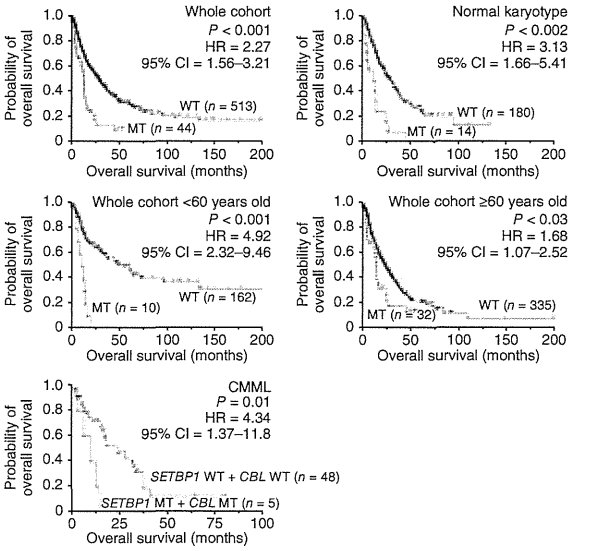

Figure 3 Impact of *SETBP1* mutations on clinical outcome. In the whole cohort, cases with *SETBP1* mutations (MT) had worse overall survival compared with those with wild-type *SETBP1* (WT). *SETBP1* mutations were poor prognostic factors for individuals with normal karyotype. *SETBP1* mutations were poor prognostic factors for individuals regardless of age (>60 and ≤60 years). In the CMML cohort, individuals with double mutations of *SETBP1* and *CBL* had worse prognosis than those with both wild-type genes. Data points indicate events and censors. The Kaplan-Meier method was used to analyze survival outcomes by the log-rank test.

dissipated after multivariate analysis (**Supplementary Tables 11** and **12**). Next, because comprehensive mutational screening identified a significant association between *SETBP1* and *CBL* mutations, we compared overall length of survival in cases with either of these mutations or with these mutations in combination (**Supplementary Figs. 12** and **13** and **Supplementary Table 13**). Overall length of survival was shorter in cases with mutation in both *SETBP1* and *CBL* compared to those with the wild-type forms of these genes, and the combination of these mutations was also unfavorable in an isolated CMML cohort in which either of these mutations alone did not affect survival (**Fig. 3** and **Supplementary Fig. 13**). However, no impact of these mutations was found in a sAML cohort, probably owing to the already very poor prognosis in this subset of individuals (**Supplementary Figs. 12** and **14**).

Previous studies demonstrated that overexpression of *Setbp1* can effectively immortalize mouse myeloid precursors[31]. Expression of Setbp1 mutants (either Asp868Asn or Ile871Thr) also caused efficient immortalization of mouse myeloid progenitors with similar phenotypes (**Fig. 4a,b** and **Supplementary Fig. 15**). Moreover, although having similar levels of Setbp1 protein expression as cells immortalized with wild-type *Setbp1*, cells immortalized with mutant *Setbp1* showed significantly more efficient colony formation and faster proliferation (**Fig. 4c,d** and **Supplementary Figs. 16** and **17**). This observation is consistent with the gain of leukemogenic function due to *SETBP1* mutation. As with overexpressed wild-type *Setbp1*, homeobox genes *Hoxa9* and *Hoxa10* represent critical targets of Setbp1 mutants, as cells immortalized by wild-type or mutant *Setbp1* expressed comparable levels of the corresponding mRNAs, and knockdown of either caused a marked reduction in colony-forming potential (**Supplementary Figs. 18** and **19**). In agreement with these findings, *SETBP1*-mutant leukemias ($n = 14$) showed significantly higher *HOXA9* and *HOXA10* expression levels compared to wild-type cases without *SETBP1* overexpression ($n = 9$; $P = 0.03$ and 0.03, respectively), supporting the notion that *HOXA9* and *HOXA10* are likely functional targets of mutated SETBP1 in myeloid neoplasms (**Supplementary Fig. 20**).

Multiple mechanisms could contribute to the enhanced oncogenic properties of *SETBP1* mutations. For instance, mutation could increase protein stability (**Supplementary Fig. 21**), resulting in greater protein amounts (analogous to upmodulation of *SETBP1* mRNA), in agreement with a previously reported observation[1]. However, we also showed that *SETBP1* mRNA overexpression *in vitro* was associated with the immortalization of progenitors and that there were primary cases of sAML with and without mutations of *SETBP1* and high levels of wild-type mRNA. Thus, although plausible, the mechanisms underlying increased *SETBP1* expression and its proto-oncogenic role may be more complicated. It is also possible that interaction of Ski and/or SnoN with SETBP1 through the SKI homology region could be affected by mutations, leading to transformation[20,32]. *SETBP1* was shown to regulate PP2A activity via binding to SET[20], and decreased PP2A activity has been described in AML[21,33]. In fact, we observed that mutant *Setbp1*–immortalized myeloid progenitors had increased tyrosine phosphorylation of Ppp2ca compared to myeloid progenitors immortalized with wild-type *Setbp1* (**Supplementary Fig. 22**), suggesting that *SETBP1* mutations could cause further PP2A inhibition.

In summary, recurrent somatic *SETBP1* mutations are new lesions that interact with previously defined pathways underlying poor prognosis and provide new insights into the process of leukemic evolution. The apparent association of *SETBP1* mutations with poor clinical outcome observed here provides an important focal point for future mechanistic studies as well as a goal for therapeutic targeting.

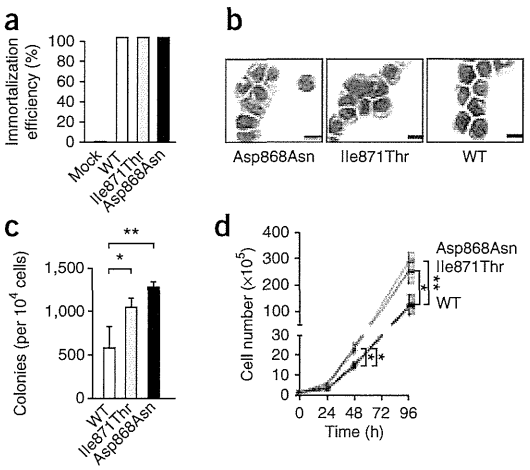

Figure 4 Immortalization of mouse myeloid progenitors by *SETBP1* mutations. (**a**) Efficiencies of empty pMYs retroviral vector (mock) or of pMYs constructs expressing wild-type Setbp1 (WT) or Setbp1 mutants (Asp868Asn and Ile871Thr) in immortalizing mouse myeloid progenitor cells in three independent experiments. (**b**) Wright-Giemsa staining of cells immortalized by transduction with retroviruses expressing the indicated wild-type or mutant Setbp1 proteins. Scale bars, 50 μm. (**c**) Mean and s.d. values for the colony-forming potentials of mouse myeloid progenitors immortalized by the expression of wild-type or mutant Setbp1 on methylcellulose medium in the presence of stem cell factor (SCF) (100 ng/ml) and interleukin (IL)-3 (20 ng/ml). Combined results from three independent myeloid progenitor populations immortalized by each retroviral construct are shown. (**d**) Expansion of myeloid progenitors immortalized by expression of wild-type or mutant Setbp1 in liquid medium with SCF and IL-3 over a 96-h period. Results from three independent populations immortalized by each retroviral construct are presented. Cell numbers were counted by trypan blue staining. Error bars, s.d. $*P < 0.05$, $**P < 0.005$; *t* tests were used for comparisons between strains.

URLs. The February 2009 human reference sequence (GRCh37) produced by the Genome Reference Consortium was used as the reference genome (UCSC Genome Browser; http://genome.ucsc.edu/cgi-bin/hgGateway). Basewise conservation scores were calculated using PhyloP in the UCSC Genome Browser. Expression array and methylation array data were extracted from Oncomine (https://www.oncomine.org/), BioGPS (http://biogps.org/) and The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/) and were analyzed by Matlab software (http://www.mathworks.com/). Somatic mutation data were searched in the Catalogue of Somatic Mutations in Cancer (COSMIC) database on the Welcome Trust Sanger Institute website (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Each potential mutation was compared against databases of known SNPs, including Entrez Gene (http://www.ncbi.nlm.nih.gov/gene) and the Ensembl Genome Browser (http://useast.ensembl.org/index.html). SAMtools (http://samtools.sourceforge.net/) and Integrative Genomics Viewer (http://www.broadinstitute.org/igv/) software were used. The Database of Genomic Variants is a publically available database of copy number variations (http://projects.tcag.ca/variation).

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Whole-exome sequencing results have been deposited in the Sequence Read Archive (SRA; BioProject accession PRJNA203580).

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H. Makishima and K.Y. designed research, performed research, collected data, performed statistical analysis and wrote the manuscript. Y.O., N.N., K.P.N., B.P., K.O.G., B.A.V., A.J., I.G.-S., Y. Shiraishi, Y.N., M.S., M.T., K.C., H.T., H. Muramatsu, H.S., S.M. and L.-Y.S. performed research and analyzed data. K.G. and H. Mori collected data. M.A.S., R.L.P., M.A.M., S.K. and Y. Sauntharajah designed research, analyzed and interpreted data, and wrote the manuscript. Y.D., S.O. and J.P.M. designed research, contributed analytical tools, collected data, analyzed and interpreted data, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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