Supplemental Materials and Methods

Western blotting. Cells were lysed in sodium dodecyl sulfate (SDS) buffer with β-mercaptoethanol, boiled and sonicated as described before [1]. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with mouse monoclonal anti- c-ABL1 (Ab-3) (Calbiochem), anti-phosphotyrosine, clone 4G10 (Upstate), and anti-β-actin (Abcam) primary antibodies followed by IRDye800-goat anti-mouse IgG and IRDye680-donkey anti-rabbit secondary antibodies. Blots were analyzed with the Odyssey Infrared Imaging System (Li-Cor).

Microarray analysis.
The whole protocol has been described before [2]. Briefly, 5,000 $Lin^-Kit^+Sca\text{-}1^+$ cells from 3 CML-CP –like mice and 3 control mice were FACS-isolated, their RNA was extracted and linearly amplified, and microarray analysis was carried out on Affymetrix Mouse Genome 430 2.0 GeneChips covering approximately 45,000 transcripts. Individual genes were analyzed using the MicroArraySuite 5.0 software for calculation of the presence or absence calls and the D-Chip Software. 300 genes were found to be changed at least 1.5-fold ($p<0.05$, Mann-Whitney test) in expression when 3 CML-CP -like mice were compared with 3 control mice.

Transgenic mice
SCLtTA/p210BCR-ABL1 mice were provided with drinking water supplemented with tetracycline hydrochloride (0.5g/L) (Sigma-Aldrich). Transgenic mice were identified by polymerase chain reaction (PCR) of tail snip DNA. DNA isolation and purification from mice tails were performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma, Saint Louis, Missouri), and genotyping for the SCLtTA and p210*BCR-ABL1* transgenes was performed using transgene-specific primers (Eurofins MWG Operon). 2X GoTaq polymerase Master Mix (Promega) was used to amplify both SCLtTA and p210*BCR-ABL1* transgenes. *BCR/ABL*-specific primers (forward: 5′-GAGCGTGCAGAGTGGAGGGAGAACA-3′; reverse: 5′-GGTACCAGGAGTGTTTCTCCAGACTG-3′) amplified a 500 basepair-long fragment using amplification conditions of 40 cycles at 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1 minute. SCLtTA-specific primers (*tTA*: 5′-TTTCGATCTGGACATGTTGG-3′; *SCL*: 5′-AGAACAGAATTCAGGGTCTTCCTT-3′) yielded a 750 basepair product using amplification conditions consisting of 40 cycles at 94°C for 40 seconds, 60.5°C for 1 minute, and 72°C for 1 minute. PCR products were run in a 1.5% agarose gel containing ethidium bromide, and visualized using the Gel Doc™ XR+ Molecular Imager® System (Bio-Rad).

Detection of CML-CP –like disease
Peripheral blood was obtained from cardiac puncture. Spleen (SPL) were removed, photographed (Sony Cyber-shot® Digital Camera W220), weighed, then mashed to obtain cells in suspension, and erythrocytes were lysed with ACK lysis buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.1mM EDTA, pH 7.2-7.4). Mononuclear bone marrow cells (BMCs) were obtained after density separation by Lympholyte M (Cedarlane). All cells were washed and resuspended in cold PBS containing 1.0% BSA and rat anti-mouse CD16/CD32 (FcRγ III/II) antibody and incubated for 10-15 minutes, followed by incubation with rat anti-mouse fluorescein isothiocyanate (FITC)-conjugated Ly-6G/C (Gr-1), phycoerythrin (PE)-conjugated CD11b (Mac-1), allophycocyanin (APC)-conjugated CD45R/B220, and PE-cyanin7 (PE-Cy™7)-conjugated CD3, or APC-conjugated Ter-119 and PE-conjugated CD41 (all from BD PharMingen) for 30 minutes in 4°C, and fixed with 1% paraformaldehyde and analyzed by FACS.

Real-time quantitative RT-PCR (qRT-PCR)
Isolation of DNase-treated RNA was performed using RNeasy® Mini Kit or RNeasy® Micro Kit for BMCs and splenocytes (Qiagen) followed by cDNA synthesis using the Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) [2]. p210*BCR-ABL* transcripts were detected by real-time TaqMan assay (Eurogentec) and TaqMan Universal PCR Master Mix (Applied Biosystems) used in combination

with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). As an internal standard, the expression level of GAPDH was used.

Oxidative DNA damage

Cytospins were incubated with monoclonal anti- 8-oxoG (Chemicon) or anti- γ-H2AX (Upstate) primary antibodies followed by Alexa Fluor® 594 or 488 goat anti-mouse IgG secondary antibody (Molecular Probes) was applied; simultaneously, DNA was counterstained with blue-fluorescent nucleic acid stain 4′,6′-diamidino-2-phenylindole (DAPI). Specific staining was visualized using an inverted Olympus IX70 fluorescence microscope (Olympus America) equipped with a 100 X/1.35 numeric aperture UPlan Apo objective and Cooke Sensicam QE camera (Cooke). A series of 2-dimensional images of each cell was stored in SlideBook software version 3.0.1 (Intelligent Imaging Innovations). Deconvolution was applied to increase the resolution and contrast of the images. A collection of 2-dimensional images describing individual cells was converted to a one 2-dimensional photomicrograph. 2-dimensional photomicrographs of 8-oxoG lesions underwent further analysis using Adobe Photoshop (Adobe Systems) before final quantification of nuclear lesions. Images from 15 - 279 individual cells were analyzed per experimental group.

Supplemental Results

Supplemental Table 1. **Characteristic of CML-CP patients as good and poor responders to TKIs.**

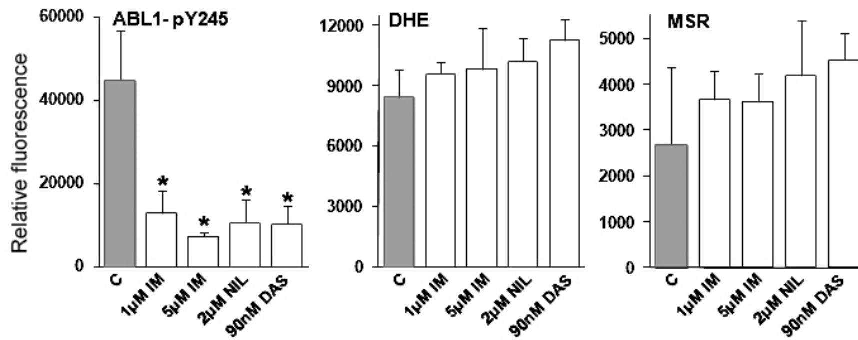
	Patients	Initial TKI	Cytogenetic and Molecular Monitoring and Treatment Changes	% Ph+ by G-band in BMCs		
				Year 1	Year 2	Year 3
GR	0027	Imatinib	achieved CCR at 12 months; CCR maintained at 24 months; BCR-ABL1:ABL1 ratio 0.006% on the international scale at 42 months/3.5 years consistent with MMR	0	0	ND
	0046	Imatinib	achieved CCR at 12 months; CCR maintained at 24 months; BCR-ABL1:ABL1 ratio 0.0% on the international scale at 36 months/3.0 years consistent with CMR	0	0	ND
	0051	Imatinib	achieved CCR at 12 months; CCR maintained at 24 & 36 months; BCR-ABL1:ABL1 ratio 0.003% on the international scale at 36 months consistent with MMR	0	0	0
	0116	Dasatinib	achieved CCR at 12 months; CCR maintained at 24 months; BCR-ABL1:ABL1 ratio 0.012% on the international scale at 30 months/2.5 years consistent with MMR	0	0	ND
	0023	Dasatinib	achieved CCR at 12 months; CCR maintained at 24 months; BCR-ABL1:ABL1 ratio 0.013% on the international scale at 36 months/3 years consistent with MMR	0	0	ND
	0025	Dasatinib	achieved CCR at 12 months; BCR-ABL1:ABL1 ratio 0.003% on the international scale at 36 months/3 years consistent with MMR	0	ND	ND
PR	0074	Imatinib	failed to achieve CCR at 12 months; BCR-ABL1:ABL1 ratio 38.65% on the international scale at 12 months; treatment changed to dasatinib at 12 months; no further follow-up as yet available	97	ND	ND
	0140	Imatinib	cytogenetic analysis failed at 12 months; BCR-ABL:ABL ratio 78.82% on the international scale at 12 months; imatinib increased to 600mg; imatinib resistant mutation confirmed at 14 months and treatment changed to dasatinib at 14 months; BCR-ABL1:ABL1 ratio 0.002% at 24 months	failed	ND	ND
	0154	Imatinib	failed to achieve CCR at 12 months; BCR-ABL1:ABL1 ratio 21.0% on the international scale at 12 months; treatment changed to dasatinib 100mg at 12 months; no further follow-up as yet available	65	ND	ND
	0165	Imatinib	BCR-ABL:ABL ratio 21.69% on the international scale at 3 months; treatment changed to dasatinib 100mg at 7 months; no further follow-up as yet available	ND	ND	ND

All CML-CP patients were recruited to the SPIRIT2 trial at diagnosis and were randomized to imatinib (400 mg daily) or dasatinib (100 mg daily) treatment.
CCR = complete cytogenetic response; MMR = major molecular response; CMR = complete molecular response; ND = not done; GR = good responders; PR = poor responders; TKI = tyrosine kinase inhibitor; Ph = Philadelphia chromosome; BMCs = bone marrow cells.

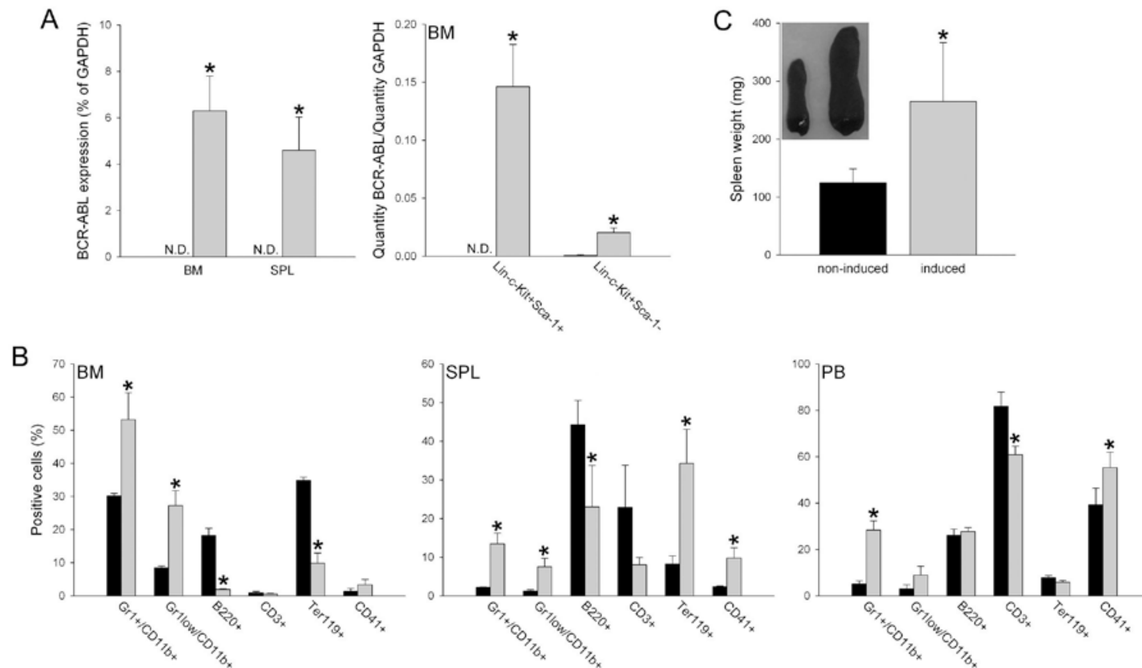
Supplemental Table 2. **Microarray analysis of the expression of genes potentially involved in generation of high levels of ROS in $Lin^-Kit^+Sca1^+$ LSCs in comparison to HSCs.**

probe set	Gene symbol	Fold change	P value	Function
1428859_at	*Paox*	1.55	0.023794	H_2O_2 synthesis
1449481_at	*Ant*	1.72	0.003562	oxidative phosphorylaion
1417607_at	*Cox6a2*	1.9	0.03907	mitochondrial electron transport
1457633_x_at	*CoxD*-like	6.3	0.017259	mitochondrial electron transport
1421259_at	*Pklr*	-1.65	0.004456	pyruvate synthesis
1418862_at	*Echdc3*	-1.84	0.021639	mitochondrial fatty acid oxidation
1416411_at	*Gstm2*	-2.01	0.012738	conjugation of ROS with glutathione

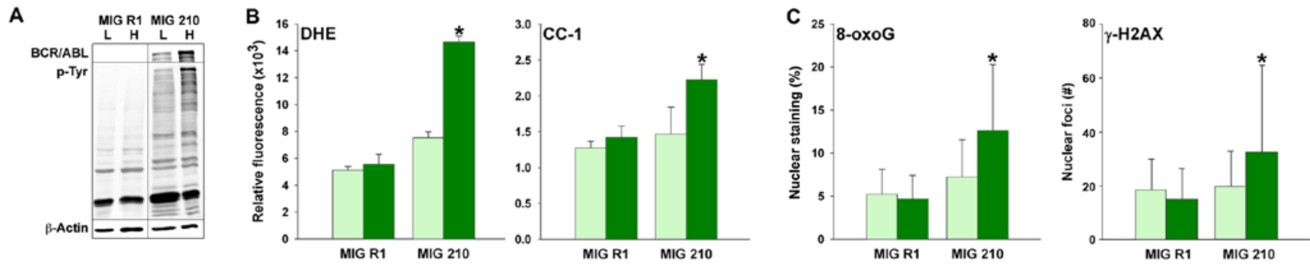
Results are representative for CML-CP –like and normal mice (3 mice per group).



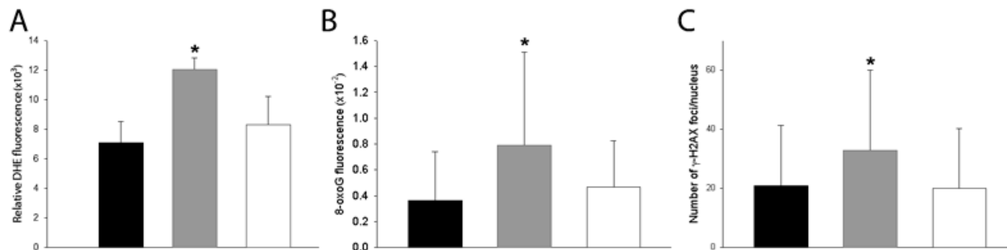
Supplemental Figure 1. **First, second, and third-generation TKIs do not reduce ROS.** Lin$^-$ CD34$^+$ CML-CP cells maintained with growth factors were untreated (C) or treated with 1μM imatinib (IM), 5 μM IM, 2μM nilotinib (NIL), and 90 nM dasatinib (DAS). BCR-ABL1 kinase activity (ABL1-pY245) and ROS [DHE detects •O_2^-, Mitosox Red (MSR) detects mitochondrial •O_2^-] were examined in annexin V-negative cells. Results represent mean ± SD from 3 patients; * $p< 0.01$ in comparison to C.



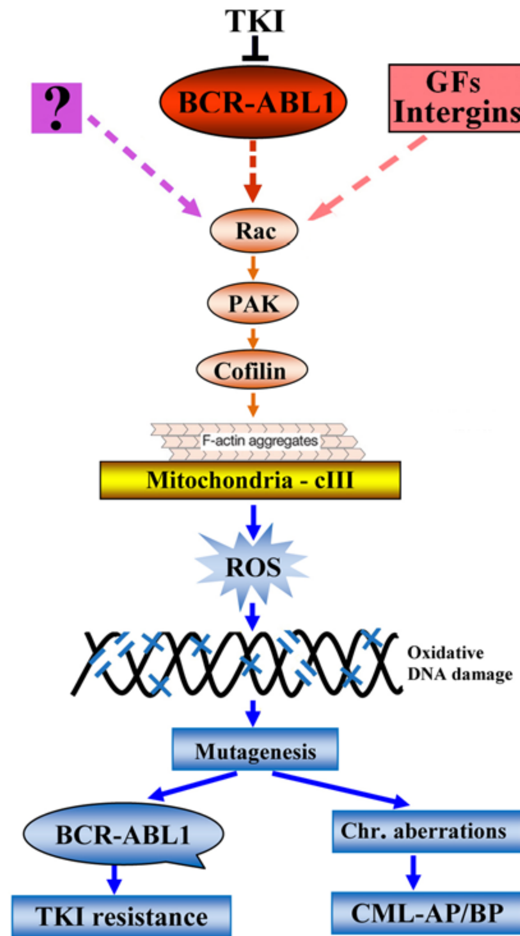
Supplemental Figure 2. **Tet-off SCLtTA/BCR-ABL1 transgenic mice exhibit CML-CP -like disease phenotype. (A)** *BCR-ABL1* mRNA expression in CML-CP –like (gray bars) and healthy (black bars) mice (3 mice per group) by qRT-PCR. *left panel*: *BCR-ABL1* mRNA expression in bone marrow (BM) and spleen (SPL) cells; *right panel*: *BCR-ABL1* mRNA expression in BM-derived HSCs/LSCs (Lin$^-$c-Kit$^+$Sca-1$^+$) and HPCs/LPCs (Lin$^-$c-Kit$^+$Sca-1$^-$). *$p<0.01$ in comparison to the corresponding normal cells (N.D. = not detected). **(B)** Percentage of mature (Gr-1$^+$CD11b$^+$) and immature (Gr-1^{low}CD11b$^+$) granulocytes, B cells (B220$^+$), T cells (CD3$^+$), erythroid cells (Ter119$^+$), and megakaryocytes (CD41$^+$) in BM, SPL, and peripheral blood (PB) of healthy (black bars) and CML-CP -like (gray bars) mice (3 mice per group); *$p<0.05$ in comparison to the cells from healthy mice. **(C)** Spleen weight in CML-CP -like (gray bar) and healthy (black bar) mice (58 and 55 mice, respectively); *$p<0.001$. Representative spleens are shown in the inset.



Supplemental Figure 3. **ROS and oxidative DNA damage is proportional to BCR-ABL1 "dosage" in BCR-ABL1 -transformed human CD34$^+$ cells.** Lin$^-$CD34$^+$ fractions of human BMCs obtained from healthy donors were infected with pMIG-p210BCR-ABL1-IRES-GFP (MIG 210) retroviral construct or empty vector (MIG R1). Cells were maintained in IMDM containing 10% FBS, 1% antibiotic-antimycotic solution, and growth factors (SCF and GM-CSF) required to promote their continuous proliferation. (**A**) Cells expressing the 20% highest (H) and lowest (L) levels of GFP were sorted and analyzed by Western blot to detect p210BCR-ABL1 protein expression (*upper panel*) and cellular protein tyrosine phosphorylation (p-Tyr; *center panel*) in L and H populations; the *lower panel* shows β-Actin for loading control. **(B)** GFP$^+$CD34$^+$ cells were incubated with redox-sensitive fluorochromes DHE and CC-1 to detect various ROS (cellular superoxide and hydrogen peroxide) in H (dark green bars) and L (light green bars) cell populations by FACS. **(C)** Oxidative DNA damage was detected by immunofluorescence in 15-31 cells (8-oxoG) and in 24-53 cells as described in Figure 3; *$p<0.05$ in comparison to MIG 210 (L) and MIG R1 (H).



Supplemental Figure 4. **Kinase-dead BCR-ABL1 K1172R mutant does not induce ROS-induced oxidative DNA damage in human CD34$^+$ cells.** Lin$^-$CD34$^+$ fractions of human BMCs obtained from healthy donors were infected with pMIG-p210BCR-ABL1-IRES-GFP (grey bar), pMIG-p210BCR-ABL1(K1172R)-IRES-GFP (white bar) or empty vector (black bar). Cells were maintained in IMDM containing 10% FBS, 1% antibiotic-antimycotic solution, and growth factors (SCF and GM-CSF) required to promote their continuous proliferation. (**A**) ROS, (**B**) 8-oxoG, and (**C**) γ-H2AX were measured. Results represent mean ± SD from 5 experiments (ROS), 16-83 cells (8-oxoG), and 32-41 cells (γ-H2AX); *$p<0.05$ in comparison to other groups.



Supplemental Figure 5. **Rac-PAK-cofilin-F-actin-mitochondria pathway generates high level of ROS leading to oxidative DNA damage and genomic instability in TKI-naïve and TKI-treated CML-CP LSCs.** In CML-CP cells BCR-ABL1, growth factors (GFs) and integrins, and/or other unknown mechanisms constitutively stimulate Rac, which in turn stably activate PAK resulting in upregulation of phospho-cofilin. This pathway promotes F-actin stabilization and modification of mitochondrial potential resulting in electron leakage from mitochondrial respiratory chain complex III (cIII) causing ROS-induced oxidative DNA damage. This effect, when combined with inefficient/unfaithful DNA repair, can facilitate the appearance of BCR-ABL1 kinase mutants encoding for TKI resistance and accumulation of chromosomal aberrations (Chr. aberrations) resulting in disease relapse and malignant progression toward CML-AP/BP.

REFERENCES

1. Rink L, Slupianek A, Stoklosa T, et al. Enhanced phosphorylation of Nbs1, a member of DNA repair/checkpoint complex Mre11-RAD50-Nbs1, can be targeted to increase the efficacy of imatinib mesylate against BCR/ABL-positive leukemia cells. *Blood*. 2007;110(2):651-660.
2. Schemionek M, Elling C, Steidl U, et al. BCR-ABL enhances differentiation of long-term repopulating hematopoietic stem cells. *Blood*. 2010;115(16):3185-3195.