ETV6/RUNX1 FUSION GENE ABROGATION DECREASES THE ONCOGENIC POTENCIAL OF TUMOUR CELLS IN A PRECLINICAL MODEL OF ACUTE LYMPHOBLASTIC LEUKAEMIA.

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31 ABSTRACT

32 Background: The t(12;21)(p13;q22), which fuses *ETV6* and *RUNX1* genes, is the most common genetic
33 abnormality in children with B-cell precursor acute lymphoblastic leukaemia. The implication of the
34 fusion protein in leukaemogenesis seems to be clear. However, its role in the maintenance of the disease
35 continues to be controversial. Aim: To eliminate the expression of the *ETV6/RUNX1* fusion gene, in order
36 to elucidate the effect in the leukaemic cells. Methods: Generation of an *in vitro* *ETV6/RUNX1* knock out
37 model using the genetic modification system CRISPR/Cas9. Functional studies and generation of edited-
38 cell xenograft model were carried out. Results: For the first time, the expression of *ETV6/RUNX1* fusion
39 gene was completely eliminated, thus generating a powerful model on which to study the role of the
40 fusion gene in leukaemic cells. *ETV6/RUNX1* inactivation caused the deregulation of cellular processes
41 that could be participating in the maintenance of the leukaemic phenotype, such as differentiation and
42 lymphoid activation, apoptosis, cell signaling and cell migration. Tumour cells showed higher levels of
43 apoptosis, lower proliferation rate and a greater sensitivity to PI3K inhibitors *in vitro* along as a decrease
44 in tumour growth in xenografts models after *ETV6/RUNX1* fusion gene abrogation. Conclusions:
45 *ETV6/RUNX1* fusion protein plays a fundamental role in the maintenance of the leukaemic phenotype,
46 thereby being making the fusion protein a potential therapeutic target.

47

48 BACKGROUND

49 The gene fusion between the transcription factors *ETV6* (*TEL*) and *RUNX1* (*AML1*) is generated by
50 t(12;21)(p13; q22), the most frequent chromosomal translocation in children with acute lymphoblastic
51 leukaemia (ALL).^{1,2} Patients carrying this translocation are associated with a good prognosis and
52 excellent molecular response to treatment. However up to 20% of cases relapse.³⁻⁷ Furthermore, the
53 response to treatment of some relapse cases is associated with resistance to treatments such as
54 glucocorticoids (GCs),⁸ and these patients must be treated with stem cell transplantation.⁹
55 *ETV6/RUNX1* (E/R) protein is known to play a role in the development of B-ALL, but by itself it is not
56 able of initiating the disease. Postnatal genetic events are required for the development of clinically overt
57 leukaemia. These second events are usually mutations or deletions, such as the loss of wild type (WT)
58 allele of *ETV6*.¹⁰ Recent studies suggest that E/R is responsible for the initiation of leukaemia and also
59 essential for disease progression and maintenance, through deregulation of different molecular pathways
60 that contribute to leukaemogenesis. E/R regulates phosphoinositide 3-kinase (PI3K)/Akt/mammalian

target of rapamycin (mTOR) (PI3K/AKT/mTOR) pathway, which promotes proliferation, cell adhesion and DNA damage response; *STAT3* pathway involved in self-renewal and cell survival and *MDM2/TP53* whose deregulation induces the inhibition of apoptosis and consequently cell survival.¹¹ Therefore, the fusion protein could thus be intervening in relapse processes and in the lack of response to treatment. However, the functional studies carried out by the silencing of *E/R* fusion gene expression, mediated by siRNA and shRNA, reveal that there is still controversy about the role of the oncoprotein in the maintenance of the leukaemic phenotype. Thus *E/R* silencing by siRNA neither induced cell cycle arrest/apoptosis nor attenuated clonogenic potential of cells. Therefore the *E/R* fusion protein may be dispensable for the survival of definitive leukaemic cells.¹² By contrast, other studies showed that *E/R* expression was critical for the survival and propagation of the respective leukaemia cells *in vitro* and *in vivo*.¹³ These results arise some doubts about the implications of the fusion protein in tumour cells. The implementation of new genetic editing strategies has allowed the development of functional studies by generation of gene and gene fusion KO models, both *in vitro* and *in vivo*.¹⁴ In this study, we completely abrogated the expression of *E/R* fusion protein in REH ALL cell line using the CRISPR/Cas9 editing system and we studied the effects of genetic ablation of the fusion protein in cellular functions. We also studied whether the suppression of *E/R* expression sensitize tumour cells to PI3K inhibitors. Finally, we generated a xenograft mouse model to study the oncogenic potential of tumours cells after *E/R* depletion. In summary, we provide evidence that fusion protein has a key role in the maintenance of the leukaemic phenotype.

MATERIAL AND METHODS

Cell lines and culture conditions

REH, obtained from DMSZ German collection (ACC 22), is a cell line established from the peripheral blood of a patient with ALL who carried t(12;21) and del(12) producing respective *E/R* fusion and deletion of residual *ETV6*. REH was maintained in RPMI 1640 (Life Technologies, Carlsbad, California, USA) supplemented with 15% fetal bovine serum (FBS) and 1% of Penicillin/ Streptomycin (P/S) (Life Technologies). Stromal HS-5 cell line was obtained from ATCC collection (CRL-11882) and maintained in DMEM (Life Technologies, Carlsbad, California, USA) supplemented with 10% FBS and 1% of P/S. Both cells lines were maintained at 37°C with 5% CO₂.

sgRNAs design and cloning

Based on the methodology of CRISPR/Cas9, two single guides RNAs (sgRNAs) (G1 and G2) were designed with the Broad Institute CRISPR designs software (<http://www.broadinstitute.org/rnai/public/analysis-tools/sgna-design>). One of them directed towards the end of exon 5 of *ETV6* and other directed towards the beginning of intron 5-6, both before the fusion point, with the intention of producing indels or deletions that modify the open reading frame of the oncogene, and, therefore, the gene expression. These sgRNAs were cloned into a vector containing the Cas9 nuclease coding sequence and GFP, pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid #48138)¹⁵ as described previously¹⁴ (Supplementary Table 1). Then they were electroporated into the REH cells.

sgRNA transfections

REH ALL cells (4×10^6 cells) were electroporated with 4 µg of both plasmid constructs¹⁴ (PX458 G1 and PX458 G2) using the Amaxa electroporation system (Amaxa Biosystem, Gaithersburg, MD, USA) according to supplier's protocol.

Flow cytometry analysis and cell sorting

Seventy-two hours after sgRNAs transfection, GFP-positive cells were selected by fluorescence-activated cell sorting (FACS) using FACS Aria (BD Biosciences, San Jose, California, USA). Single-cells were seeded in 96-well plate by FACS, establishing the different KO and control clones.

Sequencing of sgRNA targets sites

Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. To amplify the region of *E/R* fusion, PCR was performed using the following primers: forward 5'- ACCCTCTGATCCTGAACCCC- 3' and reverse 5'- GGATTTAGCCTCATCCAAGCAG- 3'. PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Basilea, Switzerland) and were sequenced by the Sanger method using each forward and reverse PCR primers (Supplementary table 2).

The editing efficiency of the sgRNAs and the potential induced mutations were assessed using Tracking of Indels by Decomposition (TIDE) software (<https://tide-calculator.nki.nl>; Netherlands Cancer Institute), which only required two Sanger sequencing runs from wild-type cells and mutated cells.

Off-target sequence analysis

The top four predicted off-target sites obtained from "Breaking Cas" website (<http://bioinfogp.cnb.csic.es/tools/breakingcas/>) were analyzed by PCR in the different clones (Supplementary table 2) before to functional and xenograft experiments.

121 **qPCR**

122 Total RNA extraction was performed with the RNeasy Kit (Qiagen) as suggested by the manufacturer.
 123 Real-time reverse transcriptase– polymerase chain reactions (RT-PCRs) were performed as described ¹⁶.
 124 The primers for *E/R* (sense, 5 - CTCTGTCTCCCCGCCTGAA
 125 -3; antisense, 5 - CGGCTCGTGCTGGCAT-3), were designed. Real-time RT-PCR data shown include at
 126 least 3 independent experiments with 3 replicates per experiment.

127 **Transcriptome sequencing**

128 RNA-seq was performed by using SMART-Seq v4 Ultra Low Input RNA kit (Clontech, California,
 129 U.S.). In all samples, RNA was analyzed following manufacturer’s recommendations for the protocol
 130 “Illumina library preparation using Covaris shearing”. Libraries were sequenced in the HiSeq400
 131 platform (Illumina) according to manufacturer’s description with a read length of 2×150 nucleotides.
 132 Briefly, bcl files were demultiplexing on BaseSpace (Illumina Cloud based resource) to generate fastq
 133 files. Raw data quality control was performed with fastQc (v0.11.8), globin contamination was assessed
 134 with HTSeq Count, FastQ screen evaluated ribosomal RNA contamination and other external possible
 135 resources of contamination (Mus musculus, Drosophila melanogaster, Caenorhabditis elegans and
 136 mycoplasma). STAR (v020201) was used for the alignment (hg19 reference genome) and FeatureCounts
 137 (v1.4.6) to generate the read count matrix. Finally, DESeq2 was used for differentially gene expression
 138 analysis. Of noted, DESeq2 model internally corrects for library size therefore normalizes the values and
 139 enables paired comparisons. Heatmap was performed in R.
 140 Go enrichment analysis (<http://geneontology.org>) to evaluate whether a set of genes was significantly
 141 enriched between the different comparisons was used. The most significant biological mechanisms,
 142 pathways and functional categories in the data sets of genes selected by statistical analysis were identified
 143 through PANTHER Overrepresentation Test.

144 **Western blotting**

145 Protein expression was assessed by SDS-PAGE and western blotting (WB). The antibodies were obtained
 146 from Cell Signaling Technology (Danvers, MA, USA) including a human anti-Bcl-2 antibody (1:1000;
 147 2872) for Bcl-2, a human anti-Bcl-xL antibody (1:1000; 2762) for Bcl-xL, a human anti-phospho Akt
 148 antibody (1:1000; 4060) for p-AKT (Ser473) and a human anti-phospho mTOR antibody (1:1000;2971)
 149 for p-mTOR (Ser2448). Anti-rabbit IgG horseradish peroxidase-conjugated (1:5000; 7074) was used as a

secondary antibody. Antibodies were detected using ECLTM WB Detection Reagents (RPN2209, GE Healthcare, Illinois, Chicago, USA). ImageJ software was used for densitometric analysis.^{17,18}

Apoptosis, cell cycle analysis and proliferation assays

Apoptosis was measured by flow cytometry with an annexin V-Dy634 apoptosis detection kit (ANXVVKDY, Immunostep, Salamanca, Spain) following the manufacturer's instructions. Briefly, 5×10^5 cells were collected and washed twice in PBS and labeled with annexin V-DY-634 and non-vital dye propidium iodide (PI), allowing the discrimination of living-intact cells (annexin-negative, PI-negative), early apoptotic cells (annexin-positive, PI-negative) and late apoptotic or necrotic cells (annexin-positive, PI-positive). In parallel, cell distribution in the cell cycle phase was also analyzed by measuring DNA content (PI labeling after cell permeabilization). These experiments were carried out after 24, 48 and 72 culture hours.

For proliferation measuring, MTT assays and labeling of cells with CellTrace CFSE Cell Proliferation Kit (Thermo Fisher) were used. In MTT assays, cells were plated on 96-well plates, cell density varied according to the days of the experiment, in a range between 3×10^4 and 5×10^3 cells (24-240 hours). MTT solution (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added at concentration of $0.5 \mu\text{g}/\mu\text{L}$ (Merck, Darmstadt, Germany). After incubation for 3-4 hours at 37°C , cells were lysed with the solubilization solution (10% SDS in 0.01M HCl) and absorbance was measured in a plate reader at 570 nm. For labeling of cells, 3×10^5 cells were stained with CellTrace-CFSE following the manufacturer's instructions and plated on 6-well plates. After 48 hours, CFSE expression was measured by flow cytometry.

B-ALL-stromal cell co-culture

HS-5 human mesenchymal stromal cells (MSCs) were plated at a density of 1×10^5 cells per coverslip in 6-well plates. After 24h, control cells and E/R KO clones were stained with Celltrace-CFSE and 3×10^5 cells placed on top of the stromal cell monolayer. Cells were co-cultured during 48h in RPMI 1640 supplemented with 15% FBS and 1% of P/S at 37°C with 5% CO₂.

Drugs and treatments

The followings drugs were used: Vincristine and Copanlisib (BAY 80-6946) were obtained from Selleckchem (Houston, USA) and Prednisolone (P6004) obtained from Merck. All drugs were prepared at the appropriate stocking concentrations in DMSO (Merck) and stored at -20°C until use.

Mouse xenograft tumourigenesis

16 four- to five-week-old female NOD/SCID/IL2 receptor gamma chain null (NSG) mice (Charles River, Barcelona, Spain) were used. 5×10^6 tumour cells from REH or control clone were subcutaneously injected into the left flank and tumour cells from KO clones (KO1, KO2 and KO3) were injected in the right flank as described previously.¹⁴ REH vs KO2 in the group 1, REH vs KO3 in the group 2, control clone vs KO1 in the group 3 and control clone vs KO2 in the group 4 (4 mice per group). The study received prior approval from the Bioethics Committee of our institution and followed the Spanish and European Union guidelines for animal experimentation (RD 53/2013 and 2010/63/UE). Tumour diameters were measured every 2–3 days with a caliper. Tumour volume was calculated as described elsewhere by the formula $a^2b\pi/6$ (a and b being, respectively, the smallest and the biggest diameters). Mice were sacrificed by anesthesia overdose when tumour volume reached 2 cm³ or 48–62 days after cell injection, upon which the tumours were collected and weighted.

Histopathology and immunohistochemistry

Excised tumours were sampled just after sacrifice and representative areas were a) formalin-fixed (24 hours) (Merck Millipore) and paraffin-embedded and (b) snap-frozen in OCT and stored at 80°C as previously described.¹⁹ Tissue sections 2μM thick were stained with hematoxylin & eosin (H&E) and prepared for immunohistochemistry (IHC). IHC was performed as previously described¹⁹ using the anti-Ki67 primary antibody (Merck Millipore). The number of mitotic figures were counted in 6 high-power field.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 Software. Differences in relative expression of E/R and cell viability after treatments were tested by Tukey's multiple comparisons test. Differences in protein expression, proliferation and apoptosis levels were tested by unpaired *t*-test. Differences in tumour masses over time were tested by non-parametric Mann Whitney U test followed by Tukey's multiple comparisons test and parametric Student's *t* test. Statistical significance at values of $P \leq 0,05$ (*), $P \leq 0.005$ (**) and $P \leq 0,001$ (***) was noted.

RESULTS

CRISPR/Cas9 edited lymphoid cell line showed a loss of E/R functionality

E/R sequence was edited by CRISPR/Cas9 and evaluated by Sanger sequencing in REH cells (Supplementary Figure 1). The edition efficiency evaluated through TIDE was 62,6% with sgRNA G1

210 and 70,6% with sgRNA G2. The most frequent generated mutations were insertions up to 4 base pairs
211 (bps).

212 Single-edited cells were seeded into a 96 well plate to obtain clones with a single edition that predicted a
213 KO sequence for the oncogene. Only 48 single cell clones proliferated in culture. These clones were
214 screened by sanger sequencing and the results revealed that more than 50% of clones (25/48) presented an
215 edited *E/R* sequence. Among them, three single-edited cell clones “KO1, KO2 and KO3 clones” with a
216 predicted *E/R* KO sequence were selected for the study. These clones had different editions in their
217 sequences. KO1 clone carried an insertion of two cytosines at the end of exon 5, near the PAM sequence
218 of the sgRNA G2, thus a frameshift mutation that generated a stop codon before finishing the exon. On
219 the other hand, KO2 and KO3 had an insertion of 5 and 3 nucleotides respectively near the PAM
220 sequence within exon 5, followed by a deletion of 100 bps approximately between both sgRNAs. These
221 alterations modified the open reading frame, generating the stop codon in the next exon. In addition, the
222 loss of the splicing region prevented the correct processing of the protein. Additionally, two single-edited
223 cell clones with WT *E/R* sequence were used as control clones “Control 1 and Control 2” (Supplementary
224 Figure 2).

225 In order to check the functionality of the *E/R* alleles carrying these clones, the expression of the fusion
226 transcript *E/R* was quantified by quantitative PCR. For that, RNAs of the different clones were extracted
227 and transcribed to cDNA. Quantification revealed a total loss of *E/R* mRNA expression in KO2 and KO3
228 clones as compared to control clones ($P<0.001$) and a leaky expression in KO1 clone ($P<0.001$) (Figure
229 1).

230 The four most likely off-target sequences from both guides were analyzed by sequencing Sanger into the
231 different clones. The study of the obtained sequences revealed the lack of editing in those regions,
232 confirming the absence of CRISPR/Cas9 off-targets (data not shown).

233 **Transcriptomic analysis of *E/R* KO lymphoid cell line generated by CRISPR/Cas9 showed a** 234 **distinct expression signature and a deregulation of its downstream signaling genes**

235 The gene expression profile analysed by total RNA-sequencing showed a distinct expression signature in
236 *E/R* KO clones as compared with REH cells and control clones. 342 genes were significantly deregulated
237 after *E/R* fusion gene depletion ($q<0.05$), 180 upregulated and 160 downregulated (Supplementary table
238 3). Some of these genes have been previously associated with ALL pathology and have also been shown

239 to be directly regulated by *RUNXI* transcript factor²⁰ (Table 1). The gene expression profile of the top50
240 of the most deregulated genes according to fold change (FC) values is shown in Figure 2.

241 In order to elucidate the effect of *E/R* fusion gene abrogation on a functional level, the significantly
242 deregulated genes were grouped into a cellular processes according to their function by enrichment
243 analysis (Supplementary table 4). These cellular processes were manually classified into 11 categories:
244 differentiation and lymphoid activation, immune response, response to stimuli, cell activation, apoptosis,
245 cell signaling, chemical homeostasis, GPTase regulation, cell migration, location of cellular components
246 and neuronal system development.

247 Apoptosis was one of the altered cellular processes. In particular, an overexpression of *miR-146* was
248 observed in the *E/R* KO clones as compared with control clones ($P<0.001$). *miR-146* can regulate the
249 expression of the apoptosis factor *STAT1*, and the anti-apoptosis factor *Bcl-xL*, thus promoting the
250 apoptosis of ALL cells.²¹ Furthermore, tumour protein P63 gene (*TP63* gene) was also upregulated in *E/R*
251 KO clones as compared with control clones ($P<0.001$). This gene is involved in an antiapoptotic pathway
252 that regulates the normal survival of B cells.^{22,23}

253 An overexpression of *RGS16* was also observed in ALL cells after *E/R* abrogation. This gene plays an
254 antiproliferative role through inhibition of the PI3K/AKT/mTOR pathway²⁴⁻²⁶ and inhibition of cell
255 migration.²⁷ On the other hand, the tumour suppressor *PTPKR* whose expression prevents the activation
256 of pathways such as PI3K/AKT/mTOR and STAT signaling pathways²⁸ was downregulated after *E/R*
257 fusion gene abrogation. This gene was found downregulated in some types of cancer such as ovarian,
258 breast and NK-T cell lymphoma,²⁹⁻³¹ but also in ALL cell lines.²⁸ However, *E/R* positive ALL patients
259 have shown an overexpression of *PTPKR*.^{32,33}

260

261 **Table 1. Most relevant deregulated genes associated with ALL pathology.** The different columns show the name of deregulated
262 gene, if it was overexpressed or infraexpressed after *E/R* abrogation, the cellular processes in which it participates and the
263 expression of this gene in ALL patients.

264

Gene	Overexpressed/ Infraexpressed (<i>E/R</i> KO)	Function	ALL patients
<i>CXCR7</i>	Infraexpressed	Cell proliferation and migration ³⁴⁻³⁹	Overexpressed ⁴⁰
<i>LCK</i>	Infraexpressed	Lymphoid activation and development, GC resistance ⁴¹⁻⁴³	Overexpressed in patients with prednisone poor response ⁴¹

<i>PTPRG</i>	Infraexpressed	Cell growth, differentiation, mitotic cycle and oncogenic transformation ^{44,45}	Infraexpressed in in high hyperdiploid childhood ⁴⁶
<i>PIK3C3</i>	Infraexpressed	Regulation of autophagy. ⁴⁷ Protection of leukaemic cells during chemotherapeutic treatment ⁴⁸	Overexpressed in E/R positive patients ^{32,48}
<i>PTPRK</i>	Infraexpressed	Tumour suppressor ²⁸	Overexpression in E/R positive patients ^{32,33}
<i>miR-146</i>	Overexpressed	Innate immunity ⁴⁹ and apoptosis ²¹	Overexpression in children ⁵⁰
<i>RXRA</i>	Overexpressed	Retinoic acid X receptor that serves as a therapeutic target ⁵¹	Overexpressed in patients with <i>IKZF1</i> alterations ⁵¹
<i>RGS16</i>	Overexpressed	Antiproliferative role ²⁴⁻²⁶ and inhibition of cell migration ²⁷	Overexpression in high hyperdiploid childhood ⁵²
<i>TLR7</i>	Overexpressed	Immune system ⁵³	Infraexpressed ^{54,55}

E/R abrogation reduces proliferative capacity and resistance to apoptosis *in vitro*

To elucidate the biological effects of abrogation of E/R expression in the KO clones, several studies were performed. MTT proliferation studies were performed at 24-hour intervals up to 240 hours. The results showed no proliferation differences between KO clones and REH cells or control clones (Supplementary Figure 3A). We simultaneously analyzed the cell cycle distribution of the different cells by permeabilization followed by PI staining. No differences were observed between the different clones (Figure 3A). No significant differences were observed through the expression of CFSE by flow cytometry (Figure 3B). However, E/R KO clones showed a significantly lower proliferation rate than control clones when they were co-cultured with MSCs (HS-5) ($P<0.05$) (Figure 3C).

Deregulation of genes such as *miR-146a* or *TP63* observed by expression analysis suggested the alteration of cellular processes such as the regulation of apoptosis. Levels of anti-apoptotic factor such as *Bcl-2* or *Bcl-xL* gene have shown to play a key role in the survival of E/R positive cells, protecting from programmed death.^{56,57} To check these findings, *Bcl-2* and *Bcl-xL* expression levels were measured through WB. Suppression of the fusion protein produced a decrease of 60% and 47% in the expression of *Bcl-2* and *Bcl-xL* proteins respectively ($P=0.003$; $P=0.043$), thus reducing the resistance to apoptosis provided by the antiapoptotic factors of this family (Figure 4). In agreement with this observation, we detected an increased in the late apoptotic levels assessed by annexin V and propidium iodide staining in E/R KO clones as compared with control clones (8.99 ± 2.08 vs 2.135 ± 0.065) ($P<0.05$) (Figure 3D).

Treatment with Vincristine (1 μ M), a drug whose efficacy has been demonstrated in the treatment of ALL

285 by induction of apoptosis in mitotic cells,⁵⁸ induced a greater late apoptotic rate in E/R KO clones as
286 compared with control clones (75.8 ± 9.59 vs 32.7 ± 2.1) ($P < 0.05$) (Figure 3E).

287 **Abrogation of ETV6/RUNX1 expression enhances sensitivity to the PI3K inhibitor Copanlisib**

288 Deregulation of *RGS16* or *PTPKR* genes also suggested the alteration of the PI3K/AKT/mTOR pathway.
289 Several studies have already suggested that E/R may be key in the maintenance of the leukaemic
290 phenotype through the activation of different pathways, including the PI3K/AKT/mTOR pathway,^{13,59}
291 resulting in proliferation and cell survival of leukaemic cells. Akt phosphorylation levels measured
292 through WB showed a reduction of 90% in Akt activity in the KO clones relative to REH cells and
293 control clones ($P = 0.003$), suggesting the decrease in PI3K/Akt/mTOR activity as a result of the
294 elimination of the expression of E/R (Figure 4).

295 A large proportion of relapsing positive E/R patients become resistant to GCs such as Prednisolone,
296 widely used in ALL treatment.⁸ Fuka's group demonstrated that the use of PI3K inhibitors can sensitize
297 positive E/R cells to GCs.¹³

298 After verifying a lower activation levels of PI3K/Akt/mTOR pathway with the elimination of E/R
299 expression, we aimed to test if these cells responded in the same way to PI3K inhibitors. For that, we
300 used Copanlisib, a PI3K inhibitor with inhibitory activity predominantly against the PI3K-alpha and
301 PI3K-delta isoforms.^{60,61} Treatment with Copanlisib (10 nM) resulted in higher decrease of viability in
302 E/R KO clones compared with REH cells and controls clones (Figure 5A). To verify if Copanlisib was
303 actually inhibiting the PI3K/Akt/mTOR pathway, we measured the phosphorylation levels of Akt and
304 mTOR by WB, before and after treatment. We observed that the phosphorylation levels of both proteins
305 decreased after treatment (Figure 5B).

306 On the other hand, treatment with Prednisolone (250 μ M) was comparable to the effect of Copanlisib on
307 E/R positive cells. We did not observe a higher decrease of cell viability in E/R KO clones as compared
308 with REH cells and control clones (Figure 5C). A joint exposure of Copanlisib (10 nM) and Prednisolone
309 (250 μ M) showed a decrease of cell viability in REH and control clones expressing *E/R* fusion gene as
310 compared with Prednisolone and Copanlisib alone. Furthermore, we also observed greater reduction of
311 cell viability in E/R KO clones as compared with REH cells and control clones (Figure 5C).

312 **E/R repression impairs the tumourigenic capacity *in vivo***

313 In order to determine the effects of E/R expression abrogation *in vivo*, 16 NSG mice were subcutaneously
314 injected with REH cells or control clone (left flank) and KO clones (right flank). Only 6 mice injected

with KO clones developed tumour growth on the right flank (6/16), whereas all those injected with REH or control clone developed a tumour (16/16). In the first group (REH cells vs KO2 clone), none of flanks injected with KO2 developed tumour (mean mass: 0 mg $\pm$ 0 vs 4872.5 mg $\pm$ 1323; $P=0.029$). In the second group (REH cells vs KO3 clone), only one of flanks injected with KO3 developed a tumour (1/4). This tumour was significantly smaller than those generated from REH cells (mean mass: 40 mg $\pm$ 69.3 vs 4212.5 mg $\pm$ 1663.9; $P=0.029$). In the third group (control clone vs KO1), we observed tumour growth in 2/4 flanks of mice injected with KO1. These tumours were significantly smaller than those generated from control clone (mean mass: 483 mg $\pm$ 354.4 vs 2470 mg $\pm$ 872.5 vs $P=0.041$). In the same way, 3/4 mice develop tumours from KO2 in the group 4 (control clone vs KO2), but these tumours were significantly smaller than those generated from control clone (mean mass: 355 mg $\pm$ 293.6 vs 2255 mg $\pm$ 1215.6; $P=0.029$) (Figure 6A and Supplementary figure 4). In general, subcutaneous tumours generated from E/R KO cells were significantly smaller than those produced by REH cells or control clone (mean mass: 202 mg $\pm$ 298.9 vs 4542.5 mg $\pm$ 1539; $P<0.001$ vs 2347.1 mg $\pm$ 1087.2; $P\leq 0.001$) (Figure 6B).

In addition, significant differences were observed in the time of appearance of the tumours. Those tumours generated through the KO clones appeared around day 42 (mean: 46.5 $\pm$ 7.8), unlike those generated by the REH cells or the control clone, which appeared around day 29 (mean: 29 $\pm$ 4.2; $P=0.001$) and day 36 (mean: 36.6 $\pm$ 5.34; $P=0.03$) respectively (Figure 6C).

Histopathological analysis of representative tumours from each group of mice revealed a higher number of mitotic figures in tumours from REH (52 vs 20, $P=0.017$) and control clones (62 vs 20; $P=0.006$) as compared to tumours from KOs clones. In KOs tumours, but not in REH and control clone tumours, we also observed the "starry sky" (macrophages containing dead apoptotic tumour cells) (Supplementary figure 5). No other morphological changes between tumours were observed.

DISCUSSION

In this study, we generated an E/R KO model in an ALL cell line carrying the t(12;21) in order to assess how the loss of fusion expression affects the tumour cells. This translocation is the most common in children with ALL^{1,2} and encodes a chimeric transcription factor that converts *RUNXI* from a transcriptional modulator to a transcriptional repressor of *RUNXI* target genes.⁶² The expression of E/R results in the generation of a persistent pre-leukaemic clone, which requires of secondary genetic

abnormalities to converts to ALL.^{57,63} However, the implication of the E/R expression in the maintenance of the disease is not quite clear. Previous studies have already silenced the fusion protein by using RNAi and shRNA, but they did not completely eliminate the levels of the protein. In addition, these studies showed contradictory results, which suggest that more studies are needed to help elucidate the true oncogenic potential of the fusion protein.^{12,13}

The recent evolution of genetic editing techniques with the CRISPR/Cas9 system has allowed, among others, the generation of KO gene models, helping us to better understanding the biology of diseases such as ALL.⁶⁴ We used CRISPR/Cas9 system to completely eliminate the expression of the E/R fusion protein. The loss of fusion expression was checked, observing an absence of mRNA coding for E/R in the KO2 and KO3 clones and a leaky expression in KO1 clone. We hypothesize that this loss of mRNA was due to nonsense-mediated mRNA decay mechanism.⁶⁵ In this way we generated a powerful model on which to study the effect of the elimination of the fusion gene on leukaemic cells. These cells maintain a series of secondary alterations that triggered the leukaemia, similar as occurs in patients.

Transcriptome analysis of different clones showed a huge number of genes significantly deregulated after *E/R* abrogation. A greater number of upregulated genes was observed which agrees with the repressive activity of the *E/R* fusion gene.^{62,66} The most of genes significantly deregulated are involved in different cellular processes such as differentiation and lymphoid activation, apoptosis, cell signaling and cell migration. These results are in agreement with previous studies, in which was demonstrated the implication of E/R in the cellular processes that may be maintaining the leukaemic state of the tumour cells.^{13,57,59,66} The expression of some of these genes has been described as a specific signature of E/R positive ALL patients.^{32,33}

Within of the top50 of the significantly deregulated genes in our study we observed a series of downregulated genes after *E/R* abrogation such as *CXCR7*, *LCK* and *PTPGR* and other upregulated genes such as *Mir-146*, *RXRA*, *RGS16*, *TP63* and *IL7R*. The reverse deregulation of these genes, often seen in ALL patients, leads to increased migratory, tumour activation or chemotherapy resistance effects.

Very subtle changes were observed in cell proliferation and cell cycle distribution after *E/R* abrogation *in vitro*, in agreement with previous studies.^{12,13} However, E/R KO clones showed a significantly lower proliferation rate when they were co-cultured with MSC. Mesenchymal cells have been shown to play a key role in the development and evolution of ALL^{67,68} and Bonilla et al. (2019) observed in a recently study that MSCs induce greater cell adhesion, higher proliferation ratio and greater migration capacity to

375 REH cells.⁶⁹ Our data show that the *E/R* fusion gene therefore participates in the interaction of leukaemic
376 cells with the microenvironment and the loss of *E/R* fusion gene expression reverses the proliferative
377 capacity that MSCs confer on leukaemia cells. E/R KO clones also showed a higher late apoptosis rate,
378 demonstrating that the fusion gene regulates the expression of antiapoptotic factors that protect leukaemic
379 cells from apoptosis. Death levels were also higher in E/R KO clones after treatment with Vincristine.
380 On the other hand, we wanted to check if the non-expression of E/R and consequently the loss of
381 activation of the PI3K/Akt/mTOR pathway, was able to sensitize the cells to PI3K inhibitors. The use of
382 PI3K inhibitors alone has shown to be an effective treatment in E/R positive cells. Furthermore, the
383 activity of these inhibitors in combination with Prednisolone, a GC widely used in the treatment of ALL,
384 has been shown to decrease the resistance offered by positive E/R cells to GCs. In our study, we observed
385 that use of Copanlisib, a PI3K inhibitor, achieved a significantly decrease of cell viability in E/R KO
386 clones as compared with E/R positive cells. We also observed that treatment with Copanlisib achieved the
387 sensitization to Prednisolone in E/R positive cells as Fuka's study described.¹³ However, in our study, this
388 sensitization was even greater in E/R KO cells. Therefore, our data demonstrate that the fusion gene may
389 be a good therapeutic target with which to improve the drug sensitivity of positive E/R cells.
390 Finally, we wanted to check if E/R abrogation also decreased the tumour potential of cells *in vivo*. For
391 that, a xenograft model was generated by injecting these cells into immunosuppressed mice, taking the
392 injection of REH cells or a control clone on the opposite flank as control. Mice injected with KO clone
393 cells did not generated tumours or generated smaller tumours than those generated by REH cells or
394 control clone. The higher rate of mitotic activity in REH and control tumours observed through the
395 histopathology analysis explains the greater growth of these tumours and reveals a greater tumoural
396 capacity of these cells carrying *E/R* fusion gen *in vivo*.
397 Together these data suggest that the *E/R* fusion gene has a key role in the maintenance of the leukaemic
398 phenotype. By eliminating E/R expression, the cells lost tumourigenic capacity, becoming more sensitive
399 to drugs and reducing their oncogenic capacity *in vitro* and *in vivo*. Therefore, although more studies are
400 needed to elucidate the mechanism of action of the fusion gene, this study demonstrates that it could be a
401 possible therapeutic target to design new drugs that prevent the correct expression of this protein.

402

403 **AUTHORS CONTRIBUTION**

404 AM, IGT, RB and JMHR designed research. AM performed the experiments, compiled data and drafted
405 the manuscript. JLO was responsible for mouse experiments and drug tests. JHS was responsible for
406 sequencing experiments. VAP carried out additional experimental work. IGT, RB, JMHR, JLO, VAP and
407 TG helped interpret the results and write the manuscript. AM wrote the paper with input from all authors.

408

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417

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427

428 **COMPETING INTERESTS**

429 The authors declare that they have no competing interests

430

431 **REFERENCES**

- 432 1 Pui, C. H., Relling, M. V. & Downing, J. R. Acute lymphoblastic leukemia. *The*
433 *New England journal of medicine* **350**, 1535-1548, doi:10.1056/NEJMra023001
434 (2004).
- 435 2 Shurtleff, S. A. *et al.* TEL/AML1 fusion resulting from a cryptic t(12;21) is the
436 most common genetic lesion in pediatric ALL and defines a subgroup of patients
437 with an excellent prognosis. *Leukemia* **9**, 1985-1989 (1995).
- 438 3 Conter, V. *et al.* Molecular response to treatment redefines all prognostic factors
439 in children and adolescents with B-cell precursor acute lymphoblastic leukemia:
440 results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood* **115**, 3206-
441 3214, doi:10.1182/blood-2009-10-248146 (2010).
- 442 4 Moorman, A. V. *et al.* Prognostic effect of chromosomal abnormalities in
443 childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK
444 Medical Research Council ALL97/99 randomised trial. *The lancet oncology* **11**,
445 429-438, doi:10.1016/S1470-2045(10)70066-8 (2010).
- 446 5 Pui, C.-H., Robison, L. L. & Look, A. T. Acute lymphoblastic leukaemia. *The*
447 *Lancet* **371**, 1030-1043, doi:10.1016/s0140-6736(08)60457-2 (2008).
- 448 6 Rubnitz, J. E., Downing, J. R. & Pui, C. H. Significance of the TEL-AML fusion
449 gene in childhood AML. *Leukemia* **13**, 1470-1471 (1999).
- 450 7 Uckun, F. M. *et al.* Expression of TEL-AML1 fusion transcripts and response to
451 induction therapy in standard risk acute lymphoblastic leukemia. *Leuk*
452 *Lymphoma* **42**, 41-56, doi:10.3109/10428190109097675 (2001).
- 453 8 Bokemeyer, A. *et al.* Copy number genome alterations are associated with
454 treatment response and outcome in relapsed childhood ETV6/RUNX1-positive
455 acute lymphoblastic leukemia. *Haematologica* **99**, 706-714,
456 doi:10.3324/haematol.2012.072470 (2014).
- 457 9 Kuster, L. *et al.* ETV6/RUNX1-positive relapses evolve from an ancestral clone
458 and frequently acquire deletions of genes implicated in glucocorticoid signaling.
459 *Blood* **117**, 2658-2667, doi:10.1182/blood-2010-03-275347 (2011).
- 460 10 Raynaud, S. *et al.* The 12;21 translocation involving TEL and deletion of the
461 other TEL allele: two frequently associated alterations found in childhood acute
462 lymphoblastic leukemia. *Blood* **87**, 2891-2899 (1996).
- 463 11 Sun, C., Chang, L. & Zhu, X. Pathogenesis of ETV6/RUNX1-positive
464 childhood acute lymphoblastic leukemia and mechanisms underlying its relapse.
465 *Oncotarget* **8**, 35445-35459, doi:10.18632/oncotarget.16367 (2017).
- 466 12 Zaliouva, M., Madzo, J., Cario, G. & Trka, J. Revealing the role of TEL/AML1
467 for leukemic cell survival by RNAi-mediated silencing. *Leukemia* **25**, 313-320,
468 doi:10.1038/leu.2010.277 (2011).
- 469 13 Fuka, G. *et al.* Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR
470 signaling and impairs reconstitution of leukemia in xenografts. *Leukemia* **26**,
471 927-933, doi:10.1038/leu.2011.322 (2012).
- 472 14 Garcia-Tunon, I. *et al.* The CRISPR/Cas9 system efficiently reverts the
473 tumorigenic ability of BCR/ABL in vitro and in a xenograft model of chronic
474 myeloid leukemia. *Oncotarget* **8**, 26027-26040, doi:10.18632/oncotarget.15215
475 (2017).
- 476 15 Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat*
477 *Protoc* **8**, 2281-2308, doi:10.1038/nprot.2013.143 (2013).
- 478 16 Martinez, N. *et al.* The oncogenic fusion protein RUNX1-CBFA2T1 supports
479 proliferation and inhibits senescence in t(8;21)-positive leukaemic cells. *BMC*
480 *Cancer* **4**, 44, doi:10.1186/1471-2407-4-44 (2004).

481 17 Abramoff, M. D., Magalhães, P. J. & Ram, S. J. Image Processing with ImageJ.
482 *Biophotonics International* **11**, 7 (2004).

483 18 Collins, T. J. ImageJ for microscopy. *BioTechniques* **43**, 25-30,
484 doi:10.2144/000112517 (2007).

485 19 Ordonez, J. L. *et al.* The PARP inhibitor olaparib enhances the sensitivity of
486 Ewing sarcoma to trabectedin. *Oncotarget* **6**, 18875-18890,
487 doi:10.18632/oncotarget.4303 (2015).

488 20 Tijssen, M. R. *et al.* Genome-wide analysis of simultaneous GATA1/2, RUNX1,
489 FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators.
490 *Developmental cell* **20**, 597-609, doi:10.1016/j.devcel.2011.04.008 (2011).

491 21 Yan, W. *et al.* The effect of miR-146a on STAT1 expression and apoptosis in
492 acute lymphoblastic leukemia Jurkat cells. *Oncology letters* **13**, 151-154,
493 doi:10.3892/ol.2016.5395 (2017).

494 22 Binsky, I. *et al.* TAp63 regulates VLA-4 expression and chronic lymphocytic
495 leukemia cell migration to the bone marrow in a CD74-dependent manner. *J*
496 *Immunol* **184**, 4761-4769, doi:10.4049/jimmunol.0904149 (2010).

497 23 Lantner, F. *et al.* CD74 induces TAp63 expression leading to B-cell survival.
498 *Blood* **110**, 4303-4311, doi:10.1182/blood-2007-04-087486 (2007).

499 24 Liang, G., Bansal, G., Xie, Z. & Druey, K. M. RGS16 inhibits breast cancer cell
500 growth by mitigating phosphatidylinositol 3-kinase signaling. *The Journal of*
501 *biological chemistry* **284**, 21719-21727, doi:10.1074/jbc.M109.028407 (2009).

502 25 Liu, T. *et al.* The retinoid anticancer signal: mechanisms of target gene
503 regulation. *British journal of cancer* **93**, 310-318, doi:10.1038/sj.bjc.6602700
504 (2005).

505 26 Vasilatos, S. N. *et al.* Crosstalk between lysine-specific demethylase 1 (LSD1)
506 and histone deacetylases mediates antineoplastic efficacy of HDAC inhibitors in
507 human breast cancer cells. *Carcinogenesis* **34**, 1196-1207,
508 doi:10.1093/carcin/bgt033 (2013).

509 27 Berthebaud, M. *et al.* RGS16 is a negative regulator of SDF-1-CXCR4 signaling
510 in megakaryocytes. *Blood* **106**, 2962-2968, doi:10.1182/blood-2005-02-0526
511 (2005).

512 28 Stevenson, W. S. *et al.* DNA methylation of membrane-bound tyrosine
513 phosphatase genes in acute lymphoblastic leukaemia. *Leukemia* **28**, 787-793,
514 doi:10.1038/leu.2013.270 (2014).

515 29 Sun, P. H., Ye, L., Mason, M. D. & Jiang, W. G. Protein tyrosine phosphatase
516 kappa (PTPRK) is a negative regulator of adhesion and invasion of breast cancer
517 cells, and associates with poor prognosis of breast cancer. *J Cancer Res Clin*
518 *Oncol* **139**, 1129-1139, doi:10.1007/s00432-013-1421-5 (2013).

519 30 Swierczewska, M. *et al.* PTPRK Expression Is Downregulated in Drug Resistant
520 Ovarian Cancer Cell Lines, and Especially in ALDH1A1 Positive CSCs-Like
521 Populations. *Int J Mol Sci* **20**, doi:10.3390/ijms20082053 (2019).

522 31 Chen, Y. W. *et al.* Receptor-type tyrosine-protein phosphatase kappa directly
523 targets STAT3 activation for tumor suppression in nasal NK/T-cell lymphoma.
524 *Blood* **125**, 1589-1600, doi:10.1182/blood-2014-07-588970 (2015).

525 32 Ross, M. E. *et al.* Classification of pediatric acute lymphoblastic leukemia by
526 gene expression profiling. *Blood* **102**, 2951-2959, doi:10.1182/blood-2003-01-
527 0338 (2003).

528 33 Yeoh, E. J. *et al.* Classification, subtype discovery, and prediction of outcome in
529 pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer*
530 *cell* **1**, 133-143 (2002).

531 34 Boudot, A. *et al.* Differential estrogen-regulation of CXCL12 chemokine
532 receptors, CXCR4 and CXCR7, contributes to the growth effect of estrogens in
533 breast cancer cells. *PLoS ONE* **6**, e20898, doi:10.1371/journal.pone.0020898
534 (2011).

535 35 Levoe, A., Balabanian, K., Baleux, F., Bachelier, F. & Lagane, B. CXCR7
536 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein
537 signaling. *Blood* **113**, 6085-6093, doi:10.1182/blood-2008-12-196618 (2009).

538 36 Li, T. *et al.* The expression of CXCR4, CXCL12 and CXCR7 in malignant
539 pleural mesothelioma. *The Journal of pathology* **223**, 519-530,
540 doi:10.1002/path.2829 (2011).

541 37 Wang, J. *et al.* The role of CXCR7/RDC1 as a chemokine receptor for
542 CXCL12/SDF-1 in prostate cancer. *The Journal of biological chemistry* **283**,
543 4283-4294, doi:10.1074/jbc.M707465200 (2008).

544 38 Yoshida, D., Nomura, R. & Teramoto, A. Signalling pathway mediated by
545 CXCR7, an alternative chemokine receptor for stromal-cell derived factor-
546 1 α , in AtT20 mouse adrenocorticotrophic hormone-secreting pituitary
547 adenoma cells. *Journal of neuroendocrinology* **21**, 481-488, doi:10.1111/j.1365-
548 2826.2009.01867.x (2009).

549 39 Zabel, B. A., Lewen, S., Berahovich, R. D., Jaen, J. C. & Schall, T. J. The novel
550 chemokine receptor CXCR7 regulates trans-endothelial migration of cancer
551 cells. *Mol Cancer* **10**, 73, doi:10.1186/1476-4598-10-73 (2011).

552 40 Melo, R. C. C. *et al.* CXCR7 is highly expressed in acute lymphoblastic
553 leukemia and potentiates CXCR4 response to CXCL12. *PLoS ONE* **9**, e85926,
554 doi:10.1371/journal.pone.0085926 (2014).

555 41 Brownlie, R. J. & Zamoyska, R. T cell receptor signalling networks: branched,
556 diversified and bounded. *Nature reviews. Immunology* **13**, 257-269,
557 doi:10.1038/nri3403 (2013).

558 42 Nika, K. *et al.* Constitutively active Lck kinase in T cells drives antigen receptor
559 signal transduction. *Immunity* **32**, 766-777, doi:10.1016/j.immuni.2010.05.011
560 (2010).

561 43 Serafin, V. *et al.* Glucocorticoid resistance is reverted by LCK inhibition in
562 pediatric T-cell acute lymphoblastic leukemia. *Blood* **130**, 2750-2761,
563 doi:10.1182/blood-2017-05-784603 (2017).

564 44 LaForgia, S. *et al.* Receptor protein-tyrosine phosphatase gamma is a candidate
565 tumor suppressor gene at human chromosome region 3p21. *Proceedings of the*
566 *National Academy of Sciences of the United States of America* **88**, 5036-5040,
567 doi:10.1073/pnas.88.11.5036 (1991).

568 45 Liu, S., Sugimoto, Y., Sorio, C., Tecchio, C. & Lin, Y. C. Function analysis of
569 estrogenically regulated protein tyrosine phosphatase gamma (PTPgamma) in
570 human breast cancer cell line MCF-7. *Oncogene* **23**, 1256-1262,
571 doi:10.1038/sj.onc.1207235 (2004).

572 46 Chatterton, Z. *et al.* Epigenetic deregulation in pediatric acute lymphoblastic
573 leukemia. *Epigenetics* **9**, 459-467, doi:10.4161/epi.27585 (2014).

574 47 Funderburk, S. F., Wang, Q. J. & Yue, Z. The Beclin 1-VPS34 complex--at the
575 crossroads of autophagy and beyond. *Trends in cell biology* **20**, 355-362,
576 doi:10.1016/j.tcb.2010.03.002 (2010).

577 48 Polak, R. *et al.* Autophagy inhibition as a potential future targeted therapy for
578 ETV6-RUNX1-driven B-cell precursor acute lymphoblastic leukemia.
579 *Haematologica* **104**, 738-748, doi:10.3324/haematol.2018.193631 (2019).

580 49 Taganov, K. D., Boldin, M. P., Chang, K. J. & Baltimore, D. NF-kappaB-
581 dependent induction of microRNA miR-146, an inhibitor targeted to signaling
582 proteins of innate immune responses. *Proceedings of the National Academy of*
583 *Sciences of the United States of America* **103**, 12481-12486,
584 doi:10.1073/pnas.0605298103 (2006).

585 50 Zhang, H. *et al.* MicroRNA patterns associated with clinical prognostic
586 parameters and CNS relapse prediction in pediatric acute leukemia. *PLoS ONE*
587 **4**, e7826, doi:10.1371/journal.pone.0007826 (2009).

588 51 Churchman, M. L. & Mullighan, C. G. Ikaros: Exploiting and targeting the
589 hematopoietic stem cell niche in B-progenitor acute lymphoblastic leukemia.
590 *Exp Hematol* **46**, 1-8, doi:10.1016/j.exphem.2016.11.002 (2017).

591 52 Davidsson, J. *et al.* Tiling resolution array comparative genomic hybridization,
592 expression and methylation analyses of dup(1q) in Burkitt lymphomas and
593 pediatric high hyperdiploid acute lymphoblastic leukemias reveal clustered near-
594 centromeric breakpoints and overexpression of genes in 1q22-32.3. *Human*
595 *molecular genetics* **16**, 2215-2225, doi:10.1093/hmg/ddm173 (2007).

596 53 Sanchez-Cuaxospa, M. *et al.* Low expression of Toll-like receptors in peripheral
597 blood mononuclear cells of pediatric patients with acute lymphoblastic
598 leukemia. *International journal of oncology* **49**, 675-681,
599 doi:10.3892/ijo.2016.3569 (2016).

600 54 Corthals, S. L. *et al.* Differential immune effects mediated by Toll-like receptors
601 stimulation in precursor B-cell acute lymphoblastic leukaemia. *Br J Haematol*
602 **132**, 452-458, doi:10.1111/j.1365-2141.2005.05893.x (2006).

603 55 Dorantes-Acosta, E. *et al.* TLR stimulation of bone marrow lymphoid precursors
604 from childhood acute leukemia modifies their differentiation potentials. *Biomed*
605 *Res Int* **2013**, 846724, doi:10.1155/2013/846724 (2013).

606 56 Bauer, E. *et al.* Cooperation of ETV6/RUNX1 and BCL2 enhances
607 immunoglobulin production and accelerates glomerulonephritis in transgenic
608 mice. *Oncotarget* **7**, 12191-12205, doi:10.18632/oncotarget.7687 (2016).

609 57 Torrano, V., Procter, J., Cardus, P., Greaves, M. & Ford, A. M. ETV6-RUNX1
610 promotes survival of early B lineage progenitor cells via a dysregulated
611 erythropoietin receptor. *Blood* **118**, 4910-4918, doi:10.1182/blood-2011-05-
612 354266 (2011).

613 58 Silverman, J. A., Reynolds, L. & Deitcher, S. R. Pharmacokinetics and
614 pharmacodynamics of vincristine sulfate liposome injection (VSLI) in adults
615 with acute lymphoblastic leukemia. *J Clin Pharmacol* **53**, 1139-1145,
616 doi:10.1002/jcph.155 (2013).

617 59 Inthal, A. *et al.* Role of the erythropoietin receptor in ETV6/RUNX1-positive
618 acute lymphoblastic leukemia. *Clin Cancer Res* **14**, 7196-7204,
619 doi:10.1158/1078-0432.CCR-07-5051 (2008).

620 60 Krause, G., Hassenruck, F. & Hallek, M. Copanlisib for treatment of B-cell
621 malignancies: the development of a PI3K inhibitor with considerable differences
622 to idelalisib. *Drug design, development and therapy* **12**, 2577-2590,
623 doi:10.2147/DDDT.S142406 (2018).

624 61 Liu, N. *et al.* BAY 80-6946 is a highly selective intravenous PI3K inhibitor with
625 potent p110alpha and p110delta activities in tumor cell lines and xenograft
626 models. *Molecular cancer therapeutics* **12**, 2319-2330, doi:10.1158/1535-
627 7163.MCT-12-0993-T (2013).

628 62 Hiebert, S. W. *et al.* The t(12;21) translocation converts AML-1B from an
629 activator to a repressor of transcription. *Molecular and cellular biology* **16**,
630 1349-1355 (1996).
631 63 Papaemmanuil, E. *et al.* RAG-mediated recombination is the predominant driver
632 of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia.
633 *Nature genetics* **46**, 116-125, doi:10.1038/ng.2874 (2014).
634 64 Montano, A., Forero-Castro, M., Hernandez-Rivas, J. M., Garcia-Tunon, I. &
635 Benito, R. Targeted genome editing in acute lymphoblastic leukemia: a review.
636 *BMC biotechnology* **18**, 45, doi:10.1186/s12896-018-0455-9 (2018).
637 65 Culbertson, M. R. & Leeds, P. F. Looking at mRNA decay pathways through
638 the window of molecular evolution. *Current opinion in genetics & development*
639 **13**, 207-214, doi:10.1016/s0959-437x(03)00014-5 (2003).
640 66 Fuka, G., Kauer, M., Kofler, R., Haas, O. A. & Panzer-Grumayer, R. The
641 leukemia-specific fusion gene ETV6/RUNX1 perturbs distinct key biological
642 functions primarily by gene repression. *PLoS ONE* **6**, e26348,
643 doi:10.1371/journal.pone.0026348 (2011).
644 67 Genitsari, S. *et al.* Biological Features of Bone Marrow Mesenchymal Stromal
645 Cells in Childhood Acute Lymphoblastic Leukemia. *Turkish journal of*
646 *haematology : official journal of Turkish Society of Haematology* **35**, 19-26,
647 doi:10.4274/tjh.2017.0209 (2018).
648 68 Nwabo Kamdje, A. H. *et al.* Mesenchymal stromal cells' role in tumor
649 microenvironment: involvement of signaling pathways. *Cancer biology &*
650 *medicine* **14**, 129-141, doi:10.20892/j.issn.2095-3941.2016.0033 (2017).
651 69 Bonilla, X., Vanegas, N. P. & Vernot, J. P. Acute Leukemia Induces Senescence
652 and Impaired Osteogenic Differentiation in Mesenchymal Stem Cells Endowing
653 Leukemic Cells with Functional Advantages. *Stem cells international* **2019**,
654 3864948, doi:10.1155/2019/3864948 (2019).
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657

658 **FIGURE LEGENDS**

659

660 **Figure 1. E/R expression levels by RT-qPCR.** Control clones showed an expression of E/R similar to it
661 was observed in the parental REH cells. In E/R KO clones, whose sequence was edited by the
662 CRISPR/Cas9 system, KO2 and KO3 showed a total loss of E/R expression and KO1 showed a leaky
663 expression. All the experiments were carried out by triplicate, the means with the standard deviations for
664 each clone were represented. *** $P \leq 0.001$ (unpaired t -test).

665

666 **Figure 2. Transcriptomic analysis of E/R KO clones.** Heat map of TOP50 differentially expressed
667 genes in E/R KO clones as compared with REH cells and control clones. Each row represents one
668 differentially expressed gene; each column represents one clone. The dendrogram on the top reveals the
669 sample clustering; the dendrogram on the left reveals the gene clustering.

670

671 **Figure 3. *In vitro* functional studies after E/R abrogation.** (A) Cell cycle distribution of control clones
672 and E/R KO cells at 48 h. (B) CFSE quantification by flow cytometry after 48 in culture. The peak on the
673 right (10^3) represents the percentage of cells that have not divided and the left peak (10^2) represents the
674 percentage of cells that have divided and therefore diluted their CFSE expression. (C) CFSE expression
675 by flow cytometry of cells co-cultured with MSC cell line HS-5 at 48h. (D) Apoptosis level quantification
676 by PI expression. The figure shows the percentage of PI negative cells (left) and PI positive cells (right) at
677 48 h. (E) Apoptosis level quantification by PI expression after treatment with Vincristine ($1 \mu\text{M}$) at 48 h.
678 On the right is represented the mean distribution of control clones (dark grey) and E/R KO clones (grey)
679 of different experiments. All the experiments were carried out by triplicate. * $P \leq 0.05$ (unpaired t -test).

680

681 **Figure 4. Western blot analysis of E/R targets expression.** Lower phospho-Akt (60 kDa), BCL-XL (30
682 kDa) and BCL-2 (28 kDa) expression levels were observed in all E/R KO clones compared with parental
683 cell line (REH) and control clones.

684

685 **Figure 5. Cell viability and protein expression measured after Copanlisib / Prednisolone treatment.**
686 (A) Cell viability was measured by the MTT proliferation assay after treatment (192 h) with Copanlisib
687 (10 nM). E/R KO clones (light grey square line) showed a higher sensitivity to Copanlisib than REH cells

688 (black square line) and control clones (grey square line). This graph represents the average of three
 689 independent experiments and in turn the average of the 2 control clones and the 3 KO clones. (B) p-Akt
 690 (60 kDa) and p-mTOR (289 kDa) expression levels decreased after treatment with Copanlisib. (C)
 691 Prednisolone (black square line), Copanlisib (dark grey square line) and Copanlisib plus Prednisolone
 692 combination (grey square line) were tested in the different clones. The relative cell viability was
 693 calculated as the percentage of untreated cells. * $P \leq 0.05$; ** $P \leq 0.005$ (unpaired *t*-test).

694

695 **Figure 6. In vivo effects of CRISPR-mediated editing of the *E/R* oncogene.** (A) External appearance
 696 of mice and developed tumours 48 - 62 days after subcutaneous cell injection. Tumours formed by KO
 697 clones (right flank) were smaller than those induced by REH cells or control clones (left flank). (B)
 698 Evolution of tumour growth measured every 2-3 days until the moment in which mice were sacrificed.
 699 (C) Representation of the mean tumour size corresponding to each clone, independently of the group.
 700 * $P \leq 0.05$; ** $P \leq 0.005$ (unpaired *t*-test).

701

E/R KO clones vs control clones – Top 50 genes

Bcl-2

β -actin

Bcl-xL

α/β -Tubulin

p-Akt

β -actin

REH

Control 1

Control 2

KO1

KO2

KO3

A **B** **C**

A **B** **C**