Dissecting the role of aberrant DNA methylation in human leukaemia

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Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized by the genetic translocation t(9;22)(q34;q11.2) encoding for the BCR-ABL fusion oncogene. However, many molecular mechanisms of the disease progression still remain poorly understood. A growing body of evidence suggests that the epigenetic abnormalities are involved in tyrosine kinase resistance in CML, leading to leukaemic clone escape and disease propagation. Here we show that, by applying cellular reprogramming to primary CML cells, aberrant DNA methylation contributes to the disease evolution. Importantly, using a BCR-ABL inducible murine model, we demonstrate that a single oncogenic lesion triggers DNA methylation changes, which in turn act as a precipitating event in leukaemia progression.

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Genetic alterations impairing cellular differentiation and growth control are widely regarded as the molecular causes of chronic myeloid leukaemia (CML) and acute myeloid leukaemia\(^1\)-\(^3\). Recent evidences suggest that epigenetic alterations such as DNA methylation can contribute to leukaemia pathogenesis\(^4\)-\(^6\). In particular, abnormal epigenetic regulation of specific genes might play an important role in both leukaemia development and therapeutic responsiveness. CML is a particular type of leukaemia characterized by the presence of the BCR-ABL oncogene. BCR-ABL is a constitutive active tyrosine kinase mediating the deregulation of several pathways involved in proliferation and differentiation\(^7\). Although the expression of BCR-ABL is considered the primarily feature associated with CML onset, other molecular mechanisms contributing to CML development remain to be elucidated. Both hypermethylation of specific genomic loci and genome-wide hypomethylation have been found to correlate with cancer development. Specifically, hypermethylation of tumour suppressor genes has been found to play a crucial role in carcinogenesis by affecting normal cell growth\(^8\). Further, aberrant DNA methylation has been linked to the onset of leukaemic clones resistant to tyrosine kinase inhibitors and deemed responsible for CML propagation and progression\(^9\). Among the genes found to be hypermethylated in CML and other lymphoid malignancies, and correlating with a poor outcome, are HOXA4 and HOXA5, both acting as differentiating genes during hematopoiesis\(^10\). Interestingly, the observation that a member of CCAAT/enhancer-binding protein (CEBP) family of transcription factors: DNA damage-inducible transcript 3 (DDIT3 gene, hypermethylated in CML\(^11\), suggests that the aberrant epigenetic regulation of the CEBP gene may also play a role in leukaemia onset\(^1\),\(^2\).

Taken together, these findings indicate that aberrant DNA methylation might contribute to leukaemia development by synergizing with acquired genetic lesions. However, the lack of suitable animal models or other tools to directly address this particular question has constrained a thorough analysis. Human-induced pluripotent stem (iPS) cells can be generated from various cell types, including cancer cells, by overexpressing a defined set of transcription factors including OCT4, SOX2, KLF4 and c-MYC (refs \(^13\)-\(^17\)). Previous studies have shown that cellular reprogramming might be able to erase tissue-specific DNA methylation and to re-establish an embryonic stem (ES)-like DNA methylation state\(^18\). Here by means of cellular reprogramming followed by \textit{in vitro} re-differentiation we tested the functional relevance of DNA aberrant methyleme in CML development. Reprogramming of CML cells into an iPS-like state was able to erase the cancer-specific DNA methylation signature and to identify a cell population no longer effective in producing CML when subsequently transplanted into immunocompromised mice. Finally, using an inducible BCR-ABL transgenic mouse\(^19\), we demonstrate \textit{in vivo} that a single genetic aberration perturbing DNA methylation profile acts as a crucial precipitating event in CML disease development.

**Results**

**Reprogramming erases leukaemia-specific methylation pattern.**

To understand the role of DNA methylation during CML development, we generated Leukaemia-iPS (LiPS) cells from two CML cell lines, K562 and KBM7, as well as from human CML primary bone marrow cells obtained from a BCR-ABL-positive CML patient. Leukaemia cells were transduced as previously reported\(^13\),\(^20\). Two weeks after infection, colonies with typical human ES-like morphology were picked and expanded on mouse embryonic fibroblast feeder layers resulting in stable ES-like cell lines: LiPS1-K562 and LiPS2-K562, both derived from the K562 cell line, and CML-LiPS1 and CML-LiPS2, derived from primary CML cells. In addition, we included the previously characterized KBM7 cell line and its reprogrammed counterpart in our analysis\(^15\). Remarkably, reprogrammed primary CML cells still preserved the BCR-ABL oncogene (Supplementary Fig. 1c and 1d). A comprehensive SNP array analysis confirmed that LiPS clones derived from K562 and KBM7 cell lines retained the same genetic alterations as the parental leukaemia cells (Supplementary Fig. 2 and Supplementary Data 1-2) ruling out the possibility that an essentially normal subclone or contaminating cell was selected during reprogramming.

Having established several LiPS cell lines, we proceeded to test whether cellular reprogramming was sufficient to reset DNA methylation of the parental leukaemic cells. Genomic DNA methylation profiles of K562, KBM7, primary CML cells and of the respective LiPS clones were assessed by Reduced Representation Bisulfite Sequencing (RRBS), which has been shown to provide high sensitivity and specificity for detecting cancer-specific changes in DNA methylation not only in CpG islands but also throughout genes and in repetitive regions\(^21\),\(^22\). Compared with human ES cells, CD34\(^+\)-derived iPS cells (CD34\(^+\)-iPS) and CD34\(^+\) cells, K562 and KBM7 cell lines exhibited widespread hypermethylation throughout the genome, including CpG islands, genes and promoters (Fig. 1a) as well as across families of repetitive elements. Primary CML cells also demonstrated significant hypermethylation in CpG islands, gene, and promoter regions, although to a lesser extent than in the cell lines. Compared with pluripotent cells, primary CML cells demonstrated hypermethylation across families of repetitive elements, similarly to CD34\(^+\) cells (Fig. 1a).

DNA methylation levels were substantially decreased throughout the genome in the LiPS cells as compared with their parental leukaemic cell types, demonstrating the ability of cellular reprogramming to reset aberrant methylome (Fig. 1a). Overall, the LiPS clones acquired DNA methylation patterns that were similar but not identical to those of ES cell lines and CD34 + iPS, supporting the notion of cell-of-origin-specific epigenetic memory in many types of induced pluripotent cells\(^23\),\(^24\). Indeed, through genome-scale DNA methylation analysis, we identified LiPS-specific DNA methylation changes of relevant pluripotent and hematopoietic-associated genes. Pluripotent genes such as SALL4 were reactivated in LiPS and exhibited decreased DNA methylation levels, particularly at the promoter regions consistent with the well-established role of DNA demethylation as a key event in cellular reprogramming (Fig. 1b and Supplementary Fig. 3)\(^18\). Epigenetic reprogramming affected not only pluripotency-associated genes but also other genomic loci, that is, hematopoietic transcription factors or tumour suppressor genes highly methylated across leukaemia cells and responding to cellular reprogramming with different extent of LiPS demethylation (Fig. 1b).

Characterization of differentially methylated regions in CML.

To characterize the changes in DNA methylation that are induced by the reprogramming of CML cells to pluripotency, we used the RRBS data to bioinformatically identify differentially methylated regions (DMRs) between LiPS clones and the cells they were derived from (see Materials and Methods). Among the 500 strongest DMRs that are associated with gene promoters, the vast majority were hypermethylated in CML cells as compared with the derived LiPS cells (Fig. 2a). This observation is consistent with the increased levels of promoter methylation that are common among cancer cells. To better understand the biological functions of the genes that are characterized by differential promoter methylation, we performed gene ontology enrichment analyses.

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The 500 gene promoters hypermethylated in CML cells as compared with LiPS cells were significantly enriched for functional categories associated with development, differentiation and signalling (Fig. 2b and Supplementary Table 1), suggesting that certain aspects of these stem cell-associated processes may be silenced by DNA methylation in CML cells. In contrast, the 500 gene promoters strongly hypermethylated in LiPS as compared with CML cells were enriched for hematopoietic regulatory gene categories such as lymphocyte activation and immune response (Fig. 2c and Supplementary Table 1), the latter recently shown to be attenuated in iPS cells.

Beyond this gene promoter centric analysis of the potential biological functions of differential DNA methylation between CML cells and LiPS cells, we also investigated the co-localization of DNA methylation changes with other epigenetic and regulatory mechanisms throughout the genome. To that end, we repeated the differential DNA methylation analysis on the 5-kb tiling regions spanning the whole genome, and we used the EpiExplorer software to compare the 500 most DMRs with the background of all tiling regions. This analysis revealed that DMRs are enriched for epigenetic marks associated with gene regulatory sequences including promoters and enhancers.
Further, consistent with previous studies demonstrating a strong association between Polycomb binding in pluripotent cells and hypermethylation in cancer\textsuperscript{28,29}, we observed that the CML hypermethylated regions were highly enriched for Polycomb binding sites (Fig. 2d).

Overall, these observations confirm that CML reprogrammed into LiPS cells undergo epigenome reprogramming that erases characteristic patterns of cancer-associated DNA methylation while establishing characteristic DNA methylation patterns of pluripotent cells.

Reprogramming restores myeloid differentiation of CML cells.

To assess whether epigenetic reprogramming observed in LiPS cells coincides with phenotypic changes in their oncogenic potential, we applied \textit{in vitro} and \textit{in vivo} strategies using both primary human CML cells and the CML model cell line K562, chosen for its high efficiency of engraftment in xenotransplantation approaches. By co-culture with OP9 stroma cells\textsuperscript{30} (Supplementary Fig. 4a), we demonstrated that CML-LiPS-derived CD45\textsuperscript{+}/CD34\textsuperscript{+} cells reacquire myeloid and erythroid differentiation capabilities giving rise to CD15\textsuperscript{+}/CD14\textsuperscript{+}/GlyA\textsuperscript{+} cells, despite the presence of BCR-ABL oncogene (Supplementary Fig. 4a-d). With a similar OP9 co-culture we proved that K562-LiPS-derived CD45\textsuperscript{+} cells exhibit a well-differentiated monocyte/macroage phenotype in contrast with the leukaemia counterpart, locked into an undifferentiated cellular state (Fig. 3a and Supplementary Fig. 4e). Indeed, while the parental leukaemia and LiPS cells showed low expression of the hematopoietic transcription factor \textit{CEBPA} (Fig. 3b), the purified CD45\textsuperscript{+} cells demonstrated the reactivation of this critical differentiation-associated gene locus and increase of its levels of expression (Supplementary Fig. 4f-g). Remarkably, when K562-LiPS-derived CD45\textsuperscript{+} cells and the parental leukaemia cells (20,000 per mouse) were injected into immunocompromised...
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice, all mice (n = 3) transplanted with leukaemic cells developed the disease and died within 30 days after the injection. In contrast, seven out of the eight mice transplanted with LiPS-derived CD45+ cells, regardless the successful engraftment, did not show any sign associated with a leukaemic phenotype, that is, spleen infiltration as observed in case of K562, during the entire 80 days of monitoring period (Fig. 3b,c).

Finally, to investigate the potential contribution of DNA methylation in CML pathogenesis, we applied 5-Azacytidine (AZA) treatment to primary CML cells. On treatment with AZA, we observed a skew in differentiation towards the granulocyte and monocyte/macrophage lineages, as shown by the presence of CD15+ and CD11b+ cells, as compared with the untreated cells (Fig. 3d,e), suggesting that DNA methylation abnormalities cooperate in disrupting hematopoietic differentiation during CML development and progression.

**BCR-ABL induction reprograms DNA methylation pattern.**

Our observations prompted us to investigate the functional relationship between the presence or absence of BCR-ABL oncogene and DNA methylation response. To address this question, we took advantage of a transgenic murine model in which BCR-ABL expression can be conditionally induced and reversibly repressed. Indeed previous studies, both in cell culture and in vivo, have shown that the overexpression of specific oncogenes such as MLL-AF9 and mutant IDH1 are capable of inducing epigenetic reprogramming comparable to that observed in human disease.

In our model, BCR-ABL is under the control of the 3’ enhancer of the murine SCL (stem cell leukaemia) gene, thus allowing BCR-ABL expression mainly in the hematopoietic stem cell (HSC) compartment and leading to development of a chronic myeloproliferative disorder similar to human CML. Induction of BCR-ABL expression by the withdrawal of tetracycline from the drinking water leads to a significant increase in Gr1 and Mac1 positive myeloid population (leukaemic mice). This phenotype can be reversed when tetracycline is added to the water (rescued mice), thus confirming a direct correlation between BCR-ABL presence and the expansion of that population (Fig. 4a and ref. 19). Using this mouse model, we analysed DNA methylation patterns in the HSCs population harvested from three distinct groups of mice: (i) the non-induced BCR-ABL mice (control); ii) the induced BCR-ABL mice (leukaemic) and (iii) the repressed BCR-ABL mice (rescued). Leukaemic mice showed moderate increase of CpG islands DNA methylation levels, indicating that a single oncogenic fusion protein can trigger characteristic perturbations of DNA methylation in a highly defined mouse model (Fig. 4b).

Upon BCR-ABL activation, the leukaemic mice acquired aberrant DNA methylation at several gene loci, one example being the hematopoietic transcription factor *Hoxb1* (Fig. 4c,d and Supplementary Table 2). Remarkably, the repression of BCR-ABL led to the widespread reversion of the BCR-ABL-induced changes in DNA methylation, supporting the hypothesis that BCR-ABL triggers DNA methylation changes. This observation could be explained by transcription factors and epigenetic regulator proteins directly downstream of BCR-ABL influencing DNA methylation, but it could also be a reflection of a broader change in epigenetic cell states when BCR-ABL transforms cells.
In summary, these data demonstrate the functional effect of a single genetic lesion in determining the aberrant methylome state, suggesting that epigenetic alterations act as a secondary precipitating event during leukaemia development.

5-Azacytidine reduces BCR-ABL cells oncogenic potential. To test whether the inhibition of BCR-ABL-induced aberrant DNA methylation could restore normal hematopoiesis during leukaemogenesis, six leukaemic mice per group were treated for 4 weeks with AZA (2 mg kg\(^{-1}\)) or with the tyrosine kinase inhibitor imatinib (200 mg kg\(^{-1}\); IM, first line treatment for CML), respectively. Mice treated with AZA exhibited no expansion of the Mac1\(^+\) Gr1\(^+\) myeloid population as compared with the untreated counterpart, closely mimicking the results observed for the IM-treated group used as a positive control. Furthermore, both AZA- and IM-treated mice displayed normal lymphopoiesis, absent in the untreated controls (Supplementary Fig. 5a). These data indicate that despite their different mechanisms of action, both AZA, at the epigenetic level by correcting aberrant DNA methylation and IM at the genetic level by blocking the BCR-ABL oncogene, are capable of re-establishing normal hematopoiesis.

To further evaluate the contribution of aberrant DNA methylation to leukaemia development in vivo, 44 irradiated B6.SJL-Ptprca Pepc\(^b\)/BoyJ (pep boys) congenic recipient mice were transplanted with murine BCR-ABL leukaemia bone marrow cells. Eight to ten mice were randomly assigned to five groups: control (not leukaemic); leukaemic untreated, treated with AZA (2 mg kg\(^{-1}\)); or IM (200 mg kg\(^{-1}\)) or combination of the two drugs IM and AZA. The treatment was administered for 4 weeks (Supplementary Fig. 5b) and the mice were followed up for a period of up to 150 days. The untreated mice developed leukaemia (as expected) with a median survival of 41 days. Strikingly, the AZA treatment provided longer survival (median survival 100 days; \(P = 0.0008\)) than the IM treatment (median survival: 56 days; \(P = 0.03\)) as compared with the untreated mice. Unfortunately, the combination of AZA and IM, at their standard dosages, resulted in haematological and gastrointestinal toxicity for the mice that did not allow us to make any conclusive observation on the potential benefits of using combination of the drugs to correct the leukaemia phenotype. Future studies will be necessary to assess optimal dosage range and scheduling mode to: (1) reduce the toxicity and (2) understand the potential synergic effect of the two drugs (Fig. 5b). Moreover, the AZA treatment efficiently targeted the stem cell compartment (LSK population from the CD45.2\(^+\) donor cells), restoring expression and promoting demethylation of loci differentially methylated between control and sick mice (Supplementary Data 2), such as the Gdf3 (Growth differentiation factor 3) (Supplementary Fig. 6), a promising target of the BCR-ABL oncogene.

Taken together, these data indicate that DNA methylation aberrations play a critical role in CML development, supporting the relevance of DNA methylation modulating drugs as therapeutic options.
Discussion

This study analysed the interconnection and functional relevance of genetic and epigenetic alterations in CML. By deriving genetically matched but epigenetically distinct LiPS cell lines from CML cell lines and human primary CML cells, and by using an inducible mouse model of CML, we show that: (1) genetic and epigenetic alterations are both required to maintain the leukaemic potential, and (2) that the BCR-ABL fusion protein is able to trigger DNA methylation changes that can act as a secondary event and contribute to leukaemia formation. Using nuclear reprogramming as an ‘epigenetic tool’ we were able to erase aberrant DNA methylation from leukaemia cells, thereby delaying the onset of the malignancy. Among the leukaemia-specific DMRs, less than half were associated with promoters, whereas the vast majority corresponded to specific histone marks such as enhancer-associated H3K4me1 and promoter-associated H3K4me3. Interestingly, genes hypermethylated in LiPS compared with CML cells belonged to functional categories of lymphocyte activation and immune response. It has been shown that iPS cells are defective in their antiviral response as a result of the downregulation of key genes involved in immune response.\textsuperscript{25,26} Similarly, attenuated antiviral response reported in human ES cells is restored by differentiation, suggesting that the impaired antiviral response of iPS cells likely stems from the pluripotent state and might promote cell pluripotency.\textsuperscript{25,26,34}

Our data show that the mice transplanted with CD45\(^+\) cells derived from LiPS do not develop leukaemia within the 80 days monitoring time (Fig. 3b). In particular, we demonstrated that leukaemia progression was susceptible not only to the genetic inhibition of the driving oncogene but also to the inhibition of the secondary DNA methylation changes, which provides strong evidence for the cooperative role of both genetic and epigenetic alterations in leukaemia.

In a related study focusing on glioblastoma, Stricker \textit{et al.}\textsuperscript{35} recently demonstrated that the reprogramming of glioblastoma cells was also able to erase aberrant DNA methylation. But in contrast to our observations for CML, these induced epigenetic changes were insufficient to compromise oncogenic potential, and neural progenitors derived from the reprogrammed glioblastoma remained highly malignant. This important difference between glioblastoma and CML supports the relevance of aberrant DNA methylation specifically in CML, consistent with the much lower rates of genetic alterations in leukaemia compared with glioblastoma\textsuperscript{36} and with the history of successful use of DNA methylation inhibitors in leukaemias. This conclusion is further supported by comparing our results in CML with a recent study focusing on cells derived from Ewing sarcoma patients, which is a solid tumour driven by an oncogenic fusion protein EWS-FLI1. On knockdown, Tomazou \textit{et al.}\textsuperscript{37} observed widespread changes in histone acetylation and histone methylation, whereas DNA methylation patterns were largely unaffected. Consistent with this observation, AZA had a much weaker effect in EWS-FLI1-expressing cells than histone deacetylase inhibitors, whereas the functional impact of AZA on BCR-ABL-expressing cells in our mouse model was striking and even exceeded those observed for imatinib.

Overall, these results highlight the specific relevance of aberrant DNA methylation in CML, which are driven by BCR-ABL expression and are at the same time essential for maintaining a strong leukaemic phenotype in a mouse model. This study provides a rationale for broadly exploring the use of demethylating agents as adjuvant treatments in leukaemia, even in subtypes with recurrent and well-characterized genetic abnormalities such as BCR-ABL.

Materials and methods

\textbf{Cell cultures and LiPS cells generation.} Human K562 leukaemia cells (ATCC CCL-243) and L929 cells (ATCC CRL-1593.2) were cultured following the manufacturer’s instructions. KBM7 cells were a kind gift from the T. R. Brummelkamp laboratory. They were cultured in DMEM 10% fetal bovine serum (FBS). iPS

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derived from CD34+ cells were a gift from the George Daley laboratory and were maintained in human ES cell medium (In Vitrogen) in the presence of FGF (Peprotech) and 20% of Replacement Serum (Invitrogen) as described in ref. 18. Primary CML total bone marrow mononuclear cells were obtained after a written informed consent in accordance with the Declaration of Helsinki in agreement with the approved protocol from the review board of the S.Orsola-Malpighi Hospital, Bologna, Italy. Primary samples were cultured in QBSF-60 (90% Biological), 20% FBS, SCF 50 ng/ml, Epo 100 ng/ml, GM-CSF 50 ng/ml, FLT3L 1 ng/ml, TPO 20 ng/ml, IL-3 1 ng/ml, and LIF 1 ng/ml. Retroviral infections were performed as described in refs 13,20. In brief, cells were transduced with a pool of the four factors Oct4, Sox2, Klf4 and c-Myc in a ratio of 1:1:1:1 for a 72 h period. At day 4 post transduction, regular medium was switched to human ES cell medium (Invitrogen) in the presence of FGF (Peprotech) and 20% of Replacement Serum (Invitrogen). After 15-16 human days, iPSC-like colonies were morphologically identified, picked and mechanically disaggregated on irradiated mouse embryonic fibroblasts (Globalstem). CML primary cells were treated with 1 μM of 5-Aza-cytidine for 72 h to allow monocyteic differentiation.

In vitro differentiation. Embryoid body (EB) formation was induced by culturing human LiPS in clumps in the absence of FGF in bacterial plates to avoid attachment to the bottom of the plates. To allow spontaneous endoderm formation, after 3–4 days, embryoid bodies were transferred onto 0.2% gelatin-coated glass six-well plates and cultured in differentiation medium (DMEM supplemented with 20% FBS, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, nonessential amino acids and penicillin-streptomycin, all from Invitrogen). LiPS cells were maintained on gelatin-coated plates in differentiated medium supplemented with 100 μM ascorbic acid (Sigma).

For ectoderm differentiation, EBs were cultured on laminin (Stemgent) coated six-well plates. In brief, after 4 days in EB medium as a floating culture, the cells were mechanically scraped, pelleted, resuspended in 100 μM of 5-Aza-cytidine for 72 h to allow monocyteic differentiation.

In vitro hematopoietic differentiation. Embryoid bodies were produced by culturing human LiPS in clumps in the absence of FGF in bacterial plates to avoid attachment to the bottom of the plates. To allow spontaneous endoderm formation, after 3–4 days, embryoid bodies were transferred onto 0.2% gelatin-coated glass six-well plates and cultured in differentiation medium (DMEM supplemented with 20% FBS, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, nonessential amino acids and penicillin-streptomycin, all from Invitrogen). LiPS cells were maintained on gelatin-coated plates in differentiated medium supplemented with 100 μM ascorbic acid (Sigma).

To induce hematopoietic differentiation, EBs were cultured on laminin (Stemgent) coated six-well plates. In brief, after 4 days in EB medium as a floating culture, the cells were mechanically scraped, pelleted, resuspended in 100 μM of 5-Aza-cytidine for 72 h to allow monocyteic differentiation.
FACS analysis and sorting. Cells were suspended in Ca\(^{++}\) Mg\(^{++}\)-free PBS containing 2% (vol/vol) PBS (Gibco/BRL). Human hematopoietic progenitor cells were identified by labelling with CD45-PE (1:50; clone H130) and/or CD45-APC (1:50; clone 561) while mature phenotypes were analysed using CD15-Pacific Blue (monocytes and granulocytes) (1:50; clone W6D3/H98), CD14-FITC (monocytes and macrophages clone 1;50; M2E2); CD11b (1:50; ICRF44; macrophages) and Glycophorin-PE/Cy5 (red blood cell membrane), and erythroid precursors (1:50; Clone HB5; all from Biogenex). Murine hematopoietic cells were identified using the following antibodies: Mac1/CD11b-PE/Cy7 (1:200; clone M1/70), Gr-1-PE (1:200; clone 8CS), B220-PE/Cy5 (1:100; clone RA3-682), Sca-1-APC (1:100; clone E13-161-7), c-Kit-APC/Cy7 (1:100; clone 2B8); CD45.1-APC (1:100; clone A20) and CD14-APC (1:100; clone M410; all from Biologex). All antibodies were incubated at the concentration suggested by the manufacturer for 30 min on ice. Non-specific signals and dead cells were excluded by appropriate fluorochrome-conjugated isotype and propidium iodide staining, respectively. Cell fluorescence was analysed using the FACSaria II (Becton Dickinson).

Quantitative reverse transcription PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and RNeasy Mini Kit (Qiagen). Genomic DNA was eliminated using DNA-free DNAse 1 (Invitrogen), and total RNA was then subjected to another round of purification by phenol solution with pH4.3 ± 0.2 (Sigma-Aldrich). For pluripotent gene expression analysis, cDNA was generated by SuperScript II reverse transcriptase (Invitrogen) with random hexamers (Invitrogen) from 1 μg of total RNA, and real-time PCR was performed with GoTag qPCR Mastermix (Promega). Expression of CEBPA was quantified using Taqman one-step qRT-PCR master mix (Applied Biosystems) relative to β-Actin. Primers used for CEBPA qRT-PCR were FW 5′-TCA GCT TCT CC-3′; Reverse 5′-ACTIN 0′-CCC TTG ACT GTG CCG TTG-3′. For isolation of bone marrow, 10,000 cells per mice. Transplanted mice were sacrificed for isolation of bone marrow on the 10th day post-transplantation.

Transplantation of immunocompromised and B6. SJL-Ptprc\(^{-}\)Pepck\(^{-}\)Boy1. Sorted Lin−PI−cD45.1\(^{+}\) cells and K562 cells were transduced by in vitro orbital injection into sublethally irradiated (150 Gy) 6-week-old recipient NOSG mice (20,000 cells per mice). Transplanted mice were sacrificed for isolation of bone marrow and spleen. Transplantation was performed in quadruplicate for Lin− cD45.1 clone and K562 and in triplicate for Lin− cD45.1 clone and K562 expressing transgenes. FACS analysis and sorting were performed as described above. Three days after transplantation, total RNA was isolated from cells using TRIzol reagent (Invitrogen), and RNeasy Mini kit (Qiagen). cDNA was synthesized with Superscript First-Strand Synthesis System (Invitrogen). qRT-PCR were performed with the following primers: Forward 5′-GAG CCC GCC GGT CAT TG-3′; reverse 5′-CCC TTG ACT GTG CCG TTG-3′; probe 5′-FAM GCG GCC GGT CAT TG-3′. The chronic myelogenous leukemia-specific transcription factor, PU.1. The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. Science 233, 212–214 (1986).

Transplantation of immunocompromised and B6. SJL-Ptprc\(^{-}\)Pepck\(^{-}\)Boy1. Sorted Lin−PI−cD45.1\(^{+}\) cells and K562 cells were transduced by in vitro orbital injection into sublethally irradiated (150 Gy) 6-week-old recipient NOSG mice (20,000 cells per mice). Transplanted mice were sacrificed for isolation of bone marrow and spleen. Transplantation was performed in quadruplicate for Lin− cD45.1 clone and K562 and in triplicate for Lin− cD45.1 clone and K562 expressing transgenes. FACS analysis and sorting were performed as described above. Three days after transplantation, total RNA was isolated from cells using TRIzol reagent (Invitrogen), and RNeasy Mini kit (Qiagen). cDNA was synthesized with Superscript First-Strand Synthesis System according to manufacturer’s instructions (Invitrogen). qPCR was performed with SYBR reagent (Bio-Rad). Primers used were: Gdf3_F1 (Forward): 5′-GACA TGA AGC AGC AGC GC-3′; Gdf3_R1 (Reverse): 5′-TCA GCT TCT CC-3′; 24. Lister, R. Human cell line methylation maps recapitulate in vivo patterns. Nature 455, 286–291 (2008).
36. Appin, C. L. & Brat, D. J. Molecular genetics of gliomas. *Cancer J.* **20**, 66–72 (2014).
37. Tomazou, E. M. et al. Epigenome mapping reveals distinct modes of gene regulation and widespread enhancer reprogramming by the oncogenic fusion Protein EWS-FLI1. *Cell Rep.* **10**, 1082–1095 (2015).
38. Loh, Y. H. et al. Generation of induced pluripotent stem cells from human blood. *Blood* **113**, 5476–5479 (2009).
39. Xi, Y. & Li, W. BSMA: whole genome bisulfite sequence MAPping program. *BMC Bioinformatics* **10**, 232 (2009).
40. Liu, Y., Siegmund, K. D., Laird, P. W. & Berman, B. P. Bis-SNP: Combined DNA methylation and SNP calling for Bisulfite-seq data. *Genome Biol.* **13**, R61 (2012).
41. Assenov, Y. et al. Comprehensive analysis of DNA methylation data with RsBeads. *Nat. Methods* **11**, 1138–1140 (2014).
42. Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, Article3 (2004).
43. Bock, C. Analysing and interpreting DNA methylation data. *Nat. Rev. Genet.* **13**, 705–719 (2012).
44. Bock, C. et al. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* **21**, 4067–4068 (2005).
45. Li, L. C. & Dahiya, R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427–1431 (2002).
46. Amabile, M. et al. Real-time quantification of different types of bcr-abl transcript in chronic myeloid leukemia. *Haematologica* **86**, 252–259 (2001).
47. Ernst, J. & Kellis, M. ChromHMM: automating chromatin-state discovery and characterization. *Nat. Methods* **9**, 215–216 (2012).

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**Author contributions**

D.G.T. supervised the project; G.A., A.D.R. and R.S.W. conceived and designed the study; G.A., A.D.R., R.S.W., A.K.E., H.Z., L.Q., M.M.L. and E.L. performed the experiments; F.M. and H.Y. analysed RRBS and microarrays data; M.E.F. assisted in the RRBS analysis; G.M. provided procurement and initial characterization of the primary CML samples; T.B. provided the near-haploid leukaemic cell line KBM7. G.A., A.D.R., C.B. and D.G.T. wrote the paper.

**Additional information**

**Accession codes**: RRBS data have been deposited in the gene expression omnibus (GEO) database under the accession number GSE50456.

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

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