HemaSphere
Powered by EHA

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Letter
OPEN ACCESS

ARHGEF4 Regulates an Essential Oncogenic Program in t(12;21)-Associated Acute Lymphoblastic Leukemia

Clemence Virely[1], Luca Gasparoli[1], Maurizio Mangolini[1], Katherine Clesham[1], Sarah Inglott[2], Darren Edwards[3], Stuart Adams[2], Jack Bartram[3], Sujith Samarasinghe[3], Philip Ancliff[3], Ajay Vora[3], Jasper de Boer[1], Owen Williams[1]

Correspondence: Owen Williams (e-mail: owen.williams@ucl.ac.uk).

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells. Although the overall survival rate is currently more than 90% it still represents one of the main causes of childhood cancer deaths. The chromosomal translocation t(12;21)(p13;q22), associated with more than 25% pediatric ALL cases, involves genes encoding two transcription factors involved in normal hematopoiesis, ETV6 and RUNX1.[1] Although the ETV6-RUNX1 fusion protein is weakly oncogenic, requiring secondary events to induce overt leukemia, its expression is nevertheless required for maintenance and propagation of disease.[2,3] The oncogenic activity of ETV6-RUNX1 appears to be dependent on deregulation of transcriptional target genes,[4] although the detailed disease mechanisms remain to be elucidated. We previously found that *ARHGEF4* expression is specifically associated with *ETV6-RUNX1*$^+$ ALL.[5] ARHGEF4 (also known as ASEF) is a member of the diffuse B-cell lymphoma (DBL) family of guanine nucleotide exchange factors (GEFs). Although originally described as a RAC1-specific GEF, more recent data suggest that its substrate is CDC42.[6] Small guanine nucleotide binding proteins (GTPases) activation is tightly modulated by GEFs and aberrant GEF regulation can contribute to their activation in cancer.[7] In this study, we investigated the function of ARHGEF4 in *ETV6-RUNX1*$^+$ ALL cells.

To confirm the association of *ARHGEF4* expression with *ETV6-RUNX1*+ ALL, we analyzed its expression in B-precursor ALL cell lines (Supplementary Fig. 1A, http://links.lww.com/HS/A97) and pediatric patient-derived xenograft (PDX) B lineage ALL samples (Supplementary Table 1 and Supplementary Fig. 1B, http://links.lww.com/HS/A97). The results showed a high correlation between *ETV6-RUNX1* status and elevated *ARHGEF4* mRNA expression, confirming the previous published data.[5,8] To investigate this correlation further, we examined *ARHGEF4* expression following shRNA-mediated *ETV6-RUNX1* silencing in REH cells.[3] Fusion gene knock-down resulted in diminished *ARHGEF4* expression (Fig. 1A). Furthermore, increased *ARHGEF4* expression was observed following overexpression of the human *ETV6-RUNX1* cDNA (Fig. 1B).[3,9,10] These data demonstrate a causal relationship between the *ETV6-RUNX1* fusion gene and elevated *ARHGEF4* expression in human B-lineage ALL, and confirm a previously reported demonstration of reduced *ARHGEF4* expression following shRNA-mediated silencing of *ETV6-RUNX1* in ALL cells.[4] This is likely specific to human cells, since we found previously that the fusion did not affect mouse *Arhgef4* expression.[5]

To determine the function of ARHGEF4 in *ETV6-RUNX1*$^+$ leukemia, we examined the survival of human leukemia cell lines following *ARHGEF4* silencing (Supplementary Fig. 1C, http://links.lww.com/HS/A97). This resulted in significant apoptosis induction after 5 days in both REH and AT2 cells (Fig. 1C). Thus, *ARHGEF4* expression is necessary for survival of *ETV6-RUNX1*$^+$ leukemia cells. In contrast, *ARHGEF4* silencing did not affect the viability of *ETV6-RUNX1*$^-$ ALL cells lines (Supplementary Fig. 1D, http://links.lww.com/HS/A97). We then examined the effect of *ARHGEF4* silencing on the ability of human *ETV6-RUNX1*$^+$ leukemic cells to form colonies in vitro and to propagate disease in vivo. REH cells were harvested three days after lentiviral shRNA transduction, at which point no effects on viability were detectable, and plated into methylcellulose cultures or transplanted into recipient mice. *ARHGEF4* silencing compromised the colony forming activity of REH cells (Fig. 1D), and significantly impaired their ability to engraft leukemia (Fig. 1E).

To determine ARHGEF4 substrate specificity, the activity of CDC42 and RAC1 were examined after *ARHGEF4* silencing in REH cells. Three independent *ARHGEF4*-specific shRNA resulted in inhibition of CDC42 activity (Fig. 1F), whereas RAC1 activity was not affected (Supplementary Fig. 1E, http://links.

[1]Cancer Section, Developmental Biology and Cancer Programme, UCL Great Ormond Street Institute of Child Health, London, UK
[2]SIHMDS-Haematology, Great Ormond Street Hospital for Children, London, UK
[3]Department of Paediatric Haematology, Great Ormond Street Hospital for Children, London, UK.
CV, LG, and MM are co-first authors.
The authors declare no conflicts of interest.
Supplemental Digital Content is available for this article.
Copyright © 2020 the Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the European Hematology Association. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.
HemaSphere (2020) 4:5(e467). http://dx.doi.org/10.1097/HS9.0000000000000467.
Received: 12 May 2020 / Accepted: 8 July 2020

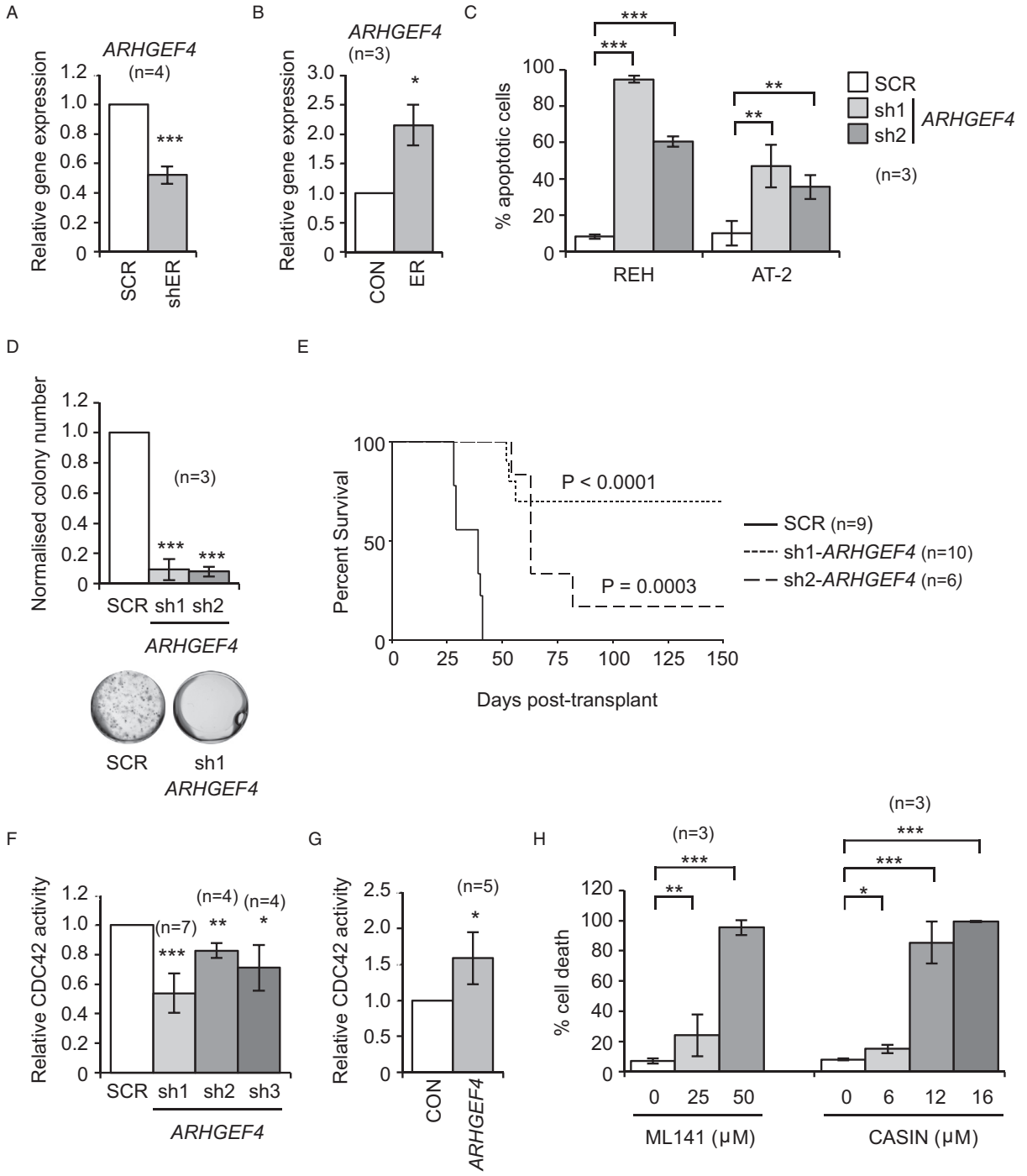

Figure 1. *ARHGEF4* is downstream of ETV6-RUNX1 and is required for t(12;21) ALL survival and disease progression by activating CDC42. (A) *ARHGEF4* gene expression in REH cells 5 days after transduction with control scramble (SCR) or *ETV6*-specific (shER) shRNA, or (B) empty vector control (CON) or the *ETV6-RUNX1* cDNA. $^{*}p<0.05$, $^{***}p<0.001$, one sample *t* test. (C) REH apoptosis 5 days following transduction with SCR or *ARHGEF4*-specific (sh1 and sh2) shRNA. $^{**}p<0.01$; $^{***}p<0.001$, unpaired Student's *t* test. (D) REH colony formation 3 days after transduction with SCR or *ARHGEF4*-specific (sh1 and sh2) shRNA. $^{***}p<0.001$, one sample *t* test. (E) Kaplan-Meier survival curve for NSG mice transplanted with 1 x 10^5 viable REH cells 3 days after transduction with SCR or sh*ARHGEF4* shRNA. p values for sh1 and sh2 versus shSCR controls are shown, Mantel-Haenszel log-rank test. (F) CDC42 activity in REH cells 3 days after transduction with SCR or *ARHGEF4*-specific (sh1, sh2, sh3) shRNA, and (G) 7 days after transduction with empty vector control (CON) or the *ARHGEF4* cDNA. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, one sample *t* test. (H) Cell death in REH cells 24 hours after treatment with CDC42 inhibitors ML141 and CASIN. $^{*}p<0.05$, $^{**}p<0.01$; $^{***}p<0.001$, unpaired Student's *t* test.

lww.com/HS/A97). Furthermore, overexpression of ARHGEF4 increased CDC42 activity in REH cells (Fig. 1G). Thus, although it has been reported that ARHGEF4 can function as a GEF for both CDC42 and RAC1, our data are consistent with a study demonstrating the specificity of purified ARHGEF4 for CDC42 in vitro.[6] We then examined the impact of pharmacological CDC42 inhibition in REH cells. The CDC42 inhibitors, ML141 and CASIN, both induced dose-dependent cell death (Fig. 1H).

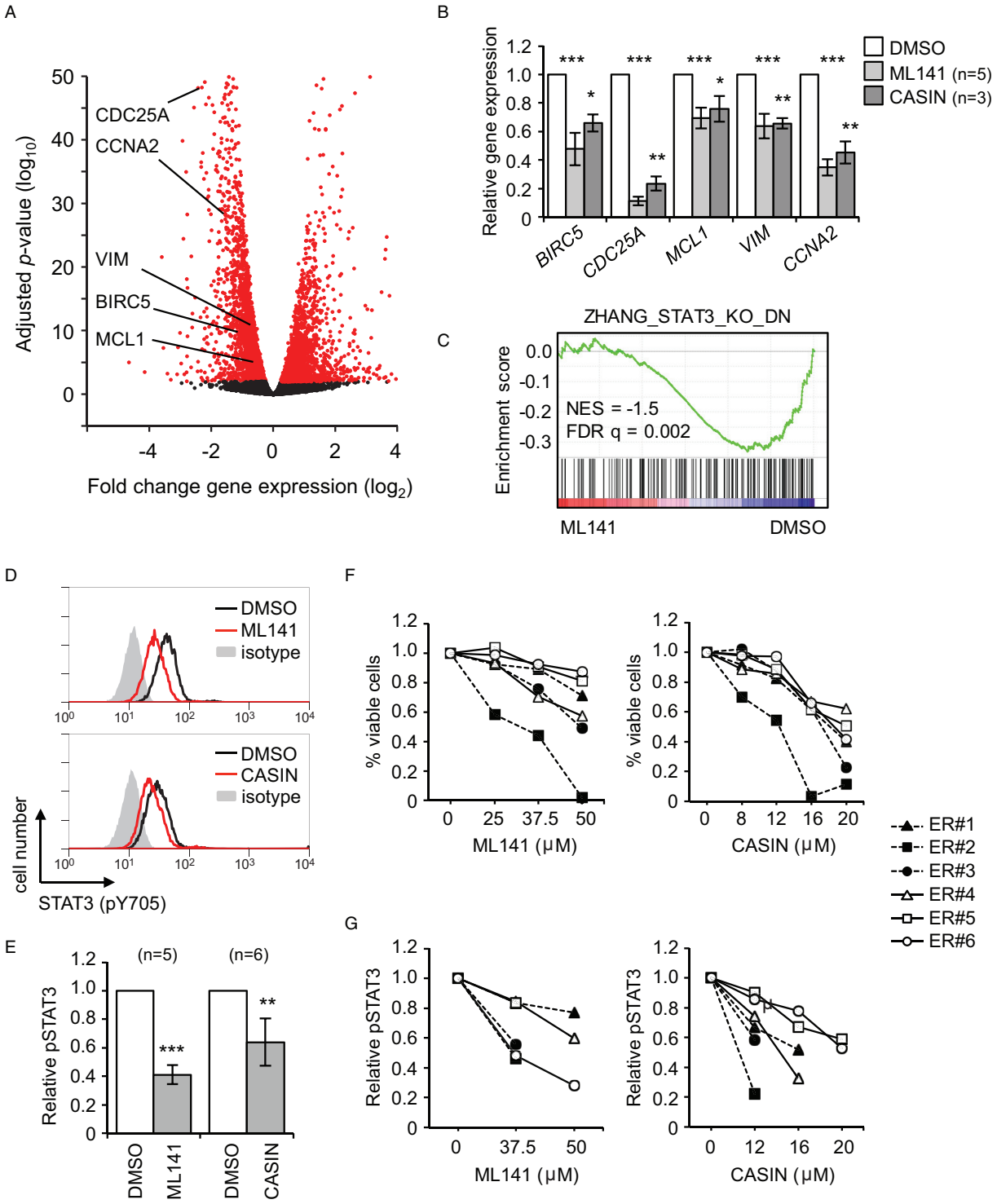

Figure 2. CDC42 mediates t(12;21) ALL-associated STAT3 activation. (A) Volcano plots of fold gene expression changes in REH cells following treatment with 25 μM ML141 or DMSO control, for 24 hours. Expression changes with $p < 0.01$ are shown in red, Wald test. (B) qPCR analysis of STAT3 target gene expression in REH cells 24 hours following treatment with DMSO, 25 μM ML141 or 10 μM CASIN. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, one sample *t* test. (C) GSEA of STAT3 target gene set, derived from a list of genes whose expression was previously shown to decrease in *Stat3*-deficient hematopoietic progenitor cells,[12] in ML141 induced gene expression changes. (D) Flow cytometry plots and (E) graphs of phospho-STAT3(pY705) expression in REH cells 24 hours after treatment with DMSO, 25 μM ML141 or 6 μM CASIN. $^{**}p < 0.01$; $^{***}p < 0.001$, one sample *t* test. (F) Viability of 6 *ETV6-RUNX1*$^{+}$ PDX samples (ER#1-ER#6) 24 hours following exposure to DMSO, ML141 (left panel) or CASIN (right panel). (G) Flow cytometry analysis of phospho-STAT3(pY705) expression in *ETV6-RUNX1*$^{+}$ PDX samples 16 hours following exposure to DMSO, ML141 (left panel) or CASIN (right panel).

These data indicate that ETV6-RUNX1 maintains CDC42 activity, and leukemia cell viability, through induction of ARHGEF4 expression.

We next determined the effect of CDC42 inhibition on the transcriptome of REH cells following treatment with ML141 or DMSO for 24 hours, by RNA sequencing (RNA-seq) (Fig. 2A).

Genes associated with apoptosis were enriched in gene expression changes induced by CDC42 inhibition (Supplementary Fig. 1F, http://links.lww.com/HS/A97), consistent with the increase in cell death observed following ML141 and CASIN treatment (Fig. 1H). The gene expression data also showed significant decreases in a number of hematopoietic STAT3 target genes (Fig. 2A, B),[11] suggesting a link between CDC42 activity and STAT3 function. This was particularly interesting, since we showed previously that *ETV6-RUNX1+* ALL cells require STAT3 activity for survival.[3] Indeed, further analysis of the gene expression data revealed negative enrichment of two STAT3 gene sets (Fig. 2C and Supplementary Fig. 1G, http://links.lww.com/HS/A97).[12,13] We next examined the impact of CDC42 inhibition on STAT3 activity directly. STAT3 (pY705) phosphorylation was found to decrease in REH cells treated with either ML141 or CASIN (Fig. 2D, E). In order to determine whether the link between CDC42 and STAT3 was also evident in patient-derived leukemia cells, we examined the effect of CDC42 inhibition in the panel of *ETV6-RUNX1*$^+$ pediatric PDX ALL cells. Both ML141 and CASIN treatment of these PDX samples resulted in induction of cell death (Fig. 2F) and inhibition of STAT3 (pY705) phosphorylation (Fig. 2G).

In summary, here we demonstrate that *ARHGEF4* expression is induced downstream of the ETV6-RUNX1 fusion protein and that it is necessary for *ETV6-RUNX1*$^+$ ALL survival and disease progression. Evidence from the literature suggests that *ARHGEF4* gene expression may be regulated directly by the fusion. RUNX1 was shown to bind to introns within the *ARHGEF4* gene in both human primary hematopoietic progenitor/stem cells[14] and human megakaryocytes.[15] This suggests that the ETV6-RUNX1 fusion protein may bind directly to the *ARHGEF4* gene, since the only DNA binding domain retained in the fusion is contained within the RUNX1 moiety. Furthermore, ETV6-RUNX1 binding to the *ARHGEF4* promoter region can be detected in previously published chromatin immunoprecipitation data from human B-precursor ALL NALM6 cells, expressing the fusion ectopically (Supplementary Fig. 2, http://links.lww.com/HS/A97).[16]

The dependence of *ETV6-RUNX1*$^+$ ALL cells on *ARHGEF4* expression can be explained by the function of ARHGEF4 in maintaining STAT3 activity, mediated by its substrate CDC42. This study provides a mechanistic explanation for the dependence of *ETV6-RUNX1*$^+$ ALL cells on STAT3 signaling and their association with elevated *ARHGEF4* expression. The association of aberrant CDC42 activity with numerous different cancers has led to a large body of research aimed at their therapeutic targeting.[17] The data reported in the current study provide critical insight into the specific regulation of CDC42 activity in t(12;21)$^+$ ALL cells by ARHGEF4, expanding the list of potential candidates for novel therapeutic targeting in this leukemia.

Sources of Funding

This work was supported by grants from Action Medical Research (GN2368) to CV; GOSHCC to MM (ICH22), JdB (W1073) and OW (V1305, V2617); Children with Cancer UK to LG (14–169,17–249) and to KC (16–232). This research was supported by the NIHR Great Ormond Street Hospital Biomedical Research Centre.

Acknowledgements

The authors thank Ayad Eddaoudi and Stephanie Canning, UCL GOS ICH Flow Cytometry Facility, for providing assistance with flow cytometry, all the staff of the UCL GOS ICH Western Laboratories for excellent animal husbandry, Tony Brooks and Paola Niola and Mark Kristiansen, UCL Genomics for providing assistance with the RNA-sequencing, Prof D. Trono for lentiviral packaging constructs, and Dr. R.W. Stam and Prof R. Panzer-Grümayer for ALL cell lines.

References

1. Sundaresh A, Williams O. Mechanism of ETV6-RUNX1 leukemia. *Adv Exp Med Biol.* 2017;962:201–216.
2. Fuka G, Kantner HP, Grausenburger R, et al. Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. *Leukemia.* 2012;26:927–933.
3. Mangolini M, de Boer J, Walf-Vorderwulbecke V, et al. STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia. *Blood.* 2013;122:542–549.
4. Fuka G, Kauer M, Kofler R, et al. The leukemia-specific fusion gene ETV6/RUNX1 perturbs distinct key biological functions primarily by gene repression. *PLoS One.* 2011;6:e26348.
5. Lyons R, Williams O, Morrow M, et al. The RAC specific guanine nucleotide exchange factor Asef functions downstream from TEL-AML1 to promote leukaemic transformation. *Leuk Res.* 2010;34:109–115.
6. Gotthardt K, Ahmadian MR. Asef is a Cdc42-specific guanine nucleotide exchange factor. *Biol Chem.* 2007;388:67–71.
7. Vigil D, Cherfils J, Rossman KL, et al. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nat Rev Cancer.* 2010;10:842–857.
8. Haferlach T, Kohlmann A, Wieczorek L, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol.* 2010;28:2529–2537.
9. Morrow M, Horton S, Kioussis D, et al. TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity. *Blood.* 2004;103:3890–3896.
10. Morrow M, Samanta A, Kioussis D, et al. TEL-AML1 preleukemic activity requires the DNA binding domain of AML1 and the dimerization and corepressor binding domains of TEL. *Oncogene.* 2007;26:4404–4414.
11. Baker SJ, Rane SG, Reddy EP. Hematopoietic cytokine receptor signaling. *Oncogene.* 2007;26:6724–6737.
12. Zhang H, Li HS, Hillmer EJ, et al. Genetic rescue of lineage-balanced blood cell production reveals a crucial role for STAT3 antiinflammatory activity in hematopoiesis. *Proc Natl Acad Sci U S A.* 2018;115:E2311–E2319.
13. Durant L, Watford WT, Ramos HL, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity.* 2010;32:605–615.
14. Beck D, Thoms JA, Perera D, et al. Genome-wide analysis of transcriptional regulators in human HSPCs reveals a densely interconnected network of coding and noncoding genes. *Blood.* 2013;122:e12–22.
15. Tijssen MR, Cvejic A, Joshi A, et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. *Dev Cell.* 2011;20:597–609.
16. Linka Y, Ginzel S, Kruger M, et al. The impact of TEL-AML1 (ETV6-RUNX1) expression in precursor B cells and implications for leukaemia using three different genome-wide screening methods. *Blood Cancer J.* 2013;3:e151.
17. Maldonado MDM, Dharmawardhane S. Targeting Rac and Cdc42 GTPases in Cancer. *Cancer Res.* 2018;78:3101–3111.