Reversibility of acute B-cell leukaemia induced by *BCR–ABL1*

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Cancer is thought to arise from multiple genetic events that establish irreversible malignancy. A different mechanism might be present in certain leukaemias initiated by a chromosomal translocation. We have taken a new approach to determine if ablation of the genetic abnormality is sufficient for reversion by generating a conditional transgenic model of *BCR–ABL1* (also known as *BCR–ABL*)-induced leukaemia. This oncogene[1] is the result of a reciprocal translocation and is associated with different forms of leukaemia[2]. The most common form, p210 *BCR–ABL1*, is found in more than 90% of patients with chronic myelogenous leukaemia[3,4] (CML) and in up to 15% of adult patients with *de novo* acute lymphoblastic leukaemia[5] (ALL). Efforts to establish a useful transgenic model have been hampered by embryonic lethality when the oncogene is expressed during embryogenesis[6,7], by reduced penetrance or by extremely long latency periods[8,9]. One model uses the 'knock-in' approach to induce leukaemia by p190 *BCR–ABL1* (ref. 10). Given the limitations of models with p210, we used a different experimental approach[11]. Lethal leukaemia developed within an acceptable time frame in all animals, and complete remission was achieved by suppression of *BCR–ABL1* expression, even after multiple rounds of induction and reversion. Our results demonstrate that *BCR–ABL1* is required for both induction and maintenance of leukaemia.

We established four *BCR–ABL1* transresponder lines (2, 3, 4 and 27) from founder animals. Transgenic mice were born with the expected mendelian frequency and developed normally, indicating that the tetracycline-responsive expression system corrects for *BCR–ABL1* toxicity in embryonic tissue[7]. So far no mouse transgenic for the transresponder construct (Fig. 1*a*) has developed a haematological disorder (with a median follow-up period of 10 months). Double transgenic mice (*BCR–ABL1*-tetracycline transactivator (tTA)) were generated by breeding female transresponder mice with male mouse mammary tumour virus (MMTV)-tTA transactivator mice[12] under continuous administration of tetracycline (0.5 g/l) in the drinking water, starting five days before mating. The genotypic distribution of double transgenic mice followed the predicted mendelian frequency in all four lines.

Withdrawal of tetracycline administration in double transgenic animals allowed expression of *BCR–ABL1* and resulted in the development of lethal leukaemia in 100% of the mice within a time frame that was consistent within each line (Fig. 1*b*). An increase in the peripheral blood leukocyte count was noted 6–10 days after withdrawal of tetracycline in animals from lines 2, 3 and 4, and within 10–20 days in line 27. Examination of blood smears at this time point revealed the appearance of cells that resembled immature lymphocytes. The condition progressed to leukaemia on the basis of the following criteria: (i) more than 90% of the cells in the peripheral blood were lymphoblasts (Fig. 2*a*,*b*), with elevations of the leukocyte count ranging from 80,000 to 150,000 cells per μl; (ii) development of massive lymphadenopathy and splenomegaly; (iii) infiltration of leukaemic cells into the skin, pleura and meninges (Fig. 2*c*); (iv) pale bone marrow with replacement of normal haematopoietic cells by lymphoblasts (Fig. 2*a*, right); (v) accompanying severe anaemia and thrombocytopenia in the peripheral blood (Fig. 2*a*, left and middle); and (vi) adoptive transfer of the leukaemia into non-irradiated recipients. In the last case, we injected lymph node cells from a donor animal from line 3 (sacrificed on day 18 after first induction) into non-irradiated wild-type littermates and pure-bred FVB/N mice. Both littermates developed B-cell leukaemia within 14 weeks after transplantation. Necropsy confirmed leukaemia accompanied by anaemia, ascites, splenomegaly and meningeal involvement. Fluorescence activated cell sorter (FACS) analysis of leukaemic cells was in complete concordance with the expression pattern of cells from the donor animal (Fig. 2*b*). The mixed background of the double transgenic mice (BL6×SJL×FVB/N) prevented transplantation into pure-bred FVB/N recipients.

To confirm the lineage of the leukaemic cells, we analysed peripheral white blood cells, bone marrow and lymph node cells by flow cytometry for expression of lineage-specific antigens.

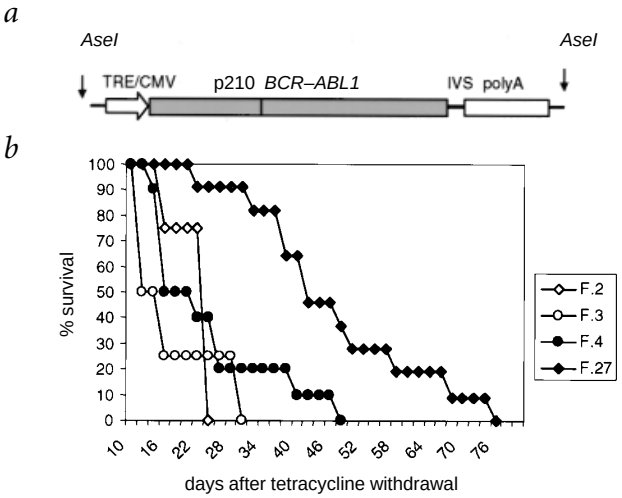

Fig. 1 Induction of leukaemia by *BCR–ABL1* under the control of the 'tet-off' system in transgenic mice. ***a***, The p210 *BCR–ABL1* transresponder construct. The p210 *BCR–ABL1* cDNA was inserted downstream of the tetracycline-responsive element fused to a minimal CMV promoter (TRE/CMV). ***b***, Survival of double transgenic animals (*BCR–ABL1*-tTA) after induction of *BCR–ABL1* expression by tetracycline withdrawal in the four transresponder lines. The major difference among the *BCR–ABL1* lines was the survival period after induction of *BCR–ABL1* expression. Double transgenic mice from lines 2, 3 and 4 succumbed to a rapidly progressing leukaemia within 3–6 weeks, whereas animals from line 27 survived for up to 11 weeks. The age of the mice at the time of induction had no impact, as leukaemia was induced in all double transgenic animals at all ages (19 days to 8 months), with no differences in the kinetics or the pathological features of the disease.

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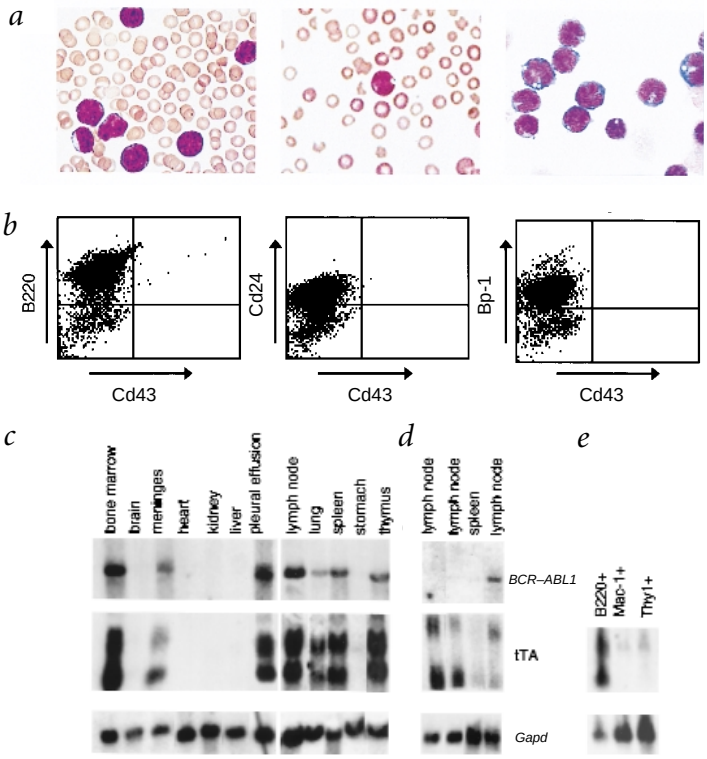

Fig. 2 Expression of *BCR–ABL1* induces an acute pre-B cell leukaemia. ***a***, Wright-Giemsa stain of cytocentrifuged peripheral blood 10 d (left) and 21 d (middle) after induction of *BCR–ABL1* expression by withdrawal of tetracycline demonstrating leukaemic lymphoblasts along with neutropenia, anaemia and thrombocytopenia. Lymphoid blasts replace other lineages in the bone marrow (right). ***b***, FACS analysis of lymph-node cells demonstrating expression of B220, Cd24 and Bp-1, but not Cd43. ***c***, Northern-blot analysis of *BCR–ABL1* and tTA mRNA in tissues from leukaemic mice from line 27. We detected two species of tTA mRNA (ref. 12). ***d***, Northern blot demonstrating that line 27 animals succumb to a *BCR–ABL1* independent leukaemia after initial remission (lanes 1–3) while on tetracyline. Lane 4 shows expression of *BCR–ABL1* in tissue from an animal that died of leukaemia before reversion. ***e***, The MMTV-LTR directs expression of tTA to the B220+ B-cell population in the bone marrow (lane 1), but not to myeloid (lane 2) or T cells (lane 3).

Most cells stained positive for B220, Cd24 and Bp-1, whereas no expression of Cd43 (Fig. 2*b*), the T-cell marker Thy1, or the myeloid markers Gr-1 or Mac-1 was observed. Southern-blot analysis of immunoglobulin rearrangement patterns showed rearrangements of the heavy chain, but no rearrangement of the κ-light chain (Fig. 3*a*,*b*). We therefore conclude that the leukaemic cells were arrested at a late stage of pro-B-cell development and that *BCR–ABL1* caused acute B-cell leukaemia (B-cell ALL; refs 5,13). We detected *BCR–ABL1* and tTA mRNAs in bone marrow, spleen and enlarged lymph nodes of diseased mice (Fig. 2*c*), and in infiltrated tissues. Western-blot analysis confirmed these data at the protein level (Fig. 3*c*). The disease may model human B-cell ALL rather than CML due to the restriction of *BCR–ABL1* expression to a subset of cells in mouse bone marrow, whereas expression of the oncogene in patients is driven by the ubiquitously active *BCR* promoter. Cell-type specificity in the tetracycline-responsive expression system is dependent on the promoter directing expression of the transactivator protein tTA. Here, the MMTV-LTR directs expression of tTA to B220+ (B cell) bone marrow cells (Fig. 2*e*). To develop a CML-like disease, we are currently developing transactivator strains of mice, which can direct tTA expression to the stem-cell compartment using a *CD34* transgene[14].

Previous transgenic and retroviral transplantation models[15] have established that *BCR–ABL1* is important in the initiation of neoplastic transformation, but not whether continuous expression of the fusion protein is required for maintenance of leukaemia. We tested whether abolition of *BCR–ABL1* might lead to a reversal of the phenotype. Leukaemic mice at advanced stages of disease (as defined by leukocytosis, splenomegaly and, in some cases, lymphadenopathy) were given tetracycline. A rapid and precipitous drop in the number of white blood cells with normalization occurred within 48–72 hours (Fig. 4*b*), and blast cells were no longer detectable in the peripheral blood. Moreover, complete regression of enlarged lymph nodes was achieved within five days (Fig. 4*a*).

The rapid disappearance of leukaemic cells from the blood suggested apoptosis rather than differentiation as the underlying mechanism for the reversion of the phenotype. To test this hypothesis, we examined peripheral leukocytes for the presence of apoptotic cells. Apoptosis was not detected in leukaemic mice that did not receive tetracycline or in wild-type mice, whereas approximately 80% of the cells in leukaemic animals underwent apoptosis within 20 hours after tetracycline administration, dropping to normal levels within 3 days after re-administration of the antibiotic (Fig. 5). These findings are consistent with studies demonstrating that cells expressing *BCR–ABL1* are protected from apoptosis[16,17].

The reversion of the leukaemic phenotype was permanent in animals from lines 2, 3 and 4 as long as administration of tetracycline was continued, but subsequent withdrawal

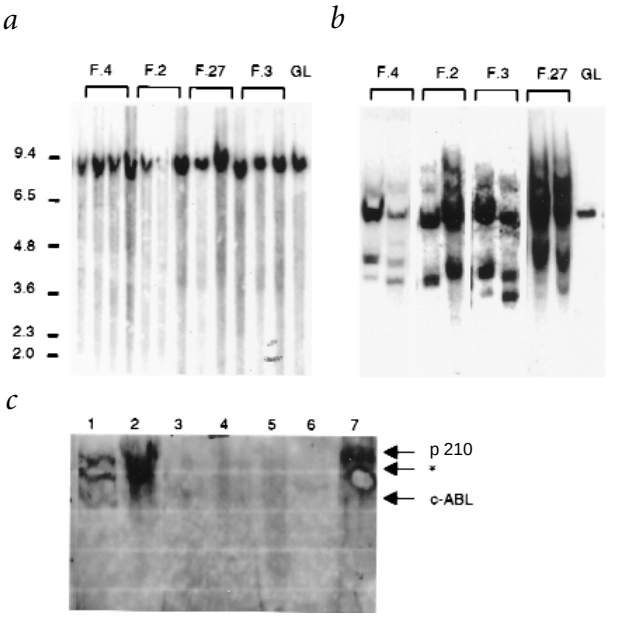

Fig. 3 Analysis of immunoglobulin rearrangements and p210 protein expression in mice from different founder lines with B-cell ALL. Genomic DNA was isolated from liver, lymph node and spleen for Southern-blot analysis and digested with *Eco*RI for analysis of heavy-chain rearrangement and with *Eco*RI/*Bam*HI for analysis of light-chain rearrangement. ***a***, No rearrangement of the light chain was detected. ***b***, All samples demonstrate rearrangement of the heavy chain. We used NIH 3T3 cells as a control for germline configuration (GL). ***c***, Western blot of p210 protein expression in lymphoid tumours. Protein was detected using an antibody against the carboxy terminus of c-Abl, which recognizes the p210 fusion protein (top), a proteolytic p210 product (*) and c-Abl (bottom). Lane 1, F3 pleural effusion; lane 2, spleen of *BCR–ABL1*-negative control mouse; lane 3, splenocytes of an animal from the F27 line, which relapsed on tet; lanes 4 and 5, lymph nodes of a second F27 that relapsed while on tet; lane 6, F4 pleural effusion; lane 7, F27 pleural effusion.

of the antibiotic again induced leukaemia. The period of time between reversion and subsequent induction had no impact, as the same results were obtained in three repeated induction experiments over one year (Fig. 4*c*). This demonstrates that the leukaemic phenotype in these animals is dependent on the continuous expression of *BCR–ABL1*. We did, however, see a difference in mice from line 27. All reverted animals (6/6) succumbed after 2–4 weeks of remission to a rapidly progressing B-cell leukaemia that was independent of *BCR–ABL1* expression, as shown by lack of detectable mRNA in tumour tissue (Fig. 2*d*) and absence of BCR–ABL1 protein (Fig. 3*c*). We hypothesize that in this founder line, which takes longer to develop leukaemia, secondary mutations were acquired during the prolonged development of the disease, causing a *BCR–ABL1*-independent leukaemia.

We describe here a new transgenic model of p210 *BCR–ABL1*-induced acute B-cell ALL that has several advantages over existing models[15]. The binary expression system allows for the generation of *BCR–ABL1* transgenic mice in which conditional expression of the oncogene causes leukaemia within an experimentally convenient latency in all animals. Re-administration of tetracycline induces the complete reversion of the phenotype. It is the first animal model to demonstrate that continued expression of *BCR–ABL1* is necessary to maintain cancer of the haematopoietic system, and that the leukaemic phenotype, even at advanced stages of disease, is completely reversible. This correlates with previous *in vitro* studies using a temperature-sensitive v-abl virus[18]. Complete reversibility in our *in vivo* model may be dependent on the length of time the cells need to be exposed to *BCR–ABL1* to develop the leukaemic phenotype. Longer exposure periods might increase the likelihood of additional genetic events, rendering maintenance of the phenotype independent of the initially transforming event, which is in concordance with a study using SV40 large T antigen[19]. Our findings suggest that complete and lasting remissions may be achieved if the genetic abnormality is abolished or silenced before secondary mutations are acquired. Our results have implications for therapies that directly target leukaemia oncogenes, with a relevant example being the use of *BCR–ABL1*-specific tyrosine kinase inhibitors[20]. Therefore, future studies using transgenic systems that allow for induction and reversion of expression will be invaluable in testing this hypothesis for other leukaemias.

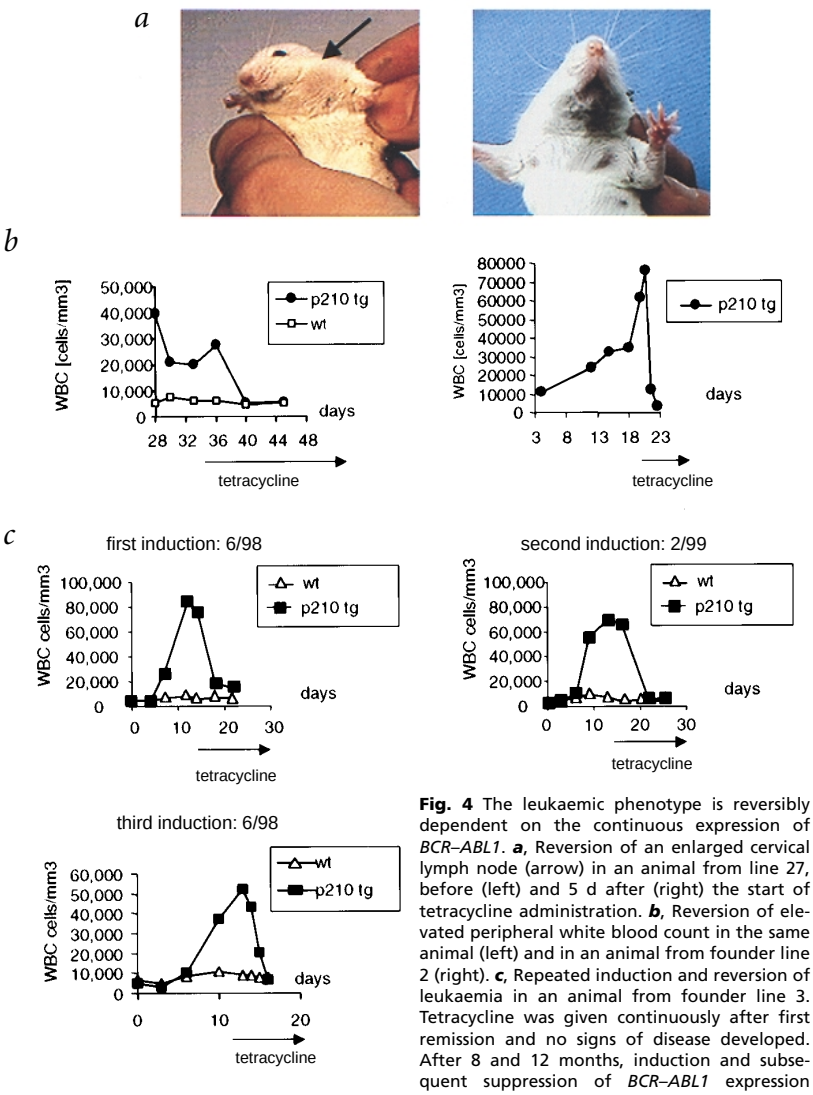

Fig. 4 The leukaemic phenotype is reversibly dependent on the continuous expression of *BCR–ABL1*. ***a***, Reversion of an enlarged cervical lymph node (arrow) in an animal from line 27, before (left) and 5 d after (right) the start of tetracycline administration. ***b***, Reversion of elevated peripheral white blood count in the same animal (left) and in an animal from founder line 2 (right). ***c***, Repeated induction and reversion of leukaemia in an animal from founder line 3. Tetracycline was given continuously after first remission and no signs of disease developed. After 8 and 12 months, induction and subsequent suppression of *BCR–ABL1* expression resulted in an increase in the number of white blood cells similar to that seen previously.

Methods

Generation of transgenic mice. The cDNA for p210 *BCR–ABL1* was released from plasmid GDp210 (ref. 21) by digestion with *Eco*RI and subcloned into the *Eco*RI site of the transresponder plasmid pUHD10-3 (ref. 11). We inserted a 720-bp fragment from rabbit *HBB* intron 8 (encoding β-globin; ref. 22) downstream of the p210 *BCR–ABL1* cDNA, with the polyadenylation site provided by pUHD10-3. Digestion with *Ase*I released the transgenic construct from the vector. We generated transgenic mice by injecting DNA into fertilized eggs from FVB/N mice. MMTV-tTA transactivator mice[12] were re-derived from a mixed BL6×SJL background into the FVB/N background. We identified transgenic mice by Southern analysis of tail snip DNA after *Eco*RI digestion. We used a 450-bp fragment from the joining region of *BCR–ABL1* generated by PCR (ref. 23) and the 1,020-bp tTA fragment isolated from the transactivator plasmid pUHD15-3 after digestion with *Bam*HI and *Eco*RI for detection of the transgenes.

Secondary transplantation of the acute B-cell leukaemia. We injected 5×10^6 cells from the enlarged lymph node of an animal with acute leukaemia into non-irradiated wild-type littermates (like the donors, mixed Bl6, SLJ and FVB/N) as well as pure-bred FVB/N recipient mice. Animals were monitored biweekly for development of macroscopic disease and leukaemia.

Monitoring of peripheral blood for signs of disease. We performed total differential and white blood cell counts biweekly on peripheral blood from double transgenic mice and single- or non-transgenic littermates kept in the same cages. We stained peripheral blood films with Wright-Giemsa for differential analysis. FACS analysis was performed by staining with lineage-specific antibodies after lysis of red blood cells with lysis buffer (0.15 mol/l NH_4Cl, 1 mmol/l $KHCO_3$, 0.1 mmol/l Na^2EDTA, pH 7.3) using the following monoclonal antibodies: anti-Thy1.1, anti-Cd4, anti-Cd8, anti-Cd43, anti-Bp-1 and anti-Cd24 (PharMingen), and anti-B220, anti-Gr-1 and anti-Mac-1 (Caltag).

Sorting of bone marrow cells. We isolated bone marrow from tibia and femur. Cells expressing the pan T-cell marker Thy1, the B-cell marker B220

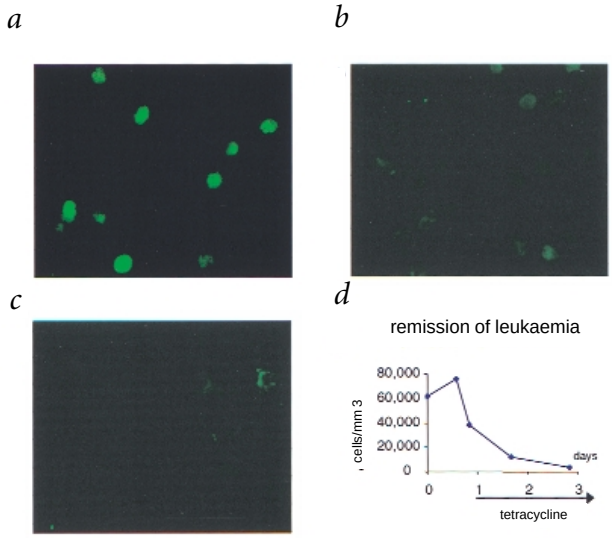

Fig. 5 Apoptosis of leukaemic cells in peripheral blood following re-administration of tetracycline. The TUNEL assay was performed with white blood cells isolated from leukaemic animals from line 2: ***a***, 14 hours after tetracycline; ***b***, 68 hours following tetracycline, at which time peripheral white blood counts had returned to normal (<10,000 cells/mm^3). ***c***, Peripheral blood of a non-transgenic littermate. Multiple TUNEL-positive cells are seen in (*a*), but not in (*b*) or (*c*). The figures show representative fields; more than 300 cells were counted from each sample. ***d***, The number of white blood cells in the peripheral blood decreased to normal levels within 3 d of tetracycline treatment.

and the myeloid marker Mac-1 were isolated using the MiniMacs system (Miltenyi Biotec) according to the manufacturer's guidelines. We performed two rounds of purification for each marker. The purity was assessed by cytospins of positive cells after Wright Giemsa staining and FACS analysis. The purity for B220-positive cells was 86% and was 95% for Mac-1 positive selection.

Isolation of RNA and northern-blot analysis. We extracted total RNA from tissues of diseased and control mice after homogenization in guanidine isothiocyanate solution (TriReagent, MRC). We separated total RNA (20 μg) on a 1% agarose gel containing formaldehyde (0.22 M) and transferred to an uncharged nylon membrane (Magnagraph, Micron Separations) by capillary transfer overnight in 10×SSC. Membranes were treated with an ultraviolet crosslinker (Stratagene) and subsequently probed with the ^{32}P-α[dCTP] radiolabelled probes for *BCR–ABL1* and tTA as described above.

Southern-blot analysis for immunoglobin rearrangements. Genomic DNA was prepared from tissues of diseased mice. We digested DNA (10 μg) with *Eco*RI alone or *Eco*RI and *Bam*HI, electrophoresed through a 0.6% agarose gel, transferred overnight onto a positively charged nylon membrane and hybridized with a radioactive probe for the immunoglobulin heavy chain[24] or light chain[25].

Western-blot analysis. Protein lysates of haematopoietic cells and tissues were homogenized with a Polytron homogenizer, resuspended in RIPA buffer with immediate addition of sample buffer and boiled for 10 min (ref. 26). Amounts of proteins loaded in each lane were standardized by Bradford protein assay as well as assessment by Coomassie blue staining. We performed SDS–PAGE and western-blot analysis with an anti-Abl antibody (8E9, Pharmingen) as described[26].

Detection of apoptotic cells in peripheral blood. We detected peripheral white blood cells undergoing apoptotic cell death with the TUNEL assay[27] on cytocentrifuged samples after lysis of red blood cells using a detection kit (Boehringer) according to the manufacturer's recommendations.

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