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REFERENCES

1 Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH *et al.* Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* 2010; **116**: 354–365.

2 Bains A, Lu G, Yao H, Luthra R, Medeiros LJ, Sargent RL. Molecular and clinicopathologic characterization of AML with isolated trisomy 4. *Am J Clin Pathol* 2012; **137**: 387–394.

3 Gupta V, Minden MD, Yi QL, Brandwein J, Chun K. Prognostic significance of trisomy 4 as the sole cytogenetic abnormality in acute myeloid leukemia. *Leuk Res* 2003; **27**: 983–991.

4 Burnett AK, Hills RK, Hunter AE, Milligan D, Kell WJ, Wheatley K *et al.* The addition of gemtuzumab ozogamicin to low-dose Ara-C improves remission rate but does not significantly prolong survival in older patients with acute myeloid leukaemia: results from the LRF AML14 and NCRI AML16 pick-a-winner comparison. *Leukemia* 2013; **27**: 75–81.

5 Burnett AK, Milligan D, Goldstone A, Prentice A, McMullin MF, Dennis M *et al.* The impact of dose escalation and resistance modulation in older patients with acute myeloid leukaemia and high risk myelodysplastic syndrome: the results of the LRF AML14 trial. *Br J Haematol* 2009; **145**: 318–332.

6 Burnett AK, Russell NH, Hills RK, Hunter AE, Kjeldsen L, Yin J *et al.* Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the medical research council AML15 trial. *J Clin Oncol* 2013; **31**: 3360–3368.

7 Gibson BE, Webb DK, Howman AJ, De Graaf SS, Harrison CJ, Wheatley K *et al.* Results of a randomized trial in children with Acute Myeloid Leukaemia: medical research council AML12 trial. *Br J Haematol* 2011; **155**: 366–376.

8 Grimwade D, Walker H, Harrison G, Oliver F, Chatters S, Harrison CJ *et al.* The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood* 2001; **98**: 1312–1320.

9 Hann IM, Stevens RF, Goldstone AH, Rees JK, Wheatley K, Gray RG *et al.* Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council. *Blood* 1997; **89**: 2311–2318.

10 Shaffer LG, McGowan-Jordan J, Schmid M. *An International System for Human Cytogenetic Nomenclature (ISCN)*. S. Karger: Basel, Switzerland, 2013.

11 Chilton L, Hills RK, Harrison CJ, Burnett AK, Grimwade D, Moorman AV. Hyperdiploidy with 49-65 chromosomes represents a heterogeneous cytogenetic subgroup of acute myeloid leukemia with differential outcome. *Leukemia* 2014; **28**: 321–328.

12 Klein K, Kaspers G, Harrison CJ, Beverloo B, Reedijk A, Bongers M *et al.* Clinical impact of additional cytogenetic aberrations, cKIT and RAS mutations and treatment elements in pediatric t(8;21)-AML: Results from an international retrospective study by the International Berlin-Frankfurt-Münster Study Group. *J Cin Oncol* 2015; **33**: 4247–4258.

13 Harrison CJ, Hills RK, Moorman AV, Grimwade DJ, Hann I, Webb DK *et al.* Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. *J ClinI Oncol* 2010; **28**: 2674–2681.

14 von Neuhoff C, Reinhardt D, Sander A, Zimmermann M, Bradtke J, Betts DR *et al.* Prognostic impact of specific chromosomal aberrations in a large group of pediatric patients with acute myeloid leukemia treated uniformly according to trial AML-BFM 98. *J Clin Oncol* 2010; **28**: 2682–2689.

15 Ferrari S, Grande A, Zucchini P, Manfredini R, Tagliafico E, Rossi E *et al.* Overexpression of c-kit in a leukemic cell population carrying a trisomy 4 and its relationship with the proliferative capacity. *Leuk Lymphoma* 1993; **9**: 495–501.

OPEN

Concurrent PI3K and NF-κB activation drives B-cell lymphomagenesis

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Aberrant activation of the PI3K and NF-κB pathways occurs frequently in human B-cell lymphomas.[1,2] Recent studies suggested reciprocal molecular interactions between these two pathways in lymphomagenesis. For example, PI3K inhibition suppresses NF-κB activity in human Burkitt's lymphoma and diffuse large B-cell lymphoma,[3,4] while blockade of NF-κB causes suppression of PI3K activity in primary effusion lymphoma cell lines.[5] Despite frequent alterations and molecular interactions of these two pathways in human lymphomas, genetic activation of anyone of these two pathways was not sufficient to initiate lymphoma development in mice.[6–8]

We recently reported that mutant mice (termed miR-17 ~ 92 TG mice) with B-cell-specific transgenic expression of miR-17 ~ 92, a cluster of six microRNAs (miRNAs) that are frequently upregulated in human cancers,[9–11] spontaneously developed B-cell lymphomas with a high incidence.[12] Subsequent molecular analyses showed that transgenic miR-17 ~ 92 expression led to constitutive activation of the PI3K and canonical NF-κB pathways by suppressing the expression of multiple negative regulators of these pathways.[12] However, it remains unclear whether functional cooperation of these two pathways is sufficient to drive lymphoma development and, thereby, to mediate the lymphomagenic effect of miR-17 ~ 92 overexpression.

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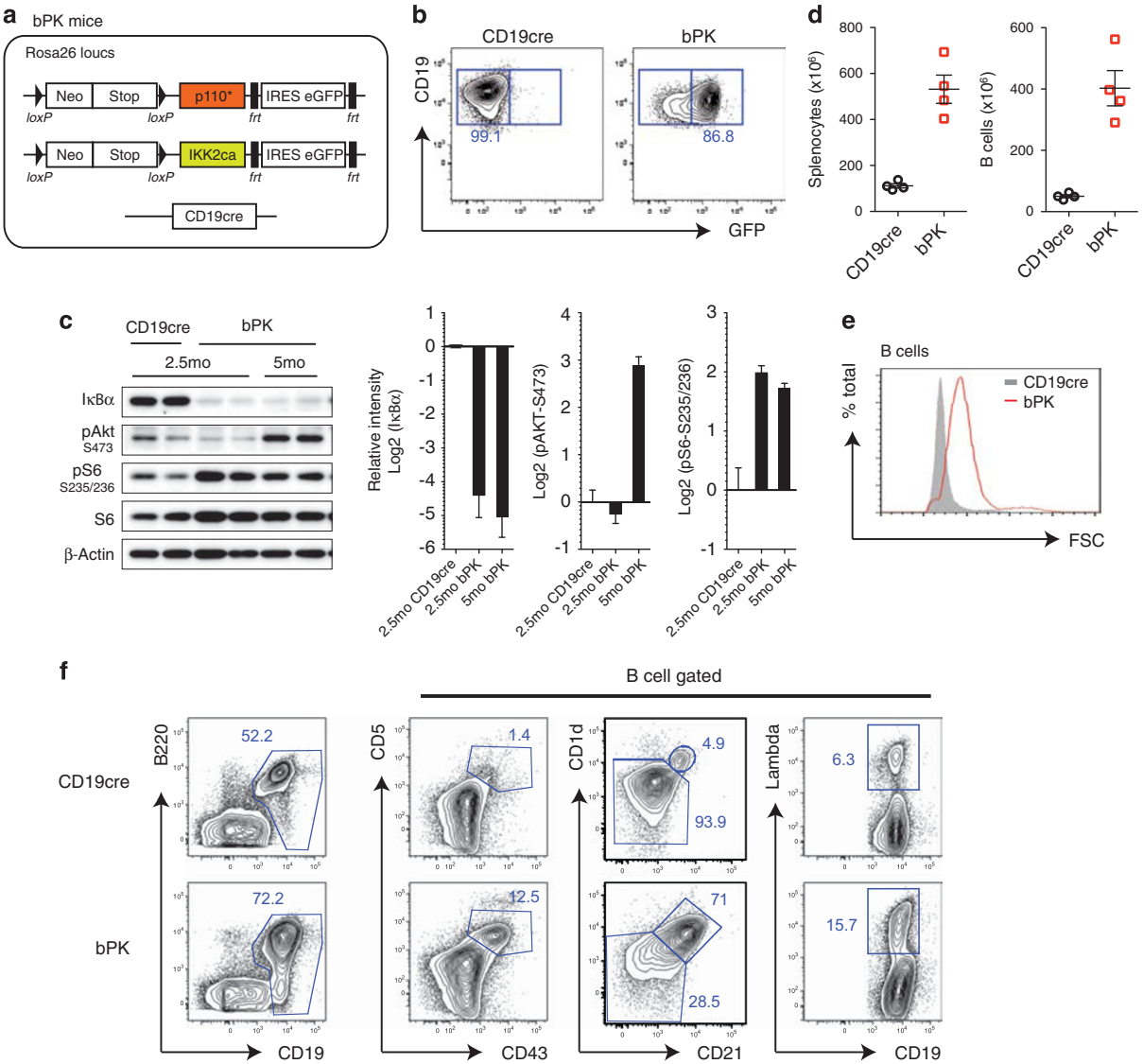

Figure 1. Characterization of transgenic mice with concurrent activation of the PI3K and NF-κB pathways in B cells. (**a**) Scheme of B-cell-specific Rosa26-Stop^{FL}-IKK2ca and Rosa26-Stop^{FL}-p110* double transgenic mice (CD19cre;p110*;IKK2ca, termed bPK mice). The endogenous Rosa26 promoter drives the expression of transgenes upon CD19cre-mediated deletion of the Neo-Stop cassette. (**b**) GFP expression in splenic B cells of bPK mice. (**c**) Immunoblot analysis of steady-state levels of IκBα, phospho-AKT (S473) and phospho-S6 (S235/236) as readouts for NF-κB and PI3K pathway activities, respectively. 2.5mo, 2.5-month old; 5mo, 5-month old. Left, representative immunoblots; right, bar graphs summarizing quantification results. (**d**) Total cell number and B-cell number in the spleen of 8-week-old bPK mice. Centered values and error bars indicate mean and s.e.m., respectively. (**e**) Increased cell size of splenic B cells in bPK mice. (**f**) Expansion of B1 cells, marginal zone B-like cells, and λ^+ B cells in bPK mice.

To directly test this, we generated B-cell-specific double transgenic mice that concurrently activate the PI3K and NF-κB pathways (CD19cre;p110*;IKK2ca mice, termed bPK mice; Figure 1a). In these mice, the PI3K pathway is activated by a p110* transgene, which encodes a constitutively active form of P110α, the catalytic subunit of PI3K,[8] while the NF-κB pathway is activated by a IKK2ca transgene, which harbors two serine-to-glutamate substitution mutations in the activation loop of the kinase domain of IKK2 and constitutively activates the canonical NF-κB pathway.[6] Both p110* and IKK2ca were knocked in at the Rosa26 locus with a loxP-flanked Neo-STOP cassette, which contains the neomycin resistance gene (Neo) and a transcriptional termination signal (STOP), inserted between the Rosa26 promoter and the transgene.[6,8] The CD19cre transgene drives B-cell-specific expression of the Cre recombinase, which deletes the Neo-STOP cassette and turns on the expression of these two transgenes and green fluorescent protein (GFP).

We first confirmed the expression of GFP and the activation of the PI3K and NF-κB pathways in B cells of young bPK mice. As shown in Figure 1b, the vast majority (~85%) of bPK B cells were GFP-positive. Consistently with previous reports,[6,8] both the PI3K and NF-κB pathways were active in these cells as indicated by increased phospho-AKT (S473) and phospho-S6 (S235/236) levels and IκBα degradation, respectively (Figure 1c). Similar to miR-17 ~ 92 TG mice, young bPK mice showed splenomegaly, increased splenic B-cell number and size, expanded B1 cell population ($CD19^+B220^{int}CD43^+CD5^+$), and higher percentage of λ^+ B cells (Figures 1d–f).[12] A majority of splenic B cells in young bPK mice were $CD19^+CD1d^+CD21^+$, a phenotype similar to marginal zone B (MZB) cells in wild-type mice, recapitulating the characteristic feature of B cells in IKK2ca single transgenic mice (Figure 1f).[6]

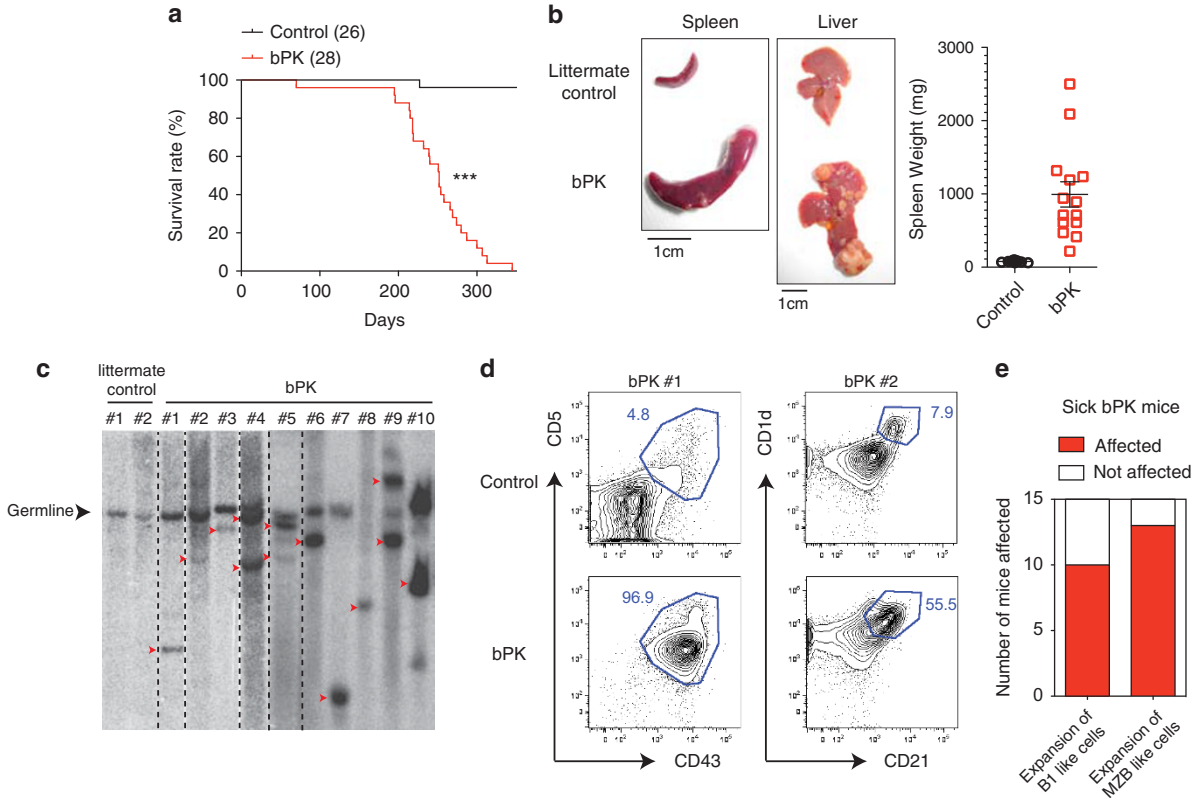

Figure 2. bPK mice developed B-cell lymphomas. (**a**) Kaplan–Meier survival curves of 28 bPK and 26 littermate control mice. The *P*-value ($P < 0.001$) was determined by Mantel–Cox log-rank test. (**b**) Splenomegaly and hepatic granulomas in aged bPK mice. Centered values and error bars indicate mean and s.e.m., respectively. (**c**) Southern blot analysis of B-cell clonality. Genomic DNA of splenic B cells was digested with *Eco*RI and hybridized with a probe corresponding to the J_H4 region of the IgH locus. Red arrowheads indicate clonal bands corresponding to VDJ or DJ rearrangements. Each lane represents one mouse. (**d**) Representative fluorescence-activated cell sorting plots of splenic B cells showing surface phenotypes of B1-like cells ($CD19^+B220^{int}CD43^+CD5^+$) or marginal zone B-like cells ($CD19^+CD1d^+CD21^+$) in different bPK mice. (**e**) The number of sick bPK mice analyzed exhibiting expansion of B1-like cells and marginal zone B (MZB)-like cells.

We next monitored a large cohort of bPK and littermate control (p110*;IKK2ca but Cre-negative) mice for lymphoma development. As shown in Figure 2a, most of the 28 bPK mice died within 1 year (average lifespan: 8 months), whereas only one of the 26 littermate control mice died in the same period. We were able to analyze 15 sick bPK mice before they succumbed to diseases (Supplementary Table 1). These mice exhibited severe splenomegaly and hepatic granulomas (Figure 2b). Southern blot analysis showed that in 77% of these mice (10 out of 13 mice) B cells underwent mono- or oligoclonal expansion, a hallmark of lymphoma (Figure 2c; Supplementary Table 1). Consistently, these lymphoma cells were highly proliferative and were much bigger than B cells in littermate control mice (Supplementary Figure 1). B cells in these sick mice exhibited a surface phenotype of B1, MZB or both (Figures 2d and e). We have previously shown that most of miR-17 ~ 92-driven B-cell lymphomas were able to establish secondary tumors in immunodeficient mice.[12] We transplanted primary B cells from seven sick bPK mice exhibiting clonal B-cell expansion into *Rag1*-/- mice. Among them, primary B cells from four sick bPK mice were able to establish secondary tumors in the spleen or lymph nodes of *Rag1*-/- mice (Supplementary Table 1). Taken together, bPK mice developed lymphomas with a high incidence and the cell surface phenotypes of B cells in sick bPK mice were similar to those of B-cell-specific miR-17 ~ 92 TG mice (Supplementary Table 1).[12]

In summary, we demonstrated that concurrent activation of the PI3K and NF-κB pathways is sufficient to drive lymphoma development in mice. Our previous study has shown that transgenic miR-17 ~ 92 expression activates these two pathways in B cells and leads to a high incidence of lymphomas.[12] Therefore, these results suggest that the PI3K and NF-κB pathways are two major downstream pathways mediating the lymphomagenic effect of miR-17 ~ 92 overexpression. Future investigations are warranted to explore the possibility of concurrently targeting these two pathways for the treatment of lymphomas driven by elevated miR-17 ~ 92 expression.[13]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HYJ, ML and CX designed the study. HYJ and ML performed cellular and molecular analysis of mice. JS assisted the experiments. HYJ and CX wrote the manuscript.

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REFERENCES

1 Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 2009; **8**: 627–644.
2 Staudt LM. Oncogenic activation of NF-kappaB. *Cold Spring Harb Perspect Biol* 2010; **2**: a000109.
3 Kloo B, Nagel D, Pfeifer M, Grau M, Duwel M, Vincendeau M *et al.* Critical role of PI3K signaling for NF-kappaB-dependent survival in a subset of activated B-cell-like diffuse large B-cell lymphoma cells. *Proc Natl Acad Sci USA* 2011; **108**: 272–277.
4 Qiao Q, Jiang Y, Li G. Inhibition of the PI3K/AKT-NF-kappaB pathway with curcumin enhanced radiation-induced apoptosis in human Burkitt's lymphoma. *J Pharmacol Sci* 2013; **121**: 247–256.
5 Hussain AR, Ahmed SO, Ahmed M, Khan OS, Al Abdulmohsen S, Platanias LC *et al.* Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. *PLoS One* 2012; **7**: e39945.
6 Sasaki Y, Derudder E, Hobeika E, Pelanda R, Reth M, Rajewsky K *et al.* Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity* 2006; **24**: 729–739.
7 Suzuki A, Kaisho T, Ohishi M, Tsukio-Yamaguchi M, Tsubata T, Koni PA *et al.* Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J Exp Med* 2003; **197**: 657–667.
8 Srinivasan L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA *et al.* PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 2009; **139**: 573–586.
9 Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S *et al.* Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 2004; **64**: 3087–3095.
10 He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S *et al.* A microRNA polycistron as a potential human oncogene. *Nature* 2005; **435**: 828–833.
11 Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S *et al.* A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* 2005; **65**: 9628–9632.
12 Jin HY, Oda H, Lai M, Skalsky RL, Bethel K, Shepherd J *et al.* MicroRNA-17 ~ 92 plays a causative role in lymphomagenesis by coordinating multiple oncogenic pathways. *EMBO J* 2013; **32**: 2377–2391.
13 Jin HY, Lai MY, Xiao CC. microRNA-17 ~ 92 is a powerful cancer driver and a therapeutic target. *Cell Cycle* 2014; **13**: 495–496.

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Somatic *PHF6* mutations in 1760 cases with various myeloid neoplasms

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Next-generation sequencing has enabled us to detect driver mutations in a sensitive manner. By whole-exome sequencing, we previously identified a somatic mutation of the plant homeodomain finger 6 (*PHF6*) gene (p.G291X) in 1 out of 29 cases with myelodysplastic syndromes (MDS).[1] Initially, germline mutations of *PHF6*, located at Xq26.2, are reported to cause congenital Börjeson–Forssman–Lehmann syndrome (BFLS) with X-linked recessive inheritance.[2] BFLS is characterized by mental deficiency, epilepsy, hypogonadism, obesity and dysmorphic features.[3] Recently, it was found that germline *PHF6* mutations are also responsible for the female cases with a congenital disorder similar to Coffin–Siris syndrome.[4] Moreover, somatic *PHF6* mutations were reported in hematological neoplasms, including T-acute lymphocytic leukemia (38% of cases were positive for mutations)[5] and acute myeloid leukemia (AML) (3%).[6] According to recent studies, somatic *PHF6* mutations were identified in 3% of cases with *de novo* AML[7] and in ~3% of those with MDS.[8,9] Nevertheless, pathophysiology due to *PHF6* defects in myeloid neoplasms remains to be fully elucidated.

In this study, we clarified the implications of somatic *PHF6* mutations in the cases with various myeloid neoplasms ($N = 1760$), including the cohort of MDS and AML that we previously reported.[8,10,11] To identify somatic mutations, we applied whole-exome sequencing to 49 cases. Subsequently, targeted sequencing (SureSelect, Agilent, Santa Clara, CA, USA) and PCR-based pool sequencing were performed in 1428 and 356 cases, respectively, 73 of which were subjected to both methods (Supplementary Table 1). Detailed methods of the sequencing were previously reported.[1,8] Written consent forms were obtained from all the patients. Genetic analysis was approved by the ethical review board in each institution. Somatic mutations were confirmed by paired DNA from tumor and germline samples (buccal smear or CD3-positive cells). In case of non-paired DNA, the nonsense and frameshift mutations were classified to be somatic, and the missense mutations were classified as somatic if they were already reported as somatic in the Catalogue of Somatic Mutations in Cancer database (http://www.sanger.ac.uk/genetics/CGP/cosmic/).

In total, we identified 62 somatic mutations of *PHF6* in 54 cases (Table 1). By copy number analysis,[1,8] deletions affecting the *PHF6* locus were identified in five cases, while no focal amplifications of *PHF6* locus were identified. Among somatic mutations, 17 were missense, 16 frameshift, 23 nonsense and 6 affecting splice sites. Therefore, mutations leading to truncated transcripts were dominant (63%, 39/62). While *PHF6* mutations were distributed to the whole coding region, 14 out of 17 (82%) missense mutations were located at the PHD2 domain and 8 (47%) were recurrent (p.R274Q) (Figure 1a). The PHD2 domain of PHF6 is rich in positively charged amino acids including arginine and lysine, which were confirmed to be essential for the DNA-binding capacity of PHF6 as recently reported.[12] Consequently, missense mutations affecting these amino acids in the PHD2 domain (p.R274Q and p.K235E) (Figure 1a) might result in loss of PHF6 function. Together with highly frequent truncating mutations and dominant deletions, most of the *PHF6* mutations (87%; 53/61) might be pathogenic in myeloid neoplasms due to loss of function.

Clinically, *PHF6* mutations were detected in the cases with AML with myelodysplasia-related changes (AML/MRC) (4/26, 15.4%), *de novo* AML (11/340, 3.2%), chronic myelomonocytic leukemia (CMML) (4/86, 4.7%), MDS (34/1139, 3.0%) and chronic

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