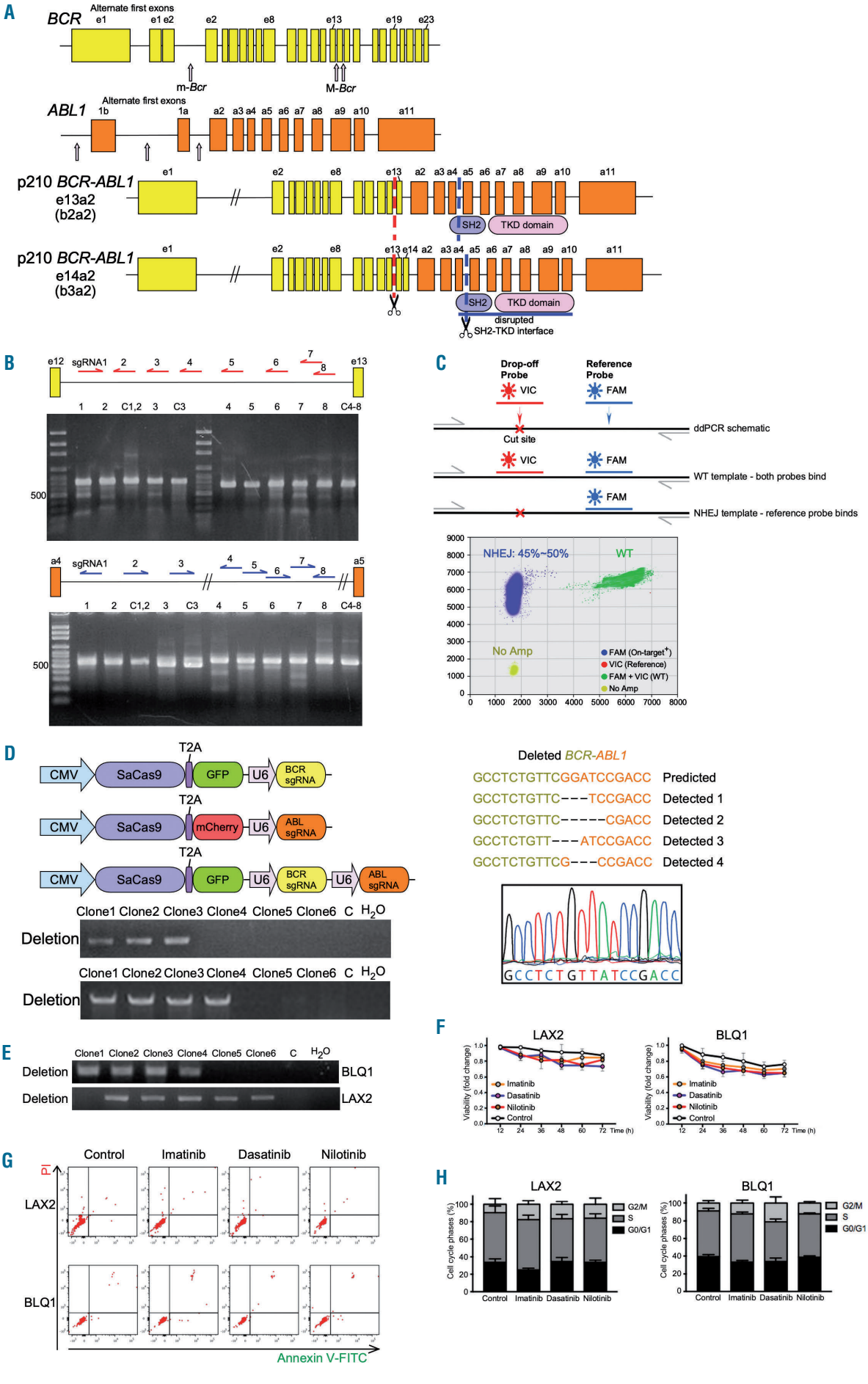
CRISPR/Cas9-mediated gene deletion efficiently retards the progression of Philadelphia-positive acute lymphoblastic leukemia in a p210 BCR-ABL1^{T315I} mutation mouse model

Philadelphia-positive acute lymphoblastic leukemia (Ph$^+$ ALL) refers to the subgroup of ALL with the Philadelphia chromosome, consisting of the t(9;22) translocation, which results in an oncogenic *BCR-ABL1* fusion gene. For decades, Ph$^+$ ALL has been regarded as an ALL subgroup with a poor outcome.[1] Although the introduction of tyrosine kinase inhibitors (TKI) has contributed to improved outcomes, one of the major challenges that lies ahead is the emergence of resistant mutations of the *BCR-ABL1* gene.[2] One point mutation in particular, the T315I mutation, which influences the gatekeeper residue Thr315, is resistant to all currently approved first- and second-generation TKI.[2,3] Ponatinib, a third-generation TKI, is the only TKI effective against the T315I *BCR-ABL1* mutation.[2] However, the recent 5-year results of the phase II PACE trial showed that more than half of Ph$^+$ ALL patients bearing the T315I mutant responded poorly to ponatinib.[4] We report here that *in vivo* delivery of CRISPR/Cas9 can effectively retard rapid progression of Ph$^+$ ALL with the T315I mutation. This CRISPR/Cas9 method only disrupts the *BCR-ABL1* fusion gene, and has the potential to destroy any point-mutated *BCR-ABL1* fusion gene that may be drug-resistant.

BCR-ABL1 is required for both induction and maintenance of leukemia, and switching-off this gene can result in rapid apoptosis of leukemic cells, a phenomenon referred to as "oncogene addiction".[5] As CRISPR/Cas9 technology has shown its undeniable power of genome editing by overcoming the limitations of earlier methods, we reasoned that disrupting the T315I-mutated *BCR-ABL1* gene via CRISPR/Cas9 might revert the leukemia phenotype. Since normal *BCR* and *ABL* genes are also expressed in non-leukemc cells in Ph$^+$ ALL patients, it is essential to destroy only the *BCR-ABL1* fusion genes while leaving the expression of normal *BCR* and *ABL* genes unimpaired. Consequently, one strategy considered was to target introns rather than exons. Conceivably, paired single guide RNA (sgRNA) that target the introns of *BCR* and *ABL* could enable ablation of the *BCR-ABL1* fusion gene while leaving the non-leukemic cells unaffected (*Online Supplementary Figure S1A, B*). Considering the fact that the CRISPR-mediated deletion frequency is inversely related to the deletion size,[6] the targeted sequences we chose for paired sgRNA were adjacent to the *BCR-ABL1* junction sequences (Figure 1A). Although *BCR-ABL1* has diverse breakpoints, the fusion hybrids from the patient-derived pre-B ALL samples in our hands express p210 *BCR-ABL1* isoforms, so we designed the sgRNA specifically against p210 *BCR-ABL1*. P210 fusion proteins usually comprise products of either the b2a2 or the b3a2 exon junction, corresponding to the fusion of *BCR* exon 13 and *ABL1* exon 2 (e13a2) or e14a2.[7]

To save the effort of distinguishing the p210 subtype before CRISPR/Cas9 editing, we selected the commonly owned intron 12 by b3a2 and b2a2 p210$^{BCR-ABL1}$ fusion gene as the target site for the BCR gene. For the *ABL* gene, the target site we chose was its intron 4, where the SH2 domain spans and is before the tyrosine kinase domain (TKD) of ABL kinase so that the size of the ablated *BCR-ABL1* fragment would be around 10 kb (Figure 1A). Another thought was that the absence of the SH2-TKD interface, caused by ablation of the SH2 domain, would disable the oncogenic potential of *BCR-ABL1*.[8] To avoid affecting RNA splicing, the targeted sites for sgRNA were at least 100 bp away from the 5' or 3' end of the introns. Publicly available tools (crispr.mit.edu and Benchling) were used to find sgRNA with minimal off-target DNA cuts.[9] We also chose SaCas9 rather than the SpCas9 to further minimize the possibility of off-target effects.[10] Eight sgRNA for either *BCR* intron 12 or *ABL* intron 4 were chosen and engineered into the GFP- or mCherry-expressing pX601 plasmids that we had previously constructed.[11] The plasmids were transfected into 293T cells followed by a Surveyor assay,[12] and the sgRNA7 for *BCR* and sgRNA4 for *ABL1* were selected for the subsequent experiments because of their comparatively higher targeting efficiency, which was between 30% and 45% (Figure 1B). The frequency of indels was determined for the top five genomic off-target locations as predicted by the design tool, and no indels were detected by the Surveyor assay (*Online Supplementary Table S1*).

Next, we examined the *BCR-ABL1* ablation by transfecting the paired plasmids, pX601-BCR-intron12-GFP and pX601-ABL-intron4-mCherry, into K562 cells, which express b3a2 p210$^{BCR-ABL1}$. GFP and mCherry double-positive cells were sorted and seeded into the individual wells, each of which contained 2000 - 3000 cells. The cells in each well were defined as "a clone" for the sake of descriptive convenience, although the clone was not necessarily a homogeneous population. The percentage of the ablated clone was defined as "ablation efficiency" and the percentage of non-homologous end joining (NHEJ) events in each ablated clone was defined as "on-target efficiency". Polymerase chain reaction (PCR) and the subsequent Sanger sequencing with PCR products were performed 48 to 72 h after transfection, and the ablated *BCR-ABL1* could be detected in about 50% of the clones (*Online Supplementary Figure S1C*). Since the K562 cell line contains multiple copies of *BCR-ABL1*, this could presumably account for the observation that only 50% of the clones were ablated. To further evaluate the NHEJ event in each ablated clone, we adopted the droplet digital PCR (ddPCR) method, which is a well-proven approach for detecting on-site editing of CRISPR/Cas9.[13] The results showed that the efficiency of NHEJ-mediated targeting was 45%~50% on either the *BCR* or *ABL1* site (Figure 1C). Of note, the majority of the cells in the seeded clones that were positive for *BCR-ABL1* ablation underwent rapid cell death within 4 to 5 days after transfection, indicating the dominant role of *BCR-ABL1* for the survival of leukemic cells addicted to this fusion gene. Considering the limited survival time of these targeted cells, it was hard to define the ablation efficiency at the single-cell level because the time required for the proliferation of a single cell to the cell population that could have enabled the PCR detection was far longer than the expected lifespan of this targeted single cell. Therefore, instead of resorting to the laborious single-cell sequencing method, we evaluated the ablation efficiency at the clonal level. Despite the acceptable efficiency, expression of two sgRNA from separate constructs would be impractical *in vivo*. We therefore incorporated two sgRNA into one construct that also simultaneously expressed SaCas9, and an improved ablation efficiency of around 55 - 65% was subsequently observed (*Online Supplementary Figure S1D*). For the purpose of *in vivo* genome editing, we engineered the lentiviral CRISPR vector encoding SaCas9 to express either one or two sgRNA. The observed ablation efficiency was around 40 - 50% for the dual vector system


A
BCR
Alternate first exons
e1
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e8
e13
e19
e23
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M-Bcr
ABL1
Alternate first exons
1b
1a
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a3 a4
a5
a6
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a8
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p210 BCR-ABL1
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SH2
TKD domain
p210 BCR-ABL1
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(b3a2)
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disrupted
SH2-TKD interface
B
e12
sgRNA1
e13
C1,2
C3
C4-8
500
a4
a5
C
Drop-off Probe
Reference Probe
VIC
FAM
Cut site
ddPCR schematic
WT template - both probes bind
NHEJ template - reference probe binds
NHEJ: 45%~50%
WT
No Amp
FAM (On-target+)
VIC (Reference)
FAM + VIC (WT)
D
T2A
CMV
SaCas9
GFP
U6
BCR sgRNA
mCherry
ABL sgRNA
Clone1 Clone2 Clone3 Clone4 Clone5 Clone6 C H2O
Deletion
Deleted BCR-ABL1
GCCTCTGTTCGGATCCGACC Predicted
GCCTCTGTTC---TCCGACC Detected 1
GCCTCTGTTC-----CGACC Detected 2
GCCTCTGTT---ATCCGACC Detected 3
GCCTCTGTTCG---CCGACC Detected 4
E
BLQ1
LAX2
F
LAX2
BLQ1
Viability (fold change)
Imatinib
Dasatinib
Nilotinib
Control
Time (h)
G
Control
Imatinib
Dasatinib
Nilotinib
Annexin V-FITC
H
Cell cycle phases (%)
G2/M
S
G0/G1

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Figure 1. CRISPR/Cas9-mediated genome editing to target p210$^{BCR-ABL1}$ with T315I mutation. (A) A schematic of the chimeric p210$^{BCR-ABL1}$ genes derived from the various breaks with the indicated loci for the CRISPR/Cas9-mediated targeting. TKD, tyrosine kinase domain. (B) Surveyor assay to detect the gene editing efficiency mediated by CRISPR/Cas9 plasmids in 293T cells. Eight single guide (sg) RNA to target either BCR (top panel) or *ABL* (bottom panel) are indicated. C, non-transfected control cells. (C) The digital droplet polymerase chain reaction (PCR) assay to detect non-homologous end joining (NHEJ) events in K562 clones with the transfection of paired CRISPR/Cas9 plasmids. Top panel: primer and probe design strategy for detection of NHEJ editing on either the *BCR* or *ABL1* targeting site; bottom panel: representative two-dimensional droplet fluorescence intensity plot of an NHEJ drop-off assay. WT, wildtype. (D) Structure of the lentiviral CRISPR vectors and detection of *BCR-ABL1* ablation in K562 cells after the transduction of two individual lentiviruses (left top panel) or the "2-in-1" lentivirus (left middle panel). Representative sequences of the PCR products derived from the "2-in-1" lentivirus-transduced K562 cells showing the correct *BCR-ABL1* ablation are displayed in the left bottom and right panels. C, non-transfected control cells. (E) PCR detection of *BCR-ABL1* ablation in LAX2 and BLQ1 sorted clones (GFP$^+$) after 3 days of transduction of "2-in-1" CRISPR/Cas9 lentivirus. Six representative clones ("Clone 1" to "Clone 6") were compared with non-transfected control cells ("C"). (F) The viability of LAX2 and BLQ1 cells was measured by CCK-8 assay after 3 days of treatment with various tyrosine kinase inhibitors (TKI). (G) Apoptosis of LAX2 and BLQ1 cells, analyzed by an annexin-V-fluorescein isothiocyanate/propidium iodide staining assay upon 3 days of treatment with various TKI. (H) The cell cycle of LAX2 and BLQ1 cells analyzed by bromodeoxyuridine incorporation assay upon 3 days of treatment with various TKI.

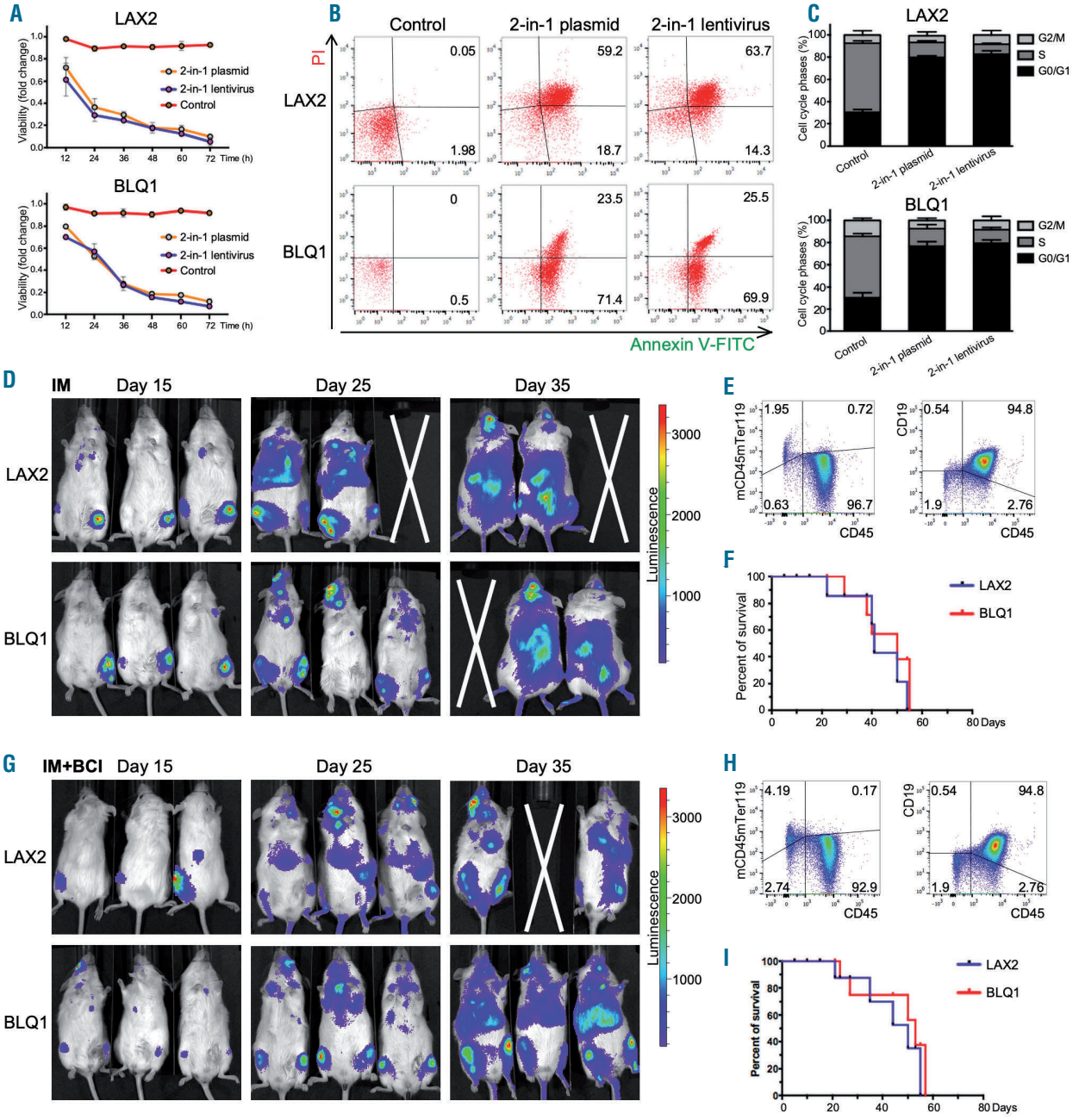

Figure 2. Reverting the tumorigenicity of Philadelphia-positive acute lymphoblastic leukemia with the T315I mutation by CRISPR/Cas9-mediated *in vivo* targeting. *(continued on the next page)*

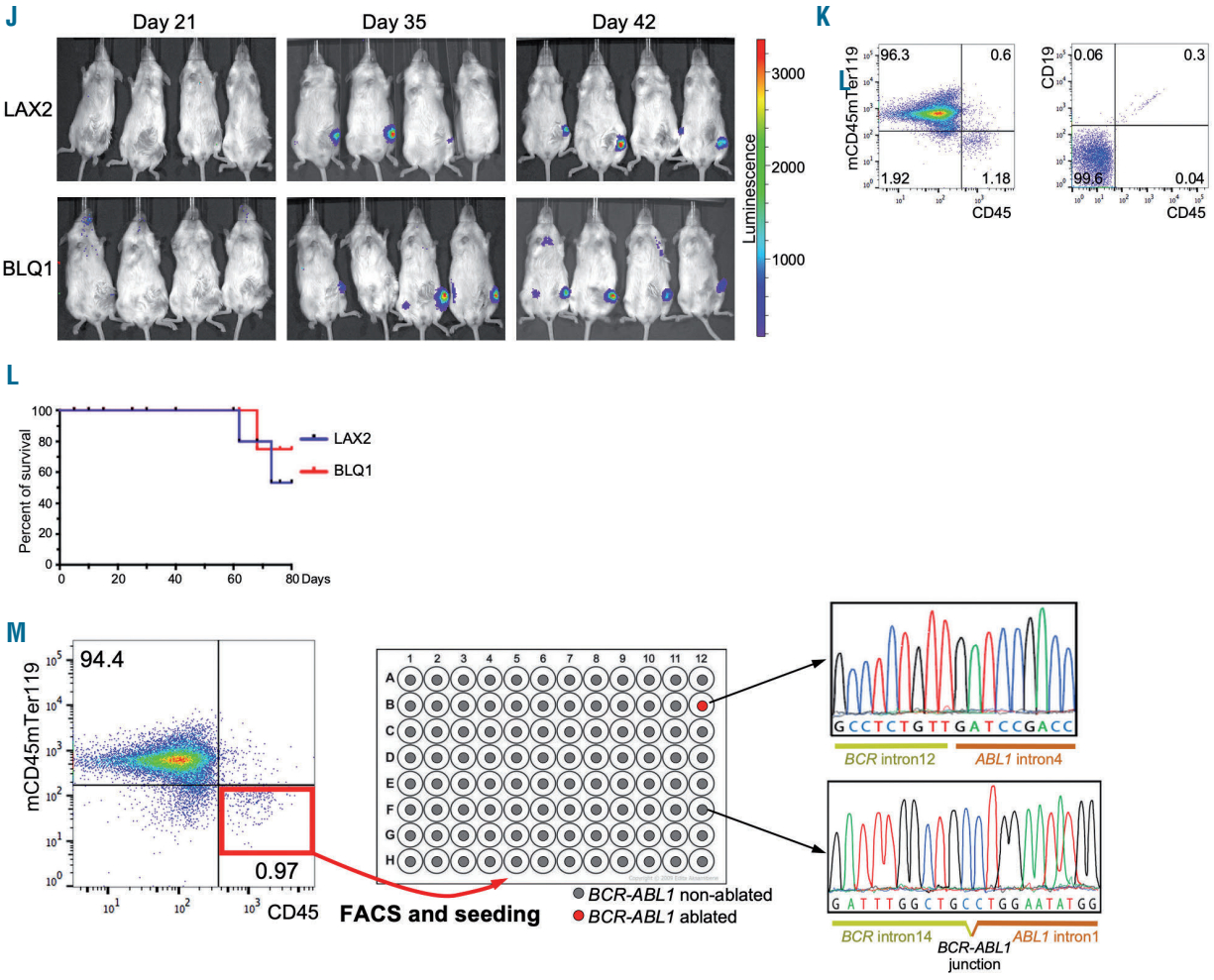

Figure 2. Reverting the tumorigenicity of Philadelphia-positive acute lymphoblastic leukemia with the T315I mutation by CRISPR/Cas9-mediated *in vivo* targeting. (A) The viability of LAX2 and BLQ1 cells upon transfection with "2-in-1" plasmid or transduction of 2-in-1 lentivirus that simultaneously targets *BCR* intron 12 and *ABL* intron 4. (B) Apoptosis of LAX2 and BLQ1 cells analyzed by an annexin-V-fluorescein isothiocyanate/propidium iodide staining staining assay upon 3 days of treatment with the "2-in-1" plasmid or lentivirus. (C) The cell cycle of LAX2 and BLQ1 cells analyzed by the bromodeoxyuridine incorporation assay upon 3 days of treatment with the "2-in-1" plasmid or lentivirus. (D) LAX2 and BLQ1 cells that were pre-infected with the lentiviral vectors expressing firefly luciferase were intrafemorally injected into sublethally irradiated NSG mice followed by an intraperitoneal injection of 1 μM/L imatinib (IM) every other day. The leukemia burden was then measured by luciferase bioimaging, and bone marrow aspiration was performed on day 35 after transplantation in order to measure the human cell chimerism ($CD45^+$) and proportion of B cells ($CD19^+$) as shown in (E). The overall survival of the recipient mice (n=8 per group) was plotted by Kaplan-Meier analysis as shown in (F). (G-I) The same experiments as described in (D-F) except for the addition of BCI (5 μM/L) to IM. (J-L) The same experiments as described in (D-F) except for the treatment methods for the recipient mice, which were given 40 μL pre-titrated "2-in-1" lentiviruses at a multiplicity of infection (MOI) of 40, injected intrafemorally for 1 month at 7-day intervals. (M) The bone marrow of recipient mice was harvested 40 days after transplantation and subjected to fluorescence-activated cell sorting for human $CD45^+$ cells, which were then seeded as individual clones in a 96-well plate. *BCR-ABL1* ablation was determined by polymerase chain reaction (PCR) analysis with subsequent Sanger sequencing of the PCR products.

and around 60 - 70% for the single vector system (named the "2-in-1" method) in K562 cells (Figure 1D), so the "2-in-1" method was used hereafter.

Next, the "2-in-1" CRISPR/Cas9 lentiviruses were used to transduce patient-derived pre-B ALL samples, LAX2 and BLQ1 cells,[14] which contain the T315I mutated $p210^{BCR-ABL1}$. The ablation efficiency of *BCR-ABL1* observed in LAX2 and BLQ1 was the same as that in K562 cells (Figure 1E). To verify the property of multidrug-resistance, LAX2 and BLQ1 were treated with imatinib, dasatinib or nilotinib prior to investigation of cell proliferation, apoptosis and the cell cycle. Expectedly, the cells did not respond to TKI treatment (Figure 1F-H), even when the TKI was combined with the small molecule BCI, a drug that has been shown to overcome conventional mechanisms of drug resistance in patient-derived pre-B ALL cells[14] (*Online Supplementary Figure S1E-G*). In stark contrast, however, the CRISPR/Cas9 plasmid-transfected or lentivirus-transduced cells underwent rapid proliferation arrest and apoptosis (Figure 2A-C).

Finally, we examined the effects of *in vivo* genome editing. By transplanting LAX2 or BLQ1 into immunodeficient NOD-$Prkdc^{scid}$IL2rg^{Tm1Wjl} mice (NSG mice, which were maintained at the University of California San Francisco in accordance with Institutional Animal Care and Use Committee-approved protocols), we established patient-derived xenograft models and observed rapid progression of pre-B ALL in both LAX2- and BLQ1-recipient mice. Specifically, the onset of ALL in the bone marrow could be observed within a week and full-blown leukemia with extremely severe extramedullary involvement in the whole body could be developed within 35 to 40 days (*Online Supplementary Figure S2A-C*). The

intraperitoneal injection of imatinib or imatinib plus BCI hardly delayed the disease onset or progression, even when the drugs were administered for 1 month at intervals of 2 days (Figure 2D-I). However, when the CRISPR/Cas9 lentiviruses were injected intrafemorally from post-transplant day 7 for 1 month at intervals of 7 days, the leukemia phenotype was significantly reverted. Specifically, the progression of leukemia was significantly delayed, such that the ALL had not spread throughout the body even at 40 days after transplantation, and animals' survival time was distinctly prolonged (Figure 2J, L). Consistent with the flow cytometry data showing that the percentage of human CD45$^+$ leukemic cells was only 1.18% on post-transplant day 35 (Figure 2K), ddPCR only detected 1.1%~1.5% of human *BCR-ABL1*$^+$ transcripts among the total bone marrow cells on post-transplant day 35 and 3.2%~4.1% on post-transplant day 42; ddPCR did not detect any *BCR-ABL1*-ablated transcripts (*Online Supplementary Figure S2D*). To further verify this, we sorted the CD45$^+$ human cell population from the bone marrow of recipient mice 40 days after transplantation and seeded them as individual clones for DNA extraction and Sanger sequencing. Expectedly, we could hardly find one positive clone for the ablated *BCR-ABL1* among the majority of negative clones (Figure 2M), confirming that the Ph$^+$ ALL cells were addicted to the existence of *BCR-ABL1* for their survival and proliferation.

In conclusion, we adopted the CRISPR/Cas9 genome editing tool, both *in vitro* and *in vivo*, to efficiently mitigate the oncogenic effects of Ph$^+$ pre-B ALL with the T315I mutation, which is a form of ALL resistant to treatment with most TKI. The results raise the possibility that the same strategy coud be used to disrupt the expression of *BCR-ABL1*, and so revert its tumorigenicity, no matter what new drug-resistant mutations the fusion gene acquires. However, the current strategy does not target 100% of leukemic cells *in vivo*, which allows the non-targeted malignant clones, even though very few at the beginning, to re-establish leukemia in the long run. Therefore, before CRISPR/Cas9 technology can be translated into a therapy for the treatment of *BCR-ABL1*-driven leukemia, the transduction efficiency of the lentiviral vector must be improved and further investigations performed on its combination with other therapeutic regimens.

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