Autonomous feedback loop of RUNX1-p53-CBFB in acute myeloid leukemia cells

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Although runt-related transcription factor 1 (RUNX1) and its associating core binding factor-β (CBFB) play pivotal roles in leukemogenesis, and inhibition of RUNX1 has now been widely recognized as a novel strategy for anti-leukemic therapies, it has been elusive how leukemic cells could acquire the serious resistance against RUNX1-inhibition therapies and also whether CBFB could participate in this process. Here, we show evidence that p53 (TP53) and CBFB are sequentially up-regulated in response to RUNX1 depletion, and their mutual interaction causes the physiological resistance against chemotherapy for acute myeloid leukemia (AML) cells. Mechanistically, p53 induced by RUNX1 gene silencing directly binds to CBFB promoter and stimulates its transcription as well as its translation, which in turn acts as a platform for the stabilization of RUNX1, thereby creating a compensative RUNX1-p53-CBFB feedback loop. Indeed, AML cells derived from relapsed cases exhibited higher CBFB expression levels compared to those from primary AML cells at diagnosis, and these CBFB expressions were positively correlated to those of p53. Our present results underscore the importance of RUNX1-p53-CBFB regulatory loop in the development and/or maintenance of AML cells, which could be targeted at any sides of this triangle in strategizing anti-leukemia therapies.

CBFB is the beta subunit of heterodimeric core-binding transcription factor which master-regulates vital subsets of genes implicated in hematopoiesis and osteogenesis. This beta subunit which lacks DNA-binding capability, facilitates the association of DNA-binding runt domain in alpha subunit with its target DNA sequences (5′-TGTGGT-3′ and much rarely 5′-TGCGGT-3′) in various gene promoters as well as enhancers. The alpha subunit is constituted of three representative members: RUNX1, RUNX2 and RUNX3. Although each of RUNX family members plays distinct physiological roles in vivo, their functions are consistently redundant in malignant cells and thus could be targeted simultaneously for anti-tumor strategies. We have recently reported that anti-tumor potential of RUNX inhibition is primarily mediated by pro-apoptotic p53-dependent cell death pathway. Based on our results, RUNX inhibition led to the transcriptional down-regulation of genes involved in the prohibition of p53 such as BCL11A and TRIM24, resulting in up-regulation of p53-mediated pro-apoptotic signaling in tumor cells. In addition, we have also demonstrated that the intracellular amount of CBFB which is equivalent to that of total RUNX (RUNX1 + RUNX2 + RUNX3), is consistently higher in malignant tissues relative to their corresponding normal ones, suggesting that CBFB is one of the ideal targets for anti-cancer therapies.

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Tumor suppressor p53, a distant relative of RUNX family, is widely known as a nuclear transcription factor that regulates the expression of stress response genes and mediates a variety of anti-proliferative processes through transactivating its downstream target genes implicated in cell cycle checkpoints, DNA damage/repair and apoptosis. The p53-responsive element has been extensively studied and the most reliable sequences are currently characterized as 10-nucleotide half-sites of RRRCWGWYYY-N-RRCWGWYYY (R = purine, Y = pyrimidine, W = A/T and N = 0 to 13-nucleotide spacer). Of note, the emerging evidence indicates that the potential p53-target sites are not always restricted to the above-mentioned original decamer half-sites, and DNA sequences with several nucleotide mismatches seems acceptable and functional, implying that number of p53-target genes might be much greater than that previously estimated. Consistent with its extreme importance as an onc suppressor, somatic mutations of p53 gene have been considered to be one of the most frequent alterations in human cancers, and most mutations are single-base substitutions found within the genomic region encoding its sequence-specific DNA-binding domain. In a sharp contrast to wild-type p53 with the extremely short half-life, mutated p53 acquires oncogenic gain-of-function properties with the extended half-life and acts as a dominant-negative inhibitor against wild-type p53. Since p53 mutations are detectable primarily within its central DNA-binding domain, it is highly likely that mutant p53 lacks sequence-specific transactivation ability or acquires a capability to induce certain set of its target genes distinct from that of wild-type p53.

In contrast to the majority of tumors, it has been described that p53 is infrequently mutated in overall de novo AML cases (less than 10%). It is worth noting, however, that its mutation rate elevates strikingly in complex karyotype de novo AML cases or therapy-related AML cases and they display a poor prognosis. Wong TN et al. have recently described that p53 mutations arise during the quite early phase of the disease progression prior to any chemotherapeutic treatments, indicating the importance of its mutations in the initiation and propagation of AML. Additionally, it has been shown that p53 mutations are strongly associated with transformation of AML in patients into myeloproliferative neoplasms, suggesting their vital involvement during the leukemic transformations.

In spite of these findings, neither the precise molecular mechanisms behind the transcriptional regulation of CBFB nor the functional/physical association between CBFB and p53 has so far remained entirely elusive. Furthermore, the actual molecular basis of how AML cells could adapt to RUNX1-attenuated environment has been largely unknown. Here, we have sought to clarify the transcriptional regulatory mechanisms of CBFB and also examined the presence of the cell-autonomous compensation mechanisms after RUNX1-inhibition therapy in AML cells.

Results
p53 transcriptionally regulates CBFB expression. To investigate RUNX1 depletion-mediated cellular responses, we have constructed tetracycline-inducible shRNAs targeting RUNX1 (sh_RUNX1 #1 and #2) and lentivirally-transduced them into AML-derived MV4-11 cells. As shown in Fig. 1a, RUNX1 gene silencing significantly induced wild-type p53 expression in MV4-11 cells as described previously. We have also found that, like p53, CBFB expression is increased upon RUNX1-knockdown. The total amount of RUNX family members (RUNX1 + RUNX2 + RUNX3), which was estimated by RT-qPCR with primer set specifically amplifying the common coding sequence of all RUNX family members, was decreased in these RUNX1-knockdown AML cells (Supplementary Fig. S1a). In addition, the simultaneous knockdown of RUNX1 plus RUNX2 and/or RUNX3 further stimulated CBFB expression as compared to that in the absence of RUNX1 alone. We also found that these CBFB up-regulations are proportional to the extent of p53 induction in these cells (Supplementary Fig. S1b).

Previously, Berardi MI, Warren AJ and Yan J et al. reported that RUNX family members and CBFB form a heterodimeric complex and this complex formation stabilizes RUNX1/CBFB complex on its target DNA, raising a possibility that depletion of RUNX1 might disrupt the stable RUNX1/CBFB complex and then destabilize CBFB. Contrary to this hypothesis, our present results clearly showed that the amount of CBFB is remarkably increased upon RUNX knockdown under our experimental conditions, indicating that the above-mentioned previous hypothesis is not the case. Considering that the anti-tumor potency of RUNX gene silencing is highly dependent on functional p53 with the sequence-specific transactivation capability, we have sought to examine a possible involvement of p53 in the transcriptional regulation of CBFB. Close inspection of the gene expression profiles in de novo AML patients from two independent studies revealed the presence of the positive correlation between the expression levels of p53 and CBFB (Fig. 1b). In a good agreement with these observations, knockdown of p53 in MV4-11 cells caused an obvious reduction in CBFB as well as RUNX1 relative to non-silencing control cells both at the mRNA and protein levels (Fig. 1c, Supplementary Fig. S2a). When MV4-11 cells were exposed to p53 inducer Nutlin-3, CBFB level was up-regulated in a dose-dependent manner (Fig. 1d). In addition, the expression level of RUNX1 was reduced in CBFB-knocked down MV4-11 cells, whereas p53 was induced in response to CBFB depletion (Fig. 1e). To further confirm these results, CBFB was overexpressed in MV4-11 cells. As expected, forced expression of CBFB resulted in a significant increase and decrease in RUNX1 and p53, respectively (Fig. 1f). These observations raise a possibility that AML cells lacking RUNX1 might survive due to the enhanced expression of CBFB. Given the positive correlation between the expression levels of p53 and CBFB in a variety of AML cases as shown in Fig. 1b, it is likely that RUNX1 depletion-mediated up-regulation of CBFB is under the control of the accumulated p53, and thus creating an autonomous RUNX1-p53-CBFB feedback loop regulatory system for AML cell proliferation (Fig. 1g).

Intriguingly, we have found out multiple p53-responsive element-like sequences within the putative CBFB promoter region (at positions from −2000 to +200 relative to the transcription start site (+1)) (Fig. 2a and b). Kenzelmann BD and Li M et al. have previously demonstrated that p53 bound to CBFB promoter region as examined by chromatin immunoprecipitation (ChIP) with anti-p53 antibody followed by DNA sequencing. In accordance with these results, the indicated CBFB genomic fragments (P1, P2, P3, P4 and P5) containing the possible p53-responsive element-like DNA sequences were detectable in anti-p53 immunoprecipitates under
our experimental conditions (Fig. 2c). Additionally, Nutlin-3 treatment markedly stimulated CBFβ transcription (Fig. 2d). In luciferase reporter assay with CBFβ promoter region (−1884 bp to +150 bp of transcriptional start site (TSS)), Nutlin-3 treatment indeed up-regulated the CBFβ promoter activity (Supplementary Fig. S3a). Together, these data strongly suggest the vital role of p53 in the transcriptional regulation of CBFβ.

**Unidirectional compensatory regulatory loop of RUNX1-p53-CBFβ.** To further confirm the existence of RUNX1-p53-CBFβ regulatory loop, we have conducted a series of gene knockdown and restore experiments in AML cells. As shown in Fig. 3a, the additional knockdown of p53 in RUNX1-depleted MV4-11 cells
reduced the expression level of CBFB to the baseline level. Together with the results shown in Fig. 1a, these data imply that p53 induced by RUNX1 gene silencing subsequently promotes the expression of CBFB in AML cells. While, knockdown of p53 led to a significant suppression of CBFB as well as RUNX1, and the decreased expression level of RUNX1 was restored by overexpression of CBFB (Fig. 3b). These observations are suggestive that CBFB induced by p53 subsequently stabilizes RUNX1 in AML cells. Finally, we have examined the expression levels of RUNX1 and p53 in CBFB-knocked down MV4-11 cells. As seen in Fig. 3c, CBFB gene silencing decreased and increased the expression levels of RUNX1 and p53, respectively. As expected, forced expression of RUNX1 in CBFB-depleted MV4-11 cells caused a reduction in p53 to the control level. These results indicate that silencing of CBFB destabilizes RUNX1, which in turn induces p53 in AML cells. Consistent with these findings, the down-regulation of RUNX1 was detectable in MV4-11 cells at 6 hours after the treatment with Ro5-3335, which has been shown to facilitate the specific dissociation of RUNX1 from CBFB27 (Fig. 3d). Nine hours after Ro5-3335 treatment, the amounts of p53 and CBFB were increased in a time-dependent manner. Collectively, these results strongly suggest the presence of unidirectional compensatory circuit of RUNX1-p53-CBFB in AML cells.

**RUNX1-p53-CBFB axis confers resistance to anti-leukemia therapy.** Although the majority of primary de novo AML cases harbor wild-type p53, a small number of AML patients do carry p53 mutations. Since over 90% of p53 mutations occur within its sequence-specific DNA-binding domain, it is indicative that mutated p53 has an ability to recognize and bind to DNA sequences distinct from those of wild-type p53. On the other hand, it has also been described in several reports that p53-target sequences remain unchanged regardless of p53 status8,9,11,13. According to our RUNX1-p53-CBFB loop working model, the stabilized mutant p53 possibly augments this feedback regulatory loop through the direct transactivation of CBFB and potentially contributes to the acquired resistance to RUNX1-inhibition therapy (Fig. 4a). We thus examined whether p53 mutants could up-regulate CBFB expression. To this end, we have generated three expression plasmids of representative
p53 mutants (R175H, R248W and R273C) and introduced them into HEK293T cells. As shown in Fig. 4b and c, like wild-type p53, these mutants significantly induced CBFB. In luciferase reporter assays using CBFB promoter (Supplementary Fig. 4a), these mutants retained ability enough to transactivate CBFB expressions. As we have expected, ChIP assay with anti-p53 antibody in p53-mutated AML cell line MV4-11NR (R248W) revealed the binding of mutant p53 to the CBFB promoter region. In addition, wild-type p53 inducer CP-3139828 and RUNX1-CBFB inhibitor Ro5-3335 have synergistically worked (combination index (CI) < 1 at fraction of affected (Fa) = 0.5) in MV4-11NR cells bearing p53 mutation, which were originally resistant to Ro5-3335 treatment (Fig. 4d). Consistent with these findings, knockdown of mutant p53 in MV4-11NR cells significantly suppressed CBFB expression (Supplementary Fig. 4c). MDM2 inhibition with Nutlin-3 didn’t induce enough p53 elevation in MV4-11NR cells, probably due to the saturated expression of stablized mutant p53 in these cells (Supplementary Fig. 4d). These findings collectively indicated that even a mutated p53 can transactivate CBFB expressions.

We have previously reported a potent RUNX inhibitor Chb-M’ and its efficacy in AML cells. Based on our previous results, Chb-M’ impaired the interaction of RUNX family members with their consensus binding sequences on the genome DNA, and then exerted its anti-leukemic effect (Fig. 4e). Consistent with our current findings, MV4-11 cells expressing an exogenous CBFB acquired the resistance to Chb-M’ (Fig. 4f). Therefore, these results indicate that p53 mutations possibly confer resistance to RUNX1-inhibition therapy through the induction of CBFB.
To further verify the relationship between p53 expression/mutation and resistance to RUNX1 inhibition, we have employed the unbiased methods. Firstly, we have cultured Chb-M’-naïve MV4-11 cells in the medium containing a gradually increasing amounts of Chb-M’, and finally established the Chb-M’-resistant MV4-11 clones (MV4-11M’R) through the continuous selection of the resistant cells in vitro for up to 4 months (Fig. 5a and b).

Intriguingly, the expression analyses demonstrated that MV4-11M’R cells expressed a larger amount of p53 and CBFB than the parental MV4-11 cells (Fig. 5c), and their expression levels have increased in response to Chb-M’ in a dose-dependent manner (Fig. 5d). We then comprehensively examined the mutation frequency of p53 in MV4-11M’R cells by using the next generation sequencing (NGS) and demonstrated that the mutation frequency of p53 at codon R248W is around 45% (Fig. 5e), implying that one of 6 hot-spot mutations of p53 (R248W) occurs in most of the Chb-M’-resistant MV4-11M’R cells. This mutated p53 (R248W) has indeed an ability to up-regulate CBFB expression as shown in Fig. 4b and c. We have also carried out the in vivo selection for Chb-M’-resistant clones (Fig. 5f) and found a marked induction of p53 and CBFB in Chb-M’-resistant AML cells (Fig. 5g). These observations strongly suggest that the RUNX inhibition-mediated treatment permits the selective proliferation of p53-mutated AML cells and these cells acquire the enhanced tumorigenicity through the potentiated p53-CBFB-RUNX feedback loop.

We have also observed that Ara-C-resistant MV4-11 cells (MV4-11AR) express a significant larger amount of CBFB than Ara-C-naïve parental MV4-11 cells (Fig. 6b and c). In addition, Ara-C exposure further augmented CBFB expression in MV4-11AR cells (Fig. 6d). In a good agreement with these results, a larger amount of CBFB...
was detectable in patients' AML samples obtained at their relapse phase than those obtained at the primary phase (Fig. 6e). Extensive analysis of the clinical datasets revealed that the overexpression of CBFB confers an accelerated disease progression and shortened overall survival periods not only to AML patients but also to various types of cancers (Fig. 6f and Supplementary Fig. S6). Taken together, our present results strongly suggest that the p53-CBFB-RUNX feedback loop is tightly engaged in the tumorigenesis as well as the acquisition of the serious resistance to chemotherapy including the RUNX-specific inhibition therapy.

Discussion
The context-dependent oncogenic and oncosuppressive functions of RUNX family members (RUNX1, RUNX2 and RUNX3) have been well-described in multiple studies. A growing body of recent evidence supports the notion that RUNX family is tightly linked to the development and maintenance of AML as well as various types of cancers (Fig. 6f and Supplementary Fig. S6). Taken together, our present results strongly suggest that the p53-CBFB-RUNX feedback loop is tightly engaged in the tumorigenesis as well as the acquisition of the serious resistance to chemotherapy including the RUNX-specific inhibition therapy.
depletion-mediated induction of CBFB. Moreover, the additional knockdown of RUNX family genes augmented RUNX1 silencing-induced expression of CBFB and p53, which prompted us to investigate the possible interactions of CBFB with p53. Previously, it has been shown that CBFB stabilizes the RUNX transcription complex and enhances its DNA-binding capability. Since the results obtained from our series of gene knockdown and overexpression experiments did not support the above-mentioned hypothesis, it is likely that there exists an alternative molecular mechanism behind the regulation of CBFB expression in AML cells. Although we have found for the first time that CBFB transcription is modulated by RUNX family members, we have failed to find any consensus RUNX-binding sequences (5′-TGTGGT-3′ or 5′-TGCGGT-3′) within the proximal promoter region of CBFB. Besides, previously-reported ChIP-sequencing (ChIP-seq) assays with anti-RUNX1 antibody showed no significant peaks in the regulatory region of CBFB. Therefore, it is unlikely that CBFB is directly transactivated by RUNX family members. On the other hand, ChIP-seq assays with anti-p53 antibody repeatedly proved p53 bindings to CBFB regulatory region, which results are consistent with our findings as we have mentioned in the Results section. Together with the present results showing that p53 directly transactivates CBFB promoter through its p53-responsive element-like sequences, it is conceivable that RUNX1 regulates the expression of CBFB not directly but indirectly via p53.

In addition to the transcriptional regulatory mechanism of CBFB, we have also demonstrated that RUNX1-p53-CBFB regulatory circuit contributes to the acquisition of treatment resistance of AML cells. Collectively, our present study identified the novel molecular mechanism behind CBFB regulation and its indispensable roles in tumorigenesis, which provides an insight into understanding how AML cells could become resistant to RUNX1-inhibition therapy (Fig. 7).

In conclusion, our present study strongly suggests that an autonomous RUNX1-p53-CBFB regulatory triangle plays a vital role in the maintenance and the acquisition of chemo-resistance of AML cells, and potentially provides novel therapeutic targets for anti-leukemia strategy.
Materials and Methods

Cell lines. AML-derived MOLM13 and MV4-11 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany and American Type Culture Collection (ATCC), USA, respectively. AML-derived MV4-11NR cells harboring TP53 R248W mutation were kind gift from Dr. T. Ikezoe (Department of Hematology and Respiratory Medicine, Kochi University, Kochi, Japan). Embryonic kidney HEK293T cells were provided from Japanese Collection of Research Bioresources (JCRB), Japan. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS) in incubators with humidified atmospheres of 5% CO2 and 95% air at 37 °C. The other cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 1% PS under 5% CO2 and 95% air at 37 °C.

IC50 evaluation. For cell survival assay, cells were seeded at a density of $1 \times 10^5$ cells/mL. The indicated concentrations of PI polyamides or drugs were added to the culture medium and cells were incubated for 48 hours. Cell viability was then assessed by WST assay using Cell Count Reagent SF (nacalai tesque, Inc.) and Infinite® 200 PRO multimode reader (TECAN) according to the manufacturer’s instructions. Percent inhibition curves were drawn and IC50 of the indicated compounds was calculated based on the median-effect method.

Real-time quantitative PCR (qRT-PCR). Total RNA was isolated by using RNeasy mini kit (Qiagen) and reverse-transcribed with Reverse script kit (TOYOBO) to generate cDNA. Real-time quantitative polymerase chain reaction (PCR) was carried out with 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer’s recommendations. The results were normalized to GAPDH levels. Relative expression levels were calculated using the 2-ΔΔCt method. Primers used for qRT-PCR were listed in Supplementary Table S1.

ChIP-qPCR. Chromatin immunoprecipitation assay (ChIP) was performed using SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, USA) according to the manufacturer’s protocols. In brief, cells were cross-linked in 1% formaldehyde in PBS for 10 min at room temperature. After glycine quenching, cell pellets were collected, lysed and then subjected to sonication with Q55 sonicator system (QSONICA, USA). The supernatant was diluted with the same sonication buffer and processed for immunoprecipitation with the following antibodies at 4 °C overnight. Anti-p53 antibody (1C12, #2524, Cell Signaling Technology). The agarose beads were washed, chromatin DNA was reverse cross-linked and purified by ethanol precipitation.
Following ChIP, precipitated DNA was quantified by qPCR using the standard procedures for 7500 Real-Time PCR System (Applied Biosystems). Primers used for ChIP-qPCR were listed in Supplementary Table S2.

**siRNA interference.** Specific shRNAs targeting human RUNX1, RUNX2, RUNX3, CBFβ and TP53 were designed and sub-cloned into pENTR4-H1tetOx1, CS-RfA-ETBsd, CS-RfA-ETV and CS-RfA-ETR vectors (RIKEN BRC). Non-targeting control shRNA was designed against luciferase (sh_Luc). The target sequences were provided in Supplementary Table S3.

**Expression plasmids.** Human RUNX1, CBFβ and p53 cDNAs were amplified by PCR and then inserted into CSII-EF-MCS-ires2-Venus, CSII-EF-MCS-ires2-hKO1 and CSIV-TRE-Ubc-KT expression vectors. Series of p53 point mutations (R248W, R175H and R273C) were created by KOD -Plus- Mutagenesis Kit (TOYOBO Co, Ltd.). All of the PCR products were verified by DNA sequencing.

**Production and transduction of lentivirus.** For the production of lentivirus, HEK293T cells were transiently co-transfected with lentivirus vectors such as psPAX2 and pMD2.G by polyethyleneimine (PEI, Sigma-Aldrich). Forty-eight hours after transfection, viral supernatants were collected and immediately used for infection, and then successfully transduced cells were sorted by flow cytometer Aria III (BD Biosciences) based on the immunofluorescence (Kusabira-Orange or Venus).

**Immunoblotting.** Immunoblotting was conducted as described previously35. Membranes were probed with the following primary antibodies: anti-RUNX1 (A-2, Santa Cruz Biotechnology, Inc.), anti-GAPDH (FL-335, Santa Cruz Biotechnology, Inc.), anti-RUNX2 (D1L7F, Cell Signaling Technology), anti-RUNX3 (D6E2, Cell Signaling Technology), anti-CBFB (FL-182, Santa Cruz Biotechnology, Inc.) and anti-p53 (1C12, Cell Signaling Technology) antibodies. HRP-conjugated anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology) were used as the secondary antibodies. Blots were visualized using Chemi-Lumi One Super (nacalai tesque, Inc.) and ChemiDoc™ XR+ Imager (Bio-Rad Laboratories, Inc.) according to the manufacturers’ recommendations. Protein levels were quantified with Image Lab Software (Bio-Rad Laboratories, Inc.).

**Next generation sequencing.** Deep sequencing of target exons of TP53 was performed as described previously with slight modifications. Briefly, tumor DNA samples prepared from Chb-M’-naïve and -resistant MV4-11 cells were analyzed for possible mutations in TP53. After the extraction of genomic DNA using DNeasy Blood & Tissue Kit (QIAGEN), its concentration was measured using the PicoGreen® reagents (Thermo Fisher Scientific) according to the manufacturer’s instructions. Next, the entire coding sequences of TP53 gene were amplified by independent genomic PCR with a NotI linker attached primer (See Supplementary Table S4 for specific PCR primers). After checking the successful amplification by agarose gel electrophoresis, the PCR products from each sample were combined together, followed by purification of DNA using FastGene Gel/PCR Extraction Kit (Nippon Genetics) and digestion with NotI. The digested DNA was purified again and an aliquot of 2.5 μg of purified DNA was ligated with T4 DNA ligase for 5 hours, sonicated into ~200 bp in length on average using Covaris®, and used for generation of sequencing libraries using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs). The libraries were then subjected to deep sequencing on Illumina Miseq® following the standard protocol.

**Luciferase reporter assay.** Putative promoter region of CBFβ (∼1884 bp to +150 bp of TSS) was cloned from the genomic DNA of MV4-11 cells using the following primers: F 5′-CCTTGAGGCTGACAATGAGAG -3′ and R 5′-CGGTTTCTCCCTTGTTCAG -3′, and then subcloned into pGL4.20 [luc2/Puro] vector (Promega). Both pGL4.20 CBFβ promoter vector and pRL-CMV control vector (TOYO B-Net Co., LTD.) were co-transfected into HEK293T cells. Promoter activities were measured using PicaGene Dual Sea Pansy Luminescence Kit (TOYO B-Net Co., LTD.) and detected by ARVO ×5 (Perkin Elmer) according to the manufacturer’s instructions.

**Statistics.** Statistical significance of differences between control and experimental groups was assessed by a 2-tailed unpaired Student’s t test, and declared if the p value was less than 0.05. Equality of variances in two populations was calculated with F-test. The results were represented as the average ± SEM values obtained from three independent experiments. In transplantation experiments, animals were randomly allocated to each experimental group and the treatments were given with blinding. To examine the overall survival of cancer patients, PrognoScan software was utilized for data extraction and calculation of minimal p value. Survival between the indicated groups was compared using the log-rank test. For the measurement of correlation between mRNA or protein expressions, Spearman’s rank correlation coefficient was used.

**Mice.** NOD/Shi-scid, IL-2RγKO (NOG) mice were purchased from Central Institute for Experimental Animals, Japan. Littermates were used as controls in all experiments.

**Xenograft mouse model.** Xenograft mouse models of human cancer cell lines were developed using NOG mice. For leukemia mice models, 2.5 × 10⁶ cells/body of MV4-11 cells were intravenously injected. Peripheral blood (PB) was then collected every week and chimerism was checked by flow cytometer using anti-human CD45 antibody (BD Biosciences). One week after injection, mice were treated with PI polyamides (320 μg/kg body weight, twice a week IV injections) or with the equivalent amount of dimethyl sulfoxide (DMSO).

**Synthesis of PI polyamides.** Synthesis of Chb-M’ was conducted as previously reported. Briefly, Py-Im polyamide supported by oxime resin was prepared in a stepwise reaction by Fmoc solid-phase protocol. The product with oxime resin was cleaved with N,N-dimethyl-1,3-propane diamine (1.0 mL) at 45 °C for 3 h. The residue was dissolved in the minimum amount of dichloromethane and washed with diethyl ether to
yield a 59.6 mg. To the crude compound (59.6 mg, 48.1 μmol), a solution of chlorambucil (32.6 mg, 107 μmol), PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (101 mg, 195 μmol), and N,N-diisopropylethylamine (100 μL, 581 μmol) in N,N-dimethylformamide (DMF) (300 μL) was added. The reaction mixture was incubated for 1.5 h at room temperature, mixed with diethyl ether and DMF for three times, and dried in vacuo. The crude product was purified by reversed-phase flash column chromatography (water with 0.1% trifluoroacetic acid/MeCN). After lyophilization, product was obtained (30.2 mg, 19.8 μmol).

Study approval. All animal studies were properly conducted in accordance with the Regulation on Animal Experimentation at Kyoto University based on International Guiding Principles for Biomedical Research Involving Animals. All procedures employed in this study were approved by Kyoto University Animal Experimentation Committee (Permit Number: Med Kyo 14332).

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**Acknowledgements**

This work was supported by Grant-in-Aid for Scientific Research (KAKENHI), We thank Dr. H. Miyoshi (RIKEN, BRC) for kindly providing lentivirus vectors encoding CSII-EF-MCS-IRES2-Venus, CSII-EF-MCS-IRES2-hKO1 and CSIV-TRE-Ubc-KT.

**Author Contributions**

K.M. designed research, performed experiments, analyzed data and wrote the manuscript. M.N. performed experiments and analyzed data. C.T., S.M., H.K., K.Y. and H.M. helped to collect data. G.K., J.T, T.B., T.O., S.O., P.L., T.N. and S.A. participated in discussions and interpretation of the data and results and commented on research direction. H.S. designed and synthesized polyamides. Y.K. initiated the study, designed, supervised research and gave the final approval for submission.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-16799-z.

**Competing Interests:** The authors declare that they have no competing interests.

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