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„Exploring ETV6/RUNX1 function in childhood acute lymphoblastic leukemia“

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ABSTRACT

The t(12;21) (p13;q22) chromosomal translocation, generating the ETV6/RUNX1 (E/R) fusion gene, is present in 25% of childhood B-cell precursor acute lymphoblastic leukemias. While the E/R gene fusion is considered a critical step for the initiation of leukemia, its functional contribution to overt leukemia is less clear. We aimed to investigate this issue by suppressing endogenous E/R fusion protein with lentivirus-transduced shRNA in the leukemic cell lines REH and AT-2. E/R suppression reduced cell proliferation, increased apoptosis and impaired leukemia outgrowth in NOD/SCID mice. These effects were accompanied by a concurrent inactivation of the PI3K/AKT/mTOR pathway. Consistently, pharmacological PI3K/mTOR inhibition diminished the phosphorylation of AKT and RPS6, significantly increased the apoptosis rate in E/R-positive leukemias and sensitized REH cells to prednisolone. We also performed genome-wide gene expression analyses upon E/R silencing in REH and AT-2 cells and identified 777 differentially regulated genes. Even though we observed similar numbers of up- and down-regulated genes, striking differences emerged when biological processes and pathways were investigated by the Database for Annotation, Visualization and Integrated Discovery (DAVID) and Gene Set Enrichment Analyses (GSEA). E/R-repressed genes resulted in a significant enrichment of the categories "cell activation", "immune response", "apoptosis", "signal transduction" and "development and differentiation" while E/R induced genes were less significantly associated with specific pathways. Most prominently enriched terms for the latter gene set indicated alterations in "PI3K/AKT/mTOR signaling" and "hematopoietic stem cells". De-regulation of pathways and molecular functions was essentially confirmed by comparison with a previously published E/R-specific profile from primary ALL. RT-qPCR was applied to validate six differentially expressed genes (PROM1, IFITM1, PECAM1, SPIB, MDM2, DDIT4), corroborating modulation of "stem cell properties" and "B-cell differentiation", and supporting the notion that "immune-response", "cell adhesion" and "DNA damage pathways" are affected by the fusion protein. Collectively, our data demonstrate that the E/R fusion protein constitutes a major driving force in overt leukemia and establish a molecular map of its putative functions.

ZUSAMMENFASSUNG

Die chromosomale Translokation t(12;21) (p13;q22) , welche die Grundlage für die Entstehung des ETV6/RUNX1 (E/R) Fusionsgens ist, ist in 25% aller akuten lymphoblastischen B-Zell Vorläufer Leukämien im Kindesalter zu finden. Die E/R Fusion wird als kritischer Schritt in der Leukämieentstehung betrachtet, ihre funktionelle Bedeutung in der klinisch manifesten Leukämie ist hingegen weitgehend unbekannt. Um Informationen darüber zu erhalten, setzten wir uns zum Ziel, das endogene E/R Fusionsprotein in den leukämischen Zelllinien REH und AT-2 mittels lentiviral-transduzierter shRNA zu unterdrücken. Dadurch wurde eine Reduktion der Zellproliferation, Erhöhung der Apoptose und Beeinträchtigung des Auftretens einer Leukämie in einem Xenotransplantationmodell mit NOD/SCID Mäusen hervorgerufen. Diese Effekte wurden von einer Inaktivierung des PI3K/AKT/mTOR Signalweges begleitet. Gleichfalls reduzierte die pharmakologische Inhibierung von PI3K/mTOR die Protein Phosphorylierung von AKT und RPS6, und bewirkte eine erhöhte Apoptoserate und Prednisolon-Sensitivität in E/R-positiven Leukämien bzw. REH Zellen. Genomweit durchgeführte Genexpressionsstudien von E/R unterdrückten REH und AT-2 Zellen identifizierten 777 differenziell regulierte Gene. Obwohl eine ähnliche Anzahl von induzierten und reprimierten Genen gefunden wurde, zeigten sich bei der Untersuchung von biologischen Prozessen und Signalwegen mittels „Database for Annotation, Visualization and Integrated Discovery“ (DAVID) sowie „Gene Set Enrichment Analyses“ (GSEA) beträchtliche Unterschiede. E/R reprimierte Gene resultierten in einer signifikanten Anreicherung der Kategorien "Zellaktivierung", "Immunantwort", "Apoptose", "Signaltransduktion" und "Entwicklung und Differenzierung". Hingegen konnten E/R induzierte Gene weniger signifikant mit bestimmten Signalwegen assoziiert werden. Die am stärksten regulierten Begriffe in letzterem Set an Genen, deuten auf eine Veränderung der Begriffe "PI3K/AKT/mTOR Signalweg" und "hämatopoetische Stammzellen" hin. Weiters wurde die differenzielle Regulation von Signalwegen und molekularen Funktionen mit dem Vergleich bereits publizierter E/R spezifischer Profile von primären akuten lymphoblastischen Leukämien bestätigt. Schließlich setzten wir RT-qPCR ein, um die differenzielle Regulation von sechs Gene (PROM1, IFITM1, PECAM1, SPIB, MDM2, DDIT4) zu bestätigen, welche die Modulation von "Stammzell Eigenschaften" und "B-Zell Differenzierung" bekräftigten, sowie die

Annahme unterstützten, dass die "Immunantwort", "Zelladhäsion" sowie "DNA Damage Signalwege" durch das Fusionsprotein kontrolliert werden. Zusammenfassend zeigen unsere Daten, dass das E/R Fusionsprotein eine bedeutende Rolle in der malignen Zelle spielt. Zusätzlich konnten wir ein molekulares Modell für dessen putative Funktionen etablieren.

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1. INTRODUCTION

1.1 Childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood and adolescence and is characterized by the expansion and accumulation of lymphoid progenitor cells. The peak incidence of ALL in children is between 2 and 5 years and represents the principal subtype of pediatric cancer in affluent societies, affecting 1 in 100.000 people up to the age of 15. Even though steady progress in treatment has led to a cure rate of more than 80%, certain subtypes are still associated with dismal prognosis and a substantial proportion of patients may suffer from treatment-related toxicities or late effects. Hence, innovative new therapeutic approaches to increase leukemia-free survival rates and reduce the toxic side-effects of current treatment regimens are required (Parkin 1988; Pui, Robison et al. 2008).

1.1.1 Etiology and pathogenesis

Only a minority of ALL (< 5%) cases are associated with predisposing genetic syndromes (Down's syndrome, Bloom's syndrome, Ataxia-telangiectasia, and Nijmegen breakage syndrome), ionising radiation or exposure to specific chemotherapeutic drugs (Pui, Robison et al. 2008). In most cases the precise pathogenic events leading to development of ALL remain unknown.

However, the development of childhood ALL has been shown to be associated with industrialisation and modern or affluent societies. Furthermore, clusters of leukemia cases have been observed especially in new towns. These observations influenced British investigators to develop two parallel leukemia development models based on infections. Kinlen's population-mixing hypothesis explains the presence of clusters of childhood ALL cases by an exposure of susceptible, non-immune individuals to common but fairly non-pathological infections after population-mixing with carriers (Kinlen 2004). In contrast, the delayed-infection hypothesis of Greaves is based on a minimal two-hit model (Greaves 2006). It suggests that some susceptible individuals with a prenatally acquired pre-leukemic clone had low or no exposure to common infections since they lived in an affluent hygienic environment. Subsequent or delayed exposure to common infections at an age usually associated with increased

proliferation of lymphoid-cells predisposes the immune system to aberrant or pathological responses.

Development of childhood ALL is associated with at least two distinct temporal “exposure” windows. The first one takes place *in utero*, when leukemia is initiated through, for instance, chromosomal rearrangements. The second - usually postnatal - window, is linked to emergence of overt disease through the acquisition of secondary genetic changes (Figure 1) (Greaves 2006). Consistent with this concept, the initiation of disease by the chromosomal translocation t(12;21) *in utero* exceeds the rate of overt ALL by 100-fold (Mori, Colman et al. 2002).

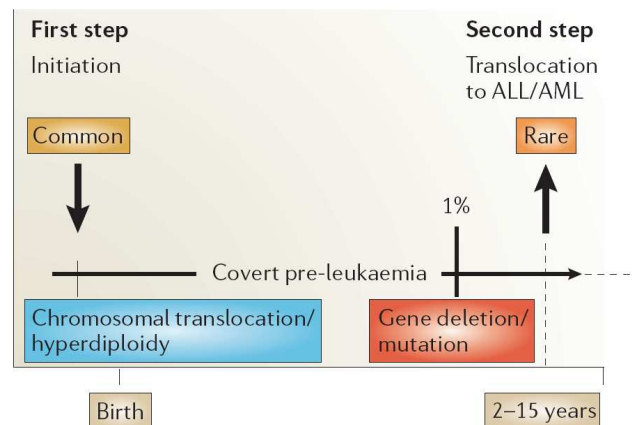


Figure 1. Two-step model of childhood acute leukemia development (Greaves 2006). Note that 1% refers to the transition frequency between covert pre-leukemia and overt clinical leukemia. This model is not applicable to infant ALL and acute myeloid leukemia where secondary genetic events are thought to take place already prenatally.

Chromosomal translocations are initiated by DNA double strand breaks, followed by non-homologous end-joining and result in the recombination of normally separate genes. Resultant in-frame chimeric or fusion proteins exhibit activated kinase activity or lead to novel transcriptional regulation (Look 1997; Rowley 1998). Importantly, each distinct chromosomal translocation contributes to leukemic transformation by altering key regulatory processes, such as the acquisition of an unlimited capacity for self-renewal, inhibition of differentiation, evading growth control and promoting resistance to apoptotic stimuli (Figure 2) (Pui, Relling et al. 2004).

The ultimate target cell for a chromosomal translocation needs to be permissive for expression of the chimeric protein and must provide an intracellular-signaling network that supports rather than inhibits clonal advantage (Greaves and Wiemels 2003).

Since different subsets of childhood leukemia show a distinct cell type or lineage phenotype, each chromosome translocation must have arisen either in a cell of the respective lineage, or in an uncommitted hematopoietic stem cell (HSC) that affects only progenies of one lineage (Reya, Morrison et al. 2001). In general, stem cells are mostly non-cycling and, as such are assumed to be protected from damage. However, they are an attractive target for chromosomal translocations as their long lifespan would allow the acquisition of secondary genetic changes necessary for leukemic cell transformation. Consistent with this notion, recent evidence in t(12;21) leukemia suggested that the leukemia initiating E/R gene fusion and subsequent chimeric protein expression may indeed already take place in an uncommitted hematopoietic stem cell (Schindler, Van Buren et al. 2009). Secondary leukemia-promoting mutations, mediated by V(D)J recombination, therefore, can only take place during early B-cell development.

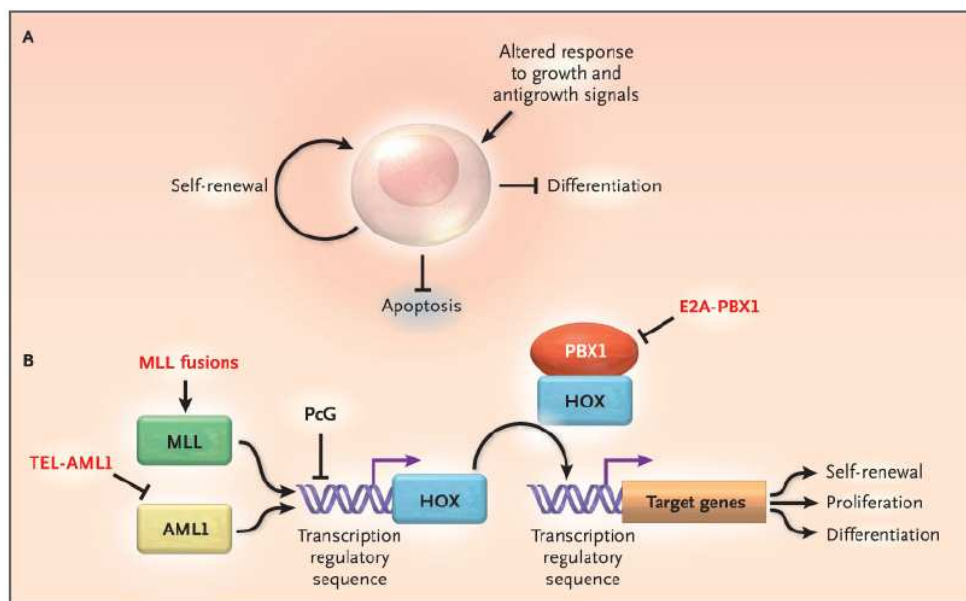


Figure 2. Contribution of chimeric proteins to leukemic cell transformation (Pui, Relling et al. 2004). During leukemogenesis, a hematopoietic stem cell or one of its committed progenitors has to elude normal mechanisms of homeostatic control, implicated in regulation of growth-factor signaling, differentiation, apoptosis, and self-renewal (panel A). Different chimeric transcription factors can commonly impact on the *HOX* gene-mediated transcriptional cascade linked to leukemic transformation. Different sites of action are indicated in red (panel B).

1.1.2 Classification of ALL

Depending on the cell type and developmental stage, childhood ALL can be divided into T-cell, mature B-cell, and precursor B-cell phenotypes. Furthermore, gross chromosomal changes representing aneuploidies and translocations, or aberrant expression of proto-oncogenes, are selectively associated with distinct leukemia immunophenotypes (Figure 3).

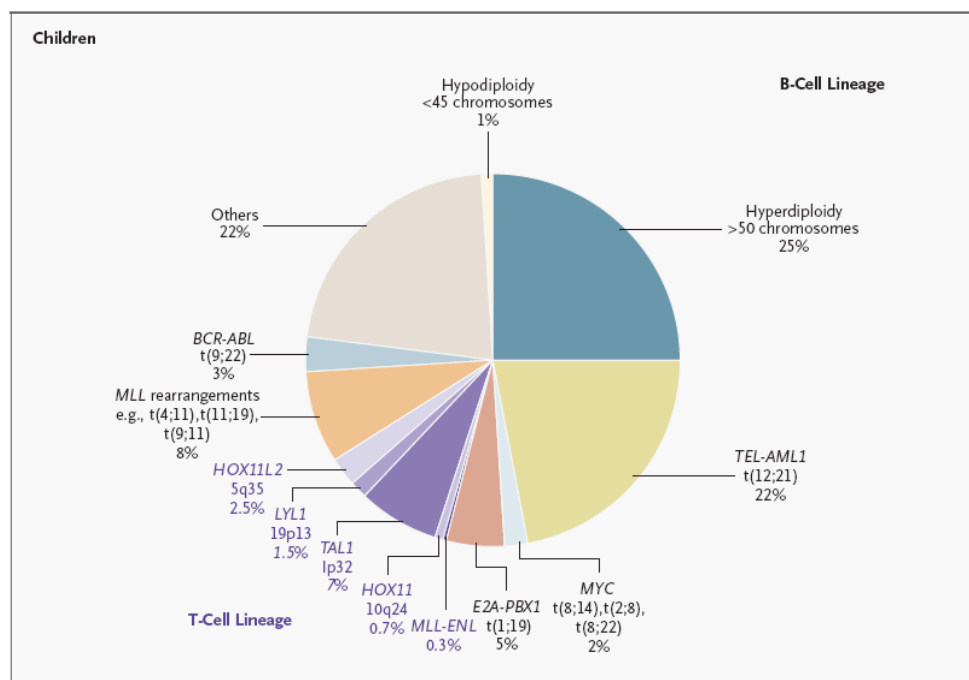


Figure 3. Genotypes and their recurrent leukemia-associated frequencies in T- and B-cell lineage childhood ALL (Pui, Relling et al. 2004).

Chromosomal translocations frequently result in the formation of chimeric transcription factors, capable of conferring altered self-renewal and differentiation to hematopoietic cells. The t(12;21)(p13;q22) chromosomal translocation, with an incidence of 20-25%, generates the *ETV6/RUNX1* (*E/R*) fusion gene (Pui, Relling et al. 2004). *Mixed lineage leukemia* (*MLL*) translocations result in the fusion of the N-terminal portion of *MLL* to the C-terminal portion of 1 of more than 40 partners and are identified in more than 80% of infant ALL cases, but are rare in older children. Furthermore, most therapy-induced leukemias, caused by topoisomerase II inhibitors involve *MLL* translocations. A small group of BCP ALL cases (5%) harbors the t(1;19) translocation, generating a fusion gene that encodes the chimeric transcription factor *E2A/PBX1*. The *BCR/ABL* gene fusion creates a constitutive tyrosine kinase and is

generated by the t(9;22) translocation which is found in 3% of BCP ALL cases. Finally, approximately 10-15% of childhood ALLs have a T-cell immunophenotype. Chromosomal translocations occur rarely in this lineage (Weng, Ferrando et al. 2004).

Specific genetic abnormalities may be associated with prognosis. This is exemplified by Kaplan Meier plots which show the event-free survival for patients that have been enrolled in four consecutive treatment protocols at St Jude Children's Research Hospital from 1991 to 2006 (Figure 4) (Pui, Robison et al. 2008). Hyperdiploid (> 50 chromosomes), *E/R* and *E2A/PBX1* fusion carrying ALL, have a favourable outcome, whereas *MLL/AF4* and *BCR/ABL* fusion positive cases have a dismal prognosis.

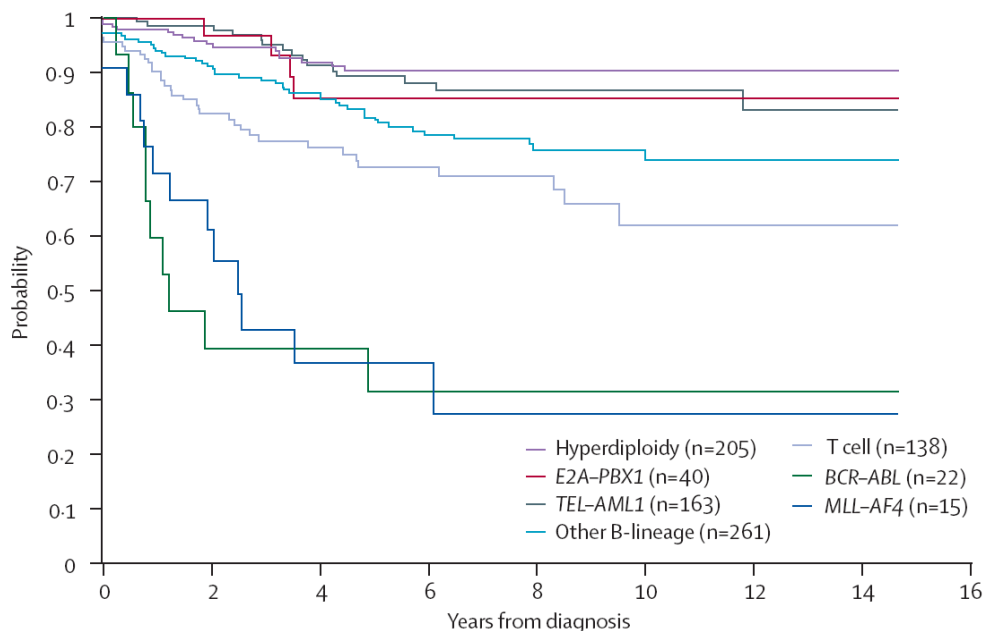


Figure 4. Kaplan-Meier event-free survival curve according to ALL subtypes; modified (Pui, Robison et al. 2008).

1.1.3 Concepts of treatment

Chemotherapy

Conventional drugs being currently applied to treat ALL inhibit nucleic acid synthesis, block protein synthesis by hydrolysing an amino acid or impair mitosis by interfering with the mitotic spindle apparatus.

The remission-induction treatment aims to eradicate more than 99% of the initial leukemic cell burden, thereby restoring normal hematopoiesis and a healthy

performance status. During this treatment, a glucocorticoid (prednisolone or dexamethasone), vincristine, and at least a third drug (asparaginase, anthracycline, or both) is administered. By this treatment, clinical remission can be achieved in 96-99% of childhood ALL cases. The induction course is followed by a remission consolidation or intensification treatment and is applied to eradicate drug-resistant residual leukemic cells. Furthermore, patients with ALL need continuation treatment to prevent or forestall relapse for reasons that currently remain unknown. Therefore, all patients receive chemotherapy for a total of 2.0-2.5 years (Pui and Evans 2006).

Stem-cell transplantation

Allogeneic hematopoietic stem-cell transplantation is considered the most intensive form of treatment. It was shown to be beneficial for selected patients with high-risk ALL, such as individuals with Philadelphia chromosome-positive disease and MLL-positive infant ALL with additional poor prognostic factors (Arico, Valsecchi et al. 2000; Mann, Attarbaschi et al. 2010).

Novel treatment concepts

Leukemic cells express lineage associated antigens representing attractive targets for treatment with monoclonal antibodies (mAb). They can be administered in unconjugated forms or conjugated to anti-leukemic drugs, immunotoxins or radioactive molecules. Examples are rituximab (Rituxan, Mabthera; Biogen Idec) and alemtuzumab (Campath, MabCampath; Genzyme Corporation) which target the cell surface molecules CD20 and CD52, respectively (Pui and Jeha 2007).

In recent years, different methods aimed to identify molecular genetic and epigenetic aberrations in leukemic cells that can be exploited for use as novel drug targets. Subsequently, the repertoire of different therapies directed against aberrantly regulated molecular pathways in cancer cells was expanded. The feasibility of drugs targeting components of the cell-cycle, gene transcription, cell motility, apoptosis and cell metabolism has been demonstrated. At least, transient responses can be obtained by inhibition of certain key enzymes such as tyrosine kinases, DNA methyltransferase, histone deacetylase, γ -secretase, serine-threonine kinases, and proteasomes.

One of the most successful small drugs is imatinib mesilate (Gleevec) which has proven effective in treating chronic lymphoblastic leukemia (Druker, George et al.

2004). In ALL, however, complete remission could not be achieved with Gleevec alone. Furthermore, rapid development of drug resistance suggests that curative treatment will need alternative concepts (Pui and Jeha 2007).

1.2 ETV6/RUNX1-positive B-cell precursor ALL

The cytogenetically not detectable t(12;21)(p13;q22), resulting in the fusion of the *ETS variant 6 (ETV6)* and *runt-related transcription factor 1 (RUNX1)* genes (also called *TEL* and *AML1*, respectively), was first described in 1995 and is present in approximately 25% of pediatric BCP ALL cases (Romana, Mauchauffe et al. 1995). In general E/R-positive ALL is associated with low risk features and, based on the application of anti-metabolites, a good prognosis, with 5-year event-free survival estimates of about 85% (Figure 4) (Pui, Robison et al. 2008).

The phenotype of the E/R-positive leukemic blasts corresponds to early CD19-positive progenitor B-cells. However, this does not rule out the possibility that the cellular targets for the initiating translocation are more primitive CD34-positive stem cells. Indeed, experiments in mice and zebrafish demonstrate that E/R oncogenic activity requires fusion gene expression from HSC onwards and, in contrast, specific expression in the B-cell lineage failed to increase the probability to develop leukemia (Andreasson, Schwaller et al. 2001; Sabaawy, Azuma et al. 2006; Schindler, Van Buren et al. 2009).

Data from identical twins shared the same E/R genomic breakpoints, suggesting prenatal spread of pre-leukemic cells from one twin to the other and, furthermore, retrospective screening of archived neonatal blood spots of ALL patients provided convincing evidence that E/R usually arises prenatally as an early or initiating mutation (Greaves and Wiemels 2003).

While E/R provides the first or initiating mutation in leukemia development, it is by itself unable to cause overt leukemia. This assumption is supported by the fact that the incidence rate of E/R fusions is 100 times the leukemia rate (Mori, Colman et al. 2002) and that the concordance rate in twins is low (~10%) (Greaves and Wiemels 2003). Furthermore, the protracted postnatal interval, or latency, before disease emergence can be up to a decade or more (Wiemels, Cazzaniga et al. 1999), corroborating the notion that additional events need to occur for the clinical manifestation of leukemia. There might not be an exclusive second genetic 'hit',

however, most cases of E/R-positive ALL have the non-rearranged *ETV6* allele deleted (Raynaud, Cave et al. 1996). Other commonly observed genetic alterations include a trisomy of chromosome 21 (Attarbaschi, Mann et al. 2004), disruptions of the *paired box 5* (*PAX5*) gene as well as loss of *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*)/*p16* and *CDKN2D/p19* (Mullighan, Phillips et al. 2008).

In vivo experiments revealed that the E/R fusion protein was able to impede the differentiation program of B-lymphocytes, leading to an accumulation of early B-cell progenitors at the expense of more mature forms. Notably, E/R seems to exert a lineage-dependent role, since no block in differentiation other than the B-lineage was observed (Tsuzuki, Seto et al. 2004). Interestingly, enforced E/R expression resulted in an enlargement of the less differentiated multipotential progenitor cell (cKit+, Sca1+, lin-) or hematopoietic stem cell compartment (Fischer, Schwieger et al. 2005; Sabaawy, Azuma et al. 2006; Schindler, Van Buren et al. 2009). Whether this very primitive compartment is responsible for the maintenance and/or expansion of a pre-leukemic clone remains to be shown.

1.2.1 The *ETV6/RUNX1* fusion

There are a number of other chromosomal translocations involving the *RUNX1* or *ETV6* genes, being detected in different subtypes of acute leukemias, affecting both the myeloid and lymphoid lineage (Rowley 1998). However, *E/R* is the only gene fusion that involves the *RUNX1* gene that has been identified in ALL.

Expression of *RUNX1* was shown to be required for the switch from yolk sac to liver hematopoiesis in the developing mouse embryo. Experiments in adult animals additionally revealed a role for *RUNX1* in the regulation of genes during hematopoietic differentiation (Dann, Fears et al. 2000). While *RUNX1* is capable of either activating or repressing its target genes, *ETV6* is generally considered to act as a transcriptional repressor (Zelent, Greaves et al. 2004). *ETV6* knockout mice are embryonic lethal, due to defects in yolk sac angiogenesis defects. Mouse chimeras with homozygous *ETV6* deletions demonstrated that *ETV6* is required for homing of hematopoietic cells to the bone marrow (Wang, Swat et al. 1998). *ETV6* also plays an important role in adult hematopoiesis by supporting cell survival and regulating megakaryopoiesis (Hock, Meade et al. 2004).

The E/R protein consists of N-terminal *ETV6* sequences and of the almost entire *RUNX1* sequence (Figure 5). Importantly, E/R is under transcriptional control of the

ETV6 promoter while specificity in DNA binding is determined by RUNX1 derived sequences (Golub, McLean et al. 1996 Aug 8; Berger 1997).

RUNX1 is located at chromosome 21q22.12, encoding a protein that consists of two large functional domains, the proximal DNA binding and protein interaction domain (or runt homology domain) and the distal transactivation domain. Additional functional domains are the mSin3A transcriptional regulator interaction domain (SID) and the VWRPY motif that is capable of binding groucho corepressors (Figure 5). RUNX1 represents a transcription factor that exerts its function in the nucleus.

ETV6 is located at chromosome 12p13.2. Functional domains of the ETV6 protein are the C-terminal ETS domain, mediating DNA binding, the central repression domain and the amino-terminal pointed domain (PD), required for oligomerization (Figure 5) (Zelent, Greaves et al. 2004).



Figure 5. Schematic presentation of full-length ETV6, RUNX and E/R proteins (Zelent, Greaves et al. 2004). Fusion sites between ETV6 and RUNX1 sequences are indicated by arrows. Numbers indicate amino acids that border functional domains.

1.2.2 Molecular functions of ETV6/RUNX1

RUNX1 binds its target genes together with its regulatory subunit core binding factor β (CBF β). It modulates transcription depending on the association with specific cofactors. For instance, binding of RUNX1 to mSin3A recruits histone deacetylases leading to reduced accessibility of a given promoter to the basal transcriptional machinery and transcriptional repression. However, this process can be reversed via dissociation of RUNX1 from mSin3A/histone deacetylases and binding to p300/histone acetylases (Figure 6) (Zelent, Greaves et al. 2004).

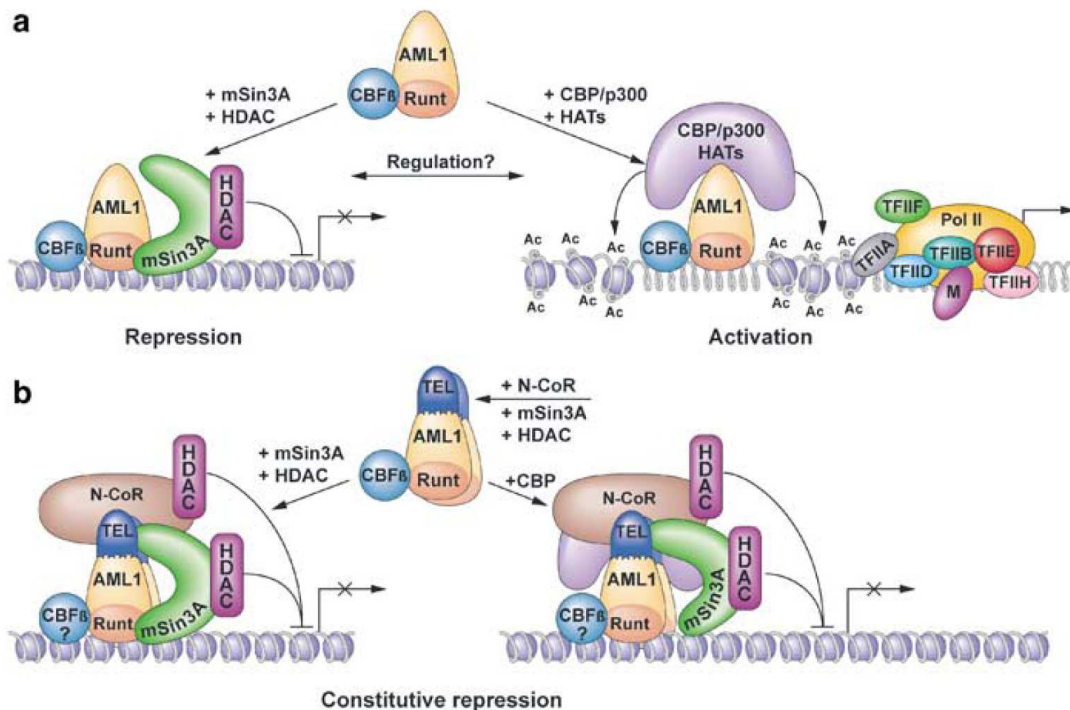


Figure 6. Molecular mechanism of wt RUNX1 and E/R (Zelent, Greaves et al. 2004). The transcription factor RUNX1 either activates or represses its target genes by recruitment of p300 or mSin3A/HDAC, respectively (panel a). By contrast, the abilities of the ETV6 moiety on E/R to dimerize and bind N-CoR and mSin3A allows formation of a very stable repressor complex (panel b), rendering the chimeric protein resistant to the regulation depicted in panel a. See text for detailed descriptions.

The E/R fusion protein can bind nuclear receptor corepressor 1 (N-CoR1) and mSin3A corepressors via its ETV6 oligomerization moiety which allows formation of a very stable repressor complex. Given that E/R retains the ability to bind the RUNX1 target sequences, it has the potential to function as a constitutive histone deacetylase dependent repressor of genes normally regulated by RUNX1 (Figure 6). Containing the pointed domain of ETV6, the fusion protein may bind to RUNX1 target sequences as a homodimer or an oligomer (Zelent, Greaves et al. 2004).

Consistent with the assumption that the fusion protein represses RUNX1 target genes in a histone deacetylase dependent mode, reporter constructs driven by regulatory regions of hematopoietic-specific genes were found to be suppressed by E/R (Hiebert, Sun et al. 1996). Furthermore, the E/R mediated repression was relieved by the histone deacetylase inhibitor trichostatin A (Fenrick, Amann et al. 1999).

Apart from its role in the nucleus, E/R has been suggested to interfere with wt RUNX1 and ETV6 function by trapping other proteins in the cytoplasm and forming non-functional complexes (Gunji, Waga et al. 2004). However, the experimental evidence for such a function is lacking.

1.3 Oncogene addiction

The phenomenon of cancer reversion by oncogene inactivation is collectively defined by the term oncogene addiction. It presumes that cancer cells, despite the acquisition of cooperating genetic abnormalities, are still dependent on the specific oncogene that initiated cellular transformation (Weinstein 2002). Oncogene activation initiates and contributes to tumorigenesis because it aberrantly activates physiologic programs to overcome growth control and bypasses checkpoint mechanisms essential for cellular mortality, self-renewal, and genomic integrity. Accordingly, following the shutdown of all safety mechanisms by secondary alterations, the inactivation of the initiating oncogene can restore some of these pathways. Possible outcomes are an arrest of proliferation, induction of cell differentiation, cellular senescence and/or increased apoptosis rates (Figure 7) (Felscher 2008).

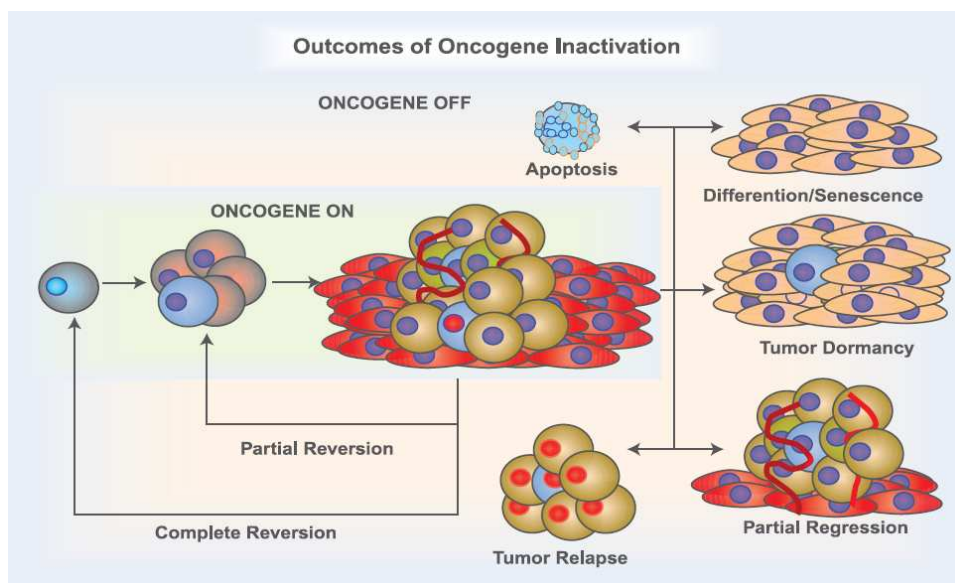


Figure 7. Possible outcomes to oncogene inactivation (Felscher 2008). The repression or inactivation of a single oncogene can either result in complete/partial tumor reversion, tumor death, dormancy, differentiation or relapse.

It also has been postulated that oncogenes induce cancer because they induce a cellular state of enforced oncogenic amnesia, and only upon oncogene inactivation the tumor becomes aware of its transgression (Felsher 2008).

Consistent with this notion, essential roles for oncogenic rat sarcoma (RAS), v-myc myelocytomatosis viral oncogene homolog (MYC) and sialidase 1 (NEU) for maintenance of viability of different types of malignancies, including solid tumors as well as hematopoietic malignancies, could be demonstrated (Chin, Tam et al. 1999; Felsher and Bishop 1999; Fisher, Wellen et al. 2001; Jain, Arvanitis et al. 2002; Moody, Sarkisian et al. 2002). Later, the effects of targeting chimeric proteins in hematopoietic malignancies were evaluated. Strikingly, in acute promyelocytic leukemia (APL) PML/RAR, generated by the t(15;17) translocation, was effectively degraded by arsenic trioxide, involving a proteasomal dependent mechanism. Treatment of patients with arsenic trioxide resulted in high rates of complete remission in both, primary and relapsed APL (Zhang TD 2001). Alternatively, experiments in acute leukemia demonstrated that targeting of the fusion proteins BCR/ABL or MLL/AF4 in ALL and MLL/ENL in AML was effective at eradicating overt disease (Thomas, Gessner et al. 2005; Kharas, Janes et al. 2008; Horton, Walf-Vorderwulbecke et al. 2009).

Based on previous results indicating that the E/R expressing leukemic cell line REH requires expression of the fusion gene for survival, we speculated that t(12;21) leukemic cells might also be addicted to E/R (Diakos, Krapf et al. 2007).

1.4 The PI3K/AKT/mTOR pathway

1.4.1 Molecular signaling mechanisms

The phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway regulates key cellular processes, such as cell survival, growth, proliferation, glucose uptake, metabolism and angiogenesis (Manning and Cantley 2007).

Different receptor tyrosine kinases (RTKs) activate PI3K, either through direct binding or following tyrosine phosphorylation of scaffolding adaptors, such as insulin receptor substrate 1 (IRS1), which then bind and activate PI3K (Figure 8). PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) and generates

phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). This reaction can be reversed by the PIP3 phosphatase and tensin homolog (PTEN).

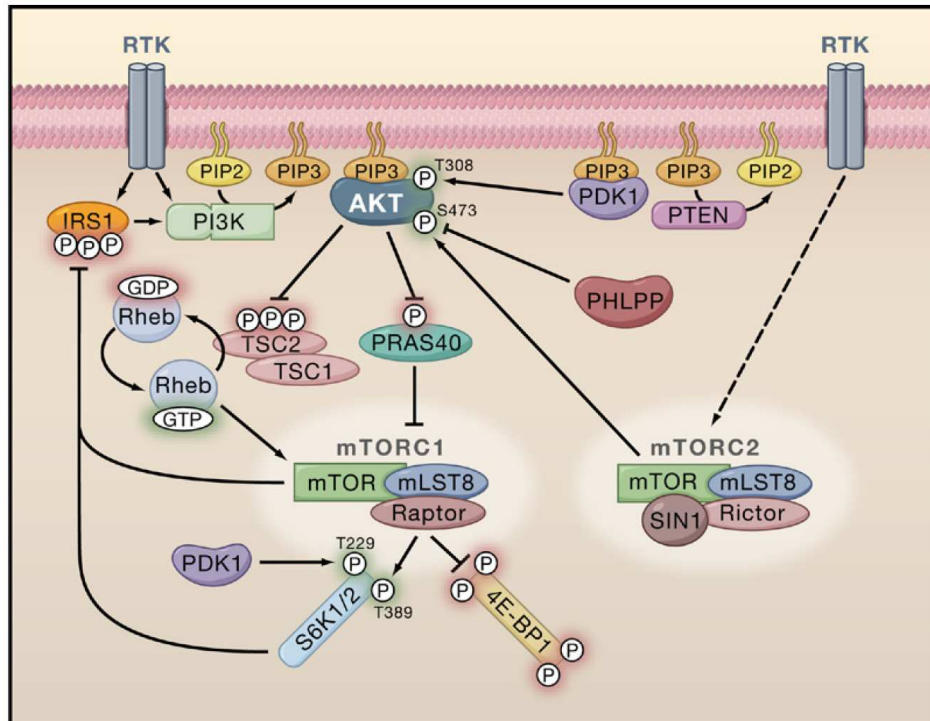


Figure 8. The PI3K/AKT/mTOR pathway (Manning and Cantley 2007). Different receptor tyrosine kinases (RTK) are capable of activating the PI3K/AKT/mTOR signaling cascade, leading to characteristic activation of downstream effector molecules. See text for detailed descriptions.

After binding to PIP3, AKT is phosphorylated and activated by pyruvate dehydrogenase kinase 1 (PDK1). Inactivation of AKT by de-phosphorylation is mediated by the pleckstrin homology domain and leucine rich repeat protein phosphatase (PHLPP). The isoforms AKT1, AKT2 and AKT3, encoded by three different genes, are ubiquitously expressed but their levels vary depending on the tissue type. In general, AKT-mediated protein phosphorylation either leads to substrate activation or inhibition. So far, over 100 non-redundant AKT substrates have been identified. For instance, tuberous sclerosis 2 (TSC2) that forms a complex together with TSC1, is phosphorylated by AKT, which blocks the ability of TSC2 to act as a GTPase-activating protein for RAS homolog enriched in brain (RHEB). This allows accumulation of RHEB-GTP, leading to activation of mTOR complex 1 (mTORC1). Furthermore, phosphorylation and inhibition of the proline-rich AKT substrate 40 (PRAS40) by AKT relieves PRAS40-mediated inhibition of mTORC1. Among the most prominent downstream targets in the mTOR pathway are the

eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase (S6K), being either repressed or activated by mTORC1 mediated phosphorylation, respectively. Alternatively, AKT can be activated by mTORC2 after its activation by RTK signaling and S6K by cytoplasmic PDK1, independently of AKT.

A negative feedback mechanism to attenuate PI3K/AKT/mTOR signaling represents phosphorylation of IRS1 by mTORC1 and S6K leading to its degradation.

1.4.2 Regulation of biological functions

Cell survival

As exemplified by the BCL2-associated agonist of cell death (BAD) in Figure 9, AKT promotes cell survival through regulation of the function or expression of several B-cell CLL/lymphoma 2 (BCL2) homology domain 3 (BH3)-only members with a pro-apoptotic function. Otherwise, their activated forms would inactivate other BCL2 family members, stimulating cell survival.

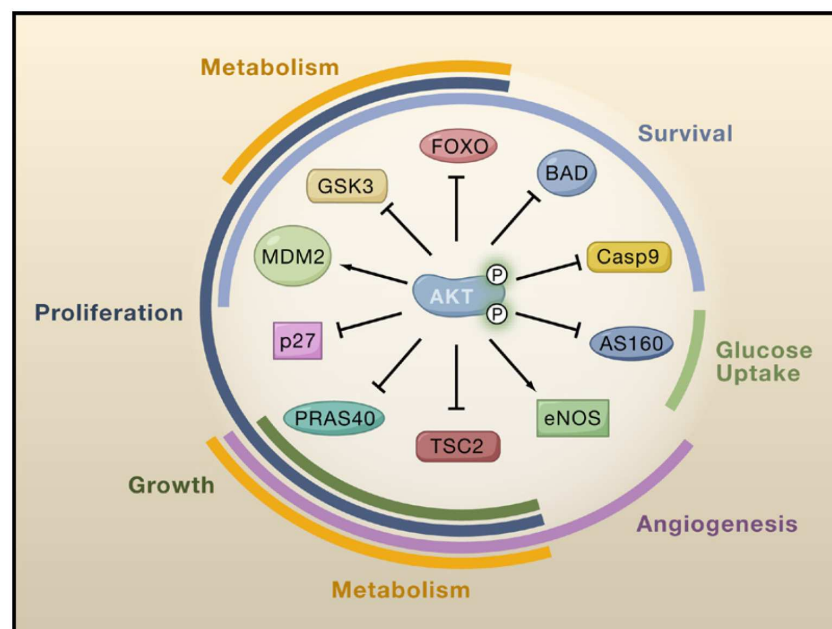


Figure 9. Biological functions of 10 representative AKT substrates (Manning and Cantley 2007). AKT-mediated phosphorylation can either result in protein activation (arrows) or inhibition (blocking arrows), controlling various cellular processes. Notably, a high degree of functional versatility and overlap exists amongst different AKT substrates. See text for detailed descriptions.

Additionally, AKT inhibits the expression of BH3-only genes indirectly through effects of forkhead box-O (FOXO) transcription factors and the E3 ubiquitin ligase mouse double minute 2 (MDM2) (Manning and Cantley 2007). Activated MDM2 triggers tumor suppressor protein p53 degradation (Mayo and Donner 2001), thereby preventing p53 dependent transcription proapoptotic genes that play an important role in p53-induced apoptosis (Villunger, Michalak et al. 2003). Furthermore, AKT inactivates glycogen synthase kinase 3 (GSK3) (Cross, Alessi et al. 1995) which prevents GSK3 isoforms to activate different substrates with pro-apoptotic roles. AKT also decreases the protease activity of procaspase-9 by phosphorylation, or indirectly by effects of BCL2 family members (Cardone, Roy et al. 1998).

Cell-survival effects of AKT can also be initiated by crosstalk with other pathways, such as the activation of nuclear factor (NF) κ B survival signaling or inhibition of c-Jun NH(2)-terminal kinase (JNK)/p38 apoptotic signaling (Nidai Ozes, Mayo et al. 1999; Kim, Khursigara et al. 2001).

Cell growth and proliferation

AKT mediated promotion of cell growth (an increase in cell mass) through activation of mTORC1 by nutrients and growth factor signaling represents one of its best conserved functions. It is well established that mTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control (Wullschleger, Loewith et al. 2006).

Different downstream targets of AKT stimulate proliferation through cell-cycle regulation (Figure 9). For instance, AKT mediated phosphorylation of CDKN1B/p27 and CDKN1A/p21 leads to their cytosolic sequestration, relieving their cell-cycle inhibitory effects (Zhou, Liao et al. 2001; Liang, Zubovitz et al. 2002). Furthermore, the transcription of CDKN1A/p21 is inhibited by the inhibitory effects of AKT on p53 signaling (Zhou, Liao et al. 2001). Additionally, the AKT targets GSK3, TSC2 and PRAS40 were described to drive cell proliferation, either through stabilization or synthesis of different proteins involved in cell-cycle entry.

AKT activity has been shown to be elevated during the G2/M phase. Strikingly, activated AKT was shown to promote progression through mitosis, even in the presence of DNA damage by phosphorylation of the DNA damage checkpoint kinase homolog 1 (CHK1) (Kandel, Skeen et al. 2002). This stimulates its translocation to the cytosol where it is sequestered from the DNA damage-sensing kinases ataxia

telangiectasia mutated (ATM) and ataxia telangiectasia Rad3 related (ATR) (Puc, Keniry et al. 2005).

Cellular metabolism

A variety of AKT downstream targets regulate nutrient uptake and metabolism in a cell-intrinsic and cell-type-specific manner (Figure 9). The main glucose transporter in most cell types is the glucose transporter type 1 (GLUT1) which is primarily regulated by differential gene expression. Hypoxia inducible factor 1 α (HIF1 α)-dependent transcription of the GLUT1 gene as well as cap dependent translation of GLUT1 mRNA is initiated by activated mTORC1 (Taha, Liu et al. 1999). Furthermore, glycogen synthesis is stimulated by AKT-mediated phosphorylation and inhibition of GSK3, preventing GSK3 from phosphorylating and inhibiting glycogen synthase. Consistent with the high glycolytic nature of tumor cells, AKT activation also increases the rate of glycolysis (Elstrom, Bauer et al. 2004).

Angiogenesis

AKT can stimulate angiogenesis through different mechanisms (Figure 9). For instance, activation of endothelial nitric oxide synthase (eNOS) through direct phosphorylation results in a release of nitric oxide and leads to vasodilation, vascular remodeling and angiogenesis (Fukumura and Jain 1998). Additionally, AKT directly phosphorylates and activates HIF1 α and thereby stimulates the secretion of vascular endothelial growth factor (VEGF) and other angiogenic factors (Gordan and Simon 2007).

Cell migration

Studies on cell migration using mouse embryonic fibroblasts deficient of specific AKT isoforms have suggested opposite effects on fibroblast migration, suggesting that different isoforms can have distinct cellular functions (Zhou, Liao et al. 2001).

1.4.3 Aberrant PI3K/AKT/mTOR pathway activation in cancer

Inappropriate activation of the PI3K/AKT/mTOR signaling pathway as a consequence of aberrant activation by cell surface receptors or somatic mutations of downstream pathway components can be observed in different forms of cancer.

For instance, activating mutations in the RTK fms-related tyrosine kinase 3 (FLT3), represent the second most frequent genetic lesions in AML. These mutations lead to ligand-independent dimerization of FLT3, following constitutive up-regulation of its tyrosine kinase activity and stimulation of downstream signaling pathways, including PI3K/AKT/mTOR (Naoe and Kiyoi 2004). Furthermore, activating mutations in the extracellular or intracellular domains of the stem cell factor ligand KIT, representing a class III receptor tyrosine kinase, were identified in 20-30% of AML patients (Frohling, Scholl et al. 2005).

PI3K is also capable of binding directly to RAS, possibly bypassing the obligate engagement with phosphotyrosines (Rodriguez-Viciano 1994). In line with this notion, RAS mutations could be associated with constitutive PI3K activation in AML and more recently in T-cell ALL (Birkenkamp, Geugien et al. 2004; Gutierrez, Sanda et al. 2009).

Somatic mutations that activate the class IA PI3K catalytic subunit p110 α , encoded by PIK3CA as well as the regulatory subunit p85 of PI3K, encoded by PIK3R1, were observed in different solid tumors and in T-cell ALL (Philp 2001; Palomero, Sulis et al. 2007).

The first described genetic abnormalities downstream of PI3K were deletions of PTEN tumor suppressor in solid tumors, followed by T-cell ALL (Li 1997; Gutierrez, Sanda et al. 2009). Later, inhibition of PTEN expression, as well as increased phosphorylation, leading to an abrogation of its enzymatic activity and increased PI3K/AKT/mTOR pathway activity was identified in AML blasts (Xu, Simpson et al. 2003) (Cheong, Eom et al. 2003) (Vazquez, Grossman et al. 2001). Furthermore, dominant-negative mutations in the SH2 domain-containing inositol phosphatase (SHIP1) may contribute to AKT activation in AML (Luo, Yoshida et al. 2003).

Importantly, studies reporting about FLT3 mutations as well as deletions of PTEN and phosphorylated PTEN in AML also correlated these with adverse effects on prognosis.

Genetic alterations involving AKT isoforms have been reported in solid tumors and T-cell ALL (Carpten 2007; Malanga 2008; Gutierrez, Sanda et al. 2009).

Apart from somatic mutations of PTEN, PIK3CA, PIK3R1 and AKT, some cancers have amplifications of AKT1, AKT2 and PIK3CA (Brugge, Hung et al. 2007).

So far, mutations within the PI3K/AKT/mTOR pathway could not be detected in BCP ALL. However, since these cells are highly sensitive for PI3K and mTOR inhibition

(Avellino, Romano et al. 2005; Levy, Kahana et al. 2009), pathway activation may result from de-regulated expression or activation of certain pathway components in the absence of genetic alterations, as shown in AML blasts (Martelli, Nyakern et al. 2006).

1.4.4 Pharmacological PI3K/AKT/mTOR pathway inhibition

Since hyperactivation of the PI3K/AKT/mTOR pathway frequently confers drug resistance to leukemic cells, small molecule inhibitors targeting components of the PI3K/AKT/mTOR pathway such as PI3K, AKT and mTOR present promising targets for cancer therapy. These pathways inhibit many tumor suppressor-like proteins such as FOXO transcription factors, BAD, GSK3 and the tuberin/hamartin complex. This diverse set of proteins negatively regulates cell survival, proliferation and growth. Therefore, blocking this pathway could simultaneously inhibit the proliferation of tumor cells and sensitize them to apoptosis. Importantly, many components in the PI3K/AKT/mTOR pathway are protein kinases which can be targeted by different classes of drugs.

LY294002 was the first synthetic PI3K inhibitor. It efficiently inhibited cell growth *in vitro* and showed a significant *in vivo* antitumor effect. Consequently, LY294002 was widely used to explore the function of PI3K. However, skin-related toxic side effects due to the induction of apoptosis and, additionally, poor solubility and low bioavailability, prevented the entry of LY294002 into clinical trials (Hu, Zaloudek et al. 2000).

In recent years, a large number of PI3K pathway therapeutics have been developed and many potential clinical scenarios exist for their application.

Specific PI3K inhibitors are further differentiated into isoform-specific or pan-PI3K inhibitors. The latter inhibit all class IA PI3Ks in a given tumor, however, the application of isoform-specific inhibitors probably reduces the risk of side effects even if administered in high doses. The major drawback of specific PI3K inhibitors is that, they might not effectively repress AKT activation in cancers with mutations or amplifications in AKT1 or AKT2 since they do not inhibit mTORC2.

In these cases, dual PI3K/mTOR inhibitors targeting different p110 isoforms as well as mTORC1 and mTORC2 might be more effective. Additionally, they are expected to effectively shut down PI3K/AKT/mTOR signaling in cancers with PTEN loss, RTK-dependent activation of AKT via mTORC2 and might mitigate feedback activation of

PI3K, as frequently observed by mTORC1 inhibition (O'Reilly 2006). For instance, the synthetic inhibitor PI-103 simultaneously inhibits PI3K and mTOR and was shown to confer antitumor activity *in vivo*, attributed to its ability to target both kinases. Importantly, PI-103 did not cause obvious side effects. *In vitro*, PI-103 inhibited growth of glioma cells at submicromolar concentrations and arrested the cell cycle at the G1 phase (Fan, Knight et al. 2006). However, pharmacokinetic studies later indicated that PI-103 was metabolized to form glucuronide, being rapidly cleared from the plasma (Raynaud 2007). Thus, optimization of its pharmacokinetic properties by structural modification is currently underway.

In addition, AKT can be targeted by either ATP mimetics or non-catalytic site inhibitors (She 2008). Isoform-specific AKT inhibitors could be useful in cancers where AKT1 and AKT2 have distinct functions.

The fourth class of inhibitors are mTOR catalytic site inhibitors. In contrast to rapamycin, they are expected to inhibit both complexes, mTORC1 and mTORC2.

1.5 Gene expression profiling in ALL

Gene expression profiling was introduced in leukemia research a decade ago, enabling the simultaneous investigation of the expression of thousands of genes. In order to better understand the diversity of genetic aberrations in acute leukemias of the myeloid as well as lymphoid lineage, independent pioneer studies demonstrated the predictability of cytogenetic and molecular subclasses by differentially expressed genes. Moreover, gene expression profiling can identify and define new prognostic subclasses within distinct leukemia subgroups. For therapy of patients with acute leukemia, gene expression profiling can promote the development of treatment-specific sensitivity assays and assist in detecting new molecular targets (Bacher, Kohlmann et al. 2009).

1.5.1 Molecular signatures of ALL subtypes

Yeoh et al first demonstrated that prognostically important leukemia subtypes can be identified by gene expression profiling (Yeoh, Ross et al. 2002).

Later, these findings were confirmed by Ross et al who identified leukemia subtype discriminating genes from a larger proportion of the human genome using new expression profiling platforms.

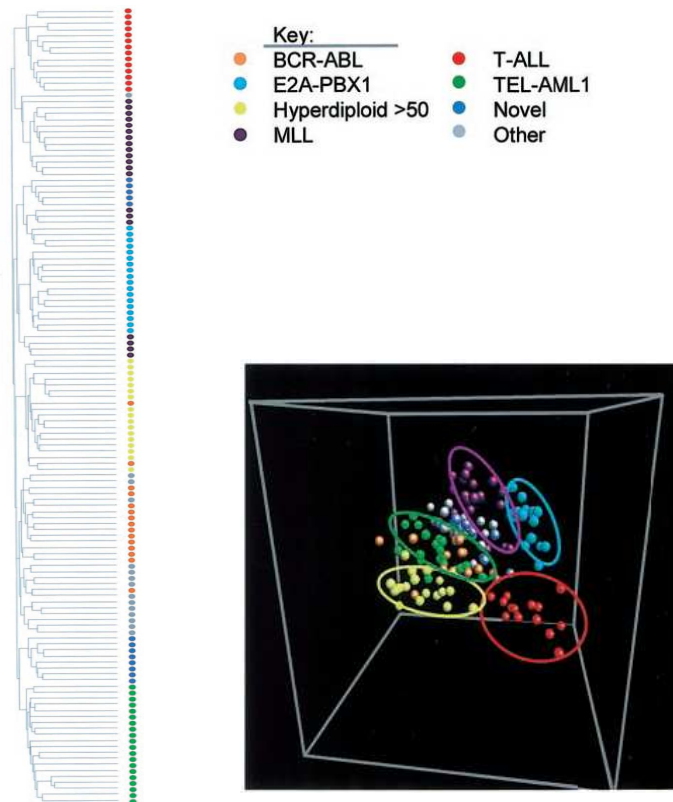


Figure 10. Expression profile based classification of distinct leukemia subtypes; modified (Ross, Zhou et al. 2003). Gene expression profiles were obtained from leukemic blasts of 132 diagnostic bone marrow aspirates. Data was analyzed by an unsupervised 2-dimensional clustering algorithm (left panel and top right panel) and principle component analysis (right panel).

These investigators applied a principle component analysis for diagnostic leukemia samples, revealing that childhood ALL cases clustered into T-cell lineage ALL and six different subtypes of B-cell lineage ALL, corresponding to rearrangements of the MLL gene, chromosomal translocations resulting in E2A/PBX1, BCR/ABL and E/R fusions, hyperdiploid leukemias with more than 50 chromosomes and a novel subgroup (Figure 10) (Ross, Zhou et al. 2003). Furthermore, leukemia subtype discriminating genes were identified. The expression patterns of the top 100 ranked genes for the six prognostically important subgroups are shown in Figure 11. As illustrated, distinct groups of genes being either over-expressed or under-expressed, clearly distinguish cases defined by the respective genetic subtype.

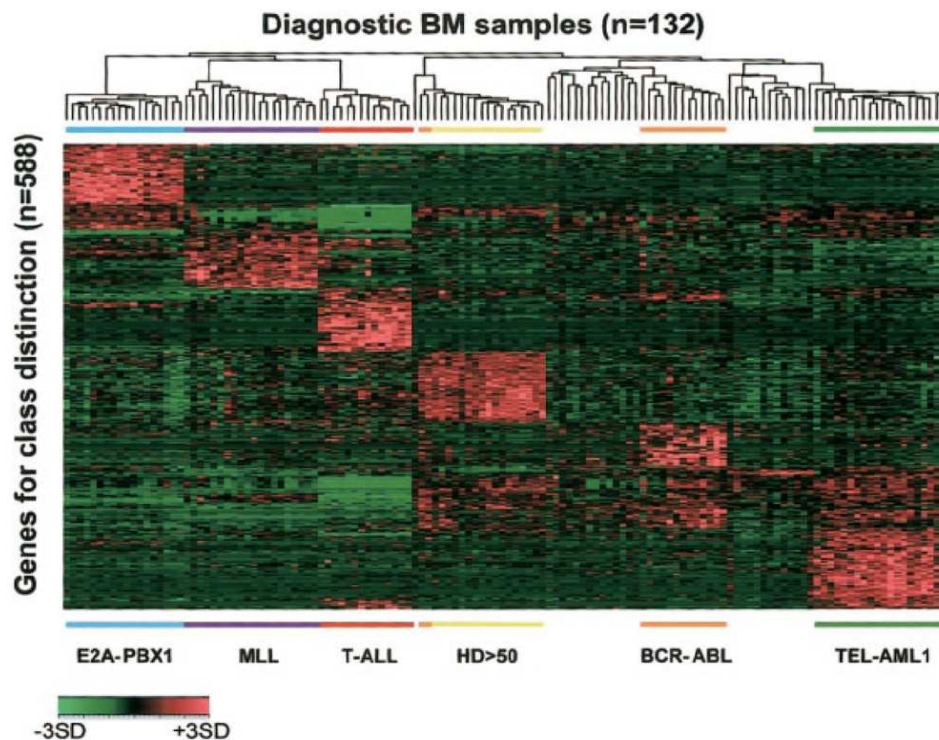


Figure 11. Two-dimensional hierarchic clustering of pediatric ALL (Ross, Zhou et al. 2003). Diagnostic leukemias (columns) vs. the top 100 chi-square ranked probe sets (rows) for the 6 subtypes of ALL, as indicated at the panel bottom. Red colours represent gene expression above the mean and green colours below the mean.

Later, Fine et al analysed gene expression in leukemic cell lines as well as primary leukemic blasts, thereby confirming many of the genes being previously associated with characteristic primary genetic change (Fine, Stanulla et al. 2004). Importantly, they highlighted a number of leukemia subtype specific features that were not immediately apparent in previous studies. For instance, they found that *BCR/ABL*-positive ALL cases have a more heterogeneous pattern of gene expression than E/R or *MLL* positive ALL cases.

Next, Anderson et al compared gene expression patterns of ALL and AML blasts to those of normal hematopoietic subpopulations of different lineages in order to show that leukemias segregate according to lineage and genetic subtype as well as identify genes that correlate with characteristic primary genetic change (Andersson, Olofsson et al. 2005). This approach also aimed to reveal de-regulation of pathogenetically important regulatory pathways. Collectively, a close relationship of lineages and maturation stages between malignant and normal hematopoietic cells could be observed. The degree of maturation of childhood leukemias with characteristic

genetic changes was visualized by projecting the primary leukemias onto the principal components determined only by the normal cells. For instance, E/R-positive cases clustered close to the precursor B-cells and, despite their common lack of the CD34 marker, E2A/PBX1-positive ALLs clustered close to the CD34/CD19 positive cells. These analyses also revealed that several genes were preferentially and highly expressed by leukemic cells and not in the various normal subpopulations, suggesting targets for future directed therapies. Obviously, leukemia cells display de-regulated activation of transcriptional programs not active in normal cells.

1.5.2 Gene expression features of ETV6/RUNX1-positive ALL

Apart from the identification of differentially regulated genes, several studies aimed to unravel molecular mechanisms of perturbed pathways in E/R-positive ALL by using either patient-derived primary cells or leukemic cell lines.

For instance, Fine et al found the erythropoietin receptor consistently highly expressed in E/R-positive samples (Fine, Stanulla et al. 2004). Later, these findings could be corroborated by our group, suggesting that this receptor might play a critical role in the development of E/R-positive ALL (Inthal, Krapf et al. 2008).

Zaza et al aimed to unravel the molecular basis of treatment associated characteristics of E/R-positive ALL (Zaza, Yang et al. 2004). These investigators correlated low rates of *de novo* purine synthesis (DNPS) in E/R-positive ALL vs. E/R-negative ALL with differential expression of 16 genes, providing a reasonable explanation for the favorable prognosis of E/R-positive ALL compared to other ALL subtypes. Importantly, anti-leukemic agents targeting DNPS, such as methotrexate or mercaptopurine are widely used.

De-regulated expression of genes involved in nucleotide metabolism was later confirmed by Andersson et al. Furthermore, they identified in E/R-positive leukemia an up-regulation of genes involved in the phosphatidylinositol signaling system, including Ca^{2+} regulators as well as the protein tyrosine phosphatases (PTP)N18, PTPRF, PTPRK and PTPRM (Andersson, Olofsson et al. 2005). The latter protein tyrosine phosphatases are also implicated in β -catenin signaling. Consistently, de-regulation of β -catenin signaling was shown to affect leukemic cell adhesion, proliferation, and survival (Chung, Hwang et al. 2002). Alterations of similar biological functions and processes in E/R-positive leukemias were later suggested by Gandemer et al. They performed gene-ontology (GO) analyses revealing that

differential expression of 16 genes tightly associated with E/R-positive leukemia, mapped to the GO terms 'cell differentiation', 'cell proliferation', 'apoptosis', 'cell motility' and 'response to wounding' (Figure 12) (Gandemer, Rio et al. 2007).

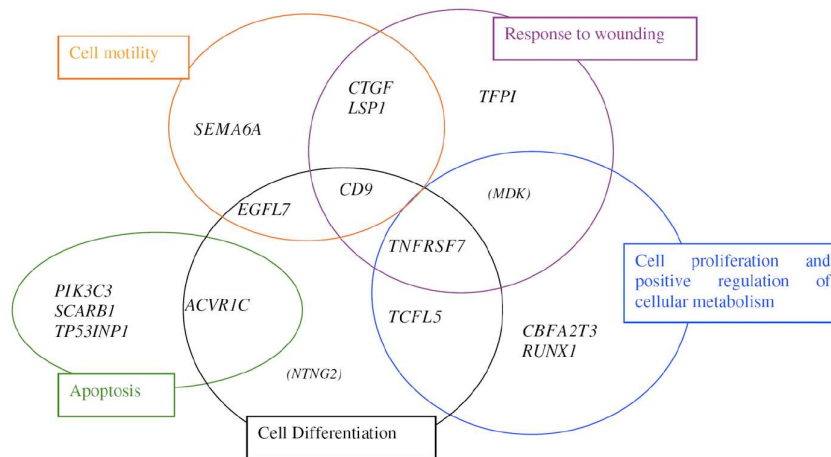


Figure 12. Schematic representation of enriched genes and associated GO terms in E/R-positive ALL; modified (Gandemer, Rio et al. 2007). Circles group genes that share common properties within relevant biological processes. Genes in brackets are expressed in a tissue-specific manner.

2. AIMS OF THE STUDY

There is overwhelming evidence that the E/R fusion protein is critical for the initiation of leukemia by inducing a pre-leukemic state and creating favourable conditions for the acquisition of obligatory secondary alterations. By contrast, the relevance of the chimeric fusion protein for maintenance of overt disease is not clearly defined. We hypothesized that repression of the endogenous fusion protein and investigation of the cellular and molecular consequences will provide important information on E/R function in the leukemic cell. In particular, the aim of our study was to develop a model for efficient and stable E/R fusion protein knockdown by lentivirus mediated shRNA transduction into the leukemic cell lines REH and AT-2.

E/R silenced cells were further used to

- I. evaluate the impact on the phenotype of the leukemic cell by assessing cell proliferation, apoptosis and cell cycle
- II. analyse their *in vivo* leukemia engraftment and outgrowth potential in xenotransplant experiments using NOD/SCID mice
- III. identify E/R regulated genes by RNA expression profiling; annotate regulated genes by DAVID and GSEA to describe suggested functions; compare gene regulation from E/R knockdown experiments with the signature from primary childhood E/R-positive ALL cases; finally validate selected target genes representative of various pathways or cellular functions by RT-qPCR
- IV. screen for modulations in cell signaling and pathways and confirmation at the functional level
- V. exploit inhibition of selected pathways activated by E/R and validate results in primary ALL cases.

3. FIRST-AUTHORSHIP PUBLICATION I

SILENCING OF ETV6/RUNX1 ABROGATES PI3K/AKT/MTOR SIGNALING AND IMPAIRS RECONSTITUTION OF LEUKEMIA IN XENOGRAFTS

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Andrea Inthal, Gerd Krapf, Ulrike Kaindl, Maximilian Kauer, Michael
N. Dworzak, MD, Dagmar Stoiber, Oskar A. Haas and Renate Panzer-
Grümayer**

Leukemia (submission approved on Mai 11th, 2011); in revision

3.1 Manuscript file

Title

Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts

Authors

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Running title

Function of ETV6/RUNX1 in childhood leukemia

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ABSTRACT

The ETV6/RUNX1 (E/R) gene fusion is generated by the t(12;21) and found in approximately 25% of childhood B-cell precursor acute lymphoblastic leukemia. In contrast to the overwhelming evidence that E/R is critical for the initiation of leukemia, its relevance for the maintenance of overt disease is less clear. To investigate this issue, we suppressed the endogenous E/R fusion protein with lentivirally transduced shRNA in the leukemia cell lines REH and AT-2 and found a distinct reduction of proliferation and cell survival. In line with the observed concurrent inactivation of the PI3K/AKT/mTOR pathway, pharmacological inhibition diminished the phosphorylation of AKT and RPS6 and significantly increased the apoptosis rate in E/R-positive leukemias. Moreover, PI3K/mTOR inhibitors sensitized glucocorticoid resistant REH cells to prednisolone, an observation of potential relevance for improving treatment of drug resistant relapses. Of note, knockdown of the E/R fusion gene also severely impaired the repopulation capacity of REH cells in NOD/SCID mice. Collectively, these data demonstrate that the E/R fusion protein activates the PI3K/AKT/mTOR pathway and is indispensable for disease maintenance. Importantly, these results provide a first rationale and justification for targeting the fusion gene and the PI3K/AKT/mTOR pathway therapeutically.

KEY WORDS

childhood ALL, ETV6/RUNX1, PI3K/AKT/mTOR pathway, RNA interference

INTRODUCTION

The *ETV6/RUNX1* (E/R) fusion gene (also known as *TEL/AML1*) is present in approximately 25% of childhood B-cell precursor acute lymphoblastic leukemia (BCP ALL) and represents the molecular equivalent of the t(12;21) [1]. Despite an overall excellent initial treatment response, up to 20% of these cases relapse [2-4]. The predominantly late occurrence of these relapses together with a considerable clonal variation between presentation and relapse implies that a postulated E/R-expressing (pre)leukemic sub-clone resists treatment and acquires distinct additional genomic aberrations during the secondary expansion and re-emergence [5-8]. In contrast to the general notion that these relapses may be as treatment sensitive as the corresponding original leukemias, recent data from our group suggest that a substantial proportion of these cases fare poorly and may even qualify for stem cell transplantation [5]. Treatment intensification may overcome resistance in some cases, but will also increase the frequency of severe side and late effects. Particularly in such forms of leukemia, new treatment modalities that only target and exploit the relevant specific genetic defect are clearly needed.

Supported by ample evidence, the multi-step model of E/R-positive leukemia currently implies that the leukemia initiating gene fusion occurs already *in utero* and is followed by several additional mutational hits before the disease becomes clinically evident [9]. Provided the chimeric protein is also required for the leukemia replenishing process, as was suggested recently, both the specific molecular alteration itself as well as any affected signaling pathway components would constitute ideal targets for specific therapeutic attacks [10].

The phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway senses and responds to mitogenic stimuli, energy and nutritional status and thereby represents an essential regulator of many cellular processes such as protein synthesis, proliferation and apoptosis. Its constitutive activation in many forms of malignancies attests its pathogenetic importance [11, 12]. Consequently, a variety of small molecules have already been developed to therapeutically target specific pathway components [13]. Although T-cell precursor ALL (T-ALL) seems to be the most frequent ALL form with a constitutive PI3K/AKT/mTOR pathway activation, only some of these cases resist therapy and consequently have a poor prognosis [14]. Despite an apparent lack of mutations in specific PI3K/AKT/mTOR pathway components in the vast majority of BCP ALL cases, analyses of gene

expression data nevertheless suggest that the PI3K/AKT/mTOR pathway is also aberrantly activated in E/R-positive ALL [15-18]. To investigate these preliminary observations further, we silenced the *E/R* fusion gene with a lentiviral vector transfer of fusion gene-specific shRNAs into leukemia cell lines, studied the evoked effects on cellular functions and affected pathways *in vitro* and compared the *in vivo* malignant potential of untreated and treated cells in NOD/SCID mice.

MATERIAL AND METHODS

Cell culture

The E/R-positive BCP leukemia cell lines REH (DSMZ, Braunschweig, Germany) and AT-2 [19] (kindly provided by J.D. Rowley, University of Chicago, IL) and primary leukemia blasts, obtained from bone marrow aspirations of children with ALL, were cultured as described previously [20]. In accordance with the Declaration of Helsinki, we obtained written informed consent from all patients' parents for using spare leukemia cells in this study. The study was approved by the ethical committees of the Children's Cancer Research Institute and St. Anna Kinderspital. LY294002 (Calbiochem Corporation, San Diego, CA) and PI-103 [21] (Selleck Chemicals, Houston, TX) were used at 40 μ M and 2 μ M concentrations, respectively. Prednisolone (Solu-Dacortin, Merck, Whitehouse station, NJ) was used at 1 mg/mL [20].

Immunoblotting

Cell lysates were prepared, resolved and transferred as described previously [20] using 90 μ g of total protein. Membranes were incubated with anti-ETV6 (SB89, kindly provided by P. Marynen, VIB, Leuven, Belgium), anti-RUNX1 (Active Motif, Carlsbad, CA) and anti-GAPDH (6C5, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary infrared dye-labeled antibodies (LI-COR Biosciences, Lincoln, NE). Membranes were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences). Intensities of bands corresponding to E/R and RUNX1 were normalized to the signal of GAPDH.

Assessment of protein phosphorylation by flow cytometry and ELISA

REH cells were kept overnight in serum-free medium before stimulation with human recombinant stem cell factor, IL-3, IL-6 and IL-7 (10 ng/mL each) (Peprotech, Rocky

Hill, NJ) for 10 minutes, or kept in culture medium containing only 10% FCS, to assess induced and basal protein phosphorylation, respectively. Fixed and permeabilized cells were incubated with fluorescence conjugated antibodies binding RPS6 (Ser235/236), AKT (Ser473), p38 (Thr180/Tyr182), NFκB (Ser529), STAT5 (Tyr694) and JNK (Thr183/Tyr185) (Cell Signaling Technology, Danvers, MA) and analyzed by flow cytometry (FACS Calibur, BD-Pharmingen, Franklin Lakes, NJ). AKT phosphorylation was also determined with the PathScan Apoptosis Multi-Target Sandwich ELISA (Cell Signaling Technology) according to the manufacturer's recommendations.

RNA interference

For E/R silencing two sequences targeting the fusion region (Figure S1) were used: shRNA A1 (5'-CACCGAATAGCAGAATGCATACTTCGAAAAGTATGCATCTGCTATT-3') has been described and tested previously [10, 22] and shRNA G1 (5'-CACCGGGAGAATAGCAGAATGCATCGAAATGCATTCTGCTATTCTCCC-3') was newly designed to confirm and extend our previous data.¹⁰ shRNA LacZ (Invitrogen, Carlsbad, CA) was used as a non-targeting shRNA control. Lentiviral particles were generated using the Block iT Lentiviral RNAi Expression System (Invitrogen) and, following ultracentrifugation, titers were assessed by ELISA-based quantification of virus associated HIV-1 p24 (Cell Biolabs, San Diego, CA) according to the manufacturer's recommendations. Leukemia cells were transduced by a multiplicity of infection of 10 in the presence of 6 µg/mL polybrene and selected for lentiviral integration with 5 µg/mL blasticidin (Invitrogen) for 2 weeks.

Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated by Trizol reagent (Life Technologies, Carlsbad, CA) and used for cDNA synthesis by SuperScript II Reverse Transcriptase (Invitrogen). RUNX1 and E/R transcripts were quantified by TaqMan RT-qPCR using the ABI Prism 7900 Detection System (Applied Biosystems, Foster City, CA). For box plot diagrams cycle threshold $\Delta(CT) \times -1$ values ($CT_{\text{target}} - CT_{\text{reference}}$) of biological replicates were depicted. Published primer probe combinations were used for E/R and GUS as endogenous reference [5, 23]. RUNX1 mRNA levels were quantified by the following primer/probe combination: forward 5'-CCTTCGTACCCACAGTGCTT-3', reverse 5'-CAACGCCTCGCTCATCTT-3', probe 5'-TCTAGAGACGTCCACGATGCCA-3'.

Cell cycle, apoptosis and viability assays

DNA synthesis and cell cycle distribution was determined by Click-iT EdU (5-ethynyl-2'-deoxyuridine) Flow Cytometry Assay (Invitrogen) according to the manufacturer's recommendations and analyzed using the FlowJo software (Tree Star, Ashland, OR). The proportion of apoptotic cells was assessed by flow cytometry using Annexin-V/propidium iodide (PI) staining, and cleaved Caspase 3 (BD-Pharmingen) staining after gating on the viable cell population based on light scatter characteristics. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as described previously [20].

Gene expression profiling

A detailed description on gene expression profiling is provided as Supplementary Information. Briefly, RNA from E/R silenced REH and AT-2 cells was used for gene expression profiling on Affymetrix HG-U133-PLUS2 (Affymetrix, Santa Clara, CA). Analyses were performed in R using Bioconductor packages [24] with mean values of biological replicates ($n = 3$) at two different time points after transduction of REH and AT-2 cells. Differential regulation of molecular functions was evaluated by gene set enrichment analysis (GSEA) using the Java tool (<http://www.broadinstitute.org/gsea/index.jsp>) [25, 26]. Data from primary childhood ALL were downloaded from the St. Jude's data base (<http://www.stjude-research.org/site/data/ALL3>) [17].

Leukemia xenograft experiments

5×10^6 REH cells stably expressing either shRNA G1 or control shRNA were injected into the tail vein of NOD/SCID mice. Diseased animals were sacrificed and bone marrow, spleen and liver analyzed for the presence of human leukemia cells by flow cytometry using anti-human CD19 (30-F11) and anti-mouse CD45 (4G7) antibodies (both BD-Pharmingen). All animal experiments were approved by the Austrian Federal Ministry for Science and Research GZ:66.009/0065-II/10b/2009.

Statistical analysis

Differences between E/R silenced and control cells were assessed by the paired two-tailed t-tests and between different model cell lines and various leukemias by the unpaired two-tailed t-tests. Statistical analysis of leukemia free survival of mice

between groups was performed using Log-Rank test. P -values ≤ 0.05 were considered statistically significant.

RESULTS

Lentiviral mediated transduction of E/R-directed shRNA expressing vectors silences the fusion gene

Long-term suppression of E/R in the leukemia cell lines REH and AT-2 was achieved by a lentiviral transfer of shRNA expressing vectors A1 and G1 that target different sequences of the E/R fusion transcript.

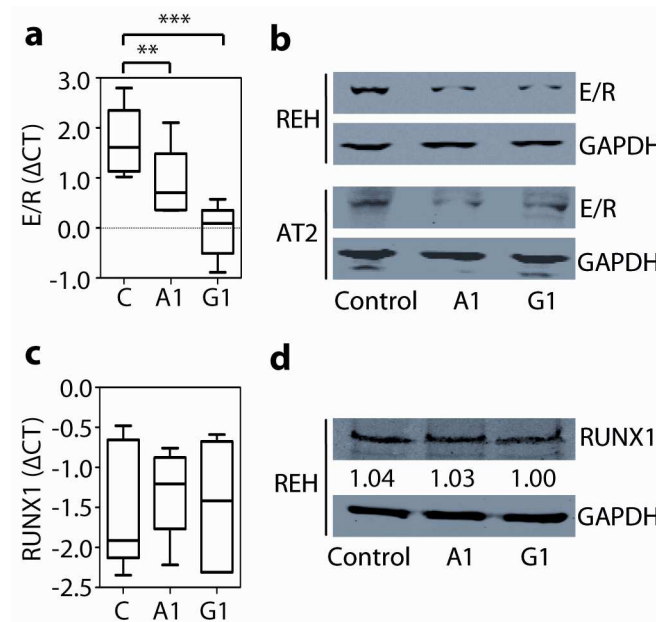


Figure 1. shRNA-mediated silencing of E/R in t(12;21) positive leukemia cell lines. Leukemia cell lines REH and AT-2 were transduced with lentiviral constructs coding for shRNA A1 (A1) or G1 (G1) targeting E/R or a non-targeting shRNA control (C). Upon selection, expression of E/R and wt RUNX1 was quantified. Quantification of E/R (a) and RUNX1 (c) transcripts in biological replicates from REH and AT-2 cells ($n = 5$); **, $P \leq 0.01$; ***, $P \leq 0.001$ (paired t-test). Protein abundance of E/R (b) and RUNX1 (d) was detected by Western blot analysis using anti-ETV6 and anti-RUNX1 antibodies, respectively. GAPDH was used as loading control. Numbers between bands represent the ratio between RUNX1 and GAPDH quantification. Data are representative of at least three independent E/R knockdown experiments.

The respective cell lines were derived from E/R-positive relapse samples, express wt RUNX1 and lack the non-rearranged *ETV6* gene. We used a GFP-expressing

construct to confirm that the lentiviral infection was more than 90% efficient (data not shown). Hence, this particular lentiviral system was employed for the delivery of shRNA A1 and G1 as well as a non-targeting control shRNA. Lentiviral titers were assessed in each batch to assure constant transduction efficacy and the corresponding knockdown effects. One week after selecting the stable transfectants, both cell lines exhibited a substantial and constant reduction of E/R transcript and protein levels with merely minor variations between different experiments and cell lines (Figure 1a and b). Of note, knockdown of the fusion gene did not repress wt RUNX1 expression (Figure 1c and d). With this standardized procedure, which satisfactorily suppressed E/R two to three weeks after viral transduction, we were therefore able to evaluate the outcome of the respective depletion.

E/R suppression abrogates cellular proliferation and survival

Regular cell counts revealed a gradual decrease of cellular growth down to 40-50% in E/R depleted versus control REH and AT-2 cell lines two week after selection, which subsequently stabilized for additional three to four weeks (Figure 2a). Growth suppression tended to be more pronounced with shRNA G1 compared to shRNA A1, most likely reflecting the differences in reduction of the fusion gene transcripts. Taking into account previous work that dealt with modulating effects of forced E/R expression on apoptosis, cell cycle and proliferation [27-30], we were especially keen to determine which of these systems would be predominantly de-regulated in our endogenous E/R knockdown model and responsible for the observed growth suppression. In line with our earlier observations that siRNA induced fusion gene silencing augments apoptosis [10], the proportion of early apoptotic cells (Annexin-V-positive/PI-negative) in E/R-silenced cells also increased 1.5 to 3-fold (Figure 2b). In contrast to our expectations that E/R depletion would unleash the break from G1 to S transition and proliferation [27-30], E/R knockdown led to a distinct decline of proliferating cells (from a mean of 37% in controls to 24% in silenced cells) (Figure 2c). Together with the cell cycle distribution showing a modest increase of the G0/G1 compartment in E/R silenced cells (Figure 2d), the decelerated proliferation rate can therefore - at least partly - be attributed to a G1 arrest with a concomitant increase in apoptosis at the transition to S that could account for the selective loss of S phase.

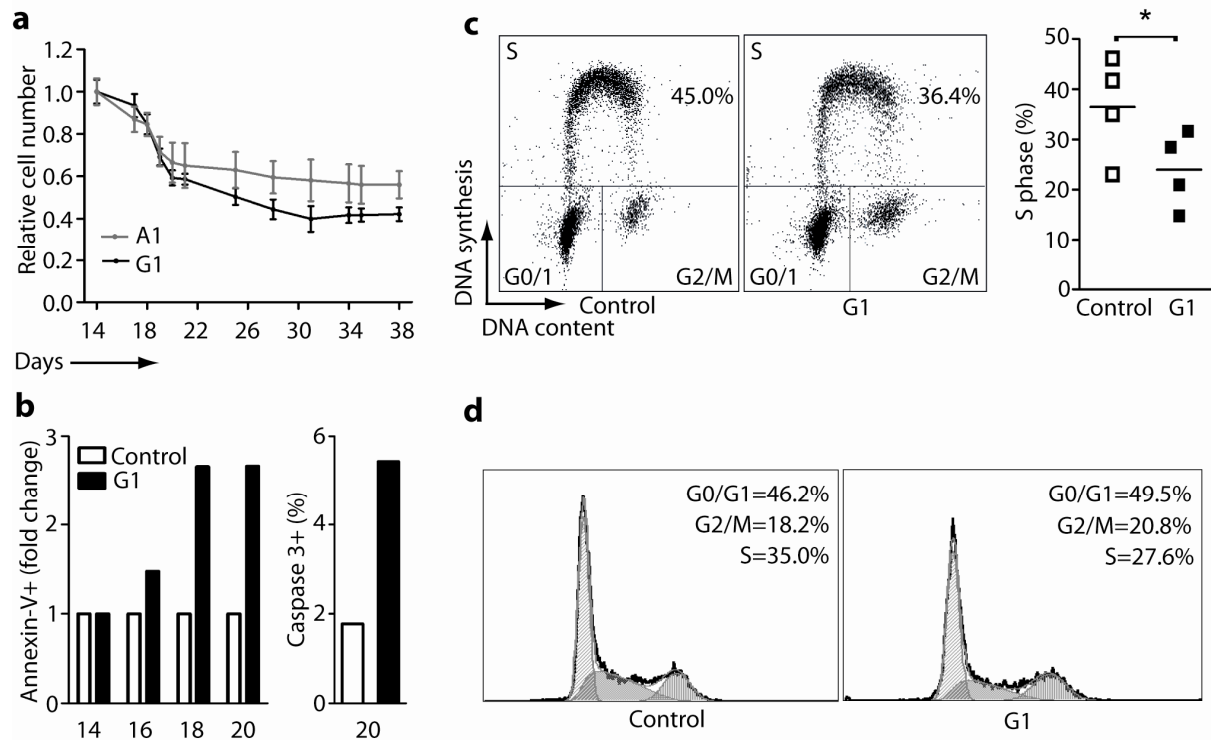


Figure 2. Suppression of E/R modulates cellular proliferation, survival and cell cycle distribution in leukemia cell lines. REH cells were subjected to shRNA-mediated silencing of E/R as described in Figure 1. (a) Cell growth was assessed by regular cell counts after transduction and is depicted for REH cells expressing shRNA A1 or G1 in relation to shRNA control. Shown are means with standard deviation of four independent E/R knockdown experiments. (b) Apoptosis was analyzed by Annexin-V/PI (left) and cleaved Caspase 3 staining (right). Bars represent fold changes of cells expressing shRNA G1 compared to shRNA control. Shown is one of four experiments from individual E/R knockdowns. (c) Evaluation of DNA synthesis by EdU incorporation. Left: Representative scatter plot; Right: Percentages of cells in S phase of the cell cycle of four independent E/R knockdown experiments. *, $P \leq 0.05$ (paired t-test). (d) Influence of E/R knockdown on cell cycle distribution. Histograms represent one of four independent E/R knockdown experiments.

A possible effect of E/R suppression on the mitotic checkpoint function [31] was addressed by phospho-histone H3 staining but did not reveal any differences between E/R depleted cells and controls (5-8% positivity in two independent E/R knockdown experiments; data not shown).

These findings suggest that E/R silencing alters the growth pattern of the leukemia cell lines REH and AT-2 by impeding proliferation and survival.

E/R suppression inhibits PI3K/AKT/mTOR activity

To identify potential causes for the observed growth retardation, we compared the

gene expression profiles from E/R-silenced REH and AT-2 cells between day 20 and 30 after viral transduction with those obtained from shRNA control cells and performed GSEA. This analysis revealed a substantial enrichment of the KEGG terms "phosphatidylinositol signaling system" (negative enrichment score = 0.426; *P*-value = 0.082) and "mTOR signaling pathway" (positive enrichment score = 0.494; *P*-value = 0.038) (Figure 3a). To further ascertain potential effects on the PI3K/AKT/mTOR cascade, we screened our microarray data for the presence of informative gene expression patterns [32]. The resulting genes along with information on their described influence on pathway regulation and/or downstream effectors [32] are listed in supplementary Table S1. Overall, E/R suppression down-regulates genes with activating and, vice versa, up-regulates those with inhibitory effects on the pathway and downstream components. A specific transcriptional regulation of these genes by E/R was further supported by inspecting publically available microarray data from primary childhood ALL [17]. In line with the results retrieved from our E/R knockdown experiments, we found an inverse correlation of the respective differentially expressed genes in the leukemias, which further corroborate the notion that E/R regulates especially pathway-associated genes with an anti-apoptotic and mitogenic function (Figure 3b).

Given that PI3K/AKT/mTOR signaling is mainly regulated at the post-transcriptional level, we investigated whether E/R silencing also affects protein phosphorylation of selected downstream substrates. E/R suppression reduced the cytokine mediated phosphorylation of AKT at Ser473 and ribosomal protein S6 (RPS6) at Ser235/236 by 50% and 30%, respectively (Figure 3c), whereas phosphorylation of other key signaling proteins that are frequently altered in hematologic malignancies remained largely unaffected [11].

The impaired phosphorylation of RPS6, a substrate of mTOR, is of specific interest, because this protein controls cell growth and proliferation through its regulation of protein synthesis [33]. A reduced AKT phosphorylation at Ser473, and additionally at Thr308, was also seen with an ELISA system (Figure 3d). Since an E/R knockdown especially affects proliferation and apoptotic processes and, vice versa, E/R expression apparently activates the PI3K/AKT/mTOR pathway, we assume that the perturbation of these cellular effects are mediated by this pathway to at least some extent. To further investigate the influence of PI3K/AKT/mTOR signaling on proliferation and apoptotic processes in E/R-expressing leukemia cells, we compared

first basal phosphorylation of PI3K and mTOR downstream effectors in REH and AT-2 cells upon E/R depletion with those of the pharmacologic pan-PI3K family inhibitor LY294002 and the small-molecule dual PI3K/mTOR inhibitor PI-103 [21].

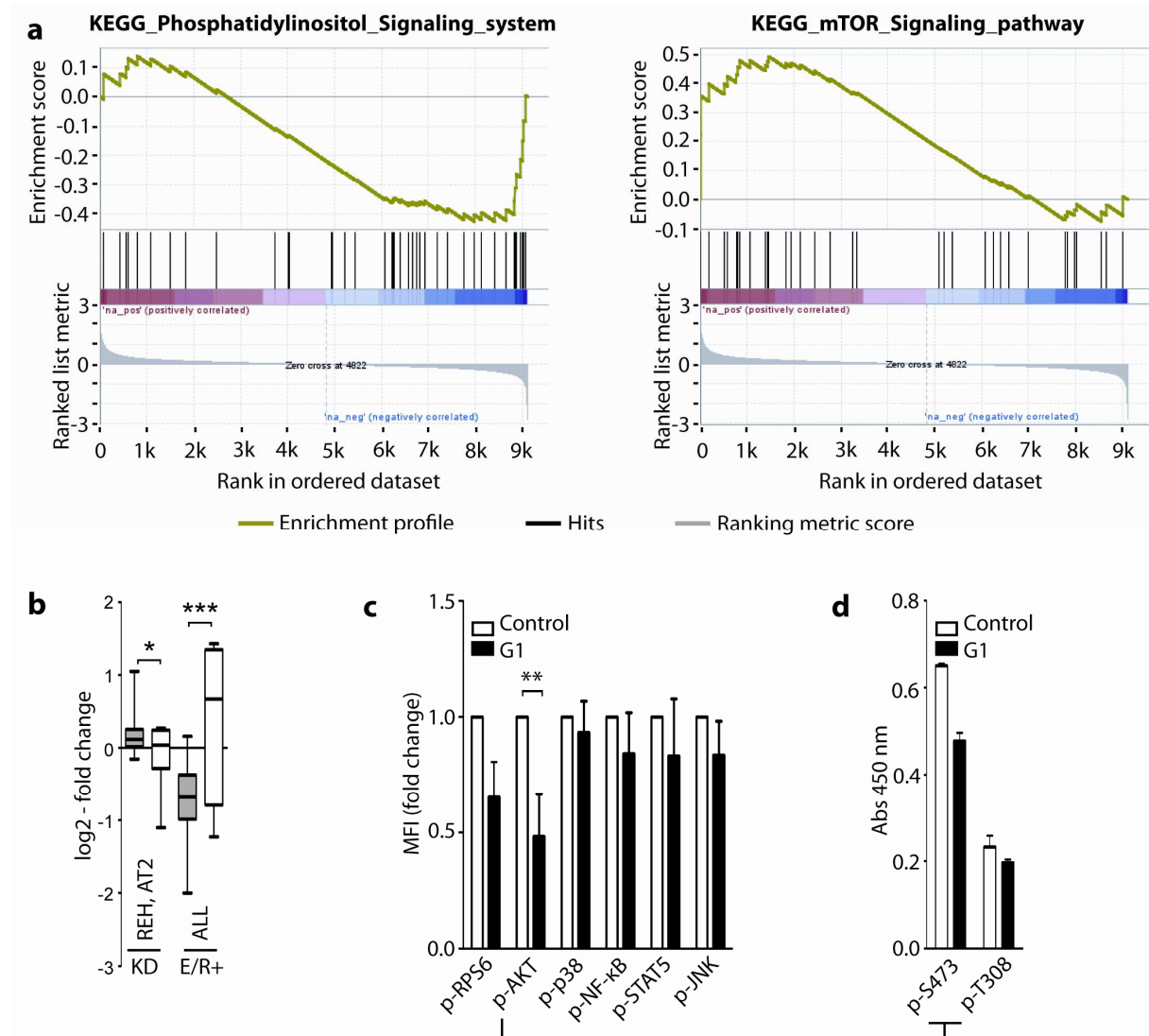


Figure 3. Suppression of E/R reveals a distinct relationship between fusion gene expression and activation of the PI3K/AKT/mTOR pathway. Expression profiling was performed from REH and AT-2 cells upon suppression of E/R by shRNA G1 compared to shRNA control. (a) GSEA plots showing the distribution of differentially regulated genes from the KD experiments for the “phosphatidylinositol signaling system” (left) and “mTOR signaling pathway” (right). Lower portions of the figure (ranking metric score) depict the distribution of the log₂-fold changes of all genes in the analysis, ordered from left with genes being highly expressed to the right with downregulated ones. Vertical black lines (hits) indicate the position of genes in ranked order. Upper portions show the enrichment score: if the peak of the graph (enrichment profile) lies on the left side, the pathway is enriched in up-regulated genes and vice versa. (b) Box plot diagram depicting the log₂-fold changes of genes involved in PI3K/AKT/mTOR signaling (grey, repressing and white, activating the pathway and/or its downstream targets). Gene sets were extracted from microarray data derived from E/R-

silenced REH and AT-2 cells vs. non-targeting controls (KD) and primary E/R-positive vs. E/R-negative BCP ALL (E/R+ ALL). Boxes cover the median and the interquartile range (25-75th percentiles) and whiskers the minimum and maximum values. *, $P \leq 0.1$; ***, $P \leq 0.01$ (unpaired t-test). (c) Cytokine mediated phosphorylation of major components of various key signaling pathways. Bars depict fold-changes of median fluorescence intensity (MFI) of specific phospho-protein stainings of E/R silenced (G1) and control REH cells (control). Controls were set to one. Shown is the median with standard deviation of three independent E/R knockdown experiments. **, $P \leq 0.05$ (paired t-test). (d) Quantification of phosphorylated AKT protein by ELISA upon cytokine stimulation. Bars indicate the mean absorbance (Abs) of triplicates with standard deviation from one of two independent E/R knockdown experiments.

Pharmacological inhibition of PI3K/AKT/mTOR signaling affects survival and proliferation of E/R-positive leukemias

LY294002 reduced phosphorylation of AKT and of RPS6 by 24% and 67%, respectively, in un-stimulated cells, which favorably compares with the 22% and 59% reduction that was achieved with E/R suppression (Figure 4a). Treatment of E/R-depleted REH and AT-2 cells with LY294002, on the other hand, did not further decrease phosphorylation of both proteins (data not shown). The small PI-103 molecule inhibited phosphorylation of AKT to a lesser but of RPS6 to a slightly higher extent than LY294002 (Figure 4a). Next, we tested whether PI3K/mTOR inhibitors are able to sensitize E/R-positive leukemia cells to prednisolone, a synthetic glucocorticoid that is widely used in ALL treatment regimens and to which relapse ALL cases may become resistant [3, 5]. Cell death and MTT assays collectively demonstrate that exposure of GC-resistant REH cells to either one of the two inhibitors or prednisolone alone affects cellular viability and apoptosis rates in a comparable manner. A joint exposure, on the other hand, significantly augments the GC-induced apoptotic effect (Figure 4b).

Finally, we investigated the potential clinical value of these findings by analyzing primary ALL samples. A 24-hour exposure of BCP ALL cells - from children who had been consecutively enrolled in the current Austrian treatment protocol - to LY294002 significantly increased the apoptotic rate of E/R-expressing cells in comparison to E/R-negative controls (Figure 4c). These data further support our notion that PI3K/AKT/mTOR signaling is aberrantly activated particularly in E/R-positive leukemias and that inhibition of this pathway could be a novel therapeutic option.

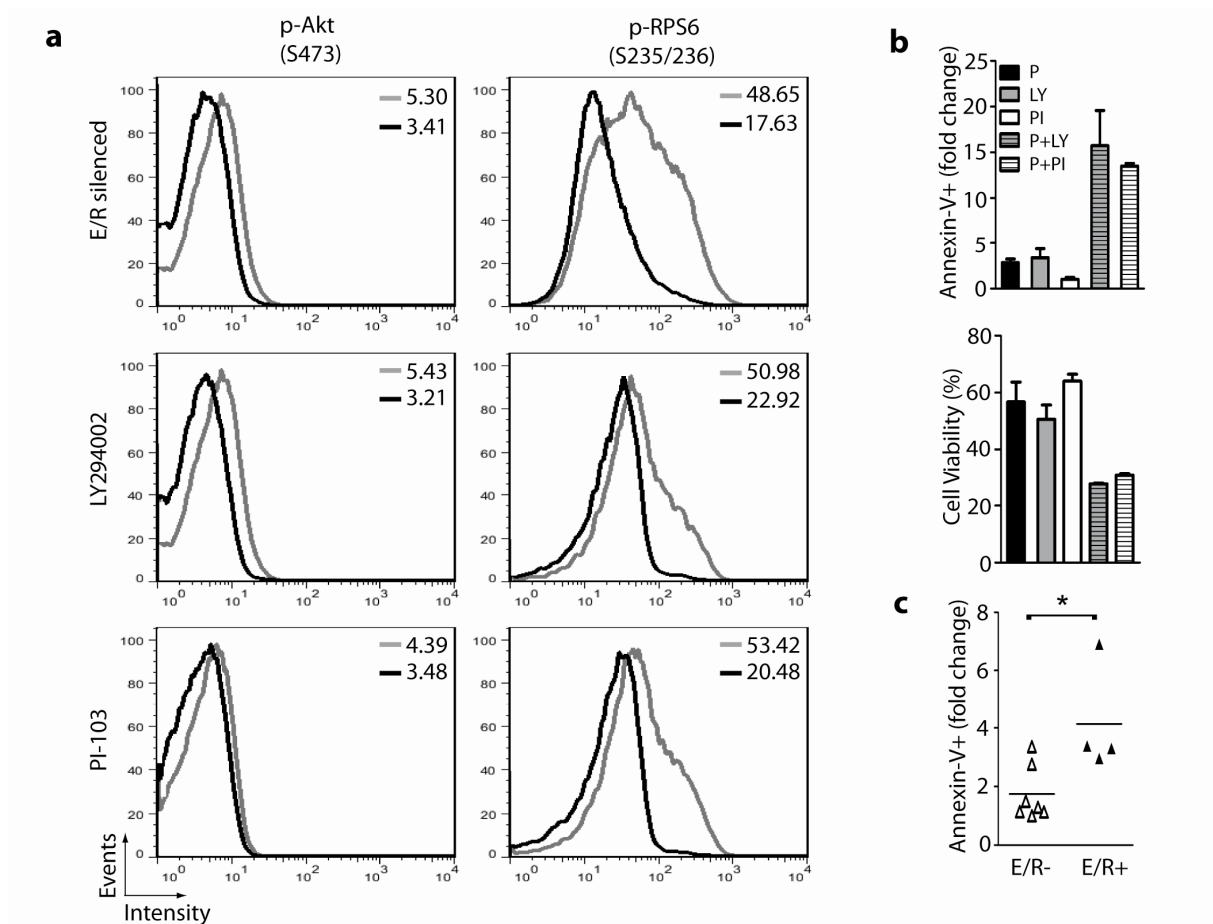


Figure 4. Activation of PI3K/AKT/mTOR signaling by E/R is blocked by fusion gene depletion or chemical inhibition. (a) Phospho-flow staining of AKT and RPS6 in REH cells cultured under standard conditions. Histograms showing phosphorylation under various conditions. Top: upon E/R silencing; middle: LY294002, and bottom: PI-103 treatment (black lines) and of respective controls (grey lines). Numbers indicate the median fluorescence intensity. Shown is one out of two independent experiments. (b) Apoptosis and viability assays of REH cells treated with either prednisolone (P), LY294002 (LY), or PI-103 (PI) alone or in various combinations for 24 hours. Percentages of Annexin-V single positive cells (upper panel) and viability by MTT assay (lower panel) are depicted as fold-change and percent of carrier control, respectively. Shown are the means of triplicates with standard deviation from one of three independent experiments. (c) Apoptosis levels in E/R-positive (E/R+) and E/R-negative (E/R-) BCP ALL samples after exposure to LY294002 for 24 hours indicated as fold-changes of carrier control. *, $P \leq 0.05$ (unpaired t-test).

Silencing of E/R impairs engraftment of leukemia in a xenotransplant mouse model

To address the functional consequences of E/R knockdown *in vivo*, we used differentially E/R-expressing REH cells in immunocompromized mice to investigate their repopulating capacity and ability to establish leukemia. We injected either E/R-

silenced or control shRNA expressing REH cells on day 15 after lentiviral transduction into the tail vein of NOD/SCID mice, which were then sacrificed either when they exhibited clinical signs of disease or electively in week 27. While all eight animals from the control group that had been transplanted with non-targeting shRNA infected REH cells, died from human leukemia, only five of eight recipients of E/R-silenced REH cells developed leukemia. The three remaining animals neither developed leukemia nor showed any signs of leukemia infiltration at the end of the observation period. Strikingly, mice receiving E/R-depleted REH cells survived for 15 weeks (median, range 8-15), whereas animals from the control group developed leukemia already 9 weeks (median, range 8-10) after injection.

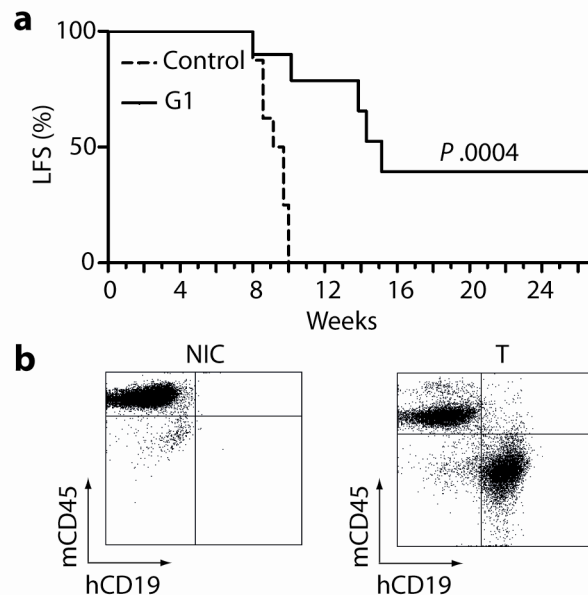


Figure 5. Reduced repopulation capacity of E/R suppressed REH cells in NOD/SCID mice. shRNA G1 or shRNA control expressing REH cells were injected into NOD/SCID mice (G1, $n = 8$; control, $n = 8$). (a) Kaplan Meier plots depicting leukemia free survival (LFS) of animals until week 27 after leukemia injection. Statistical analysis was done by the Log-Rank test. (b) Leukemia was confirmed in all sacrificed mice by flow cytometry. Staining of bone marrow cells was performed using anti-human CD19 and anti-mouse CD45 antibodies for the detection of human REH and mouse hematopoietic cells, respectively. Scatter plots represent examples from a non-injected control (NIC) and a transplanted animal with human leukemia (T).

Figure 5a shows a Kaplan Meier plot indicating a significantly prolonged leukemia free survival of recipients of E/R-depleted cells. Light microscopy and flow cytometry confirmed the presence of human leukemia in the bone marrow (Figure 5b) and peripheral blood and the dissemination of leukemia cells into the spleen irrespective of E/R silencing in diseased animals (data not shown).

These results provide first evidence that E/R expression essentially governs the repopulation capacity of leukemic REH cells and may thus be a critical factor for leukemia persistence in patients.

DISCUSSION

In continuation of our previous work we investigated the long-term consequences of RNAi-mediated E/R fusion gene silencing [10]. For this purpose, we used a lentiviral shRNA transduction system and vectors targeting the fusion site in cell lines that are accepted models for the respective types of leukemia because of their similar molecular make-up [34]. We found that suppression of the E/R fusion gene with specific shRNAs ablates the respective chimeric protein, slows down proliferation, promotes apoptosis and consequently leads to a profound reduction of treated cells. Yet despite their propensity to undergo apoptosis, a significant proportion of E/R-depleted cells still can survive and continue to grow *in vitro* at a low stable rate for many weeks. On the molecular level, E/R knockdown impedes the PI3K/AKT/mTOR signal pathway, which is aberrantly activated in E/R-positive childhood leukemias. Of particular practical and clinical relevance is the fact that an identical effect can also be achieved with pharmacological pathway inhibition in both systems. An indispensable role of the E/R fusion gene for cell survival and continuous cell replenishment is further corroborated by a diminished *in vivo* repopulation capacity of E/R-suppressed REH cells in a xenotransplant NOD/SCID mouse model. In case these mice develop leukemia at all within 27 weeks they did so significantly later than those receiving only mock treated cells.

First preliminary evidence that experimental introduction of E/R fusion gene sequences into cells may activate their PI3K/AKT/mTOR signaling pathway originally derives from transfected BA/F3 cells (unpublished observation). Our E/R silencing experiments together with the analysis of published gene expression data confirm this finding, since we were able to prove that the aberrant activation of this particular pathway is a specific outcome of the E/R gene fusion and therefore exclusively confined to the affected type of B-cell precursor ALL also in the natural setting [16-18, 35]. Conversely, the results of our experiments also preclude that any other type of genomic aberration, especially accompanying mutations of PI3K/AKT/mTOR pathway components, may be the cause of its constitutive activation [11]. As

evidenced by the drug resistance and poor outcome of some T-ALL cases with an activated PI3K/AKT/mTOR pathway, however, the extent of its activation as well as the ensuing biological effects are certainly also influenced by the particular cellular context in which they occur and are probably also modified by accompanying relevant mutations [14].

Apart from E/R's specific influence on the PI3K/AKT/mTOR signaling cascade, it is conceivable that it also enhances some upstream signaling components, such as the receptors for erythropoietin (EPOR) and the stem cell factor (KIT), which may further augment subsequent pathway effects. In a recent paper, we already demonstrated that up-regulation of the EPOR concurs with a survival advantage of E/R-positive cells and PI3K/AKT pathway activation [20]. Whether KIT - based on its over-expression in E/R-positive leukemias and differential regulation upon E/R knockdown (unpublished data) - also plays a similarly prominent role in E/R-positive leukemias as it does in acute myeloid leukemia, mastocytosis and gastro-intestinal stromal tumors, still remains to be shown. However, first preliminary evidence that this might indeed be the case was recently provided by the observation that at least RUNX1 can regulate KIT [36, 37]. In consideration of the central position that the PI3K/AKT/mTOR signaling cascade occupies in many cellular tasks that are especially critical for neoplastic behaviour in general [11, 38], it is very likely that, as implied by the results of our experiments, its aberrant activation is not only essential for initiation but also for the maintenance of E/R-positive leukemias. This assumption is further supported by the specific expression signature of E/R-positive leukemias, which also suggests the involvement of other PI3K/AKT/mTOR-regulated processes, including above all cell adhesion and DNA damage response [15, 39].

Pharmacological inhibition of the aberrantly activated PI3K/AKT/mTOR pathway has emerged as one of the therapeutic options for overcoming drug resistance [13]. Although E/R-positive leukemias are in general highly treatment-sensitive at initial presentation, a substantial proportion of them apparently become drug-resistant when they relapse [2, 5, 40]. As a proof of principle, we show that in GC-resistant cells prednisolone sensitivity can be partially restored with PI3K/mTOR pathway inhibition. In line with similar problems that are encountered in T-ALL cases whose drug resistance results from activating mutations in particular PI3K/AKT/mTOR pathway components, we thus consider PI3K/mTOR inhibition as a potential, plausible and useful accessory therapeutic option for resistant E/R-positive ALL [14].

The significantly impaired repopulation capacity of E/R-silenced REH cells in a xenotransplant NOD/SCID mouse model - which renders them unable to reconstitute leukemia at all or at least only after a substantial delay - is also of utmost biological and clinical importance. These results again provide additional evidence that the E/R fusion gene is not only relevant for initiation but also essential for sustaining the leukemic process and that therefore, disease regression can be achieved through knockdown of its activity. The idea that products of tumor-initiating mutated genes are subsequently also required for the continuation and maintenance of the neoplastic process is not restricted to E/R-positive leukemias. Based on similar observations in several other tumors and tumor cell lines, which experience comparable proliferation holdups and the propensity to undergo apoptosis, this phenomenon has also been labeled as oncogene addiction [41]. In contrast to a recent report, which suggested that except for a modest increase in S phase fraction and higher proliferation rate E/R down-regulation has no other discernable effect on cell behavior or gene expression [42], all evidence presented herein implies otherwise and proves that E/R has to be added to the growing list of oncogenes that not only initiate but also nourish the leukemia [43-45].

In conclusion, we have provided evidence herein that E/R expression is critical for the survival and propagation of the respective leukemia cells *in vitro* and *in vivo*. These findings have important biological and clinical implications. Based on what is known so far, the E/R gene fusion is the earliest and most specific alteration in this type of leukemia. Therefore, the fusion gene together with its products provides a tremendously interesting therapeutic target, because one need not pay attention to the extreme sub-clone variegation encountered in the E/R-positive cell pool [46]. The fact that key components of the PI3K/AKT/mTOR signaling pathway, which is under the control of the respective fusion gene, constitute already pharmaceutically accessible elements should therefore be further exploited experimentally.

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AUTHORSHIP

GF designed and performed research, compiled data and drafted the manuscript. RG, AI, GK and UK carried out additional experimental work. HPK and DS were responsible for mouse experiments, MK conducted bioinformatic analyses, MND assisted in phospho-flow stainings, OAH interpreted data and wrote the manuscript and RP-G conceived the study, analyzed and interpreted data and wrote the manuscript. All authors read and approved the manuscript.

CONFLICT-OF-INTEREST DISCLOSURE

The authors have no conflict of interest to disclose.

Supplementary information is available at the Leukemia journal website.

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3.2 Supplemental file

Figure S1 depicts the binding regions of shRNA A1 and G1 on the E/R fusion transcript region. Table S1 lists genes being either involved in the PI3K/AKT/mTOR pathway or represent downstream effectors. Supplementary materials and methods describe technical aspects of gene expression analyses and functional gene annotation.

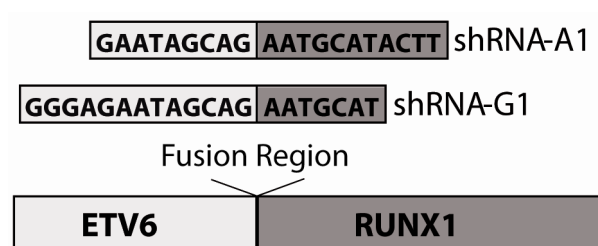


Figure S1. Sequence of shRNA A1 and G1 targeting position within the fusion region.

Table S1. Regulatory effects of differentially expressed genes on the PI3K/AKT/mTOR pathway and/or its downstream effectors.

Gene Symbol	Gene name	E/R KD ¹	E/R+ ALL ²	Regulatory Effect ³
AKT1	AKT1	0.09	-1.23	+
AKT1S1	AKT1 substrate 1	0.12	ND	-
AKT2	AKT2	0.20	ND	+
AKT3	AKT3	0.26	-0.79	+
BAD	BCL2-associated agonist of cell death	0.11	-0.62	-
CASP9	caspase 9, apoptosis-related cysteine peptidase	0.03	-1.13	-
CDKN1A	cyclin-dependent kinase inhibitor 1A	1.05	-0.67	-
CDKN1B	cyclin-dependent kinase inhibitor 1B	0.24	-0.86	-
CHEK1	CHK1 checkpoint homolog	0.14	-0.85	-
EIF4E	eukaryotic translation initiation factor 4E	0.27	1.43	+
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	0.30	-0.68	-
FOXO1	forkhead box O1	-0.15	-0.05	-
FOXO3	forkhead box O3	0.09	0.16	-
GSK3B	glycogen synthase kinase 3 beta	0.67	-2.00	-
MDM2	Mdm2 p53 binding protein homolog	-1.1	1.35	+
PDK1	pyruvate dehydrogenase kinase, isozyme 1	-0.29	0.72	+
PTEN	phosphatase and tensin homolog	0.02	-0.26	-
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	0.00	-0.77	-
RHEB	Ras homolog enriched in brain	-0.01	0.23	+
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	-0.26	0.67	+
TSC1	tuberous sclerosis 1	-0.09	-0.48	-
TSC2	tuberous sclerosis 2	0.14	-1.11	-

Numbers indicate the log2-fold ratios. Of note, log2-fold transformation distributes up- and down-regulated genes around zero.

KD, Knockdown; ND, corresponding probe sets to KD not determined on microarrays used for E/R+ ALL.

¹E/R-KD vs. controls (mean of E/R silenced REH and AT2 cells).

²E/R-positive vs. E/R-negative BCP ALL cases (E/R+ALL).

³(+), activating and (-), inhibiting regulatory effect.

SUPPLEMENTARY MATERIAL AND METHODS

Gene expression analysis by microarray technology

Changes in gene expression profiles upon knockdown of E/R were followed on Affymetrix HG-U133-PLUS2 arrays (Affymetrix, Inc., Santa Clara, CA). cRNA target

synthesis and GeneChip® processing were performed according to standard protocols (Affymetrix, Inc.). All further analyses were performed in R statistical environment using Bioconductor packages [1].

Affymetrix CEL files for all experiments were normalized together using the gcrma logarithm [2]. For further analysis probe sets (ps) were called present or absent using the Bioconductor package “panp”, and ps were excluded which showed very low expression across all arrays ($P > 0.02$). Subsequently ps were excluded that did not pass a non-stringent variability cut-off across all arrays (standard deviation > 0.05). Thereby ps were excluded that did not show any variation across all experiments. Finally, for each gene one ps was selected for further analysis by the criterion of maximizing the expression variation across arrays and thereby the most informative ps was chosen for each gene. This procedure yielded a final number of 9498 ps that were used for all further analysis.

Differentially expressed genes were determined using a moderated t-test in the R package “limma” [3]. All P -values were corrected for multiple testing using the “Benjamini-Hochberg” correction method. Significantly differentially expressed genes in the E/R knockdown vs. control experiments were determined by calculating ratios for each gene between the two conditions for each experiment separately, thus yielding five biological replicates of relative expression for each gene (REH $n = 3$, AT-2 $n = 2$). Overall, relative gene expression was positively correlated with each other in all biological replicates. Then, for each gene, significance was determined using a weighted one-sample t-test against the null hypothesis of no expression change ($\mu = 0$).

Functional annotation

Gene set enrichment analysis was performed using the Java tool provided on the homepage of the BROAD institute (<http://www.broadinstitute.org/gsea/index.jsp>). The program was run in the ranked gene list mode with default parameters, where the input was the list of all genes from the array that passed initial filtering (see above) and their respective mean log2-fold change between the knockdown and control experiments.

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4. FIRST-AUTHORSHIP PUBLICATION II

**THE LEUKEMIA-SPECIFIC FUSION GENE ETV6/RUNX1 PERTURBS
DISTINCT KEY BIOLOGICAL FUNCTIONS PRIMARILY BY GENE
REPRESSION**

**Gerhard Fuka, Maximilian Kauer, Reinhard Kofler, Oskar A. Haas,
and Renate Panzer-Grümayer**

PLoS ONE (submission approved on June 1st, 2011); under review

4.1 Manuscript file

Title

The Leukemia-specific Fusion Gene ETV6/RUNX1 Perturbs Distinct Key Biological Functions Primarily by Gene Repression

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Running title

Molecular Function of ETV6/RUNX1 in Leukemia

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ABSTRACT

Background: *ETV6/RUNX1* (*E/R*) (also known as *TEL/AML1*) is the most frequent gene fusion in childhood acute lymphoblastic leukemia (ALL) and also most likely the crucial factor for disease initiation; its role in leukemia propagation and maintenance, however, remains largely elusive. To address this issue we performed a shRNA-mediated knock-down (KD) of the *E/R* fusion gene and investigated the ensuing consequences on genome-wide gene expression patterns and deducible regulatory functions in two *E/R*-positive leukemic cell lines. **Findings:** Microarray analyses identified 777 genes whose expression was substantially altered. Although approximately equal proportions were either up- (KD-UP) or down-regulated (KD-DOWN), the effects on biological processes and pathways differed considerably. The *E/R* KD-UP set was significantly enriched for genes included in the "cell activation", "immune response", "apoptosis", "signal transduction" and "development and differentiation" categories, whereas in the *E/R* KD-DOWN set only the "PI3K/AKT/mTOR signaling" and "hematopoietic stem cells" categories became evident. Comparable expression signatures obtained from primary *E/R*-positive ALL samples underline the relevance of these pathways and molecular functions. We also validated six differentially expressed genes representing the categories "stem cell properties", "B-cell differentiation", "immune response", "cell adhesion" and "DNA damage" with RT-qPCR. **Conclusion:** Our analyses provide the first preliminary evidence that the continuous expression of the *E/R* fusion gene interferes with key regulatory functions that shape the biology of this leukemia subtype. *E/R* may thus indeed constitute the essential driving force for the propagation and maintenance of the leukemic process irrespective of potential consequences of associated secondary changes. Finally, these findings may also provide a valuable source of potentially attractive therapeutic targets.

INTRODUCTION

The *ETV6/RUNX1* (*E/R*) fusion gene (also known as *TEL/AML1*) is the hallmark of one of the most common genetic subtypes of B-cell precursor acute lymphoblastic leukemia (BCP ALL) in children [1, 2]. The fusion gene encodes a chimeric transcription factor that comprises the N-terminal portion of ETV6 and the almost entire RUNX1 protein and is thought to convert RUNX1 from a transcriptional modulator to a transcriptional repressor of RUNX1 target genes [3]. The current multistep model implies that this gene fusion occurs already during fetal development and constitutes the initiating - although not sufficient - event for neoplastic transformation [4, 5]. The idea that the ensuing gene product might perhaps also be relevant for maintenance of the malignant phenotype is derived from the results of recent experiments, which showed that RNAi-mediated silencing of the endogenous fusion gene reduces *in vitro* cell proliferation and cell survival as well as impairs the *in vivo* repopulation capacity of the treated cells [6] (Fuka et al. manuscript submitted). Microarray technologies made it possible to define the specific gene expression signatures of specific ALL subgroups, including those with an *E/R* fusion gene [7-12]. These diagnostically and clinically relevant molecular patterns derive from the comparison of a differentially expressed set of genes in a given type of leukemia relative to other subgroups included in such analyses. Since particular genetic subgroups can be clearly delineated and distinguished with this approach, it seems likely that primary underlying genetic defects, as for instance *E/R*, are the main determinants of the respective gene expression signature, although the transcriptional derangements will most likely also be modified to a certain extent by other factors, such as secondary genetic alterations. To investigate the specific impact of the chimeric E/R protein on overall gene expression, we knocked down the endogenous fusion gene in two leukemia cell lines utilizing fusion transcript specific short hairpin RNAs (shRNA) and compared the native and suppressed gene expression signatures. We also compared the E/R KD signature with that obtained from primary childhood ALL cases and validated the expression of selected target genes that represented various pathways or cellular functions, which were identified with this approach.

RESULTS AND DISCUSSION

Defining target genes of E/R knockdown

We silenced the endogenous fusion protein by lentiviral transduction of shRNA-encoding vectors in the leukemia cell lines REH and AT-2. Detailed information on the experimental design is provided in the Supplemental Material and Methods section. Expression profiling was performed in cells that were selected for viral integration and stable fusion gene suppression (Figure S1). Differentially expressed genes were determined by microarray analyses using three and two biological replicates from independent knock-down (KD) experiments of the REH and AT-2 cell lines, respectively, as well as appropriate control cells that were transduced with a non-targeting shRNA vector (GEO, Accession numbers: will be provided during review).

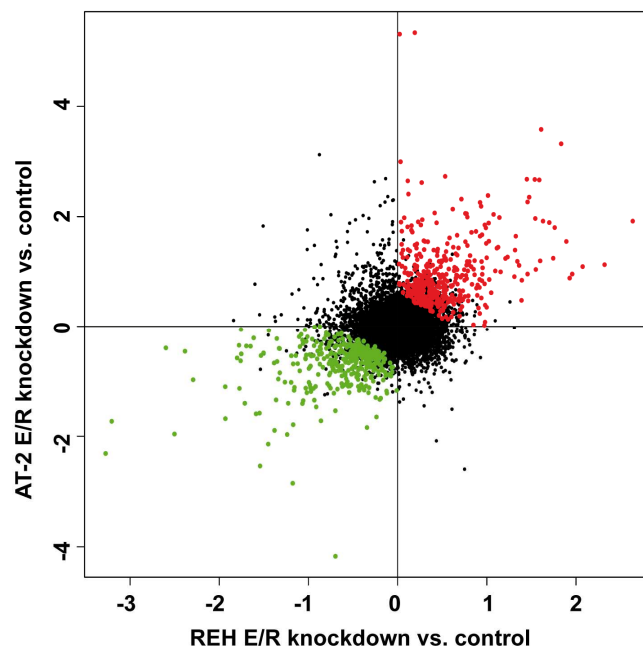


Figure 1. Scatter plot of differential gene expression values upon E/R KD in two cell lines. Each dot represents the mean expression value (log₂-fold change of E/R-repressed versus control cells) of three and two replicates, for REH and AT-2 cell lines, respectively. x-axis: REH cell line, y-axis: AT-2 cell line. Negative values indicate a decrease and positive values an increase in gene expression upon fusion gene KD. Green and red dots depict concordantly and significantly modulated genes in a joint analysis of both cell lines.

Despite the dissimilar genetic background imposed by different secondary changes in the two cell lines there was a significant correlation of differential gene expression in both models ($r = 0.31$, $P < 0.0001$) (Figure 1).

Table 1. Top 50 regulated genes from microarray analysis upon E/R KD.

Entrez Gene ID	Gene name	Gene symbol	E/R KD mean	E/R KD REH	E/R KD AT-2
6689	Spi-B transcription factor	SPIB	2.60	1.61	3.59
931	membrane-spanning 4-domains, subfamily A, member 1	MS4A1	2.58	1.83	3.32
28755	T cell receptor alpha constant	TRAC	2.28	2.64	1.92
197358	NLR family, CARD domain containing 3	NLRC3	2.13	1.59	2.67
4852	neuropeptide Y	NPY	2.11	1.54	2.68
100132169	LOC100132169	LOC100132169	2.06	1.45	2.68
7124	tumor necrosis factor	TNF	1.91	1.47	2.35
6696	secreted phosphoprotein 1	SPP1	1.86	1.46	2.27
28514	delta-like 1	DLL1	1.80	1.70	1.89
7168	tropomyosin 1 (alpha)	TPM1	1.78	1.76	1.80
3759	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	1.77	1.63	1.92
1117	chitinase 3-like 2	CHI3L2	1.76	1.54	1.97
9892	synaptosomal-associated protein, 91kDa	SNAP91	1.72	2.32	1.13
7940	leukocyte specific transcript 1	LST1	1.72	1.89	1.55
3398	inhibitor of DNA binding 2	ID2	1.70	1.01	2.39
131583	family with sequence similarity 43, member A	FAM43A	1.63	0.53	2.73
140706	chromosome 20 open reading frame 160	C20orf160	1.59	0.92	2.26
971	CD72 molecule	CD72	1.58	2.07	1.09
54541	DNA-damage-inducible transcript 4	DDIT4	1.56	1.14	1.98
4067	v-src-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	1.56	0.94	2.19
5790	protein tyrosine phosphatase, receptor type, C-associated protein	PTPRCAP	1.56	1.07	2.04
374403	TBC1 domain family, member 10C	TBC1D10C	1.52	0.71	2.32
7490	Wilms tumor 1	WT1	1.50	1.74	1.25
54510	protocadherin 18	PCDH18	1.49	1.32	1.65
8519	interferon induced transmembrane protein 1	IFITM1	1.46	1.96	0.96
6275	S100 calcium binding protein A4	S100A4	1.45	1.02	1.88
9639	Rho guanine nucleotide exchange factor 10	ARHGEF10	1.44	0.27	2.62
4330	meningioma 1	MN1	1.41	0.98	1.85
2014	epithelial membrane protein 3	EMP3	1.41	0.77	2.05
10870	hematopoietic cell signal transducer	HCST	1.41	0.76	2.06
51523	CXXC finger 5	CXXC5	1.41	1.93	0.88
170302	aristaless related homeobox	ARX	-1.42	-2.38	-0.46
3983	actin binding LIM protein 1	ABLIM1	-1.45	-1.77	-1.13
54549	sidekick homolog 2	SDK2	-1.48	-1.17	-1.79
10579	transforming, acidic coiled-coil containing protein 2	TACC2	-1.50	-2.60	-0.40
55107	anoctamin 1	ANO1	-1.52	-1.93	-1.10
8842	prominin 1	PROM1	-1.56	-1.71	-1.40
57556	semaphorin 6A	SEMA6A	-1.56	-1.55	-1.58
9687	growth regulation by estrogen in breast cancer 1	GREB1	-1.59	-1.59	-1.59
8642	dachsous 1	DCHS1	-1.60	-1.24	-1.97
650	bone morphogenetic protein 2	BMP2	-1.63	-2.29	-0.98
55303	GTPase, IMAP family member 4	GIMAP4	-1.64	-1.38	-1.89
83698	calneuron 1	CALN1	-1.80	-1.45	-2.14
147700	kinesin light chain 3	KLC3	-1.81	-1.93	-1.68
5729	prostaglandin D2 receptor	PTGDR	-2.01	-1.18	-2.85
5175	platelet endothelial cell adhesion molecule	PECAM1	-2.04	-1.54	-2.54
9619	ATP-binding cassette, sub-family G, member 1	ABCG1	-2.23	-2.50	-1.96
5121	Purkinje cell protein 4	PCP4	-2.43	-0.70	-4.17
8470	sorbin and SH3 domain containing 2	SORBS2	-2.47	-3.21	-1.73
5142	phosphodiesterase 4D, cAMP-specific	PDE4B	-2.79	-3.27	-2.31

Depicted are genes found to be significantly de-regulated in the E/R knockdown. Columns 1-3: Gene identifiers; columns 4-6: log2-fold change values for the mean of AT-2 and REH (column 4), REH (column 5), AT-2 (column 6).

A joint analysis identified 777 genes that were significantly ($P < 0.05$) and concordantly up- (KD-UP; $n = 403$) and down-regulated (KD-DOWN; $n = 374$) after the knockdown of the E/R fusion gene (Table S1). The top 50 regulated genes are listed in Table 1, along with the log2-fold changes from the array analysis. They include, for instance, the two direct RUNX1 targets *ID2* and *PTPRCAP*. *ID2* encodes a proposed inhibitor of tissue-specific gene expression and *PTPRCAP* is a key regulator of lymphocyte activation (Table S1) [13, 14]. Consistent with the notion that E/R acts as a constitutive repressor of RUNX1 target genes [3], these two genes are repressed in E/R-positive leukemias and up-regulated upon fusion gene KD. In contrast to our findings, Wotton et al. report that RUNX1-induced repression of *ID2* is abrogated by E/R. This seemingly controversial result might possibly be explained by a context dependent gene regulation, since Wotton et al. used 3T3 murine fibroblast cells in their experiments. In line with our data, *PTPRCAP* transcription was found to be repressed by *RUNX1-MTG8* and *-MTG16* fusion genes, two *RUNX1* fusions that are frequently found in acute myeloid leukemia [14]. Furthermore, the regulation of two other genes that are differentially expressed in E/R-positive ALL, also concords with our E/R KD results. *CALN1*, a brain-specific member of the calmodulin superfamily, is exclusively over-expressed [10], while *MS4A1* (CD20), a regulator of B-cell activation and proliferation, appears repressed in E/R-positive ALL [15].

Functional annotation and pathway analysis of differentially expressed genes in the KD model

To systematically assess the molecular functions that are modulated by E/R, we annotated all significantly regulated genes from the E/R KD experiments according to their regulation by the fusion gene. For this purpose, we used the “Database for Annotation, Visualization and Integrated Discovery” (DAVID) [16] to classify gene lists into functionally related gene groups. The raw output from DAVID, derived from the analysis of up- and down-regulated genes (Table S2 and Table S3), was further parsed to work out more clearly the significance levels and affiliation to broader functional groups of annotation terms (Figure 2). First inspection of these functional annotations revealed a large discrepancy between E/R KD up- and down-regulated

genes (Figure 2; right and left panel, respectively). While up-regulated genes significantly associate with various cellular functions and pathways, the down-regulated gene set, after correction for multiple testing, yielded no significant annotation term at all (the highest ranking term with $P < 0.3$ was the KEGG pathway 04070:Phosphatidylinositol signaling system). These striking differences indicate that despite the similar number of up- and down-regulated genes only the KD-UP ones relate, to a high degree, to similar functions and were therefore enriched by the DAVID analysis. The KD-DOWN genes, on the other hand, do not cluster into common functions and therefore not a single term was found to be significant. Hence, the channeling of KD-UP genes to specific pathways suggests that E/R exerts its distinct and relevant gene de-regulation through repression of specific classes of target genes. Conversely, the general lack of such a KD-DOWN-related “pathway-channeling” implies that the E/R-associated up-regulation of genes might be biologically far less relevant. Alternatively, KD-DOWN genes may encode signaling pathway components that are mostly regulated by posttranslational modifications, as is, for instance, the case in the phosphoinositide-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. Considering that E/R probably acts as transcriptional repressor of RUNX1 target genes [3], we looked for RUNX1 consensus motifs in the promoter region of the KD-UP genes, but did not find an enrichment of such motifs (data not shown). This observation suggests that also indirect late effects of gene regulation result either from an initial, or more likely, continuous E/R-mediated gene repression.

Given their apparent biological relevance, we focused our further analysis on the 403 KD-UP genes and their molecular functions as well as involvement in pathways. Based on the gene-level clustering, the top 100 annotation terms were manually curated into 14 functional meta-groups (Figure 3). Note that the name of the meta-groups reflects only the most prominent annotation terms that are comprised in the respective meta-group. A list including all terms within the 14 meta-groups is shown in Table S2. Applying stringent statistical criteria ($P < 0.05$), only the meta-groups “cell activation”, “immune response”, “apoptosis”, “development and differentiation”, “GTPase regulation”, and “protein phosphorylation and phosphate metabolism” were found to contain at least one significant annotation term (Figure 3A). The regulation of individual genes within the top six meta-groups upon E/R KD is shown in Figure 3B.

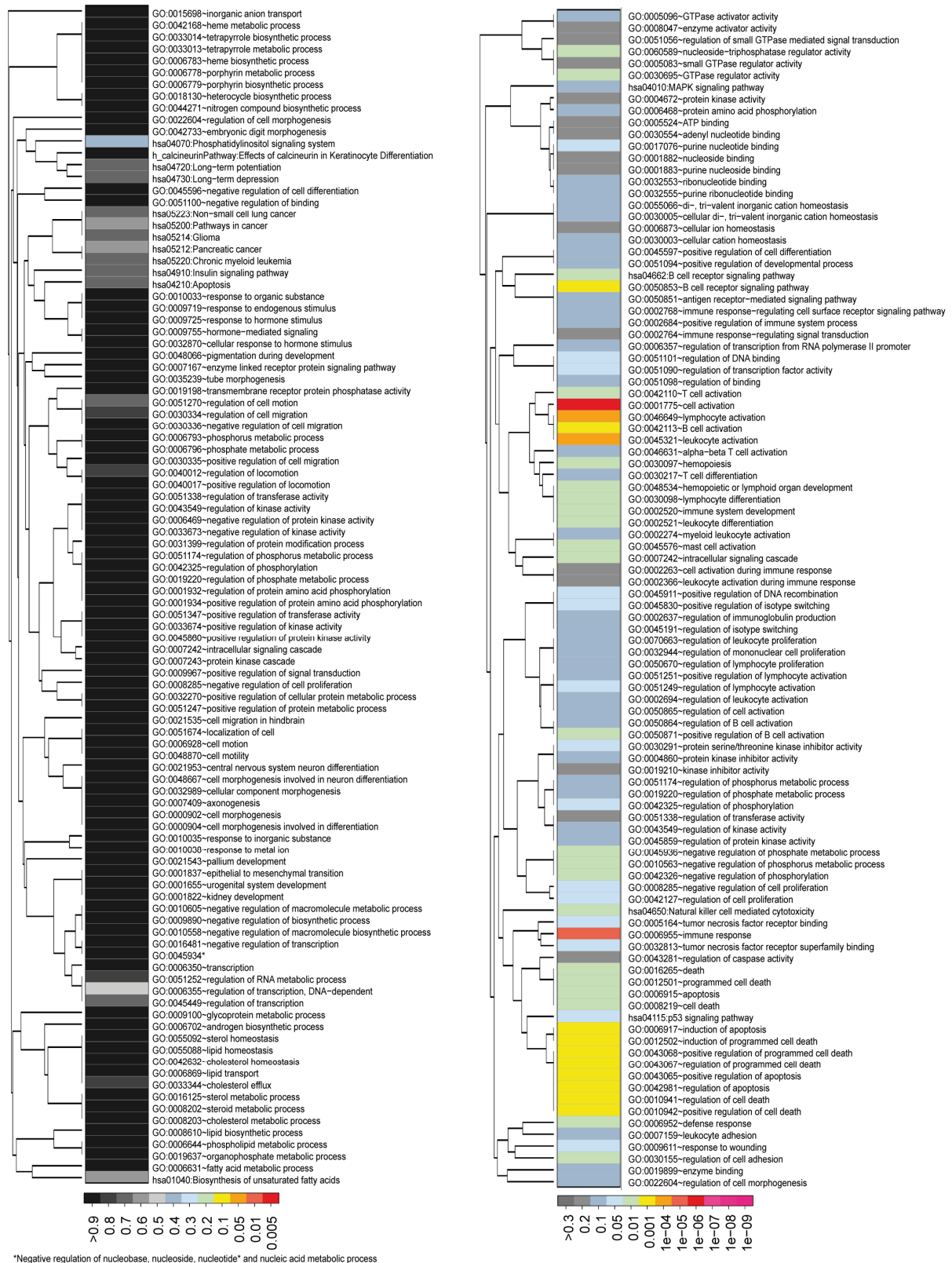


Figure 2. Functional annotation clustering of differentially expressed genes upon E/R KD. Visualization of the similarity of functional annotations that were determined by DAVID for down- (left) and up-regulated (right) genes upon E/R KD. The 100 most significant terms (ranked by P-value) are shown for both sets of annotation terms. Significance levels of functional terms are indicated by a color

code shown at the bottom of the figure. Due to large differences in the range of *P*-values for the functional groups resulting from up- and down-regulated genes upon KD, a different color-scheme was used in each panel.

The remaining groups ("cell proliferation", "response to wounding", "nucleic acid binding", "DNA damage response", "cell adhesion and migration", "chemical homeostasis", "RNA synthesis" and "enzyme binding") contained no nominally significant annotation term.

The DAVID pathway analysis was based on the overrepresentation of "significant genes" in certain gene sets and pathways. To assess the functional impact of differentially expressed genes from the E/R KD experiments independent of a specific *P*-value threshold, we performed gene set enrichment analysis (GSEA). This analysis resulted in many more up-regulated GO terms (147) from KD-UP than down-regulated terms (13). Importantly, these GO terms largely mapped to the same meta-groups identified in the DAVID analysis (Table S4).

The same discrepancy (324 vs. 49 gene sets) held true for a large collection of > 2,500 gene sets that were obtained from experimental data ("curated gene sets, C2" from MSigDB) (Table S5). The conclusions from the DAVID analysis were thus qualitatively confirmed by GSEA. Moreover, in the GSEA analysis the "Jaatinen hematopoietic stem cell UP" signature emerged as the most significantly down-regulated gene set. This result may be an indicator for an intriguing new function of E/R, namely that it induces genes that are normally expressed in cord blood-derived hematopoietic stem cells [17]. To corroborate these findings, we supplemented our comparison with two other gene sets that were obtained from sorted CD34+/lineage-negative versus CD34- normal bone marrow cells designated "Andersson-UP" and "Andersson-DOWN" (microarray data were kindly provided by Andersson et al [10]). In line with the above results, the "Andersson-UP" gene set also scored significantly in the GSEA analysis (Table S5). Combining the data from Jaatinen's and Andersson's gene sets, *CALN1*, *PROM1*, *KIT* and *CDK6* were the most highly up-regulated genes and they are similarly induced by E/R. With the exception of *CALN1*, whose function in the hematopoietic system is currently not known, all other genes are considered to be associated with hematopoietic stem or progenitor cells [18]. To the best of our knowledge, only one other group has previously analyzed the expression patterns of primary E/R-positive ALL cases and assigned them to GO categories [11].

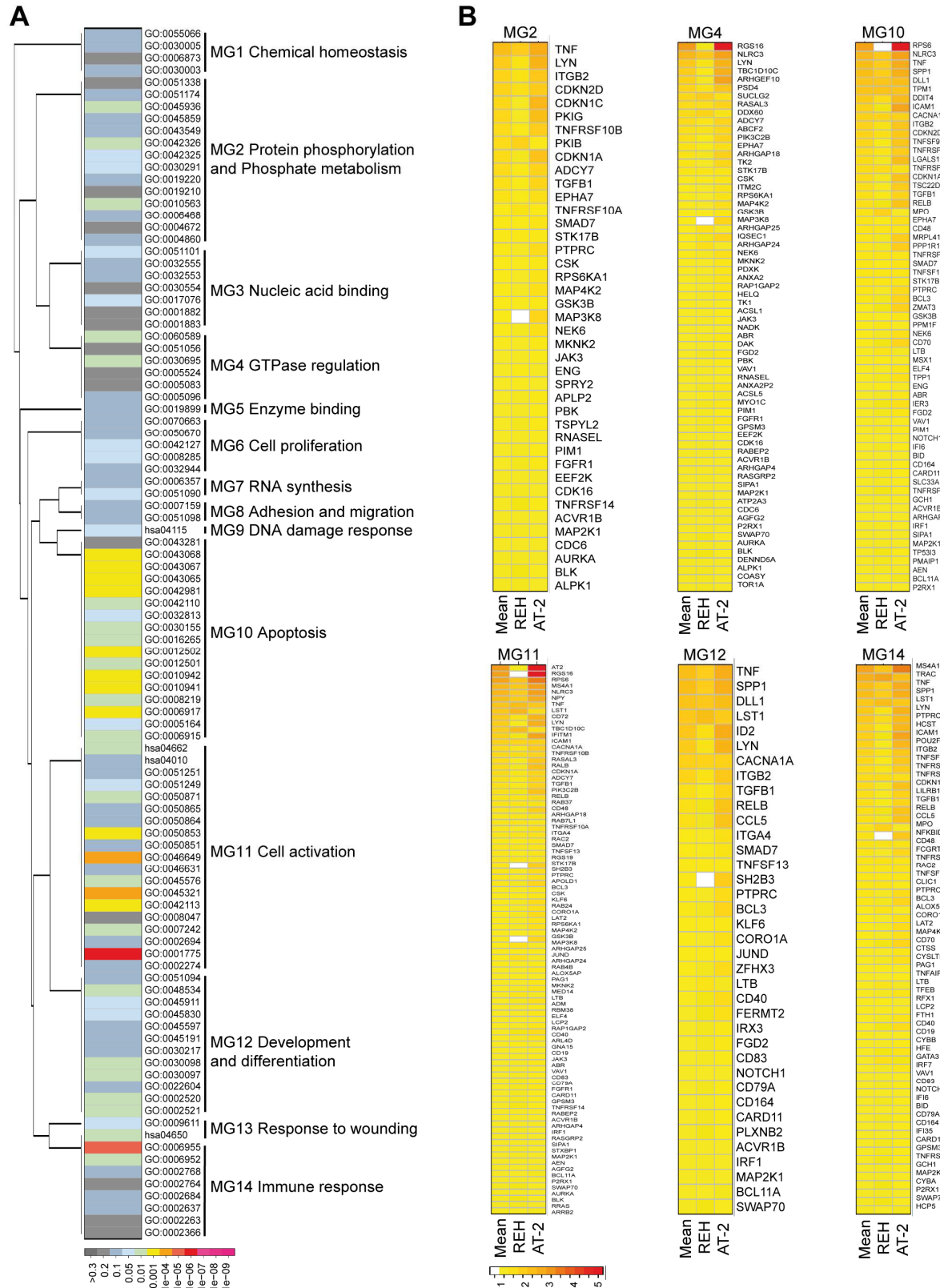


Figure 3. Meta-groups of functional annotations for up-regulated genes upon E/R KD. Meta-groups were curated based on gene-clustering of annotation terms. A: Top 100 annotation terms from up-regulated genes, their *P*-values and their affiliation to meta-groups. Similarity of the meta-groups was based on the number of shared genes. For distance calculations between the meta-groups genes

from all contributing terms were taken together. B: Change in expression of individual genes in meta-groups that contain significant annotation terms. The color code at the bottom of the figure indicates the extent of log2-fold changes in gene expression.

Consistent with our data, they also observed a distinct association with the categories “cell differentiation”, “cell proliferation”, “apoptosis”, “cell motility” and “response to wounding”. Moreover, ectopic E/R expression in a 3T3 mouse cell line model induced the categories “adhesion” and “survival” [13].

Genes concordantly modulated by E/R KD in leukemia model cell lines and primary ALL

Next we investigated to which extent gene expression changes that result from an E/R KD might also be reflected in a reciprocal fashion in primary ALL samples. For this purpose we used previously published data sets [8] that were generated by comparing expression profiles from *E/R*-positive with *E/R*-negative BCP ALL cases. The ensuing “E/R ALL signature” comprised 1.980 differentially regulated genes ($P < 0.05$, moderated t-test), 1.008 of which were under- and 972 over-expressed in *E/R*-positive ALL. Combining the data sets of both the E/R KD and primary ALL experiments, a total of 5.119 genes were represented on both microarray platforms and passed initial quality filters (Table S6). This gene set comprised approximately 50% of genes from the E/R KD signature ($n = 409$; 175 KD-DOWN and 234 KD-UP). Taking into account the specific regulation of these genes in primary ALL, we identified a set of genes whose expression is inversely correlated in the KD and ALL signatures. This set comprises 66 of the 175 KD-DOWN and 71 of the 234 KD-UP genes and they account for approximately one third (137/409) of the E/R signature genes present in both data sets (Table S7). The top 50 regulated genes of this set are listed in Table 2. They are associated with the categories “cell activation” (*TRIB*, *FYB*, *LYN* and *CD72*), “immune response” (*CXCR7*, *FAIM3*, *CD48*, *CD72*, *FKBP5*), “development and differentiation” (*SPIB*, *CD72*, *S100A4/13*, *PLP2*), “cellular proliferation” (*LGALS1*, *CXCR7*, *SOX11*, *E2F5*, *GAB1*, *CDKN1A*, *EMP3*, *LYN*, *DDIT4*, *CD72* and *LGALS1*), “cell survival” (*DRAM1*, *MDM2*, *GAB1*, *INPP5D*, *FAIM3*, *CDKN1A*, *LGALS1*, *DDIT4*, *CD72*), “proliferative signaling” (*GIMAP4*, *RAC2*, *ARHGEF4*, *PSD4*), “cell adhesion and/or migration” (*DCHS1*, *CXCR7*, *PCDH*, *ITGA4*, *LGALS1*, *ITGB2*, *EMP3*, *S100A4*, *LYN*) and “DNA damage response” (*DRAM1*, *MDM2*, *CDKN1A*, *PSD4*).

Table 2. Top 50 E/R regulated genes from microarray analysis of KD experiments and primary ALL.

Entrez gene ID	Gene name	Gene symbol	E/R KD mean	E/R+ vs. E/R- ALL
6689	Spi-B transcription factor	SPIB	2.60	-1.83
28755	T cell receptor alpha constant	TRAC	2.28	-1.03
971	CD72 molecule	CD72	1.58	-0.80
54541	DNA-damage-inducible transcript 4	DDIT4	1.56	-1.37
4067	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	1.56	-1.26
6275	S100 calcium binding protein A4	S100A4	1.45	-1.80
2014	epithelial membrane protein 3	EMP3	1.41	-0.88
3689	integrin, beta 2	ITGB2	1.31	-1.35
23550	pleckstrin and Sec7 domain containing 4	PSD4	1.30	-0.35
26112	coiled-coil domain containing 69	CCDC69	1.25	-1.50
3956	lectin, galactoside-binding, soluble, 1	LGALS1	1.11	-2.00
64777	required for meiotic nuclear division 5 homolog B	RMND5B	1.06	-0.21
1026	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	1.05	-0.56
51063	calcium homeostasis modulator 2	CALHM2	0.99	-1.09
9404	leupaxin	LPXN	0.95	-1.55
5355	proteolipid protein 2	PLP2	0.90	-1.47
2289	FK506 binding protein 5	FKBP5	0.89	-0.95
962	CD48 molecule	CD48	0.89	-0.81
55501	carbohydrate (chondroitin 4) sulfotransferase 12	CHST12	0.86	-0.72
9679	family with sequence similarity 53, member B	FAM53B	0.86	-1.07
10437	interferon, gamma-inducible protein 30	IFI30	0.85	-0.55
3676	integrin, alpha 4	ITGA4	0.83	-1.08
5880	ras-related C3 botulinum toxin substrate 2	RAC2	0.82	-0.53
6284	S100 calcium binding protein A13	S100A13	0.81	-0.75
272	adenosine monophosphate deaminase 3	AMPD3	0.80	-0.34
9331	beta 1,4- galactosyltransferase, polypeptide 6	B4GALT6	-0.72	1.68
10656	KH domain containing, RNA binding, signal transduction associated 3	KHDRBS3	-0.75	2.04
5101	protocadherin 9	PCDH9	-0.77	1.68
1875	E2F transcription factor 5	E2F5	-0.81	0.68
50649	Rho guanine nucleotide exchange factor 4	ARHGEF4	-0.81	3.32
9214	Fas apoptotic inhibitory molecule 3	FAIM3	-0.84	0.74
6664	SRY (sex determining region Y)-box 11	SOX11	-0.84	2.39
10402	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	ST3GAL6	-0.87	0.74
26011	odz, odd Oz/ten-m homolog 4	ODZ4	-0.88	0.49
57007	chemokine (C-X-C motif) receptor 7	CXCR7	-0.91	1.19
2533	FYN binding protein	FYB	-0.91	2.48
8349	histone cluster 2, H2be	HIST2H2BE	-0.91	0.94
5095	propionyl CoA carboxylase, alpha polypeptide	PCCA	-0.94	1.38
3635	inositol polyphosphate-5-phosphatase	INPP5D	-1.03	0.37
2549	GRB2 associated binding protein 1	GAB1	-1.05	1.23
54847	SID1 transmembrane family, member 1	SIDT1	-1.06	0.92
10221	tribbles homolog 1	TRIB1	-1.09	1.11
4193	mouse double minute 2	MDM2	-1.11	0.56
55332	DNA-damage regulated autophagy modulator 1	DRAM1	-1.16	2.28
950	scavenger receptor class B, member 2	SCARB2	-1.21	0.46
57556	semaphorin 6A	SEMA6A	-1.56	1.95
8642	dachsous 1	DCHS1	-1.60	1.18
650	bone morphogenetic protein 2	BMP2	-1.63	1.53
55303	GTPase, IMAP family member 4	GIMAP4	-1.64	1.14
5142	phosphodiesterase 4D, cAMP-specific	PDE4B	-2.79	0.78

Depicted are genes found to be significantly and concordantly de-regulated by E/R in KD experiments and *E/R*-positive vs. *E/R*-negative primary BCP ALL from Ross et al. (2003). Columns 1-3: Gene identifiers; columns 4-5: log2-fold change values for the mean of AT-2 and REH (column 4) and *E/R*-positive vs. *E/R*-negative primary BCP ALL (column 5).

The above pathways and functions match almost perfectly with those identified in the KD model, which underscores their specific relevance for *E/R*-positive leukemia.

Two thirds (272/409) of the *E/R* KD signature genes that concurred with the ALL data set were not specific for *E/R*-positive ALL, but were also evident in the other subgroups. This observation evokes two, not mutually exclusive explanations, namely that these genes are either de-regulated in a similar fashion in a variety of ALL subtypes or that they represent a kind of basic but essential "BCP-ALL housekeeping gene set". The notion that other initiating genetic events can elicit a similar gene de-regulation effect as *E/R* is, for instance, supported by the fact that *PROM1* is also up-regulated in *MLL*-rearranged and high-hyperdiploid ALL cases, thereby counterbalancing its low expression in other ALL subtypes. Consequently, *PROM1* de-regulation was not considered as being a specific feature of *E/R*-positive ALL (data not shown).

Validation of selected *E/R* target genes by RT-qPCR

We validated the differential expression of several selected candidate genes contained in the KD signature, which were previously either not associated with *E/R*-positive ALL (*PROM1*, *PECAM1*, *IFITM1*; Figure 4A) or concordantly regulated in both systems (*SPIB*, *MDM2* and *DDIT4*; Figure 4B).

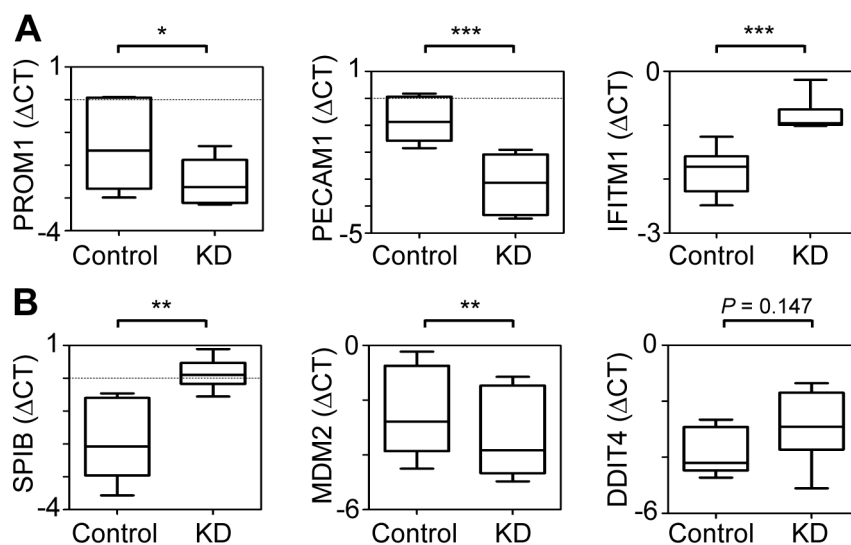


Figure 4. Validation of selected differentially expressed genes from the KD experiments by RT-qPCR. Quantification of transcripts of differentially expressed genes. A: Concordantly de-regulated by

E/R in the KD and primary leukemias. B: Only regulated in the KD experiments. Boxes cover the median and the interquartile range (25-75th percentiles) and whiskers the minimum and maximum values. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (paired t-test).

These genes were chosen because of their potential biological relevance, since they play an important role in the context of stemness and differentiation, adhesion and migration, immune response, DNA damage response as well as apoptosis. Notably, their differential expression in the context of *E/R* is novel. Quantification results of these transcripts in both cell lines from independent KD experiments concurred with those of the microarray experiments (Figure 4).

Functions of selected *E/R* regulated genes and potential implications for leukemia pathogenesis

Given that GSEA analysis of *E/R* KD regulated genes highlighted gene sets that are also up-regulated in hematopoietic stem cells, we chose *PROM1* (CD133) as the most prominent and attractive candidate from this set. *PROM1* is implicated in maintaining stem cell properties by suppressing differentiation and has recently gained much attention as a marker of tumor-initiating cells in a variety of human cancers [19]. The fact that *E/R* might regulate the expression of this gene is new and intriguing and provides additional arguments to the ongoing debate dealing with the structural hierarchy of ALL and its potential replenishment from rare leukemic stem cells [20]. In favor of this notion is a recent observation, which indicates that primitive leukemia-initiating cells with long-term *in vitro* and *in vivo* proliferation capabilities are exclusively found in the CD133+CD19-CD38- cell compartment [21]. However, this observation is in contrast to the scenario proposed by le Viseur et al., which suggests that the vast majority of ALL blasts may maintain the propensity to reconstitute leukemia *in vivo* [22]. The ER-induced "stemness" expression signature, represented for instance by *PROM1* and the stem cell factor ligand *KIT* in our model, therefore supports the later view.

The *E/R*-induced overexpression of stem cell markers in the respective leukemias can either be interpreted as a residual relict of a transformed primitive stem cell or, more likely, as the reflection of a continuously active stem cell program [23]. Although neither possibility excludes that the gene fusion process already occurs in a primitive hematopoietic stem cell [24], the latter requires that inappropriate stemness genes remain active or become perhaps reactivated at the level of maturation in which the

bulk of the leukemic cells is arrested. This interpretation is supported by the fact that - similar to the Andersson data - *E/R*-positive leukemias cluster best with normal large pre-B II cells even after suppression of the fusion gene (data not shown) [10, 25]. It is thus tempting to speculate that up-regulation of *PROM1* may play a critical role in *E/R*-positive ALL. This possibility is also relevant for our recent finding that the *E/R* fusion gene is apparently required for the *in vivo* propagation of the respective cells (Fuka et al. manuscript submitted).

PECAM1 (CD31) encodes a homophilic adhesion receptor that mediates adhesion between endothelial cells and leukocytes and could therefore probably influence adhesion and migration of leukemic cells across the micro-vascular endothelium in various niches [26]. Since it is also contained in Andersson's CD34+ stem cell signature, we envision that its over-expression also contributes to the stem cell properties of *E/R*-positive ALL.

In contrast to the up-regulation of stem cell signature genes, genes encoding B lineage differentiation markers are frequently repressed in BCP-ALL. Thus, it is not surprising that *SPIB*, a B-lymphoid restricted transcription factor, is one of the genes that is strongly suppressed by *E/R*. Being directly induced by *paired box 5* (*PAX5*), the master regulator of B-lineage commitment, *SPIB* is a key player in B-cell development and B-cell receptor signaling [27]. This *SPIB* down-regulation could thus contribute to the impaired B-cell differentiation in *E/R*-positive ALL.

The *E/R*-associated down-regulation of *IFITM1*, a transcriptional target of interferon gamma ($\text{IFN}\gamma$) [28], fits also well into one particular point of the current concept of childhood ALL etiology, namely the one which suggests that certain forms of childhood ALL may be the unfortunate consequence of an abnormal immune response to common infections [29]. The proposed mechanism implies that inflammatory cytokines suppress the growth of normal hematopoietic cells, whereas they do not exert such an effect on, for instance, *E/R*-expressing cells. Consequently, fusion gene carrying cells may experience a relative growth advantage. In support of this notion it was recently shown that *E/R*-expressing cells are more resistant to the anti-proliferative effects of transforming growth factor ($\text{TGF}\beta$) [30]. Since $\text{TGF}\beta$ and $\text{INF}\gamma$ are both key modulators of the immune system, one expects that the suppression of *IFITM1* either concurs with or even augments these effects in response to an interferon release during common infections. In line with the proposed

function of TGF β , the suppression of IFITM1 may thus additionally fuel the expansion of an E/R-expressing leukemic clone [5].

Taking into account further mechanisms that might impair an INF γ associated inhibition of proliferation, it is noteworthy that CDKN1A is induced via the tumor suppressor protein p53 pathway activation and leads to a G1 cell cycle arrest [31]. The attenuation of the p53 activity together with the transcriptional repression of its direct target p21, the gene product of *CDKN1A*, either by E/R-mediated repression of IFITM1 or up-regulation of the p53 inhibitor MDM2, as implied in our KD model, opens another fascinating layer of complexity to the E/R-mediated gene regulation process. Given that p53 acts as a gatekeeper of genome integrity [32], p53 down-regulation by any of the above outlined means may thus favor leukemia development. Intriguingly, MDM2 is induced by RUNX1-RUNX1T1 and may therefore be involved in the route of transformation in a similar fashion in other *RUNX1*-associated leukemias, as for instance the *E/R*-positive ones [33]. Furthermore, MDM2 may also promote tumorigenesis via a p53 independent mechanism [34]. Such findings are not only crucial for our understanding of leukemia development per se, but may be particularly helpful for the identification of especially relevant targets for tailored future therapies.

Another E/R-down-regulated gene that is involved in the p53 pathway is *DDIT4* (also known as *REDD1*). It is primarily induced by stress and negatively regulates the mTOR pathway. DDIT4 is activated by DNA damage via p53-dependent and -independent mechanisms, but also by hypoxia or energy stress [35]. Particularly this latter feature is interesting in the context of *E/R*-positive leukemia, because the majority of affected children are anemic at diagnosis, which seemingly grants hypoxic conditions a central role in their pathogenesis [36]. Noteworthy, E/R-associated DDIT4 suppression may further contribute to the observed PI3K/AKT/mTOR pathway activation and an improved cell survival [37]. Whether this suppression is a direct p53-related consequence that, as recently observed in breast cancer, also leads to hypoxia inducible factor (HIF)1 α accumulation, is currently not known [38].

Taken together, the above clues reinforce the essential role that E/R plays in the entire process of leukemia development and maintenance: i) It induces genes that confer stem cell properties endowing cells with unlimited self renewal capability and simultaneously represses genes that otherwise promote differentiation; ii) it alters the DNA damage response by attenuating the p53 pathway, which in addition enables

the survival and clonal expansion of cells with accumulating secondary genetic changes; iii) it triggers proliferation and cellular growth via PI3K/AKT/mTOR pathway activation, which in turn adapts extracellular signaling as well as stress and hypoxia response accordingly; iv) it also attenuates the response to inflammatory signals. All these features, sustaining proliferative signaling, evading growth suppression, resisting cell death, and induced genome instability, are typical and well established hallmarks of cancer in general [39].

Based on the analyses of our KD model, we have established a functional map of the consequences of *E/R* expression in an endogenous background. The modulation of various specific and more general key processes that are pivotal for leukemia pathogenesis was thus highlighted. These processes include "development and differentiation", "apoptosis", "adhesion and migration" as well as "DNA damage response". Finally, these data provide also a valuable source of interesting targets and pathways whose functional validation will provide further insights into the biology of *E/R*-positive leukemia and possibly also promote the identification of novel targets for treatment.

MATERIAL AND METHODS

Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated from biological replicates of *E/R*-silenced REH ($n = 3$) and AT-2 ($n = 3$) cells obtained from independent KD experiments by Trizol reagent (Life Technologies, Carlsbad, CA). cDNA was synthesized by SuperScript II Reverse Transcriptase according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Transcripts were quantified by TaqMan RT-qPCR using the ABI Prism 7900 Detection System (Applied Biosystems, Foster City, CA). The following primer/probe combinations were used: SPIB 5'-GGGCCACACTTCAGCTGTCT-3', 5'-CAGTCCAGTCCCACAGGGAG-3', 5'-CCTGGACAGCTGCAAGCATTCCA-3'; MDM2 5'-CACGCCACTTTTTCTCTGCT-3', 5'-CCTGATCCAACCAATCACCT-3', 5'-CCACCTCACAGATTCCAGCTTCGG-3'; DDIT4 5'-CTGGACAGCAGCAACAGTG-3', 5'-CATCAGGTTGGCACACAAGT-3', 5'-CCGGAGGAAGACACGGCTTA-3'; PROM1 5'-TTGTGGCAAATCACCAGGTA-3', 5'-TCAGATCTGTGAACGCCTTG-3', 5'-CCCGGATCAAAGGAGTCGGA-3'; PECAM1 5'-AGGTGTTGGTGGGAAGGAGTG-3', 5'-GTGTATTGGGGCCTTTTCCT-3', 5'-AGGCCATCCAAGGTGGGATCGT-3' and IFITM1 5'-GGCTTCATAGCATTCGCCTA-3', 5'-ATGAGGATGCCCAGAATCAG-3',

5' TCCACCGCCAAGTGCCTGAA-3'. GUS was detected by a previously published primer/probe combination [40] and used as endogenous reference. Shown are cycle threshold $\Delta(CT)$ values.

Gene expression analysis by microarray technology

Gene expression changes upon knockdown of E/R were followed on Affymetrix HG-U133-PLUS2 arrays (Affymetrix, Inc., Santa Clara, CA). cRNA target synthesis and GeneChip® processing were performed according to standard protocols (Affymetrix, Inc., Santa Clara, CA). Microarray data were performed in compliance to MIAME guidelines and submitted to GEO - accession numbers will be provided during review. All further analyses were performed in R statistical environment using Bioconductor packages [41].

Affymetrix CEL files were preprocessed as described previously [42], yielding a final number of 9.498 probesets that were used for all further analyses.

Differentially expressed genes were determined using a moderated t-test in the R package “limma” [43]. All *P*-values were corrected for multiple testing using the “Benjamini-Hochberg” correction method. Significantly changing genes in the E/R KD vs. control experiments were determined by calculating ratios for each gene between the two conditions for each experiment separately, thus yielding five biological replicates of relative expression for each gene (REH, *n* = 3; AT2, *n* = 2). Then, for each gene, significance was determined using a weighted one-sample t-test against the null hypothesis of no expression change ($\mu = 0$).

For the re-analysis of the Ross data set, CEL files were downloaded from the St. Jude's data server and microarray data was pre-processed as described previously [42], generating a data set of 12.068 genes. To test *E/R*-positive vs. *E/R*-negative BCP ALL samples, a moderated t-test (Bioconductor package. “limma”) was performed. All *P*-values were corrected by the Benjamini-Hochberg method.

Functional annotation

The “Database for Annotation, Visualization and Integrated Discovery” (DAVID) was used to annotate the 403 up- and 374 down-regulated genes from the joint analysis of the E/R knockdown in REH and AT-2 cells. The “Functional Annotation Tool” in the online version of DAVID was run (<http://david.abcc.ncifcrf.gov/>) using the default parameters and focusing on the categories Gene Ontology-Molecular Function,

Gene-Ontology-Biological Process and KEGG/Biocarta Pathways. All annotation terms that met the inclusion criteria were downloaded as “Functional Charts”.

Hierarchical clustering of annotation terms

For further analysis and visualization of the similarity among annotation terms, the functional charts were first sorted by their *P*-value (corrected for multiple testing by the Benjamini-Hochberg method) and then, to determine the relationships of the top 100 annotation terms, similarity between all terms was measured by the number of their shared genes (gDist as described in Kauer et al. 2009) [42]. The matrix of pair wise gDist values (as dissimilarities: 1-gDist) for the 100 most significant terms was used as input for hierarchical clustering using the R function “hclust” in combination with the “average linkage” algorithm. Finally, the similarity among the annotation terms was visualized as dendrogram in combination with a heatmap indicating significance levels of the clustered terms. Names of meta-groups were chosen or modified from upstream gene ontology terms (<http://www.geneontology.org/>).

Gene set enrichment

To define functional categories of de-regulated genes independent of a *P*-value cutoff for “significant genes”, we performed gene set enrichment analysis (GSEA) using the “pGSEA” package in the Bioconductor/R environment [44-46]. Gene-wise log2 expression ratios (logFC) of knockdown versus control for the cell lines REH and AT-2, and for the mean of their logFCs, were used as input for pGSEA. Gene sets were downloaded from the MSigDB v3.0 (<http://www.broad.mit.edu/gsea/msigdb/> Cambridge, USA). We tested two different gene set collections available from MSigDB: curated gene sets from canonical pathways and experimental data (C2) and GO terms (C5). To validate the enrichment on genes involved in hematopoietic stem cells, we added two more gene sets to the C2 group: genes up- and down-regulated in the Andersson et al. 2005 data set (CD34+/lineage negative vs. CD34-hematopoietic cells) [10].

FINANCIAL DISCLOSURE

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AUTHORSHIP

GF designed and performed research, compiled data and drafted the manuscript. MK conducted bioinformatics analyses, analyzed data and contributed to the writing of the manuscript. RK conducted microarray experiments and analyses. OAH interpreted data and critically revised the article. RP-G conceived the study, analyzed and interpreted data and wrote the manuscript. All authors read and approved the manuscript.

CONFLICT-OF-INTEREST DISCLOSURE

The authors have no conflict of interest to disclose.

DEPOSITION OF MICROARRAYS

Microarray data are available online at GEO (www.ncbi.nlm.nih.gov/geo/), accession number will be provided during review.

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4.2 Supplemental file

SUPPLEMENTARY MATERIAL AND METHODS

Cell lines

The E/R-positive BCP leukemia cell lines REH (DSMZ, Braunschweig, Germany) and AT-2 [1] (kindly provided by J.D. Rowley, University of Chicago, IL) were both established from relapsed leukemia. Cells were cultured in RPMI 1640 medium with Glutamax (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (all PAA Laboratories, Pasching, Austria) and kept in humidified incubators at 37°C and 5% CO₂.

RNA interference

shRNA G1 (5'-CACCGGGAGAATAGCAGAATGCATCGAAATGCATTCTGCTATTCTCCC-3') is directed against the fusion region of E/R and was newly designed to repress the endogenous fusion protein REH and AT-2 cells. The non-targeting shRNA LacZ (Invitrogen, Carlsbad, CA) was used as a control. Lentiviral supernatants were produced with the Block iT Lentiviral RNAi Expression System (Invitrogen), followed by quantification of lentiviral titers by ELISA-based quantification of virus associated HIV-1 p24 (Cell Biolabs, San Diego, CA) according to the manufacturer's recommendations. Leukemia cell lines were transduced and selected for lentiviral integration with 5 µg/ml blasticidin for two weeks.

Immunoblotting

Cell lysates were obtained with RIPA buffer supplemented with 1mM NaVO₄ and 1% protease inhibitor cocktail (Roche, Basel, Switzerland) and 90 µg of total proteins were resolved by 12% SDS PAGE gel electrophoresis and transferred to nitrocellulose membranes (Whatman, Kent, UK). Non-specific binding on the membranes was blocked with PBS containing 5% dry milk. Membranes were incubated with anti-ETV6 (SB89, kindly provided by P. Marynen, VIB, Leuven, Belgium), anti-RUNX1 (Active Motif, Carlsbad, CA) and anti-GAPDH (6C5, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary infrared dye-labeled antibodies (LI-COR Biosciences, Lincoln, NE). Membranes were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences). Intensities of bands corresponding to E/R and RUNX1 were normalized to the signal of GAPDH.

SUPPLEMENTARY FIGURES

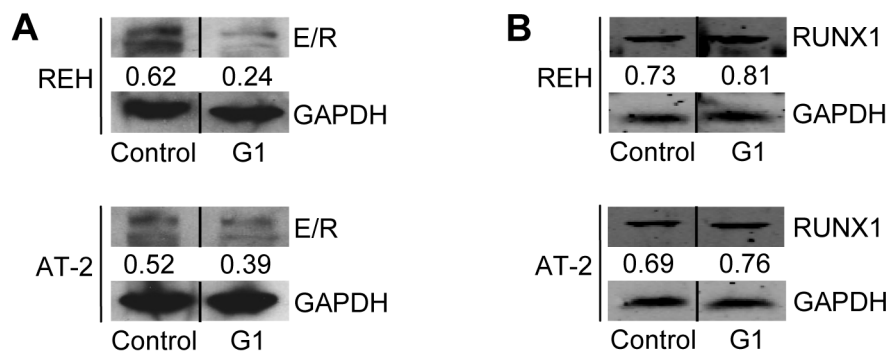


Figure S1. shRNA mediated silencing of E/R leads to chimeric protein depletion. The E/R-positive leukemia cell lines REH and AT-2 were transduced by lentiviral constructs encoding either non-targeting shRNA (control) or shRNA G1 (G1), targeting the E/R fusion transcript region. Protein levels of E/R (A) and RUNX1 (B) were detected by immunoblotting using anti-ETV6 and anti-RUNX1 antibodies, respectively. GAPDH was used to ensure equal loading. Numbers between bands represent the ratio between tested proteins and GAPDH quantification. Shown is one of at least three independent E/R knockdown experiments per cell line.

SUPPLEMENTARY TABLES*

Table S1. Genes found to be significantly de-regulated on Affymetrix HGU-133-PLUS2 arrays from E/R knockdown experiments. Columns1-3: Gene identifiers;

* Due to extensive size of excel sheets, supplementary tables are only available online upon manuscript acceptance.

columns 4-6: log2-fold change values (mean of AT-2 and REH) (column 4), REH (column 5), AT-2 (column 6); column 7: *P*-value for the mean log2-fold changes of the E/R KD (from column 4) corrected by the Benjamini-Hochberg method.

Table S2. Output from the DAVID analysis (version 6.7) for significantly UP-regulated genes upon E/R knockdown. The Categories “GO-Molecular Function”, “GO-Biological Process” and “Pathways” were selected for testing. Description of columns: “Category” – categories from DAVID; “Term” – specific terms within DAVID-categories; “Metagroup” – affiliation of “Term” into a metagroup, “Gene symbols” – genes from the tested gene list involved in “Term”; “Fold enrichment” – enrichment of genes involved in “Term” over random expectation; “*P*-value” – *P*-values for enrichment calculated by the EASE method used in DAVID; “Benjamini” – *P*-values corrected for multiple testing by the Benjamini Hochberg method. For details of the calculations: <http://www.nature.com/nprot/journal/v4/n1/pdf/nprot.2008.211.pdf>.

Table S3. Output from the DAVID analysis (version 6.7) for significantly DOWN-regulated genes upon E/R knockdown. Column descriptions as for Table S2.

Table S4. Results from the GSEA analysis for GO gene sets (MSigDB: C5). Column 1: name of the MSigDb gene set; columns 2-4: z-scores of from the pGSEA algorithm; columns 5-7: *P*-values from the pGSEA algorithm; column 8: genes involved in gene set; columns 9-12: descriptions of MSigDB gene set from MSigDB.

Table S5. Results from the GSEA analysis for “curated gene sets” from MSigDB (C2). Column descriptions as for Table S4.

Table S6. All 5119 probe sets that passed quality filters that were present on both, the HGU-133-PLUS2 arrays from our E/R knockdown experiments and E/R-positive vs. E/R-negative primary BCP ALL (from Ross et al. 2003; HGU-133-A arrays). Columns1, 2: Gene symbol (column 1) and Probe set (column 2) identifiers; columns 3, 4: log2-fold change values (column 3) and *P*-values (column 4) for the mean value of the E/R KD; column 5, 6: log2-fold change values (column 5) and *P*-values (column 6) for E/R-positive vs. E/R-negative primary BCP ALL. Mean log2-fold change and *P*-values were calculated as described in the M&M section.

Table S7 Overlap of significantly de-regulated genes from the E/R KD signature and E/R-positive vs. E/R-negative primary BCP ALL (from Ross et al. 2003). The subset of 137 genes from Table S4 with $P < 0.05$ for both, the mean from E/R knockdown vs. control and E/R-positive vs. E/R-negative primary BCP ALL. Column 1, 2 descriptions as for Table S1 and column 3-6 descriptions as for Table S6.

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5. DISCUSSION

This PhD thesis describes the consequences of RNAi-mediated endogenous E/R fusion gene suppression on the leukemic phenotype, cell signaling pathways and the expression signature of t(12;21) positive leukemia.

Specific shRNAs directed against endogenous E/R slowed down cell proliferation and enhanced the apoptosis rate in leukemic cell lines. E/R KD also impaired the repopulation capacity, since leukemia outgrowth in a murine xenotransplant model was either absent or delayed. Importantly, these effects were accompanied by a concurrent inactivation in PI3K/AKT/mTOR signaling. Similarly, pharmacological PI3K/AKT/mTOR pathway inhibition in E/R-positive cell lines and primary leukemic cells induced apoptosis and also sensitized resistant cells to prednisolone. Genome-wide gene expression studies upon E/R silencing of REH and AT-2 cells identified a significant de-regulation of 777 genes. Gene annotation by DAVID and GSEA revealed an enrichment of several terms for the E/R KD up-regulated genes that were further clustered into the metagroups “cell activation”, “immune response”, “apoptosis”, “protein phosphorylation and phosphate metabolism” and “development and differentiation”. By contrast, E/R KD down-regulated genes affected only a few terms associated with “phosphatidylinositol signaling” and “stem cell signatures”. We validated the E/R KD signature by comparison with the E/R expression signature from published primary childhood ALL cases and finally by RT-qPCR of genes representative of various pathways or cellular functions.

In a previous study, we used siRNA to suppress endogenous E/R in one leukemic cell line (Diakos, Krapf et al. 2007). However, with such an approach, suppression of the fusion protein has been of transient nature since siRNA is usually rapidly degraded and diluted with cell division. To evaluate long term effects of endogenous E/R suppression, we decided to use lentivirally delivered shRNA for this study.

Even though endogenous shRNA expression has tremendous potential, it also possesses some intrinsic risks that should be considered. For instance, similar to the externally introduced synthetic siRNA, endogenous expression of siRNAs can lead to an activation of the innate immune system as a consequence of an interferon response (Hornung, Guenther-Biller et al. 2005). Furthermore, both siRNA and shRNA molecules can induce off-target effects. However, off-target effects can be

minimized by proper shRNA design and appropriate controls (Manjunath, Wu et al. 2009). It should also be noted that shRNAs partially share the machinery used for generation and export of other non-coding RNAs such as cellular miRNAs. Excessive shRNA synthesis could therefore lead to competitive effects.

In order to achieve efficient shRNA transfer into E/R-positive cell lines, which are generally difficult to transfect, we established a lentiviral delivery system. Lentiviruses are regularly used for shRNA expression and have proven effective in transducing dividing and non-dividing cells in a variety of organs. Importantly, the viral DNA gets incorporated in the host genome and induces long-term transgene expression (Wiznerowicz and Trono 2005). The constructs being commonly used are usually non-replicating and generate a virus that, after a single round of infection, ensures stable integration of the shRNA with only a minimal component of the viral genome. Consistently, following selection for transduced cells with the antibiotic blasticidin, we achieved a stable E/R knockdown. We decided to use HIV-based vectors since, due to the extensive characterization of the basic biology of HIV replication, their design has been steadily improved in the past (Cockrell and Kafri 2007).

Stable E/R suppression opened the possibility to evaluate the consequences of fusion protein depletion *in vivo*. These experiments revealed a severely delayed or even absent repopulation capacity of E/R repressed leukemic cell lines upon transplantation, suggesting a reversion of neoplastic nature due to oncogene depletion. Apparently, leukemic cells remain dependent on E/R expression. However, this observation is not restricted to t(12;21) leukemia. To date there is an extensive list of other tumors as well as tumor cell lines which display comparable alterations in cell proliferation, propensity to undergo apoptosis and impairment of repopulation capacity. In hematological malignancies, essential roles have been described for the fusion proteins BCR/ABL, MLL/AF4 in ALL and MLL/ENL in AML, since targeting these molecules was effective to eradicate overt disease (Thomas, Gessner et al. 2005; Kharas, Janes et al. 2008; Horton, Walf-Vorderwulbecke et al. 2009). Weinstein et al defined the phenomenon that a cell becomes dependent on a mutated gene, representing an early or initiating event during the cell transformation process, by the term oncogene addiction (Weinstein 2002). This suggests that oncogenes induce a distinct and complex signaling state that, following oncogene inhibition, results in synthetic lethality. From this point of view, our data suggests that

the E/R fusion protein should be added to the growing list of oncogenes that not only initiate but also play an important role in leukemia maintenance.

We also applied gene expression profiling to characterize the impact of E/R on gene expression in t(12;21) positive leukemias. This technique has been used extensively in biological research in the past decade and has contributed to significant advances in the understanding of the molecular mechanisms of complex diseases including hematological malignancies. However, application of this technique has certain risks and limitations that should be considered (Kim, Zakharkin et al. 2010). First of all, one should keep in mind that biological systems are dynamic and constantly changing and microarray experiments present only snapshots of a biological system at a given time point. Since biological variations were shown to be the greatest source of experimental variation (Zakharkin, Kyoungmi et al. 2005), we decided to perform microarray analyses from several replicates obtained from different time points after lentiviral shRNAs transduction in different cell lines and transduction experiments. Importantly, the rates of synthesis and degradation for different mRNAs also vary. Certain mRNAs are synthesized more slowly than others but are more stable and accumulate at higher levels. By contrast, some mRNAs are degraded rapidly, leading to an inability to detect them (Cheadle, Fan et al. 2005). For these reasons, we aimed to keep activities of RNases low and kept samples during RNA isolation and processing steps, prior to gene expression analyses, on ice.

We also considered published gene expression data following proposed guidelines concerning the performance of microarray experiments in leukemia (Staal, Cario et al. 2006) for validation of the E/R KD. For instance, these guidelines propose purification of leukemic cells (when tumor loads are less than 90%), especially when signaling pathways are analyzed. However, data comparison turned out to be demanding, since most studies were performed with primary ALL blasts and for analyses, compared one leukemic subtype with the mean of all others, obviously varying in composition and sample sizes.

In general, cross-platform comparisons are nowadays regarded as certainly possible, assuming standardized protocols are considered (Staal, Cario et al. 2006). Notably, due to differences in the numbers of probe sets as well as total number of probes on each array, we could not correlate the expression levels of every gene of the E/R KD with E/R-positive primary ALL from Ross et al.

Upon E/R KD, gene expression profiling identified similar numbers of up- and down-regulated genes. However, up-regulated genes mapped to a variety of similar functions, while repressed genes mapped to more diverse functions, appearing thus not significantly enriched and might therefore be of less biological relevance. Since it has been postulated that the E/R fusion converts RUNX1 from a conditional transcriptional activator and repressor to a constitutive repressor of its target genes (Fenrick, Amann et al. 1999), the significant association with functions/processes from up-regulated genes upon E/R KD, i.e. candidates for E/R repressed genes, might result, at least partly, from initial direct gene regulation. However, since our data represent expression profiles from stable KD of E/R, we did not identify an enrichment of potential directly regulated genes by RUNX1 consensus binding site search.

Given that many more genes were identified upon E/R KD compared to the signature of primary ALL cases, we thus could identify genes being also regulated in other ALL subtypes. For instance, PROM1, which plays a role in maintenance of stem cell properties by suppressing differentiation, was also enriched in leukemias harbouring rearrangements of the *MLL* gene. The potential regulation of PROM1 by these chimeric proteins that are considered as initial events in leukemia pathogenesis supports the view that malignant cells are replenished from rare leukemic stem cells (Bomken, Fiser et al. 2010). Consistently, a recent study has demonstrated that primitive leukemia-initiating cells with long-term *in vitro* and *in vivo* proliferation capabilities are exclusively found in the CD133+CD19-CD38- cell compartment (Cox, Diamanti et al. 2009).

Transcriptional levels do not always correlate with the amount of protein being translated in the cell. Even more importantly, they do not provide any evidence for the presence and quantity of post-translational protein modifications. Therefore, we determined the phosphorylation state of important signaling nodes. These experiments confirmed preliminary evidence obtained from gene expression profiling that E/R specifically influences PI3K, downstream AKT and mTOR signaling as well as modulates the expression of several pathway effectors.

Given that the PI3K/AKT/mTOR pathway is of fundamental importance for many cellular tasks that are critical for neoplastic behaviour (Hanahan and Weinberg 2000; Steelman, Pohnert et al. 2004), pathway activation could potentially contribute to leukemia development and maintenance. Regulation of the PI3K/AKT/mTOR

pathway by E/R in our KD model precludes that activating mutations are present, at least in the investigated cell lines. Whether E/R leads to an activation of PI3K/AKT/mTOR pathway components, upstream signalling molecules, or both, remains to be further evaluated. However, a recent study from our group showed an up-regulation of the erythropoietin receptor in E/R-positive cells which concurred with a survival advantage via PI3K/AKT/mTOR pathway activation (Inthal, Krapf et al. 2008). Furthermore, gene expression data of E/R-positive leukemias as well as E/R silenced cells indicated a regulation of KIT by the fusion protein that would also lead to PI3K/AKT/mTOR activation. Importantly, a recent study also reported that RUNX1 regulates KIT expression (De Vita, Canzonetta et al. 2010). Even though the experimental evidence for the relevance of KIT in E/R-positive leukemias remains to be shown, prominent roles for this receptor have been described in other cancers like acute myeloid leukemia, mastocytosis and gastro-intestinal stromal tumors (Masson and Ronnstrand 2009).

Altogether, the observation that E/R expression is mandatory for the survival and propagation of the respective leukemic cells could also be of important clinical relevance. It has been postulated that targeted cancer therapy may only be effective when directed against mutant molecules that represent initiating events in leukemia development, being also present in persistent pre-leukemic clones. This notion may especially be valid for E/R-positive ALL cases because targeting the *E/R* gene fusion, presumed to represent a prenatal and initiating event, could circumvent the extremely high sub-clone variegation and variation between diagnosis and relapse encountered in these leukemias (Anderson, Lutz et al. 2011; Kuster, Grausenburger et al. 2011). Experimental evidence supporting this treatment concept was presented for RUNX1/ETO in acute myeloid leukemia. Selective interference with the oligomerization domain of ETO abrogated the ability of RUNX1/ETO to form high molecular weight complexes which are a functional characteristic of this fusion protein and attenuated its leukemogenic effects by inducing apoptosis (Wichmann, Chen et al. 2007). For E/R, the importance of oligomerization domains analogous to RUNX1/ETO remains to be further evaluated.

We recently evaluated genome-wide copy number alterations of 18 matched diagnosis and relapse samples, revealing high frequencies of deletions in genes being implicated in the response to glucocorticoids, including the BCL2 modifying

factor (*BMF*), the glucocorticoid receptor NR3C1 and components of the mismatch repair pathways (Kuster, Grausenburger et al. 2011). For *BMF* deletions, we could provide evidence that these correlate with poor response to glucocorticoids being frequently observed in relapse leukemias. Consistently, in those E/R-positive relapses without *BMF* deletions, the expression levels were also found to be reduced. In this study, we explored a potential benefit of the PI3K/AKT/mTOR pathway inhibition for glucocorticoid resistant leukemias. Given that PI3K/AKT/mTOR signaling impacts on several BCL2-family members, pathway inhibition may also prove effective against relapsed E/R-positive ALL (McCubrey, Steelman et al. 2008).

Apart from the described influence of E/R on the PI3K/AKT/mTOR pathway, we consider global gene expression analyses upon fusion protein suppression to contribute to a better understanding of the mechanisms of leukemia biology. The novelty of our study, compared to previous concepts, was the investigation of E/R mediated gene expression alterations in the context of a fully developed leukemia. Consistent with the observation that E/R expression is mandatory for propagation of leukemic cells, functional gene annotation suggested that the chimeric protein impacts on important biological functions such as cellular adhesion and stem cell features. Furthermore, our data suggested that E/R could modulate DNA damage response by inducing expression of MDM2, a potent negative regulator of p53 function. Following DNA damage, overexpressed MDM2 could provide a mechanism to evade tumor suppressor protein p53 induced apoptosis. Importantly, MDM2 already represents a potential target for leukemia therapy, since its inhibitor Nutlin has already been tested in clinical trials.

Even though our E/R KD approach contributed to study the influence of E/R on the leukemic cell phenotype and correlate alterations with differential gene expression, our approach was not capable of identifying direct E/R target genes. Therefore, we next aim to establish an inducible E/R KD model, suitable to perform chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-Seq) that could represent a tool to identify gene regulatory regions under the control of the fusion protein.

Finally, we envision that findings from E/R could be beneficial for other cancers, especially those involving other RUNX1 fusion proteins.

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APPENDIX A: Co-AUTHORSHIP PUBLICATION I

**ETV6/RUNX1-POSITIVE RELAPSES EVOLVE FROM AN
ANCESTRAL CLONE AND FREQUENTLY ACQUIRE DELETIONS OF
GENES IMPLICATED IN GLUCOCORTICOID SIGNALING**

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ETV6/RUNX1-positive relapses evolve from an ancestral clone and frequently acquire deletions of genes implicated in glucocorticoid signaling

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Approximately 25% of childhood acute lymphoblastic leukemias carry the *ETV6/RUNX1* fusion gene. Despite their excellent initial treatment response, up to 20% of patients relapse. To gain insight into the relapse mechanisms, we analyzed single nucleotide polymorphism arrays for DNA copy number aberrations (CNAs) in 18 matched diagnosis and relapse leukemias. CNAs were more abundant at relapse than at diagnosis (mean 12.5 vs 7.5 per case; $P = .01$) with 5.3 shared on average. Their patterns revealed a direct clonal relationship with exclusively new ab-

errations at relapse in only 21.4%, whereas 78.6% shared a common ancestor and subsequently acquired distinct CNA. Moreover, we identified recurrent, mainly nonoverlapping deletions associated with glucocorticoid-mediated apoptosis targeting the Bcl2 modifying factor (*BMF*) ($n = 3$), glucocorticoid receptor *NR3C1* ($n = 4$), and components of the mismatch repair pathways ($n = 3$). Fluorescence in situ hybridization screening of additional 24 relapsed and 72 nonrelapsed *ETV6/RUNX1*-positive cases demonstrated that

***BMF* deletions were significantly more common in relapse cases (16.6% vs 2.8%; $P = .02$). Unlike *BMF* deletions, which were always already present at diagnosis, *NR3C1* and mismatch repair aberrations prevailed at relapse. They were all associated with leukemias, which poorly responded to treatment. These findings implicate glucocorticoid-associated drug resistance in *ETV6/RUNX1*-positive relapse pathogenesis and therefore might help to guide future therapies. (*Blood*. 2011;117(9):2658-2667)**

Introduction

The *ETV6/RUNX1* (*E/R*) fusion gene (also known as *TEL/AML1*) results from the chromosomal translocation t(12;21) and is present in approximately 25% of childhood B-cell precursor acute lymphoblastic leukemia (BCP ALL).¹ This leukemia subtype is generally associated with good prognostic parameters and excellent molecular response to treatment.²⁻⁶ Although almost all children can be treated successfully, predominantly late relapses occur nevertheless in up to 20% of the patients.⁷⁻⁹

In the multistep model of E/R leukemia development, the gene fusion represents the first transforming event. It virtually always takes place during fetal development but is per se unable to generate overt disease.^{10,11} One of the earliest and most prominent of the many currently recognized lesions that drive the disease process is the deletion of the second, nonrearranged *ETV6* allele, which is found in approximately two-thirds of the cases.^{12,13} Already a decade ago, the observation of distinct *ETV6* deletions together with incongruent clonotypic immunoglobulin (*Ig*)/T-cell receptor (*TCR*) rearrangement patterns at diagnosis and relapse suggested that the majority of relapses do not represent a genuine reemergence of the original clone. Rather, both the initial and relapse leukemia seemed to evolve from a shared ancestral clone.¹⁴⁻¹⁹ These observations further implied that such preleukemic/

leukemic ancestral subclones must resist treatment to be able to once more expand to a full-blown, treatment-responsive, disease.

Genome-wide single nucleotide polymorphism (SNP) genotyping serves to identify recurrent copy number aberrations (CNAs) that may contribute to the pathogenesis of childhood BCP ALL.²⁰⁻²² CNAs compose deletions, duplications, and amplifications that preferentially target genes involved in key cellular signaling pathways, in particular the development and differentiation of B cells, cell-cycle regulation, DNA repair, and drug resistance. Although initial analyses focused especially on the evaluation of CNA in diagnostic samples, consecutive studies also compared diagnostic and relapse CNA patterns.²³⁻²⁵ The latter are frequently more pronounced and affect regions containing genes that are also functionally relevant in the same pathways. The fact that several such abnormalities, which were apparently exclusively present in relapse, were subsequently also identified in a minute cell compartment at diagnosis, proves that relapses can indeed originate from small preexistent subclones.²³ So far, CNA studies of childhood ALL composed the whole heterogeneous spectrum of genetic subgroups and therefore paid no particular attention to potentially relevant subgroup-specific characteristics.

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Table 1. Clinical characteristics of 18 E/R-positive relapse cases that were analyzed with SNP arrays

Patient ID	Sex	Age, y	Remission, mo	Relapse site	MRD	Outcome, mo	Source
1	Male	3.7	46	BM	NA	SCT, relapse,*	Germany
2	Male	4.2	51	BM	NA	SCT, remission 96	Germany
3	Male	2.3	19	BM	NA	SCT, relapse*	Austria
4	Male	3.7	78	BM	IR	SCT*	Austria
5	Male	4.3	50	BM	LR	Remission 126	Austria
6	Male	3.4	16	BM	IR	SCT, remission 86	Austria
7	Male	2.1	43	BM	NA	SCT, remission 78	Austria
8	Male	4.7	40	BM	NA	Remission 21	Austria
9	Female	3.6	32	BM	IR	SCT, remission 23	Austria
10	Male	9.3	29	BM	IR	SCT, relapse, remission 26	Austria
11	Male	3.3	79	Testis	IR	SCT, remission 69	Austria
12	Male	NA	NA	BM	NA	NA	St Jude #48
13	Female	NA	NA	BM	NA	NA	St Jude #49
14	Male	3.2	68	BM	LR	Remission 14	Germany
15	Male	4.5	27	BM	IR	Remission 35	Germany
16	Male	NA	NA	BM	NA	NA	St Jude #10
17	Male	NA	NA	BM	NA	NA	St Jude #44
18	Female	NA	NA	BM	NA	NA	St Jude #50

MRD indicates minimal residual disease; NA, not available; SCT, hematopoietic stem cell transplantation; IR, intermediate risk; and LR, low risk.

*Death.

To gain further insights into the molecular mechanisms that contribute to the development of relapses in E/R-positive leukemias, we assessed genome-wide CNA changes of 18 matched diagnosis and relapse samples and, for comparison and further evaluation, complemented them with previously published SNP array data from 47 nonrelapsed cases.²⁰

Methods

Patients

This study was composed of 18 cases of E/R-positive relapsed BCP ALL. Thirteen were selected according to the availability of material and a blast count of more than 85% in leukemic samples. Five cases were taken from an earlier study.²³ All patients, patients' parents, or legal guardians gave written informed consent, in accordance with the Declaration of Helsinki, for inclusion into this study. The study was approved by the ethical committee of the CCRI and St Anna Kinderspital. Nine patients were from Austria (3-11) and 4 from Germany (1, 2, 14, and 15). The SNP array data of 5 further patients (12, 13, 16-18; IDs: TEL-AML1-SNP-#48/#48GL/#48R, TEL-AML1-SNP-#49/#49GL/#49R, TEL-AML1-SNP-#10/#10R, TEL-AML1-SNP-#44/#44R, and TEL-AML1-SNP-#50/#50R) were kindly provided by C.G. Mullighan, St Jude Children's Research Hospital.²³ The Austrian and German patients were enrolled in the ALL-BFM 90 and 95 treatment protocols.⁴ Seventeen of the 18 patients had a bone marrow (BM) relapse and one (patient 11) an isolated relapse in the testis. In the latter case, we isolated leukemic cells from the testis for SNP profiling. To determine the constitutional genotype, early remission BM samples were available in cases 1 to 14.

Median age of patients 1 to 11, 14, and 15 at diagnosis was 4.0 years (range, 2.1-9.3 years), and median first remission duration was 44.5 months (range, 16-79 months). Clinical findings are summarized in Table 1. To evaluate the potential relevance of CNA and affected genes of our cases, we compared the 14 cases with paired remission samples and the diagnosis- or relapse-specific CNA of the remaining 4 cases with previously published data from 47 E/R-positive cases.²⁰ Although 3 of these 47 patients were reported to have relapsed later,²³ this did not influence our statistical evaluation.

DNA extraction and sample preparation

DNA was extracted from BM mononuclear cells at diagnosis, remission, and relapse and, in one instance, from leukemic cells of the testis as

described previously.²⁶ Hybridization to Affymetrix GeneChip Human Mapping 250K Nsp and 250K Sty arrays (n = 6) or to Affymetrix Genome-Wide Human SNP Array 6.0 (n = 7) were performed by imaGenes.

Copy number analysis

Raw signal intensities were analyzed for CNA with the Partek Genomic Suite software, Version 6.4 (Partek). We applied the genomic segmentation algorithm for detection of CNA-containing regions and normalized segmented DNA copy numbers to a median of 2 copies.²⁷ Segmentation parameters were chosen as follows: 5 minimum genomic markers per segment (defining how small a region can be), a *P* value threshold of .001 (controlling for outlying probes), and a signal-to-noise value of 1. Diagnosis- and relapse-specific somatic CNAs were identified by comparison with the matched germline remission pattern. In case this was not possible, a HapMap reference, consisting of 270 or 794 HapMap samples (depending on array type), was used for comparison, and detected CNA were compared with known copy number variations that were provided in a Copy Number Variant Database from Partek. Copy number changes detected by genomic segmentation were verified by comparison with the log₂ ratio visualization in the Partek Genome Browser.

In the final tables, figures, and counts, we excluded all those deletions and duplications that might have resulted from known copy number variation or Ig and *TCR* gene rearrangement-related processes at 2p11.2 (*IGK*), 7p14.1 (*TCRG*), 7q34 (*TCRB*), 14q11.2 (*TCRDA*), 14q32.33 (*IGH*), and 22q11.22 (*IGL*). Deletions of the latter region were only excluded if they derived from an *IGL* rearrangement but not if they resulted from a focal deletion of *VPREB1*.

FISH for *BMF* and *NR3C1* gene deletions

BMF and *NR3C1* copy numbers were determined by fluorescence in situ hybridization (FISH) on methanol/acetic acid fixed cells using the following probes: BAC clone CTD-2251F23 (122kb; obtained from Invitrogen Research Genetics), which encompasses the entire *BMF* gene, was hybridized together with the α -satellite centromere-specific reference probe D15Z1; *NR3C1* gene locus-specific BAC clones RP11-278J6 (165 kb; obtained from imaGenes) and RP11-138C1 located at 5p13. FISH was performed according to standard procedures, and at least 100 interphase nuclei per sample were scored. The cut-off for heterozygous deletions was more than 20%.

Table 2. Frequency of somatic CNAs in 14 E/R-positive cases at diagnosis and relapse

Patient ID	CNAs								
	Losses			Gains			All		
	Diagnosis	Relapse	Shared	Diagnosis	Relapse	Shared	Diagnosis	Relapse	Shared
1	4	7	0	2	2	1	6	9	1
2	6	12 (1)	6	0	0	0	6	12 (1)	6
3	7	6	6	0	2	0	7	8	6
4	4	9	3	0	0	0	4	9	3
5	1	7 (1)	0	2	1	0	3	8 (1)	0
6	6	10 (1)	6	2	3	2	8	13 (1)	8
7	3	5	2	0	1	0	3	6	2
8	7 (1)	9	5	0	1	0	7 (1)	10	5
9	10	11	7	0	1	0	10	12	7
10	14 (1)	13	10	1	7	0	15 (1)	20	10
11	8 (1)	8 (1)	2 (1)	1	0	0	9 (1)	8 (1)	2 (1)
12	3	14 (1)	2	0	0	0	3	14 (1)	2
13	10 (1)	18 (1)	10* (1)	2	2	2	12 (1)	20 (1)	13 (1)
14	9	16 (3)	8	3	10	2	12	26 (3)	10
Total	92 (4)	145 (9)	67 (2)	13	30	7	105 (4)	175 (9)	74 (2)
Mean	6.6	10.4	4.8	0.9	2.1	0.5	7.5	12.5	5.3

Included are only cases with available remission samples. Indicated are all CNAs in the respective leukemia; in case of biallelic deletions, the number is given in parentheses.

*For simplicity reasons, 2 deletions at relapse overlapping with 1 deletion at diagnosis were counted as 1 deletion (details about the affected regions can be found in supplemental Tables 1 and 5).

BMF mRNA quantification

Total RNA was isolated either using the RNeasy Mini Kit (QIAGEN) or Trizol Reagent (Invitrogen). Random hexamer priming and SuperScript II (Invitrogen) were used to generate cDNA. Quantification of *BMF* mRNA abundance was done using TaqMan real-time RT-PCR. *ABL* and *GUS* were used for endogenous control amplification. Primer sequences, PCR conditions, and interpretation of results are available as supplemental data (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Mutational screening of *BMF*

Mutational screening of *BMF* was performed using primers flanking the coding exons 3, 4, and 5 (details provided in supplemental data).

shRNA-mediated knock-down of *BMF* in REH cells

ETV6/RUNX1-positive REH cells were cultured as described previously.²⁸ Lentiviral vectors expressing shRNA sequences directed against the human (pHR-THT-sh-hBMF-SFFV-eGFP) *BMF* or murine (pHR-THT-sh-mBmf-SFFV-eGFP) *Bmf*, nontargeting the human *BMF*,²⁹ were used to transduce REH cells. Details on shRNA sequences and lentiviral supernatant production and transduction are provided in the supplemental data.

Glucocorticoid treatment and apoptosis assay

REH cells transduced with shRNA against *BMF* or control shRNA were treated with 1 mg/mL prednisolone (SoluDacortin, Merck) over a time period of 96 hours. Prednisolone treatment was performed 11 days after lentiviral transduction and apoptosis assessed by annexin V/propidium iodide staining as reported previously.³⁰

Information on bioinformatic analysis, pathway analysis targeted by presentation- or relapse-specific CNA, and statistical analysis are provided as supplemental data.

Results

Comparison of CNA in matched diagnosis and relapse samples

The results of our genome-wide copy number analysis of the 14 cases with paired remission samples are summarized in Table 2,

Figure 1A, and supplemental Table 1. CNAs in paired samples were identical, partially shared, or completely different (Table 2). Overall, 73% of copy number losses and 54% of copy number gains remained stable. In 13 of the 14 cases, several of the lesions that were observed at diagnosis were also seen in relapse, although all cases also lost and/or acquired some lesions in relapse. Only in one patient (patient 5), the 2 CNA patterns were completely different. Overall, 31 of 105 CNAs present at diagnosis were lost in relapse, whereas 101 were gained. Thus, the mean diagnosis-specific CNA number per case was significantly lower (mean, 2.2; range, 0-7) than the relapse-specific one (mean, 7.2; range, 2-16; $P = .0001$, Welch unpaired t test). Moreover, 3 cases (21.4%) acquired new CNA at relapse but did not lose any, whereas 11 (78.6%; including the one case with the complete change) lost as well as acquired several alterations. Data from patients without remission sample are similar to the paired samples, as they show similar patterns of distinct CNA between diagnosis and relapse but tend to have higher shared CNA counts, which most probably can be explained by the contribution of copy number variation (supplemental Tables 1, 2). Comparison of high-quality heterozygous SNP calls showed concordance between paired samples in all cases.

Comparison of CNA in relapse and nonrelapse cases

The CNA frequency of our 14 diagnosis and relapse samples was comparable with the one established in the 47 nonrelapse cases (Table 3). As regards specific CNA in individual cases, however, on average only median 75% (range, 25%-100%) remained identical on both occasions. Approximately 30% of the diagnosis samples contained deletions of tumor suppressor and cell cycle regulator genes. Heterozygous and homozygous *CDKN2A/B* and *CDKN1B* deletions, in particular, were present with an equal frequency in diagnosis and relapse samples (Figure 1B; supplemental Tables 1, 2). Because *ETV6*, *VPREB1*, *CDKN2A/B*, *CD200*, *BTLA*, *TBL1XR1*, and *PAX5* deletions already prevail at diagnosis and recur mostly unchanged in relapse samples, they are presumed to reflect a very early and essential event in leukemia development. The fact that specific patterns of individual deletions were only identical in 50%

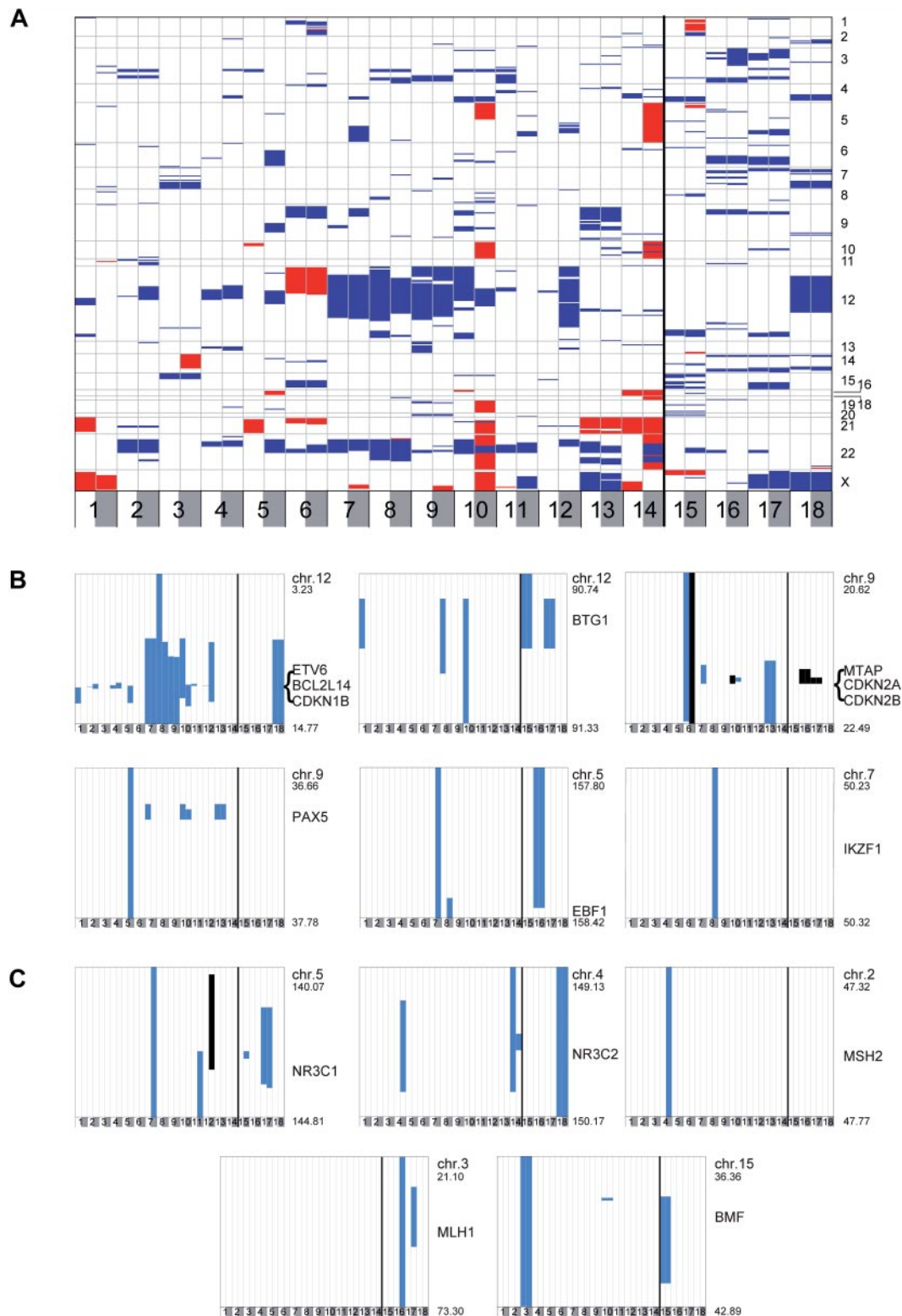


Figure 1. Heatmaps of CNAs in 18 relapsed E/R-positive leukemias. (A) Overview of somatic CNA in diagnosis and relapse samples. Copy number changes are mapped according to their chromosomal position and indicated by color (blue represents losses; and red, gains) for each chromosome from 1 to X (row) and each sample (column). Each row represents a segment of the genome with a CNA across samples. Regions in the genome without CNA events are not shown in the heatmap. Each column is labeled with the patient IDs at the bottom of the graph, and sample types are indicated by the color bars: white represents diagnosis; and gray, relapse. Samples with and without paired remission samples are separated by a bold vertical line. (B-C) Heatmaps for recurrent genomic deletions were constructed by zooming in on genes of interest and plotting along chromosomal coordinates (y-axis) color-coded presence (blue for monoallelic/black for biallelic) and absence (white) of deletions. Deletions of tumor suppressor genes and cell differentiation markers at 12p13.2 (*ETV6*, *BCL2L14*, *CDKN1B*), 12q21.33 (*BTG1*), 9p21.3 (*CDKN2A/B*), 9p13.2 (*PAX5*), 5q33.3 (*EBF1*), and 7p12.2 (*IKZF1*) are shown in panel B, and deletions of genes implicated in GC signaling and MMR at 5q31.3 (*NR3C1*), 4q31.22 (*NR3C2*), 2p21 (*MSH2*), 3p21.3 (*MLH1*), and 15q15.1 (*BMF*) are shown in panel C. Genome position (Mb) according to the University of California Santa Cruz Genome Browser (NCBI Build 36.1) is given for each graph.

Table 3. Frequency of recurrent CNAs in 14 E/R-positive relapse patients at diagnosis and relapse with paired analysis compared with those observed at diagnosis in 47 nonrelapse patients

Cytoband	Gene	47 without relapse		14 with relapse					
		Diagnosis		Diagnosis		Relapse		Shared	
		N	%	N	%	N	%	N	%
Losses									
12p13.2	ETV6	33	70	8	57	8	57	7	88
22q11.22	VPREB1	32	68	8 (3)	57 (38)	10 (5)	71 (50)	8 (2)	100 (20)
9p21.3	CDKN2A	12	26	3 (1)	21 (33)	4 (1)	29 (25)	3 (0)	100 (0)
3q13.2	CD200, BTLA	7	15	5	36	4	29	3	60
9p21.3	CDKN2B	NA	NA	3 (1)	21 (33)	3 (1)	21 (33)	2 (0)	67 (0)
3q26.32	TBL1XR1	6	13	4	29	3	21	3	75
9p13.2	PAX5	13	28	3	21	3	21	2	67
12p13.1	ATF7IP	NA	NA	5	36	6	43	4	80
12p13.3	VWF	NA	NA	4	29	1	7	1	25
12p13.2	BCL2L14, CDKN1B	NA	NA	5	36	5	36	3	60
9p21.3	MTAP	NA	NA	2 (0)	14 (0)	3 (1)	21 (33)	2 (0)	100 (100)
12q21.33	BTG1	6*	13*	3	21	0	0	0	0
15q15.1	BMF	0	0	2†	14	2†	14	2†	100
6q21	ARMC2, SESN1	7	15	1	7	1	7	0	0
5q31.3	NR3C1	6	13	0	0	3‡ (1)	21 (33)	0	0
21q22.12	RUNX1	3§	6§	2	14	2	14	2	100
9p21.3	IFN cluster	0	0	1	7	1	7	1	100
12p13.2	GABARAPL1	NA	NA	4	29	4	29	3	75
5q33.3	EBF1	5	11	0	0	2	14	0	0
7p12.2	IKZF1	0	0	0	0	1	7	0	0
4q31.22	NR3C2	5¶	11¶	0	0	2	14	1	50
2p21	MSH2	0	0	0	0	1	7	0	0
3p21.3	MLH1	0	0	0	0	0#	0	0	0
Gains									
21q	Includes RUNX1	4	9	5	36	4	29	3	60

Numbers represent cases/percentages with genomic aberrations; number of cases with biallelic deletions in parentheses.

NA indicates not applicable.

*3' of *BTG1*.

†One additional case with a conserved *BMF* deletion in the group with unpaired analysis.

‡One additional case with a relapse-specific deletion among the cases with unpaired analysis.

§Immediately distal to *RUNX1*.

||Analyzed in only 13 patients.

¶Telomeric to *NR3C2*.

#Two cases with a relapse-specific deletion among the cases with unpaired analysis (supplemental Tables 1 and 2).

to 80% of matched samples (Table 3; supplemental Figure 2) seems to indicate that they may also arise at a later stage during the course of the disease. Therefore, the sequence of such events may actually be quite variable.

Irrespective of the apparently equal frequency of recurrent CNA at diagnosis and relapse, we were able to discern particular alterations that might be either predictive or characteristic of relapse. Apart from the gene deletions that are described in more detail in the next paragraph, a deletion of the *EBF1* gene, which encodes an early transcriptional regulator of B-cell development, was exclusively seen in 2 cases at relapse. In one of these cases (patient 8), it concurred with a deletion of the *IKZF1* gene, which encodes the lymphoid transcription factor IKAROS (Table 3, Figure 1B, supplemental Tables 1-2, and supplemental Figure 3).

Deletions of genes involved in GC receptor signaling, drug resistance and MMR pathways

Of great interest were deletions of genes that so far had not been directly associated with E/R-associated leukemogenesis (Figure 1C; supplemental Table 1). They encode factors of the nuclear receptor subfamily 3 (*NR3C1*, *NR3C2*) and key components of the mismatch repair (MMR) pathway (*MSH2*, *MLH1*). Although the development of relapses from drug-resistant clones is well known

in childhood ALL, this particular matter is not considered to pose a significant therapeutic problem in the E/R-positive subgroup.^{7,24,31-33}

For the following analyses, also relapse-specific CNA encompassing these gene loci from patients without paired remission samples were included. Deletions of the *NR3C1* gene that encodes the glucocorticoid (GC) receptor were seen exclusively at relapse in 4 of the cases, including one homozygous deletion (patient 12). The *NR3C1* deletion in patient 7 was confirmed with FISH (supplemental Figure 4). The *NR3C2* gene, encoding the mineralocorticoid receptor, was deleted in 2 cases, in one of them only at relapse (Figure 1C, Table 3, supplemental Figure 5).

MLH1 and *MSH2* genes encode key components of the MMR pathway. Deletions of the one or the other of these genes were found only in the relapse samples of 3 cases (Figure 1C, Table 3, supplemental Tables 1, 2, supplemental Figure 4). The respective MMR proteins form different heterodimers and initiate repair by binding to DNA mismatches.³⁴ MMR gene deletions concurred with that of the *NR3C2* gene in one case (patient 4).

Deletion of *BMF*

SNP profiling also revealed a novel deletion at 15q15.1 in both the diagnosis and relapse samples of 3 of 18 cases (Figure 1C; Figure 2A-B). *BMF*, the only gene located in the overlapping

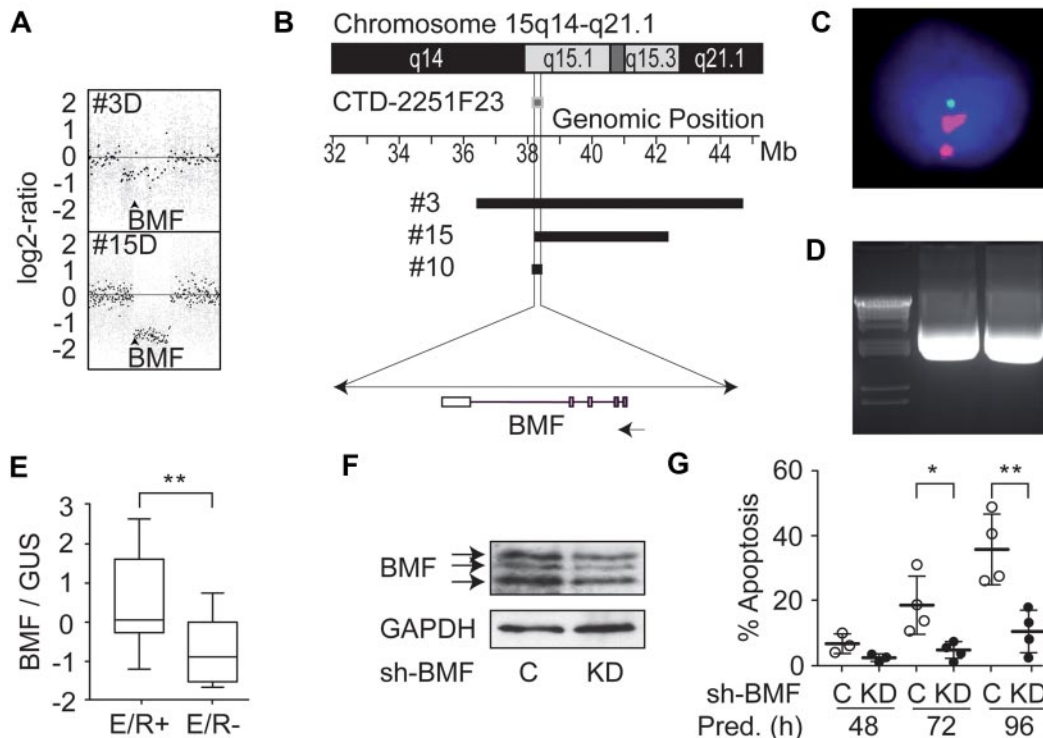


Figure 2. *BMF* deletions and functional consequences of reduced *BMF* expression in E/R-positive ALL. (A) Log₂-ratio copy numbers of genomic deletions encompassing *BMF* (indicated by an arrow) are shown for 2 patients with a deletion at 15q15.1. (B) Segmental deletions at 15q15.1 in 3 cases (3, 10, and 15). (Top) A partial ideogram of the 15q region and the position of the FISH probe used for confirmation of the deletions. The scale indicates genomic positions of the deletions in megabases according to the University of California Santa Cruz Genome Browser (NCBI Build, Version 36.1). Each black horizontal bar represents a deletion in a single patient as inferred by SNP array. Vertical lines indicate the overlapping region encompassing the only commonly deleted gene (*BMF*) (bottom). (C) Verification of the *BMF* deletion by FISH in patient 10 in 57% of nuclei (green represents *BMF*; and red, chromosome 15 centromere). (D) Genomic breakpoint of the *BMF* deletion from case 10 was cloned (details provided in supplemental data; accession code #GU984796) and sequence information used for nested PCR. The resulting PCR products from initial diagnosis (middle lane) and relapse (right lane) are shown. Left lane indicates size marker. (E) Quantification of *BMF* expression in E/R-positive (E/R+, n = 15) and E/R-negative (E/R-, n = 15) BCP ALL cases without relapse. Data represent relative expression values of *BMF* compared with *GUS*. Boxes include values between the 25th and 75th percentile, and whiskers indicate the minimal and maximal values. The black line indicates the median *BMF* expression of all samples. For statistical evaluation, Welch unpaired *t* test was used. (F) Western blot analysis of REH cells on *BMF* repression by RNAi. Protein lysates were prepared from green fluorescent protein-sorted cells expressing the shRNA vector targeting human *BMF* (KD) or a nontargeting control shRNA (C). *BMF* isoforms (*BMF*1, *BMF*2, and *BMF*3, top-down) are marked with arrows. GAPDH levels were used as a loading control. (G) Glucocorticoid-induced apoptosis on *BMF* repression in REH cells. *BMF* silenced cells were treated with prednisolone for the indicated times, and apoptosis was assessed by annexin V/propidium iodide staining in green fluorescent protein-positive cells carrying the lentiviral shRNA construct. Apoptosis was calculated by subtraction of annexin single-positive cells in untreated from prednisolone-treated cells. Percentages of 4 independent experiments from 3 separate infections are shown. Bars represent mean $\pm$ SD. **P* < .05 (paired Student *t* test). ***P* < .01 (paired Student *t* test).

minimal deleted region, encodes a member of the BH3-only group of Bcl2-related proteins, which together with other proapoptotic proteins plays a role in mitochondrial regulation of apoptosis, cancer development, and drug resistance.³⁵

FISH screening of patients 3 to 11, 14, and 15 verified the presence of heterozygous *BMF* deletions in patients 3, 10, and 15 in 50% to 80% of nuclei (Figure 2C). To further assess the frequency of *BMF* deletions in a larger cohort of E/R-positive leukemias, we screened another 24 relapse as well as 72 consecutively recruited nonrelapse cases. The identification of 4 additional heterozygous *BMF* deletions in the relapse and another 2 in the nonrelapse group proved that the frequency of *BMF* deletions is indeed higher in relapse cases (16.6% vs 2.8%; *P* = .02, Fisher exact test). Because 4 cases with a *BMF* deletion experienced an early and 3 a late relapse (defined as shorter and longer than 30 months in remission), the presence of such a deletion could, however, not be used to predict time to relapse. The remission of the 2 cases in the relapse-free cohort, on the other hand, has already lasted almost 10 years, and we consider it therefore unlikely that they will still relapse. In consideration of the potential tumor suppressor function of *BMF*, a notion that is based on the accelerated development of thymic lymphomas in *Bmf* deficient mice,³⁶ we also amplified and

sequenced all coding exons (exons 3-5) of *BMF* at the genomic level in 15 relapsed cases (including 5 with a deletion) but did not detect any mutations.

Quantitative RT-PCR of E/R-positive and E/R-negative BCP ALL (in long-term remission) revealed that *BMF* is significantly higher expressed in E/R-positive leukemias (Figure 2E). However, within this genetic subgroup, mRNA levels were significantly lower in the relapse cohort, irrespective of their *BMF* deletion status (supplemental Figure 6). Because *BMF* is induced by GC before GC triggered cell death and GC is used as a systemic one-week induction monotherapy in ALL BFM-treatment protocols, we examined the sequential expression profiles of sorted leukemic cells (with > 95% purity), which were obtained from 9 E/R-positive and 13 E/R-negative BCP ALL cases during their in vivo GC treatment.²⁹ In line with the quantitative RT-PCR data, basal *BMF* transcription (ie, before GC treatment) was significantly higher in E/R-positive than in E/R-negative BCP ALL (*P* = .05, Welch unpaired *t* test). Although statistically not significant, there was a more pronounced induction of *BMF* expression in E/R-positive than in E/R-negative cases (data not shown). Thus, E/R-positive blasts exhibit higher *BMF* expression and this is, at least in some cases, increased during initial GC therapy.

Finally, to assess a potential functional consequence of a heterozygous *BMF* deletion, we transduced REH cells with lentiviral vectors expressing 2 different shRNA targeting *BMF* and subjected these cells to glucocorticoid treatment. As shown in Figure 2F and supplemental Figure 7A, a significant reduction of *BMF* levels was achieved, which resulted in increased resistance to prednisolone (Figure 2G; supplemental Figure 7B). Thus, these data suggest that impaired *BMF* function may also have an impact on GC-induced apoptosis in primary leukemias.

Comparison of CNA with clone-specific *Ig/TCR* gene rearrangement patterns

Given the frequent differences of clone-specific *Ig/TCR* rearrangements between initial diagnosis and relapse in E/R-positive ALL,^{14,15,18} we investigated to which extent the respective *Ig/TCR* rearrangement and CNA patterns correlate. Although recurrent CNAs are part of leukemogenesis, clone-specific *Ig/TCR* rearrangements are not. They arise from V(D)J recombination, the process that mediates the formation of a functional immune receptor during lymphoid development. Hence, *Ig/TCR* rearrangements are frequently assessed and used as a clone-specific marker for the detection of minimal residual disease.

Data from 9 cases (patients 3-11) are given in supplemental Tables 3 and 4. A total of 28 identical and 2 related rearrangements of the 40 rearrangements present at initial diagnosis were preserved at relapse (75%), and identical *Ig/TCR* rearrangements significantly outnumbered related and changed rearrangements ($P = .0001$, Welch unpaired t test).

When viewed separately, both the *Ig/TCR* rearrangements and CNA patterns revealed a clear relationship between the original predominant and relapse clone in all but one case (patient 5). *Ig/TCR* rearrangements were identical, modified by ongoing recombination, or completely different. However, it was not possible to merge the results of the 2 investigations into a single clonal development scheme because the information derived from the *Ig/TCR* rearrangement analyses did not concur with that of the CNA.

Nevertheless, we propose that, in the majority of cases, both the CNA and *Ig/TCR* rearrangement-generating mechanism must at least be operative during the same period of time (provided in supplemental Figure 8). A rare complete clonal change (patient 5), on the other hand, might indicate that distinct subclones were derived from an E/R-rearranged hematopoietic stem cell. This notion is supported by recent findings demonstrating that this gene fusion and its subsequent expression may indeed transform already uncommitted hematopoietic stem cells.^{37,38} Because many of the consecutive secondary leukemia-promoting changes are mediated by V(D)J recombination, they can only take place during early B-cell development.^{20,39,40}

Discussion

To elucidate the routes leading to disease recurrence in E/R-positive leukemias, we compared SNP array-derived CNA configurations of 18 matched diagnosis and relapse samples. The changes of CNAs in the majority of relapse cases (89%) was the result of a mixture of genuinely acquired novel deletions and duplications as well as to the absence of deletions that were originally present in the corresponding diagnostic sample. Only one case had a complete clonal change. Despite the considerable discordance of various recurrent deletions in the diagnosis and relapse samples of indi-

vidual cases, the overall frequency of CNAs affecting genes that are involved in B-cell development and differentiation as well as cell cycle regulation was nevertheless comparable. This observation suggests that, although these clones are closely related, in most instances the relapse clone does not evolve directly from the one identified at diagnosis, but rather that both clones are derived from a shared ancestor. Except for a few specific and intriguing exceptions, the overall CNA frequency and pattern did also not significantly differ from nonrelapse cases, thereby precluding their application as predictive prognostic markers.

Two cases had acquired a deletion encompassing the *EBF1* gene; one of them even had an *IKZF1* deletion. Although this constellation has been reported previously in E/R-negative relapse cases in which the respective genes were deleted in 4.2% and 8.9% versus 25% and 35% at diagnosis and relapse, respectively,^{24,41} it has, to the best of our knowledge, not yet been documented in E/R-positive relapse cases. Given that both *EBF1* and *IKZF1* expression is required for early B-cell development and precedes *PAX5* expression during lineage commitment, the presence of these deletions in the recurrent leukemia suggests that the relapse clone probably originates from a slightly less differentiated cell. In support of this notion, one of the relapse cases actually had a *PAX5* deletion at diagnosis that was, however, absent in the *EBF1*-deleted relapse clone. Moreover, and at least in this particular case, the concurrence of other deletions in both samples further suggests that impairment of B-cell differentiation seems to occur late during the transformation process. However, the immunophenotype as well as clonotypic *Ig/TCR* rearrangements of these cases appeared unchanged. Whether late-appearing *IKZF1* deletions confer a poor prognosis similar to those already present at diagnosis in BCR/ABL-positive or "BCR/ABL-like" ALL cases^{13,20,24,40,41} needs to be assessed in a large group of relapse patients.

Recent studies reported deletions of *CDKN2A/B*, which encode the p16^{INK4A}/p14^{ARF} and p15^{INK4B} proteins, respectively, in the diagnostic samples of poor prognosis E/R-negative cases, on the one hand, and a higher frequency of such deletions in the relapse samples,^{23,24,31-33} on the other hand, whereas another study did not.⁴² In line with the latter, the frequency of *CDKN2A/B* and *CDKN1B* (p27^{KIP}) deletions at diagnosis in our cohort concurs with that at relapse as well as with the 26% overall frequency of nonrelapse E/R-positive cases.²⁰ Notably, the fact that *CDKN2A/B* deletions at diagnosis did not forecast a shorter time to relapse, as has been proposed for E/R-negative leukemias,²⁴ further emphasized the lack of any prognostic relevance in our E/R-positive leukemia cohort.

The deletion of the nonrearranged *ETV6* gene, a transcription factor required for bone marrow hematopoiesis, is one of the most frequent secondary aberrations that probably also collaborates with E/R in leukemia development.¹² Even though this deletion is presumably one of the earliest genomic alterations, we identified different deletions in the diagnosis and relapse samples of 3 cases, a finding that precludes the relapse clone as having evolved directly from the one originally present. (The genomic boundaries of the various deletions are provided in supplemental Table 6.) These properties concur with similar earlier observations, which also demonstrated that, although both clones derive from the same source, some of their genetic features might, nevertheless, differ completely.^{14,16,19} Taking into account that, even in cases without a deletion of the nonrearranged *ETV6* gene is generally not expressed,^{43,44} it appears likely that *ETV6* inactivation is a critical step in the pathogenesis of E/R-positive leukemia.

Other noteworthy findings in our study were the identification of novel deletions affecting *BMF*, *NR3C1*, and genes encoding key proteins of the MMR pathway. *BMF* deletions were in all instances detected already at diagnosis and preserved at relapse, whereas *NR3C1* and MMR gene deletions either prevailed or were only detected at relapse. All these genes play a pivotal role in apoptosis, GC, and MMR pathways and contribute to GC-induced apoptosis. This association suggests that drug resistance is perhaps also relevant for disease recurrence in these cases. *BMF* is a central player in GC-mediated induction of apoptosis, as its down-regulation renders leukemic cell lines GC resistant.^{29,35} In line with these reports, our data provide the first evidence that *BMF* deletions lead to haploinsufficiency and resistance to GC treatment in E/R-expressing leukemias. Conversely, forced expression of *BMF* makes leukemic cells highly drug-sensitive,^{29,35} and the observed increase of *BMF* transcripts in E/R-positive leukemias together with its induction after in vivo GC treatment,²⁹ correlates well with the known GC sensitivity of E/R-positive leukemias and might thus contribute to their favorable prognosis.⁷ In E/R-positive relapses, however, expression seems to be relatively decreased, irrespective of whether *BMF* is deleted or not. It is therefore conceivable that a reduced *BMF* expression may attenuate GC response³⁵ and raises the possibility that also epigenetic mechanisms could inactivate *BMF* in these cases. In line with this notion, it was recently shown that histone deacetylase-inhibitors preferentially up-regulate *BMF* expression in a broad range of cancer cell lines and that hyperacetylation of the *BMF* promoter region induces its transcriptional activation.⁴⁵

The physiologic role of *BMF* in the human system is largely unknown. However, *Bmf* deficiency in mice results in an abnormal resistance of B cells to apoptotic stimuli and the development of a B-cell restricted lymphadenopathy.³⁶ In light of these data, it is therefore possible that, apart from an increased resistance to apoptosis induction, *BMF* deletions, which seem to occur in an early stage of leukemia development, also confer a survival advantage to preleukemic cells, particularly in growth factor-restricted unfavorable conditions. Moreover, *BMF* may also act as a tumor suppressor, as evidenced by the accelerated development of thymic lymphomas in *Bmf*-deficient mice.³⁶ There are only indirect signs for a similar function in humans. *BMF* was found to be lost in breast and lung cancers,³⁵ and it may be affected in those rare childhood ALL cases with 15q13–15 deletions.⁴⁶ Along this line is the considerably higher frequency of *BMF* deletions in E/R-positive relapse compared with nonrelapse cases, be it at diagnosis or relapse, as seen in our analysis.

NR3C1 deletions were found almost exclusively at relapse in 28% of our patients. This comparatively high frequency differs from that obtained in an earlier study in which such GC receptor deletions were far less common.⁴⁷ Whether this difference is the result of the higher resolution of the SNP arrays used in our study or whether it results from a selection bias remains elusive. However, in another study of 61 relapsed ALL that merely included 5 E/R-positive cases, Mullighan et al also identified only 2 cases with *NR3C1* deletions at diagnosis (conserved at relapse) and in additional 3 cases solely at relapse.²³ Of further interest is an earlier study of 242 childhood ALL cases that incorporated 47 E/R-positive ALL cases. The fact that 6 of 9 *NR3C1* deleted cases were E/R-positive corroborates our observations and the notion that *NR3C1* deletions are characteristic of this particular genetic subgroup.²⁰ Although the mode of GC resistance has been the subject of controversial discussions for a long time, recent evidence suggests that the GC receptor expression level, as well as the

GC-induced regulation of both proapoptotic and antiapoptotic BCL2 family member-dependent pathway components, plays a major role.^{29,48} Minimal residual disease response evaluation in 3 of our *NR3C1*-deleted cases revealed a poor blast cell clearance in the BM after 2 months of treatment with equal or more than 10^{-3} cells, a leukemic cell load that is currently used as indication for stem cell transplantation in BFM-based therapy protocols.⁴⁹ *NR3C1* deletions acquired at relapse were not considered as sole progression markers because other genomic alterations that were present at diagnosis had not been retained. Although it is not known when the *NR3C1* deletions had actually taken place, their identification only at relapse does not necessarily preclude that they already preexisted in a minute cell fraction much earlier, as was previously recorded in a single late relapse case.⁴⁷

Defects in the MMR pathway can alter the apoptotic response to DNA-damaging agents and can therefore also cause resistance to drugs that are used for treatment of childhood ALL.³⁴ Binding of an MSH2 and MSH6 heterodimer (MutS α complex) to the mismatch sets off DNA repair, followed by binding of an MLH1 and PMS2 heterodimer (MutL α complex), which then initiates excision and replacement of the respective mismatch. The third MSH2 and MSH3 complex (MutS β) detects small insertion or deletion loops in the DNA. Thus, attenuation of either component of these 3 heterodimers, for instance, because of the heterozygous MSH2 and MLH1 gene deletions encountered in 3 of 18 relapse patients, is expected to lead to a reduced MMR function. In support of this notion, Yang et al reported MSH6 and MSH2 deletions together with a decreased MSH6 expression in 2 of 20 E/R-negative ALL that concurred with mercaptopurine and, of particular interest in the context of our findings, also with GC resistance, even though MMR is apparently not directly related to this specific pathway.²⁴ However, it remains to be shown whether chemotherapy is indeed a relevant factor for the selection of MMR-defective clones and to which extent certain commonly used drugs may even directly cause such defects and thereby contribute to disease recurrence. Although E/R-positive leukemias are in general exceptionally drug sensitive,^{2–6} it is not yet clear to which degree this is also true for relapses. Even though the currently available clinical data seem to implicate that the majority of E/R-positive relapses achieve a second remission with an overall good outcome, only long-term follow-up studies, currently not available, could substantiate this view because in this particular subgroup relapses may still occur after long remission periods.⁷ Neither CNA nor GC response nor molecular minimal residual disease parameters, alone or in combination, were able to provide consistent and conclusive information with regard to disease resistance at first presentation.⁶ Although minimal residual disease persisted beyond day 33 of initial treatment in 7 of 8 analyzed cases, which included 6 with deletions of the aforementioned genes, no concordance with any of the particular deletions or first remission duration became evident. Of note, 5 of the 7 *BMF*-deleted cases (3 identified with SNP arrays and 4 with FISH) experienced a second relapse, 4 of 5 after stem cell transplantation and another one as a result of resistant disease. Even though these observations leave the impression that certain CNAs could indeed be of predictive value, they still have to be interpreted with caution because they derive from a small number of patients. Nevertheless, these preliminary findings seem to justify further investigations of this issue in a larger cohort of unselected patients with sufficiently long follow-up periods.

In conclusion, the heterogeneous and divergent CNA patterns in E/R-positive diagnosis and relapse leukemias unequivocally prove that both diseases can frequently evolve from a common ancestral clone. A substantial proportion of the associated deletions affect genes that play an important role in B-cell development as well as the GC and MMR pathways, which in turn may explain, to some extent, disease recurrence and a poorer treatment response in these patients. Of note in this context is the high incidence of *BMF* gene deletions in patients who experienced a second relapse even after hematopoietic stem cell transplantation. Particularly for this patient group, novel treatment approaches seem to be warranted. In the future, such alternative therapy options may include, for instance, BH3 mimetics or histone deacetylase-inhibitors, which are currently already evaluated in clinical trials and which will help to restore the function of the GC pathway.^{50,51}

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Authorship

Contribution: L.K. performed SNP array analysis, compiled data, and contributed to the writing of the manuscript; R.G. conducted lentiviral-mediated *BMF* silencing; G.F., U.K., G.K., C.M., and R.M. carried out additional experimental work; A.H. provided SNP arrays and contributed to the analysis of arrays; A.I. contributed to the analysis of SNP arrays and performed experiments; G.M., M.M., L.H.M., and J.H. provided patient samples and clinical data; S.S. was responsible for FISH studies; M.K. conducted bioinformatic analysis; J.R. and R.K. provided expression data; O.A.H. provided expertise in the interpretation of data and assisted in writing the paper; R.P.-G. conceived the study, analyzed and interpreted data, and wrote the manuscript; and all authors read and approved the manuscript.

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APPENDIX B: Co-AUTHORSHIP PUBLICATION II

ETV6/RUNX1 ABROGATES MITOTIC CHECKPOINT FUNCTION AND TARGETS ITS KEY PLAYER MAD2L1

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SHORT COMMUNICATION

ETV6/RUNX1 abrogates mitotic checkpoint function and targets its key player MAD2L1G Krapf¹, U Kaindl¹, A Kilbey², G Fuka¹, A Inthal¹, R Joas¹, G Mann³, JC Neil², OA Haas³ and ER Panzer-Grümayer^{1,3}¹Children's Cancer Research Institute, Vienna, Austria; ²Molecular Oncology Laboratory, Institute of Comparative Medicine, Faculty of Veterinary Medicine, Glasgow, UK and ³St Anna Kinderspital, Vienna, Austria

Approximately 25% of childhood B-cell precursor acute lymphoblastic leukemia have an *ETV6/RUNX1* (*E/R*) gene fusion that results from a t(12;21). This genetic subgroup of leukemia is associated with near-triploidy, near-tetraploidy, and trisomy 21 as rather specific types of secondary changes. Here, we show that, unlike various controls, *E/R*-expressing Ba/F3 clones acquire a tetraploid karyotype on prolonged culture, corroborating the assumption that *E/R* may attenuate the mitotic checkpoint (MC). Consistent with this notion, *E/R*-expressing diploid murine and human cell lines have decreased proportions of cells with 4N DNA content and a lower mitotic index when treated with spindle toxins. Moreover, both *RUNX1* and *E/R* regulate mitotic arrest-deficient 2 L1 (*MAD2L1*), an essential MC component, by binding to promoter-inherent *RUNX1* sites, which results in down-regulation of *MAD2L1* mRNA and protein in *E/R*-expressing cells. Forced expression of *E/R* also abolishes *RUNX1*-induced reporter activation, whereas *E/R* with a mutant DNA-binding site leads to only minor effects. Our data link for the first time *E/R*, MC, and *MAD2L1* and provide new insights into the function of the *E/R* fusion gene product. Although tetraploidy is an almost exclusive feature of *E/R*-positive leukemias, its rarity within this particular subgroup implies that further yet unknown factors are required for its manifestation.

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Introduction

The t(12;21)(p13;q22) with its molecular counterpart, the *ETV6/RUNX1* (*E/R*) (also known as *TEL/AML1*)

gene fusion, characterizes approximately 25% of childhood B-cell precursor ALL cases. *E/R* is unique among the fusion genes involving *RUNX1*, as it is associated with acute lymphoblastic leukemias rather than myeloid leukemias (Speck and Gilliland, 2002). Accumulating evidence suggests that this fusion gene can initiate leukemia development, and although it is not sufficient to cause overt leukemia *per se*, it is necessary for its maintenance (Zelent *et al.*, 2004; Diakos *et al.*, 2007; Hong *et al.*, 2008). *E/R* encodes a chimeric protein that is composed of the N-terminal non-DNA-binding region of *ETV6* and almost the entire *RUNX1* protein. It retains the runt domain of *RUNX1* that is required for DNA binding and heterodimerization and, therefore, also provides an essential function of *RUNX1* fusion genes (Hiebert *et al.*, 1996; Morrow *et al.*, 2007; Roudaia *et al.*, 2009; Wolyniec *et al.*, 2009). The *E/R* protein acts as an aberrant transcription factor and represses or disrupts the regulation of *RUNX1* target genes in a cellular context-dependent manner (Pui *et al.*, 2004; Zelent *et al.*, 2004; Wotton *et al.*, 2008).

Approximately 15% of *E/R*-positive leukemias acquire an extra chromosome 21 as a secondary change (Attarbaschi *et al.*, 2004). Furthermore, near-tetraploid and near-triploid karyotype patterns in childhood B-cell precursor ALL were recently recognized as rather specific secondary abnormalities, although they occur in only approximately 5% of *E/R*-positive cases (Attarbaschi *et al.*, 2006; Raimondi *et al.*, 2006).

On the basis of these observations, we reasoned that *E/R* can affect chromosome segregation, most likely by compromising the surveillance mechanisms of the mitotic checkpoint (MC) (Weaver and Cleveland, 2005; Peters, 2007). The MC guarantees that all sister chromatids are properly attached to the spindles before cell-cycle progresses through mitosis. A central component of this checkpoint is the 'mitotic arrest-deficient 2' (*MAD2*) protein. Partial loss of function as well as over-expression of *MAD2L1* leads to the abrogation of MC, chromosome mis-segregation, aneuploidy, and failure to arrest mitosis in the presence of microtubule poisons (Sotillo *et al.*, 2007). The unexpected finding of a tetraploid karyotype of Ba/F3 cells on stable expression of *E/R* prompted us to pursue this issue further. We have, therefore, investigated the influence of *E/R* on MC and especially on its major component *MAD2L1*.

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Results and discussion

To assess the function of E/R, we used Ba/F3, a murine IL-3-dependent putative pro B-cell line, that is frequently used as a model cell line to study the effects of genes involved in the development of B-cell precursor ALL. For this purpose, we have created stable E/R-expressing Ba/F3 clones as reported earlier (Diakos *et al.*, 2007) and selected those with low expression of the chimeric protein. After continuous culture for approximately 2 months, all E/R-expressing clones

($n=5$) acquired a tetraploid karyotype, whereas cells containing plasmids encoding either full-length ETV6, a truncated form of ETV6 (ETV6-F, with dominant-negative effect over ETV6 wt function) (Sasaki *et al.*, 2004), RUNX1, GFP, or an empty vector remained diploid. This change in ploidy was confirmed by DNA content analysis and cytogenetics of several individual clones (Figures 1a and b). To corroborate the specificity of this effect, E/R-positive Ba/F3 clones and controls were synchronized by a short exposure to low doses of nocodazole and accumulated—unlike control cells—at

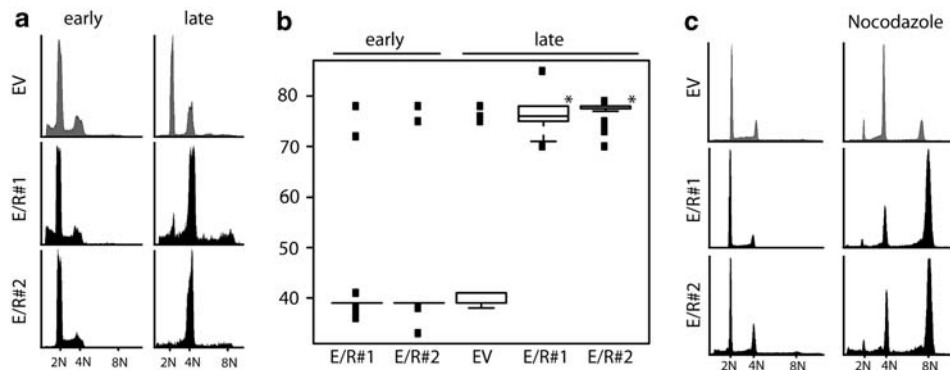


Figure 1 E/R expression induces a tetraploid karyotype in Ba/F3 cells. Representative histograms visualizing DNA content analysis (PI) of two E/R + clones (#1 and #2) and empty vector (EV) control clones at early and late passages. (b) Box blot analysis depicting chromosome numbers of E/R-expressing or EV Ba/F3 cells from cultures as in (a). Welch's *t*-test, $*P \leq 0.05$. (c) DNA content analysis of untreated (left part) and synchronized E/R and EV Ba/F3 clones on nocodazole treatment (right part). Cells were released from the first arrest (nocodazole 75 ng/ml for 14 h) for 3 days before they again were exposed to nocodazole. One of at least three independent experiments is shown.

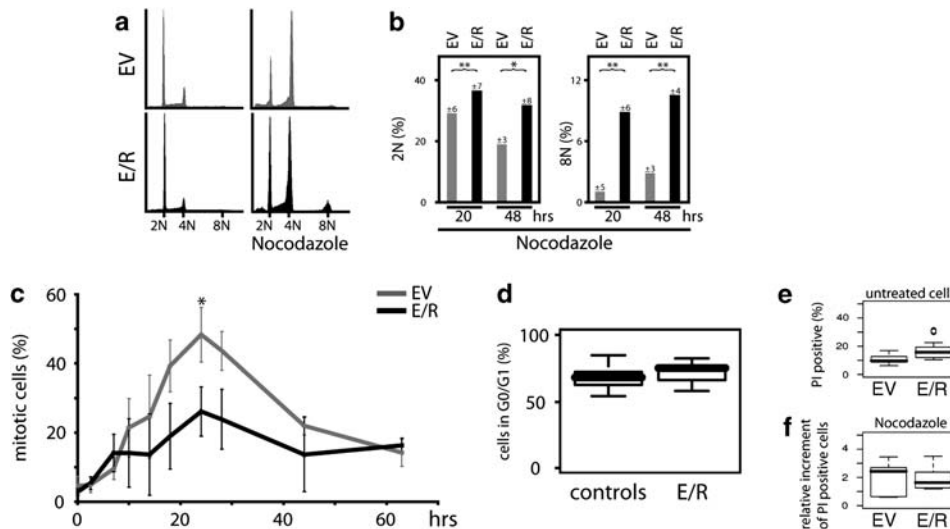


Figure 2 E/R attenuates the MC. Cell-cycle distribution of a mixture of three Ba/F3 clones that stably expressed E/R or contained an empty vector (EV) by DNA content analysis. (a) Shown are representative histograms in the absence (left) and presence (right) of nocodazole (333 ng/ml for 48 h). (b) Bar graphs indicate percentages of E/R-expressing and EV Ba/F3 cells with 2N and 8N after treatment with nocodazole for 20 and 48 h. The median and standard deviation (indicated at the top of each bar) are taken from five different experiments. Welch's *t*-test, $*P \leq 0.05$; $**P \leq 0.01$. (c) Mitotic index of stably E/R-expressing and EV Ba/F3 cells was determined by phospho-histone H3 staining on MC activation by nocodazole for the indicated times. Experiments depict the average and standard errors of the mean of three individual experiments. Welch's *t*-test, $*P \leq 0.05$. (d) Cell-cycle distribution of Ba/F3 clones stably expressing E/R or various control vectors (ETV6, truncated ETV6, RUNX1, GFP, or EV) by DNA content analysis with PI. Box blots depict the percentage of cells in G0/G1 of E/R + clones and controls from 5 and 7 independent experiments, respectively. (e/f) Apoptosis rates were assessed by PI staining in stably E/R-expressing and EV Ba/F3 cells. Percentages of PI-positive cells are depicted under optimal culture conditions (e) and on nocodazole treatment for 24 h relative to untreated cells (f). Welch's *t*-test, $P=0.8$.

8N on a second exposure to the spindle toxin (Figure 1c). These findings indicate that tetraploidization is a specific effect of E/R-expressing Ba/F3 cells in long-term cultures.

Hence, we used diploid E/R-expressing Ba/F3, and several E/R-positive and E/R-negative leukemia cell lines to investigate the influence of the chimeric E/R protein on MC. Consistent with the notion that cells with an attenuated MC are unable to fully arrest their cell cycle in mitosis when treated with spindle poisons (Kops *et al.*, 2005), the proportion of E/R-positive cells with a 2N and 8N DNA content was significantly increased under the influence of nocodazole as compared with controls (Figures 2a and b; Supplementary Figures 1a and b). The higher percentage of E/R-positive cells with 8N implies that these cells did not accurately segregate their chromosomes and/or terminate cytokinesis. Determination of mitotic cells with the phospho-histone H3 staining, which is tightly correlated with chromosome condensation during mitosis, revealed consistently lower mitotic indexes in nocodazole-treated E/R-expressing Ba/F3 cell lines and leukemias, but not in E/R-negative model and leukemia cell lines (Figure 2c; Supplementary Figure 1c).

Given that the cell-cycle distribution assessed by DNA content analysis of E/R-positive Ba/F3 cell lines showed only a minor—statistically not significant—G0/G1 increase compared with several controls (empty vector, expression vectors for RUNX1, ETV6, ETV6-F, or GFP) (Figure 2d), its deregulation is unlikely to be the cause of the low mitotic index, especially as cells were also measured after a long exposure to nocodazole. These findings accord with the observed reduction of G1 phase cells by RUNX1 and its increase by CBF oncoproteins (Lou *et al.*, 2000; Strom *et al.*, 2000; Ford *et al.*, 2009).

Of note and in line with a latent MC destabilization (Kops *et al.*, 2005), the spontaneous apoptosis rate of both E/R-positive Ba/F3 as well as E/R-positive leukemic cell lines were already slightly, but not significantly, higher than those of the respective parental Ba/F3 and E/R-negative control cell lines under optimal culture conditions (Figure 2e; Supplementary Figure 1d). We also excluded the possibility that nocodazole *per se* might selectively increase the apoptotic rate of mitotic E/R-positive cells by showing that the proportion of PI-positive cells did not increase considerably over basic levels (Figure 2f; Supplementary Figure 1e). The E/R-dependent MC impairment was further confirmed by the reduced levels of the checkpoint-associated protein securin, whose degradation at the start of anaphase initiates sister chromatid separation (Pines, 2006), and thus concurs with the lower mitotic index (Supplementary Figure 1f). These findings strikingly resemble those recently obtained in similar experiments using acute myeloid leukemia samples and cell lines with a truncated RUNX1/ETO, a fusion protein that is closely related to E/R (Boyapati *et al.*, 2007; Wolyniec *et al.*, 2009).

Having shown the influence of E/R on MC function, we set out to unravel its potential molecular mechanism. For this purpose, we interrogated publicly available

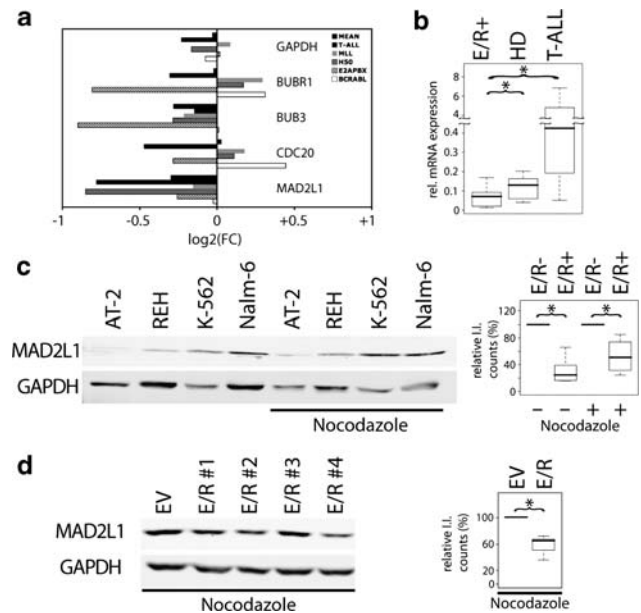


Figure 3 E/R represses MAD2L1 expression. (a) Micro-array analysis of MAD2L1 expression in various subgroups of childhood ALL. Published data were analyzed for M-values (log2(FC)) of probe sets from genes instrumental in MC function. Shown is the MAD2L1 expression from E/R-positive leukemias relative to their E/R-negative counterparts in each leukemic subgroup. Mean, the mean of all leukemic groups; T-ALL, T-ALL; MLL, ALL with MLL fusion genes; H50, high hyperdiploid ALL; E2APBX, TCF3/PBX1 (E2A/PBX1) gene rearrangement positive ALL; BCRABL, ALL with BCR/ABL fusion gene. (b) MAD2L1 mRNA was quantified by TaqMan qRQ-PCR and the ABI Prism 7900 detection system (Applied Biosystems, Foster City, CA, USA) in 10 cases each of the after distinct childhood ALL subgroups: E/R+, high hyperdiploid (HD) and T-cell precursor (T-ALL). The ABL gene was used as a standard reference for normalization. Box plots display the relative expression of MAD2L1 using STS 2.2 software. Shown are the median and interquartile range, 95% of the values are within the range of a whisker. Median values were used for statistical analysis; Welch's *t*-test, $*P \leq 0.05$. (c) Western blot analysis was performed from whole cell lysates of E/R-positive (REH, AT-2) and E/R-negative leukemic cell lines (K562 and Nalm-6). The MAD2L1 protein was detected with a specific anti-MAD2L1 antibody (ab 3632, Abcam, Cambridge, UK). Anti-GAPDH (6C5, Santa Cruz Biotechnology, CA, USA) was used as loading control. Left: protein levels under standard culture conditions and on nocodazole treatment (50 ng/ml nocodazole, 18 h). Right: box plots show the quantification of MAD2L1 protein in the respective groups from four independent experiments. MAD2L1 protein was normalized to GAPDH expression using the LICOR software (I.I., Integrated Intensity counts). Welch's *t*-test, $*P \leq 0.05$. (d) Western blot analysis (left) and quantification (right) of MAD2L1 in E/R+ Ba/F3 clones and EV controls. One of three independent experiments is shown. Welch's *t*-test, $*P \leq 0.05$.

Affymetrix data sets from childhood ALL (Ross *et al.*, 2003) for the expression of genes implicated in MC function. MAD2L1 and BUB3 (budding uninhibited by benzimidazole 3), two key players of MC, were consistently repressed in E/R-positive leukemias compared with all other ALL subgroups (Figure 3a). The finding that the MAD2L1 promoter region contained four perfect matches for RUNX binding (TGT/CGGT) and 13 sites with 85% homology within 3 kb upstream of the transcription start site makes it a likely candidate

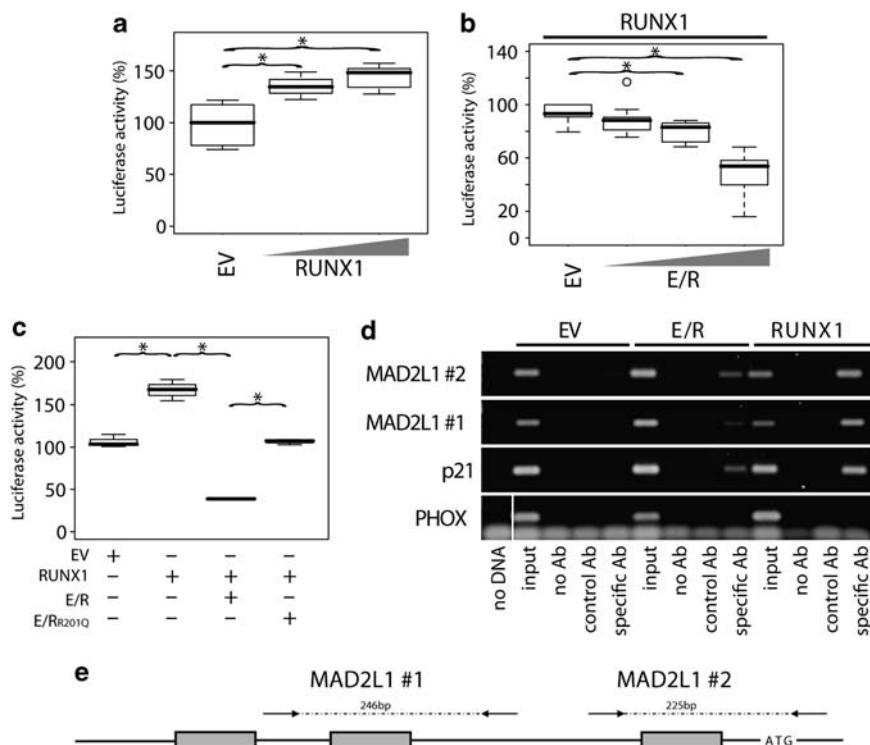


Figure 4 Direct regulation of MAD2L1 by RUNX1 and abrogation of this effect by E/R. Luciferase assays were performed using a 1.2kb MAD2L1 genomic reporter fragment containing three perfect RUNX1-binding sites (**a–c**). NIH3T3 cells were transfected (JetPEI Polyplus transfections, Illkirch, France) with RUNX1, E/R, E/R_{R201Q}, or a combination thereof. Cells were cotransfected with CBFβ (12.5 ng) to enhance RUNX1 binding and TK Renilla for transfection control. The total amount of DNA for each experiment was kept constant. Values obtained from EV control samples were set to 100%. Luciferase activity is depicted using (**a**) increasing amounts of RUNX1 pcDNA3.1-expression vector (100 and 500 ng) or (**b**) E/R (200, 400, and 800 ng) in addition to RUNX1 (400 ng) to show dose-dependent reporter activation by RUNX1 and its repression by E/R, respectively. Box plots of three representative experiments are depicted. Welch's *t*-test, **P* ≤ 0.05. (**c**) NIH3T3 cells were transfected using various combinations of 267 ng empty vector (EV), E/R, or a DNA-binding mutant E/R_{R201Q}, and/or 100 ng RUNX1-expression vector. One of three representative experiments is depicted. Welch's *t*-test, **P* ≤ 0.05. (**d**) RUNX1 and E/R occupation of MAD2L1 promoter region *in vivo* was assessed by ChIP. Chromatin from Myc-tagged E/R, Myc-RUNX1, or Myc-tagged EV stably expressing HEK293 cells was immunoprecipitated with anti-c-Myc (9E11, Abcam) or control antibody (anti-N cadherin, 610920, BD). Aliquots of the isolated DNA fragments (without antibody (noAb), with control or specific Ab and input DNA) were subjected to PCR with specific primers that amplify regions of the MAD2L1 promoter (Supplementary Methods) and analyzed by DNA gel electrophoresis. Specific primers were used to amplify specific regions of p21^{WAF1} and PHOX promoters as positive and negative controls, respectively. The gel from one of two independent experiments is shown. A vertical line has been inserted to indicate where the gel lane was cut. These gels came from the same experiment. (**e**) Schematic diagram of the 1.2 kb of the promoter region of the MAD2L1 gene cloned into the pGL3. The three perfect RUNX1-binding motifs (TGT/CGGT) are indicated by gray boxes. Arrows flanking the two RUNX1 sites (#1 and #2) indicate primer positions used for PCR amplification of isolated DNA fragments after ChIP. Numbers refer to the size of the expected PCR product. Graph is not to scale.

for a direct target of RUNX1 and also E/R. In contrast, no perfect RUNX1-binding site was present within 3 kb of the promoter region of BUB3.

We, therefore, focused our further work on MAD2L1 and validated the expression of *MAD2L1* by quantitative RT-PCR in primary childhood ALL samples and confirmed a significant difference between E/R-positive and E/R-negative leukemias (Figure 3b). In accordance with mRNA expression, protein expression of *MAD2L1* was less abundant in E/R-positive leukemic and model cell lines (Figures 3c and d). Conversely, *MAD2L1* mRNA expression was up-regulated after E/R silencing in AT-2 and REH leukemic cell lines, further emphasizing its regulation by E/R (our unpublished observation).

To test the possibility that *MAD2L1* expression is directly regulated, we determined its transcriptional activity in the context of RUNX1 and E/R. Two

constructs that contained endogenous promoter sequences with one or three perfect RUNX1 consensus sites were used for luciferase-based reporter assays (Guardavaccaro *et al.*, 2008). Both promoter constructs revealed a dose-dependent activation on RUNX1 transfection and E/R-associated repression of RUNX1-induced MAD2L1 activity in NIH3T3 cells (Figures 4a and b). Similarly, forced expression of RUNX1 led to induction of MAD2L1 promoter activation and coexpression of E/R to its abrogation in HEK293 cells (data not shown). In contrast, transfection of NIH3T3 cells with an expression vector with a point mutation in the runt domain (E/R_{R201Q}) that had been earlier shown to diminish DNA binding, but not heterodimerization with CBFβ (Song *et al.*, 1999; Li *et al.*, 2003), resulted in only partial reduction of RUNX1-induced activation (Figure 4c). These data accord with earlier studies

and indicate that DNA binding is essential for E/R activity and that E/R modulates RUNX1-induced gene regulation (Hiebert *et al.*, 1996; Friedman, 1999; Morrow *et al.*, 2007; Wotton *et al.*, 2008; Roudaia *et al.*, 2009).

We then went on to show a direct *in vivo* interaction between E/R and the MAD2L1 promoter by ChIP analysis. Using Myc-RUNX1 or Myc-E/R stably expressing HEK293 cells, we were able to prove that both RUNX1 and E/R bind to the chromatin at two consensus RUNX1 sites, which were also present in the constructs used for the reporter assays (Figures 4d and e).

Collectively, these data suggest that direct suppression of MAD2L1 by E/R binding specifically adds to the destabilization of the MC (Perez de Castro *et al.*, 2007). However, as only a small subgroup of E/R-positive leukemias acquires non-random-specific numerical chromosomal abnormalities (Attarbaschi *et al.*, 2004, 2006; Raimondi *et al.*, 2006), it seems unlikely that MAD2L1-associated MC attenuation alone is responsible for this phenomenon. Furthermore, it remains to be investigated whether the E/R-induced deregulation of MAD2L1 or any other MC components might also contribute in other ways to leukemogenesis, such as perhaps p53 activation or deregulation of the DNA damage response pathways (Michel *et al.*, 2004; Kops *et al.*, 2005; Fang *et al.*, 2006; Ha *et al.*, 2007; Perez de Castro *et al.*, 2007).

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)

APPENDIX C: Co-AUTHORSHIP PUBLICATION III

ROLE OF THE ERYTHROPOIETIN RECEPTOR IN ETV6/RUNX1- POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Grümayer**

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Role of the Erythropoietin Receptor in ETV6/RUNX1-Positive Acute Lymphoblastic Leukemia

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Abstract Purpose: We explored the mechanisms leading to the distinct overexpression of EPOR as well as the effects of EPO signaling on *ETV6/RUNX1*-positive acute lymphoblastic leukemias.

Experimental Design: *ETV6/RUNX1*-expressing model cell lines and leukemic cells were used for real-time PCR of *EPOR* expression. Proliferation, viability, and apoptosis were analyzed on cells exposed to EPO, prednisone, or inhibitors of EPOR pathways by [³H]thymidine incorporation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and Annexin V/propidium iodide staining. Western blot analysis was done to detect activation of signaling proteins. Serum EPO levels and sequences of the EPOR (*n* = 53) as well as hemoglobin levels were taken from children with acute lymphoblastic leukemia enrolled in Austrian protocols.

Results: We show here that ectopic expression of *ETV6/RUNX1* induced *EPOR* up-regulation. Anemia, however, did not appear to influence EPOR expression on leukemic cells, although children with *ETV6/RUNX1*-positive leukemias had a lower median hemoglobin than controls. Exposure to EPO increased proliferation and survival of *ETV6/RUNX1*-positive leukemias *in vitro*, whereas blocking its binding site did not alter cell survival. The latter was not caused by activating mutations in the *EPOR* but might be triggered by constitutive activation of phosphatidylinositol 3-kinase/Akt, the major signaling pathway of EPOR in these cells. Moreover, prednisone-induced apoptosis was attenuated in the presence of EPO in this genetic subgroup.

Conclusions: Our data suggest that *ETV6/RUNX1* leads to *EPOR* up-regulation and that activation by EPO might be of relevance to the biology of this leukemia subtype. Further studies are, however, needed to assess the clinical implications of its apoptosis-modulating properties.

The t(12;21)(p13;q22) with its molecular counterpart, the *ETV6/RUNX1* (also known as *TEL/AML1*) fusion gene, is present in ~25% of childhood B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) and generally implies a good prognosis (1, 2). Accumulating evidence suggests that the fusion gene formation occurs already *in utero* based on its presence at birth in the majority of children with *ETV6/RUNX1*-positive ALL (3). The cell, however, in which the fusion gene originates is not known, but it is presumably at a progenitor stage preceding the transition from pro-B to pre-B (4, 5). Based on studies in healthy newborns and animal models, the fusion gene alone is not sufficient for the clinical

manifestation of the leukemia, which requires secondary events (4, 6–10).

Gene expression profiling has been employed to characterize the different genetic subgroups of leukemias (1, 11). By this means, a relatively high expression of *EPOR* was found to distinguish the *ETV6/RUNX1*-positive leukemias from the other subgroups of ALL. It was therefore assumed that signaling by EPOR might be important for the pathogenesis of this leukemia subtype (11).

Expression of the *EPOR* and its activation by EPO has, for a long time, been exclusively associated with the erythroid lineage in which EPOR signaling is pivotal for differentiation, proliferation, and survival of progenitor cells (12, 13). Over the last years, it has become well established that the EPOR is also expressed in many normal and malignant tissues, suggesting a potential influence on cell survival (12, 13). This fact turned out to be an important issue when recombinant human EPO became available for clinical use to alleviate cancer-associated anemia and its side effects. Consequently, many *in vivo* and *in vitro* studies have been initiated to explore the effect of EPO on its apoptosis-modulating function in tumor cells. These efforts have led to contradictory results, potentially reflecting distinct biological features and activated pathways of the different malignancies (12, 13).

The signaling cascade triggered by EPO has been widely studied in the erythroid lineage. On engagement of EPO with its receptor, Janus kinase 2 is activated, which, in turn, phosphorylates the EPOR. The ensuing signaling cascade

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Translational Relevance

The data presented here suggest that the *EPOR* is over-expressed as a function of *ETV6/RUNX1* in BCP ALL with a chromosomal translocation t(12;21). *EPOR* signaling promotes survival and evasion from prednisone-induced apoptosis in *ETV6/RUNX1*-positive leukemias *in vitro*.

Recent developments in the treatment of cancer-related anemia by recombinant erythropoietin and its consequences have raised concerns about the safety and potential adverse effects of the drug, such as the promotion of tumor growth. The data provided here suggest that although signaling via *EPOR* is not pivotal for the overt leukemia, as exemplified in cell lines and a limited number of primary leukemic cells, it may enhance proliferation and survival of *ETV6/RUNX1*-positive leukemic cells. Moreover, EPO mitigated the apoptosis rate induced by prednisone, an essential drug in virtually all treatment protocols for ALL, in *ETV6/RUNX1*-positive leukemias *in vitro*. Of note, the concentrations of EPO used in our experiments exceed those reached under physiologic conditions. Such considerations may, however, not be relevant in situations when erythropoiesis-stimulating agents are applied to the patients given the chance that EPO levels might differ considerably from normal. EPO analyses in patients receiving erythropoiesis-stimulating agents are currently still missing.

includes the STAT5, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K)/Akt pathways conferring proliferative and antiapoptotic function (12, 13). At present, there is only little information on the signaling pathways affected by the *EPOR* in leukemia cells.

Herein, we provide first evidence that the *EPOR* is expressed as a function of the *ETV6/RUNX1* fusion protein. EPO promotes proliferation of *ETV6/RUNX1*-positive leukemias and interferes with prednisone-induced apoptosis *in vitro*. The PI3K/Akt pathway appears to be involved in the observed EPO-mediated survival advantage.

Materials and Methods

Leukemic cells and cell culture. REH, AT-1, and AT-2 (*ETV6/RUNX1*-positive BCP leukemia cell lines), (AT-1 and AT-2 were kindly provided by J.D. Rowley, University of Chicago), Nalm6 and SEM (both BCP ALL), K562 (erythroid blast crisis of chronic myeloid leukemia), and Jurkat (T-ALL) cells were cultured in RPMI 1640 with Glutamax (Life Technologies/Invitrogen) supplemented with 10% heat-inactivated FCS, 100 IU/mL penicillin, and 100 g/mL streptomycin (all PAA).

Mouse pro-B Ba/F3 cells were grown in RPMI 1640 containing 10% FCS and 5% WEHI-3B conditioned medium as a source for interleukin-3. Stably *ETV6/RUNX1*-expressing Ba/F3 clones and empty vector controls were established as reported previously (14).

HEK 293 cells, transformed human embryonic kidney cells, were grown in RPMI 1640 containing 10% FCS. *ETV6/RUNX1* cDNA was inserted into a pcDNA3.1-myc expression vector (Invitrogen). Stably expressing *ETV6/RUNX1* clones were obtained after single-cell dilution and clonal expansion of transfected and G418 (900 µg/mL)-selected cells.

Primary leukemic cells were obtained from bone marrow aspirations from children with ALL. Written informed consent was obtained from

the patients or their parents. The study was approved by the ethical committees of the Children's Cancer Research Institute and the St. Anna Kinderspital. Cells were isolated by density-gradient centrifugation before further processing. For positive selection, mononuclear cells containing >95% of leukemic blasts were incubated with anti-CD10 FITC antibody (DakoCytomation) followed by incubation with anti-FITC magnetic beads and magnetic field separation using MACS separation columns (Miltenyi Biotec) according to the manufacturer's recommendation and cultured within 4 h after aspiration in IMDM with 20% FCS, 100 IU/mL penicillin, and 100 g/mL streptomycin at 37°C in 5% CO₂ in a humidified incubator.

For stimulation with growth factors and treatment with pathway inhibitors, cells were washed in PBS and serum-deprived overnight in RPMI 1640 containing 0.1% bovine serum albumin (Invitrogen). The PI3K and Jak kinase inhibitors Ly294002 and AG490 (Calbiochem) were used at 25 and 10 µmol/L concentrations, respectively. To assess cell proliferation and viability, cells were plated in triplicates at a density of 1×10^5 to 2×10^5 in flat-bottomed 96-well plates (Iwaki) in 100 µL RPMI 1640 without supplements and stimulated with different concentrations of EPO (10-100 units/mL; Neorecormon; Roche). The monoclonal anti-human *EPOR* antibody MAB307 (R&D Systems), which binds to the extracellular part of the *EPOR* and was shown to block specifically EPO-mediated effects, was used as a blocking antibody at a concentration of 30 µg/mL as reported previously (15). Exposure to drugs was done as described above in the presence of 10% FCS with the addition of prednisone (Solu-Dacortin; Merck) in LC₅₀ concentrations (REH, 1 mg/mL; AT-1 and AT-2, 1 µg/mL; Nalm6 and SEM, 0.5 mg/mL; primary leukemia cells, 50 µg/mL).

SDS-PAGE and Western blot analysis. Whole-cell lysates were prepared with radioimmunoprecipitation assay buffer (50 mmol/L Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA) supplemented with 1 mmol/L NaVO₄ and 1% protease inhibitor cocktail (Roche) as described previously (14, 16). For equal loading, the protein concentration of each sample was determined by Bio-Rad protein assay kit. Proteins were resolved by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Whatman). Nonspecific binding on the membranes was blocked with PBS containing 5% dry milk. The membranes were probed with antibodies specific for phospho-AKT, AKT (Cell Signaling Technology), poly(ADP-ribose) polymerase (PARP; C210; Becton Dickinson), and glyceraldehyde 3-phosphate dehydrogenase (G6C5; Santa Cruz Biotechnology) or tubulin (DM1A; Calbiochem) in 1% dry milk in PBS at 4°C overnight. Using infrared dye-labeled secondary antibodies (LI-COR Biosciences), the membranes were directly scanned with Odyssey Infrared Imaging System (LI-COR Biosciences). Bands were quantified using LI-COR Odyssey Software.

Proliferation, viability, and apoptosis assays. Cells were cultured as described above with different agents for 48 h. [³H]thymidine (1 µCi/well; Hanke Laboratory Products) was added for 24 h. Cells were harvested onto filters and counted in a scintillation counter.

In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, a 5 mg/mL stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (20 µL; Sigma) were added according to the times indicated. After 3 to 5 h, 100 µL stop solution [10% SDS and 50% formamide (pH 4.7) with acetic acid] was added to dissolve formazan crystals. The absorbance was read at 562 nm in a microplate reader. To assess survival of cells, the mean value of cells cultured with the respective agent was divided by the mean value of control cells × 100%.

The presence of early and late apoptotic cells was determined by flow cytometry (FACSCalibur; BD Biosciences) with anti-Annexin V FITC-labeled antibody (BD Pharmingen) and propidium iodide. Cells were washed in 10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂. Antibody and propidium iodide were added and incubated for 15 min in the dark before analysis. All experiments were done in triplicates and reproduced at least three times in independent experiments.

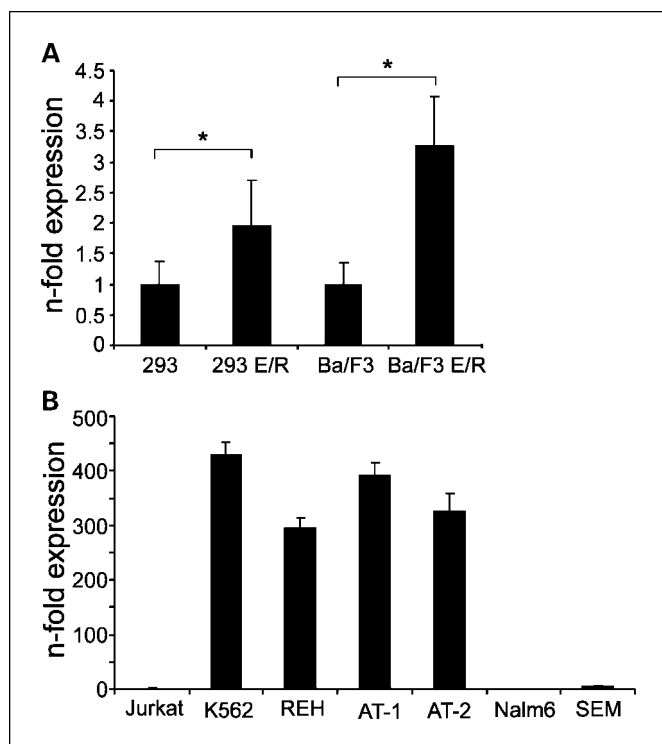


Fig. 1. *EPOR* expression in ectopic *ETV6/RUNX1* transfectants and leukemic cell lines. Quantification of *EPOR* mRNA expression by real-time quantitative PCR. **A**, *EPOR* expression in ectopically *ETV6/RUNX1* (E/R)-expressing HEK 293 and Ba/F3 cells. **B**, *EPOR* expression in *ETV6/RUNX1*-positive (REH, AT-1, and AT-2) and *ETV6/RUNX1*-negative (SEM, Nalm6, Jurkat, and K562) leukemic cell lines. *EPOR* mRNA levels were determined at least in triplicates and normalized to either *HPRT* in Ba/F3 cells or β_2 -microglobulin expression in the remaining cell lines. The *n*-fold expression was calculated from *ETV6/RUNX1*-expressing cells relative to control vector-containing cells in **A** or to Jurkat cells in **B**. Bars, SD. *, $P < 0.01$.

Sequencing of the *EPOR* gene. Genomic DNA was isolated with QIAamp DNA Blood Mini Kit (Qiagen). Specific primers for exons 2 to 4 and 6 to 8, located at the respective flanking intronic sequences, were used for amplification.

The nucleotide sequence of the *EPOR* was determined by sequencing analysis (VBC Genomics) and compared with public database sequences.⁴

EPO serum levels and *EPOR* mRNA quantification. The EPO serum levels were determined by an automated chemiluminescent immunoassay (DPC Immulite; ref. 17).

Random hexamer priming and SuperScript II (Invitrogen) were used to generate cDNA. Quantification of *EPOR* mRNA abundance in primary leukemias was done as reported previously (11). β_2 -Microglobulin was used for endogenous control gene amplification. The quantitative PCRs were done in a total volume of 25 μ L containing 12.5 μ L TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nmol/L forward and reverse primers each, and 100 nmol/L Taqman probe. The reactions were carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

For amplification of *EPOR* mRNA expression in cell lines, SYBR Green PCR was done. Primers and conditions were the same as for TaqMan PCR. For murine cell lines, the following *EPOR* and *HPRT* (for control amplification) primers were used: *EPOR* forward 5'-GCC-CCCTCTGTCTCTACT-3', *EPOR* reverse 5'-TCCCAGAAACACACCAAGTCTT-3', *HPRT* forward 5'-GGGGGCTATAAGTCTTTGC-3', and *HPRT* reverse 5'-TCCAACACTTCGAGAGGTCC-3'.

⁴ Sanger Vega Gene ID: <http://vega.sanger.ac.uk/index.html> and ENSG00000187266.

Blood counts including measurement of hemoglobin (Hb) from patients was analyzed by Advia 120 Hematology System (Bayer).

Statistical analysis. Differences of Hb levels in various leukemia subgroups were assessed by the Wilcoxon two-sample test. The Welch *t* test was used to analyze the increase in *EPOR* mRNA expression in cells stably transfected with *ETV6/RUNX1*, proliferation assays, and cell viability assays. Probability values ($P < 0.05$) were considered statistically significant.

Results

***ETV6/RUNX1* induces up-regulation of *EPOR* mRNA expression.** Based on the high expression of the *EPOR* in *ETV6/*

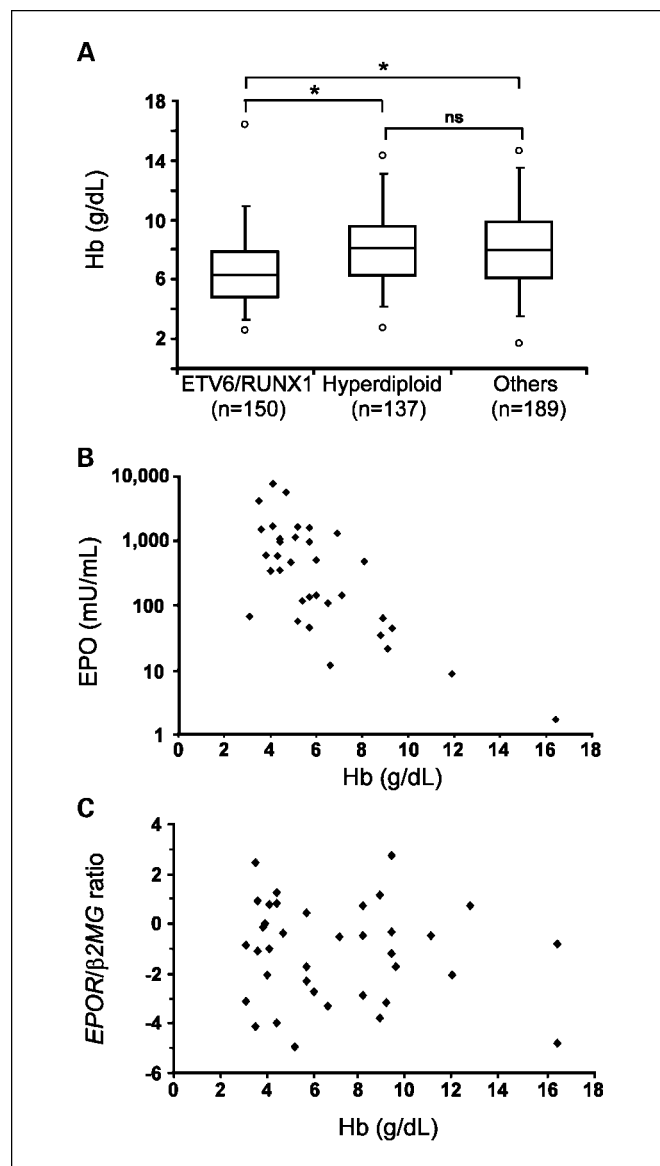
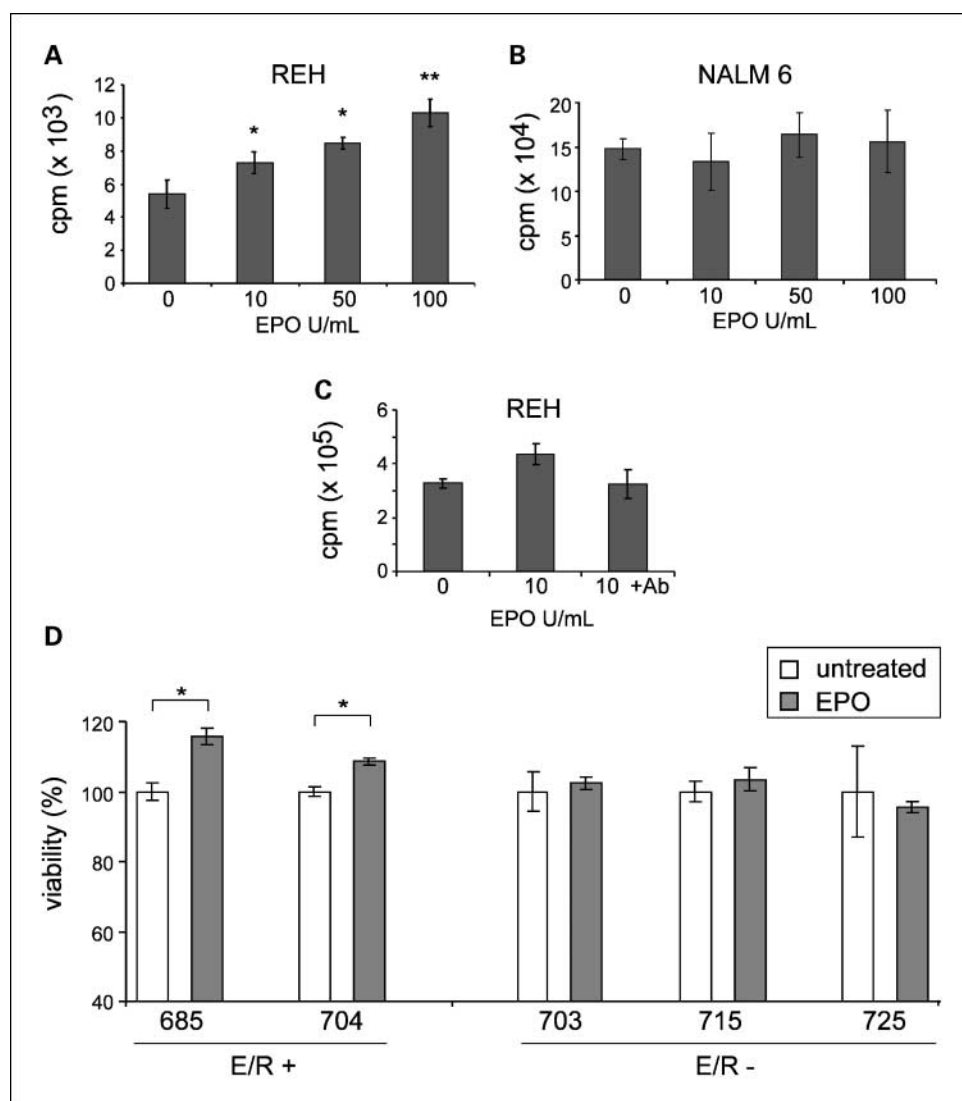


Fig. 2. Low Hb levels inversely correlate with serum EPO levels but not with *EPOR* mRNA expression in *ETV6/RUNX1*-positive leukemias. **A**, Hb levels from patients with various subtypes of BCP leukemia. The box shows the median and the interquartile range (25–75th percentiles). Ninety-five percent of the values are within the range of a whisker and the circles indicate the minimum and maximum values in each group. *, $P < 0.001$ (Wilcoxon two-sample test). **B**, correlation between EPO and Hb levels in patients with *ETV6/RUNX1*-positive leukemia. **C**, comparison of individual patients' Hb level, as a surrogate parameter of EPO, with relative expression levels of *EPOR*, normalized to β_2 -microglobulin (β_2 MG) and shown on a log₂ scale, in matched leukemic cells.

Fig. 3. EPO promotes proliferation and survival of ETV6/RUNX1-positive leukemia cells. Cell proliferation was determined by [^{3} H]thymidine incorporation in REH (A) and Nalm6 (B) cells cultured in the presence of increasing concentrations of EPO and in REH cells grown with EPO and anti-EPOR antibody (C). One representative example of at least three independent experiments is shown. (The difference in counts between A and C results from different cell numbers used for these experiments). D, differences in the metabolic status of cells used to assess cell viability (%) was determined in ETV6/RUNX1-positive (E/R+) and ETV6/RUNX1-negative (E/R-) primary leukemias by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after exposure to EPO (50 units/mL) for 72 h. Leukemic cells were isolated and cultured within 4 h after bone marrow aspiration. *, $P < 0.05$; **, $P < 0.01$.



RUNX1-positive leukemias, we assessed whether EPOR is up-regulated as a result of the fusion gene expression. Stably ETV6/RUNX1-expressing clones of human embryonic kidney HEK 293 cells and murine pro-B Ba/F3 cells showed a 2- to 3.5-fold difference in mRNA expression compared with empty vector controls (Fig. 1A).

Next, we determined the expression levels of EPOR in our model cell lines. In line with data by Fine et al. (11), quantification of mRNA revealed that the EPOR is highly expressed in all ETV6/RUNX1-positive BCP ALL cell lines but barely detectable in the ETV6/RUNX1-negative BCP and T-ALL cell lines (Fig. 1B). Jurkat was used for normalization and K562 was used as a positive control for EPOR overexpression. We further evaluated whether these transcriptional differences between ETV6/RUNX1-positive and ETV6/RUNX1-negative leukemias would also be detectable at the protein level. Because lack of specificity of several EPOR antibodies cautions its usage for Western blotting (18, 19), the directly labeled EPOR antibody (MAB307-PE), which competes with EPO for the binding to its receptor (20), was used for surface protein detection by flow cytometry. There was, however, no clear

difference between ETV6/RUNX1-positive and ETV6/RUNX1-negative leukemias (data not shown) possibly due to generally low abundance of EPOR expression on the cell surface (19).

Low Hb is associated with the ETV6/RUNX1 genetic subtype of ALL. We also considered the possibility that, in ETV6/RUNX1-positive leukemias, EPOR expression is, to some extent, further influenced by other factors known to up-regulate the receptor (21), as, for example, hypoxemia, the fundamental physiologic stimulus that causes a rapid increase in renal production of EPO (22). It was thus assessed whether children with ETV6/RUNX1-positive ALL have lower Hb levels than those with other subgroups of BCP ALL. For this purpose, 476 patients with BCP ALL, who were consecutively registered in the Austrian BFM 95 and 2000 studies (23, 24), were included. BCP leukemias were grouped in ETV6/RUNX1-positive cases, leukemias with a high hyperdiploid chromosome number (subsequently designated as hyperdiploid leukemias), and "others" ($n = 150, 137$, and 189 , respectively). The latter are characterized by the lack of the previous group characteristics. As shown in Fig. 2A, the median Hb level of children with ETV6/RUNX1-positive ALL is significantly lower ($P < 0.001$)

compared with those with hyperdiploid and "other" leukemias, whereas the difference between the latter two groups is not significant ($P = 0.77$). Next, we confirmed that for this particular genetic subgroup of leukemias, Hb and EPO are inversely correlated (Fig. 2B), indicating that EPO production is adequate for the Hb level, a finding that has been reported previously for genetically undefined cases of childhood ALL (25). This allowed us to use Hb levels as a readily available correlate for the amount of EPO in the serum. Hence, to evaluate an influence of EPO on its receptor expression, patients were selected according to their Hb level to fit into

one of the three arbitrarily chosen groups (Hb levels 3-5, >5 to 8, and >8 to 15 g/dL). This approach should enable the analysis of a wide range of physiologic EPO concentrations in the serum (0.01-10 units/mL). As illustrated in Fig. 2C, the amount of *EPOR* mRNA did not change as a function of Hb, supporting our view that EPO does not play a major role in *EPOR* mRNA up-regulation in *ETV6/RUNX1*-positive leukemia.

Lack of activating mutations of the EPOR in ETV6/RUNX1-positive leukemias. Based on the observation that overexpressed receptor kinase genes (e.g., *FLT3*, *C-KIT*, and *NOTCH1*) are frequently constitutively activated by mutations in acute

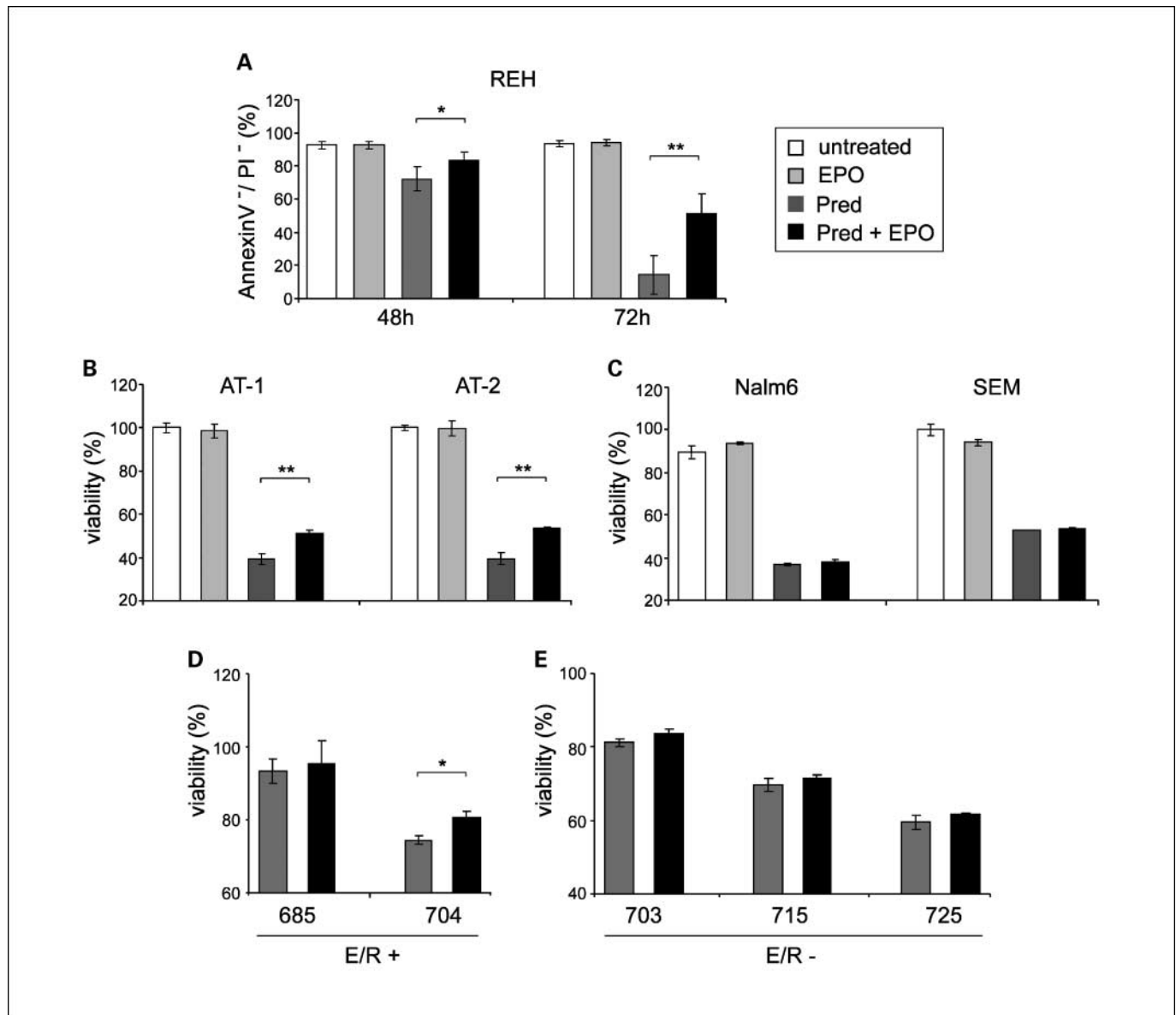


Fig. 4. EPO attenuates prednisone-induced apoptosis in *ETV6/RUNX1*-positive leukemias. **A**, apoptosis rates were determined by Annexin V/propidium iodide staining in REH cells cultured for 48 and 72 h in the presence of prednisone (Pred; 1 mg/mL) and EPO (50 units/mL). The percentage of viable, Annexin V-positive/propidium iodide-positive cells is depicted. Mean $\pm$ SD of four experiments. Evasion from prednisone-induced apoptosis by EPO in AT-1 and AT-2 cell lines (**B**), Nalm6 and SEM (**C**), and *ETV6/RUNX1*-positive and *ETV6/RUNX1*-negative primary leukemias (**D** and **E**). Cells were exposed to prednisone (50 μ mol/L) in the presence and absence of EPO (50 units/mL) and viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 72 h. The increase in cell viability by EPO is indicated in percentage. Mean $\pm$ SD from triplicates (in cell lines, one of three independent experiments, in primary leukemic cells from one experiment). *, $P < 0.05$; **, $P < 0.01$.

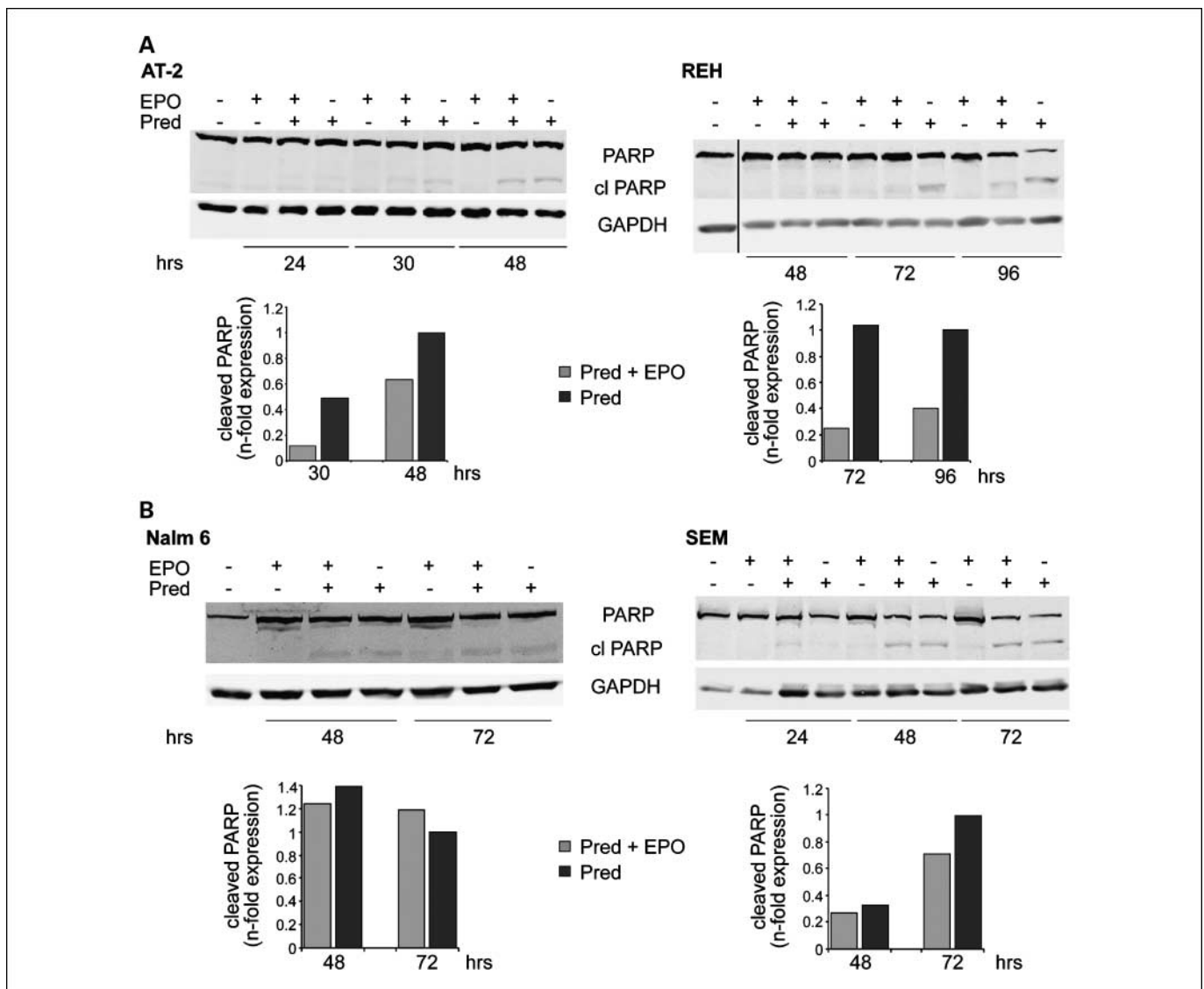


Fig. 5. EPO reduces PARP cleavage in *ETV6/RUNX1*-positive leukemias exposed to prednisone. PARP cleavage was determined by Western blot analysis in AT-2, REH, Nalm6, and SEM cells after exposure to prednisone and EPO for the indicated times. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control. PARP cleavage was normalized to glyceraldehyde 3-phosphate dehydrogenase and values are shown as *n*-fold expression at the bottom of the graph.

leukemia (26–28) and that activating mutations of the *EPOR* are present in polycythemia vera (29), we evaluated whether such mutations would also occur in *ETV6/RUNX1*-positive ALL. Exons 2, 3, and 6 to 8 of the *EPOR* were chosen for sequencing based on their functional importance and the presence of previously identified mutations in these regions (29, 30). As no genomic variations in the *EPOR* sequence were, at least in the dominant clone of the leukemic population, detected in *ETV6/RUNX1*-positive leukemias (primary leukemias, *n* = 53; leukemic cell lines, *n* = 3), we assume that activating mutations are rarely present in these leukemias, if they occur at all.

EPO enhances proliferation of *ETV6/RUNX1*-positive leukemias and attenuates the sensitivity to prednisone-induced apoptosis in vitro. After that, we evaluated whether the overexpression of *EPOR* provides a proliferative or survival advantage to *ETV6/RUNX1*-positive leukemias. REH was

compared with Nalm6, an *ETV6/RUNX1*-negative BCP leukemia cell line. Cells were cultured under reduced serum conditions in the presence of different doses of EPO for 72 h. REH cells exhibited a dose-dependent increase in proliferation on addition of EPO (Fig. 3A), whereas Nalm6 did not (Fig. 3B). Specificity of these effects was confirmed by an anti-*EPOR* blocking antibody, which abrogates EPO-induced effects, as shown in Fig. 3C. Enhanced viability by EPO was also observed in two *ETV6/RUNX1*-positive primary leukemias but in none of the three *ETV6/RUNX1*-negative cases (Fig. 3D). Collectively, these data suggest that the *EPOR* conveys a survival advantage in *ETV6/RUNX1*-expressing leukemic cells.

It has been reported previously that EPO may modulate the response of malignant cells to drugs (12, 13). We therefore explored the effects of EPO on drug-induced apoptosis in REH cells as a model system. Different drugs (prednisone, vincristine,

daunorubicine, methotrexate, and etoposide), which are currently used in treatment protocols for children with ALL, were tested at published LC_{50} concentrations (ref. 31; data not shown). EPO (50 units/mL) reduced only prednisone-induced apoptosis by 10% to 16% (Fig. 4A). This effect was Jak dependent, because AG490, a Jak kinase inhibitor, completely abolished the effects of EPO by blocking the direct downstream signaling proteins of EPOR (data not shown). A survival advantage by EPO was also reproduced in two other *ETV6/RUNX1*-positive leukemic cell lines, AT-1 and AT-2, by 12% to 14% (Fig. 4B). AT cell lines are, similar to the majority of *ETV6/RUNX1*-positive primary leukemias (32) but unlike REH cells, sensitive to glucocorticoids. In the *ETV6/RUNX1*-negative BCP cell lines Nalm6 and SEM, EPO had no effects on prednisone-induced apoptosis (Fig. 4C). The alleviation of glucocorticoid-induced apoptosis by EPO was also confirmed in primary *ETV6/RUNX1*-positive leukemias. To exclude a possible effect of normal EPO-responsive erythroid progenitor cells, which might be present in density-gradient separated mononuclear cells, highly purified ($CD10^+$) primary leukemic blast cells were prepared for these experiments. One of the two *ETV6/RUNX1*-positive leukemias (patient ID 704) was responsive to glucocorticoid concentrations used for *in vitro* experiments and displayed a reduced prednisone-induced apoptosis in the presence of EPO (Fig. 4D), whereas the other (patient ID 685) did not respond at all. There was no EPO-mediated influence on *ETV6/RUNX1*-negative leukemias ($n = 3$) detectable (Fig. 4E). Of note, all leukemias used for these experiments had a good prednisone response *in vivo*. It is therefore concluded that EPOR signaling may lead to an increased survival of *ETV6/RUNX1*-positive leukemia cells when exposed to prednisone *in vitro*.

For glucocorticoid-induced apoptosis, the engagement of caspases that cleave substrates at aspartate residues is required

(33). We consequently tested whether the EPO-mediated rescue from glucocorticoid-induced apoptosis can be visualized by cleavage of caspase-3 or its downstream target PARP. As shown in Fig. 5, PARP cleavage was reduced by 30% to 50% in AT-2 and REH cells, but not in Nalm6 and SEM cell lines, when EPO was added to the culture medium. We did, however, not detect caspase-3 activation (data not shown), which suggests that probably another effector caspase mediated apoptosis in these cells, as has been reported recently (33).

EPO signals via the PI3K/Akt pathway in *ETV6/RUNX1*-positive cell lines. In a next step, we set out to identify the pathways that are involved in the EPOR-mediated survival advantage. Despite the fact that extracellular signal-regulated kinase 1/2 and STAT5 are commonly activated in erythroid progenitor cells on stimulation with EPO but also in many other cells (13), neither of the two pathways was activated in REH cells (data not shown). Further, PI3K and nuclear factor- κ B signaling, implicated in glucocorticoid-mediated apoptosis (33), were evaluated. On addition of EPO, the PI3K pathway was activated in all three *ETV6/RUNX1*-positive cell lines (Fig. 6), whereas a strong EPO-independent phosphorylation of the p65 nuclear factor- κ B subunit, a prerequisite for the activation of the pathway, indicates that the nuclear factor- κ B pathway was constitutively activated (data not shown). These results imply that activation of the PI3K/Akt pathway is most likely involved in the proliferative and antiapoptotic effects exerted by EPO.

Discussion

The overexpression of EPOR in *ETV6/RUNX1*-positive leukemias has become a well-recognized aspect of this leukemia subtype since its description by Fine et al. (11). The underlying mechanisms, however, as well as the biological consequences for pathogenesis and implications for the clinic have not been investigated thus far. In this study, we provide first evidence that EPOR expression is up-regulated by *ETV6/RUNX1*. On engagement with EPO, signaling by the receptor exerts a proliferative stimulus and evasion from glucocorticoid-induced apoptosis in *ETV6/RUNX1*-positive leukemias. Low Hb levels of children at the time of diagnosis emerged as a new clinical feature for *ETV6/RUNX1*-positive leukemia. It did not result from lack of EPO production, which was appropriate for the degree of anemia. High EPO levels, however, did not translate into higher EPOR mRNA expression in leukemic cells.

The data presented here strongly suggest a fusion gene-dependent up-regulation of EPOR expression in model systems. Although a RUNX1 consensus binding site (TCTGGT) was found 300 bp upstream of the EPOR promoter region, we rather assume that EPOR up-regulation is an indirect effect of the fusion protein based on its proposed repressor function on RUNX1 target genes (34). Support for the EPOR overexpression as a result of *ETV6/RUNX1* in leukemias also results from siRNA-mediated silencing of the fusion gene in REH cells, which leads to down-regulation of the receptor.⁵ Such a

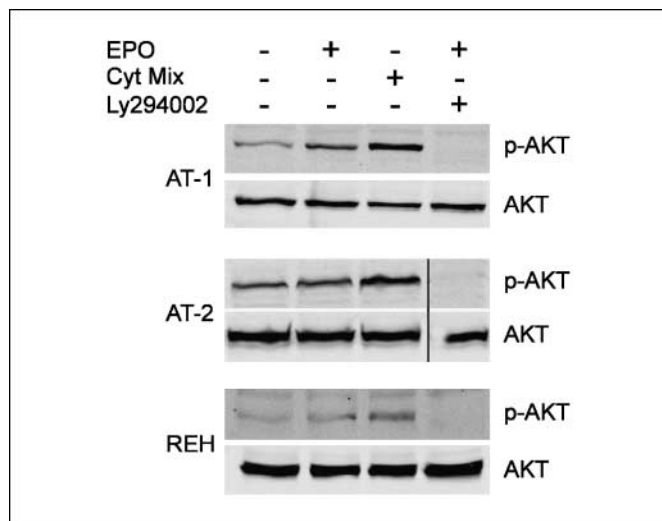


Fig. 6. EPO triggers signaling via PI3K/Akt pathway in *ETV6/RUNX1*-positive leukemic cell lines. AT-1, AT-2, and REH cells were stimulated with 50 units/mL EPO or a cytokine mix (Cyt.mix) for 15 min. The PI3K inhibitor Ly294002 was used to show specificity of signaling. Whole-cell extracts were analyzed by Western blotting and assessed for phosphorylation of AKT by a phospho-AKT antibody. Total AKT was used as loading control. A vertical line has been inserted to indicate where the membrane was cut. These membranes came from the same experiment.

⁵ C. Diakos and E.R. Panzer-Grümayer, unpublished data.

scenario would be consistent with the distinct overexpression of this receptor in the *ETV6/RUNX1*-positive subgroup of leukemias, although it does not preclude other mechanisms being operative as well.

We therefore considered the possibility that the cell in which the *ETV6/RUNX1* fusion gene occurs first might be a progenitor cell with erythroid potential. Based on the proposed existence of a lymphoid-primed multipotent progenitor, which retains the potential for erythroid gene expression (35), the overexpression of GATA2 and the transferrin receptor together with EPOR in *ETV6/RUNX1*-expressing leukemias would be compatible with such a model.⁶ GATA2 is also highly expressed at the stem and progenitor cell stage (36) and might thus, alternatively, reflect the activation of a stem cell self-renewal program. The issue of an erythroid component in *ETV6/RUNX1*-positive leukemias has been addressed already before by Andersson et al. comparing leukemia signatures with those of normal hematopoietic cells (37). These authors did, however, not detect overlapping gene expression with the erythroid lineage. A possible explanation for missing the erythroid lineage-associated genes that have emerged from our analysis may lie in the selection of the top 200 differentially regulated genes from the *ETV6/RUNX1*-positive leukemias for their analysis.

The finding of a significantly lower median Hb level in children with an *ETV6/RUNX1*-positive leukemia compared with other BCP ALL in this study is of particular interest for two reasons. First, it may mean that the erythroid lineage is affected by the fusion gene, as is the case for the structurally and functionally related fusion gene, *RUNX1/RUNX1T1* (also known as *AML1/ETO*), resulting from the t(8;21) in acute myeloid leukemias (38). In these leukemias, *RUNX1/RUNX1T1* leads to a differentiation arrest not only of the myeloid lineage but also of erythroid precursor cells, suggesting the transformation of a multipotent myeloid progenitor cell. These fusion gene-dependent changes are clinically apparent both in the fully leukemic and the myelodysplastic stage in patients as well as in mouse models (38). In contrast, similar alterations of normal hematopoiesis may not be detectable in children with *ETV6/RUNX1*-positive ALL because normal hematopoiesis is virtually completely replaced by leukemic cells at the time of diagnosis. In mouse and zebra fish models for *ETV6/RUNX1*-positive leukemia, however, no evidence for a similar phenomenon has been described (8–10, 39), implying that, even if in humans the *ETV6/RUNX1* fusion originated in a stem cell, differentiation of the erythroid lineage may not be impaired. Second, a low Hb in children with *ETV6/RUNX1*-positive leukemia might also influence EPOR expression in leukemic cells, in keeping with the up-regulation of the EPOR by its hormone (21). As shown here, an increased serum EPO in children with low Hb did not result in a higher EPOR expression in leukemic cells compared with patients with normal Hb levels. These data exclude a major effect of the actual EPO levels on the expression of its receptor in these leukemias.

We further evaluated the influence of EPO on the fully leukemic cells. Although EPO led to a dose-dependent proliferation, cell growth and survival were not altered when the EPO-binding site of the receptor was specifically blocked. Constitutive activation of EPOR and its downstream kinase, Jak2, seems an unlikely cause for abrogating these effects because we and others did not detect mutations of these two genes in *ETV6/RUNX1*-positive leukemias (40, 41). Instead, the PI3K/Akt pathway is constitutively activated in these leukemias, which could explain the lack of downstream effects on EPOR blockage (37, 42). If the compensation for receptor signaling by a downstream molecule was also a potential scenario *in vivo*, the overexpression of EPOR may provide a survival advantage during leukemia development before the occurrence of this modification.

Recent developments in the treatment of cancer-related anemia by recombinant erythropoietin and its consequences have raised concerns about the safety and potential adverse effects of the drug, such as the promotion of tumor growth (12, 13). The data provided here suggest that, although signaling via EPOR is not pivotal for the overt leukemia, as exemplified in cell lines and a limited number of primary leukemic cells, it may enhance proliferation and survival of *ETV6/RUNX1*-positive leukemic cells. Of particular interest, EPO mitigated the apoptosis rate induced by prednisone, an essential drug in virtually all treatment protocols for ALL (33), in *ETV6/RUNX1*-positive leukemias *in vitro*. Importantly, this effect was not restricted to glucocorticoid-resistant REH cells but was also reproduced in glucocorticoid-sensitive cell lines and leukemias. A potential limitation of our findings lies in the concentrations of EPO used in our experiments, which exceeds values reached under physiologic conditions (43). These concerns may not be relevant in situations when erythropoiesis-stimulating agents are applied to the patients given the chance that EPO levels might differ considerably from normal. Such analyses are currently still missing.

Collectively, our data suggest that the EPOR is overexpressed as a function of *ETV6/RUNX1*. It promotes survival and evasion from prednisone-induced apoptosis in *ETV6/RUNX1*-positive leukemias *in vitro*. Although we are tempted to speculate that EPOR signaling may provide an evolutionary advantage during leukemia development, it is too early to judge whether our findings are of clinical relevance. In particular, we do not know whether EPO levels that may be reached during treatment with erythropoiesis-stimulating drugs would support the survival of the fully malignant clone or the sustained growth of preleukemic cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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⁶ A. Inthal et al., unpublished observation.

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EDUCATION AND RESEARCH

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Oct. 2006 - Feb. 2007	Cambridge University Department of Pathology, Immunology Division Project title: "The role of NK cells in Toxoplasma gondii infection"
Feb. 2005 - Apr. 2006	University of Vienna Master thesis Department of Vascular Biology and Thrombosis Research Project Title: "An experimental approach to target angiogenesis via expression of soluble extracellular domains of human VE cadherin"
Aug. 2004 - Jan. 2005	Uppsala University Erasmus/Sokrates exchange program of the European Union

Oct. 2000 – Feb. 2005	University of Vienna Basic study period in Biology Courses included: Zoology, Botanics, Chemistry, Physics, Maths Advanced study period (masters equivalent) in Microbiology and Genetics
Jan. 2000 – Jul. 2000	Maria Theresien Kaserne, Vienna Military service at the Garde
Jun. 1999	University entrance Diploma Subjects: German, English, Maths, Biology, Psychology
Sep. 1994-Jun. 1999	Grammar school Marchettigasse, Vienna Focus: Natural Sciences and Maths
Sep. 1990-Jun. 1994	Grammar school Linzerstrasse, Vienna
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LINGUISTIC PROFICIENCY

German	Native speaker
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LABORATORY SKILLS

Recombinant Adenovirus and Lentivirus production, RT qPCR (Taqman and Cybergreen), Immunoblotting, Immunofluorescence, Confocal Microscopy, ELISA, Cell culture techniques, Molecular cloning techniques, Flow cytometry, RNA expression profiling

COMPUTATIONAL SKILLS

MS office (Word, Excel, Powerpoint), Adobe Illustrator/ Photoshop, Endnote, Flow-Jo Software, Graph Pad Prism, LICOR software

SCIENTIFIC PUBLICATIONS

Fuka G, Kantner HP, Grausenburger R, Inthal A, Krapf G, Kaindl U, Kauer M, Dworzak MN, Stoiber D, Haas O, Panzer-Grümayer R.

Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts

Manuscript submitted

Fuka G, Joas R, Kofler R, Kauer M, Panzer-Grümayer R.

Transcriptomic effects of ETV6/RUNX1 in ALL

Manuscript submitted

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INTERNATIONAL CONFERENCES

Jahrestagung der Kind-Philipp-Stiftung für Leukämieforschung, Germany, Jun. 2010

EMBO conference "RUNX proteins in development and disease", Oxford, UK, Jul. 2009

American society of hematology (ASH) meeting, Atlanta, Georgia, Dec. 2007

FEBS Congress "Molecular Machines", Vienna, Austria, Jul. 2007