

## LYMPHOID NEOPLASIA

Molecular basis of *ETV6*-mediated predisposition to childhood acute lymphoblastic leukemia

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## KEY POINTS

- Leukemia predisposition variants in *ETV6* lead to dramatic loss of transcription repressor activity, mainly by disrupting DNA binding.
- Germline *ETV6* variants influence ALL transcriptional profile with a striking resemblance of *ETV6*-*RUNX1* ALL but unique somatic mutations.

There is growing evidence supporting an inherited basis for susceptibility to acute lymphoblastic leukemia (ALL) in children. In particular, we and others reported recurrent germline *ETV6* variants linked to ALL risk, which collectively represent a novel leukemia predisposition syndrome. To understand the influence of *ETV6* variation on ALL pathogenesis, we comprehensively characterized a cohort of 32 childhood leukemia cases arising from this rare syndrome. Of 34 nonsynonymous germline *ETV6* variants in ALL, we identified 22 variants with impaired transcription repressor activity, loss of DNA binding, and altered nuclear localization. Missense variants retained dimerization with wild-type *ETV6* with potentially dominant-negative effects. Whole-transcriptome and whole-genome sequencing of this cohort of leukemia cases revealed a profound influence of germline *ETV6* variants on leukemia transcriptional landscape, with distinct ALL subsets invoking unique patterns of somatic cooperating mutations. 70% of ALL cases with damaging germline *ETV6* variants exhibited hyperdiploid karyotype with characteristic recurrent mutations in *NRAS*, *KRAS*, and *PTPN11*. In contrast, the remaining 30% cases had a diploid leukemia genome and an exceedingly high frequency of somatic copy-number loss of *PAX5* and *ETV6*, with a gene expression pattern that strikingly mirrored that of ALL with somatic

*ETV6*-*RUNX1* fusion. Two *ETV6* germline variants gave rise to both acute myeloid leukemia and ALL, with lineage-specific genetic lesions in the leukemia genomes. *ETV6* variants compromise its tumor suppressor activity in vitro with specific molecular targets identified by assay for transposase-accessible chromatin sequencing profiling. *ETV6*-mediated ALL predisposition exemplifies the intricate interactions between inherited and acquired genomic variations in leukemia pathogenesis. (*Blood*. 2021;137(3):364-373)

## Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer and a significant cause of childhood morbidity and mortality.<sup>1-3</sup> Historically, hereditary predisposition has not been considered a major risk factor contributing to ALL development; however, there is a growing body of evidence indicating that

genetic susceptibility plays a much larger role than previously thought. For example, genome-wide association studies have identified common polymorphisms associated with ALL risk affecting, including *IKZF1*, *ARID5B*, *CEBPE*, *CDKN2A*, *BMI1-PIP4K2A*, and *TP63*, and studies of leukemia-prone kindreds or children with the low hypodiploid ALL subtype have revealed

rare damaging germline variants in *PAX5*, *IKZF1*, and *TP53*.<sup>4-12</sup> More recently, we and others described germline variants in *ETV6*, the gene encoding the essential hematopoietic transcriptional repressor erythroblast transformation specific (ETS) variant 6, among families exhibiting autosomal-dominant thrombocytopenia and preponderance of ALL.<sup>13-15</sup> These *ETV6* variants were absent from the general population and cosegregated with thrombocytopenia in all cases. Initial functional evaluation of the small number of index variants revealed that they decreased *ETV6* transcriptional repressive activity, and the majority impaired DNA binding and altered subcellular localization.<sup>13-15</sup> Additional studies have recently identified familial leukemia cases with inherited structural alterations in the *ETV6* gene, further highlighting the role of this gene in ALL predisposition.<sup>16,17</sup>

To explore the contribution of germline variation in *ETV6* more broadly in childhood ALL, we subsequently sequenced 4405 childhood B-ALL cases and identified potential leukemia-related variants in ~1% of patients.<sup>18</sup> Of these, nearly half were located in the essential DNA-binding domain, including 9 variants predicted to be most deleterious by the combined annotation-dependent depletion scores. Nevertheless, the pathogenicity of these purported ALL risk variants remained unclear without experimental characterization of their functional consequences. More importantly, the mechanisms of germline *ETV6*-mediated leukemogenesis remained undefined.

Here, we systematically characterize how these germline *ETV6* variants affect the transcriptional repressor function of the encoded *ETV6* protein and elucidate their impacts on downstream gene expression in vitro and in human primary ALL samples. We also comprehensively describe the somatic genomic landscape of a large cohort of childhood leukemia cases with *ETV6* risk variants and explore the functional interactions between specific somatic lesions and germline *ETV6* variants during oncogene-mediated transformation.

## Methods

Thirty-one B-ALL and 2 acute myeloid leukemia (AML) samples with germline *ETV6* variants from 32 unique patients at St. Jude Children's Research Hospital, Dana-Farber Cancer Institute, or the Children's Oncology Group were included in this study. Genomic DNA was extracted from diagnostic bone marrow or peripheral blood samples using QIAamp DNA Midi or Maxi kit (Qiagen) and total RNA extracted using TRIzol (Thermo Scientific). Bone marrow or peripheral blood collected during clinical remission was used as the source for germline DNA. Whole-genome sequencing of matched germline and tumor DNA was performed on 30 of 32 cases, while in 2 cases, only tumor DNA was sequenced. Whole-exome sequencing was applied on 1 paired sample. Whole-transcriptome sequencing was performed on 22 of these 33 samples (supplemental Table 1, available on the *Blood* Web site). In addition, 231 children with ALL from the Ma-Spore ALL clinical trial<sup>19,20</sup> and 30 *ETV6*-*RUNX1* ALL cases from St. Jude Children's Research Hospital were included for whom whole-transcriptome and whole-genome sequencing data were used to compare with those of *ETV6* ALL, respectively. Somatic and germline variant data as well as transcriptome sequencing data for 13 ALL cases with somatic *ETV6* variants

(6 nonsense, 4 frameshift, 1 splice site, and 2 missense variants) were obtained and analyzed from St. Jude Children's Research Hospital and TARGET<sup>21,22</sup> (supplemental Table 2). This study was approved by the respective institutional review boards, and informed consent was obtained from the parents, guardians, and/or patients, as appropriate.

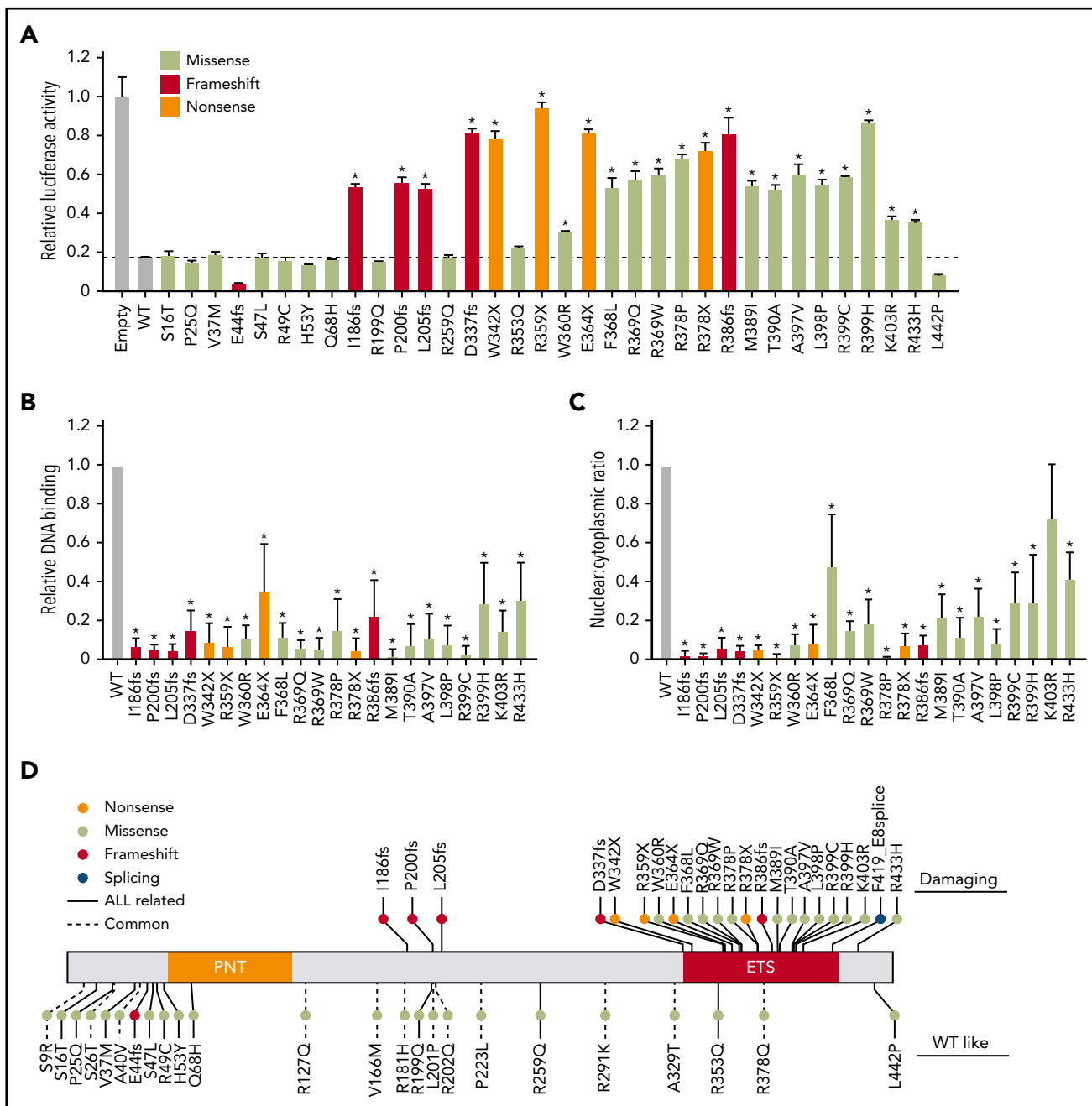
Additional details for the genomic profiling, functional assays, and statistical analyses are included in supplemental Methods.

## Results

As a transcription factor, *ETV6* primarily functions to repress the expression of a wide spectrum of target genes, many of which are highly regulated during hematopoiesis.<sup>23-25</sup> Using in vitro biochemical assays, we first sought to systematically characterize the impacts of 34 nonsynonymous germline *ETV6* variants that have been identified as ALL associated thus far.<sup>18</sup> By directly measuring transcription repressor activity with a luciferase reporter assay in HEK293T cells, we observed that wild-type (WT) *ETV6* robustly repressed PF4 promoter-driven transcription, whereas 22 of the 34 variants tested (65%) exhibited significant impairment of transcription repression; in all cases, expression of mutant *ETV6* was detectable and comparable to the level of WT *ETV6* (not shown) (Figure 1A; these variants are hereafter referred to as "damaging"). In contrast, the other 12 variants functioned similarly to or better than WT *ETV6* (these are referred to as "WT-like").

We also tested 12 common *ETV6* variants as defined previously<sup>18</sup> and found that these exhibited WT levels of transcription repression (supplemental Figure 1). Transcription repressor activity of *ETV6* variants tracked closely with bioinformatic prediction score derived from the REVEL algorithm,<sup>26</sup> with 11 of 13 damaging missense variants exhibiting a REVEL score >0.5 (supplemental Figure 2A). We then compared the prevalence of *ETV6* variants (nonsense/frameshift alterations or missense variants with REVEL score  $\geq 0.5$ ) in ALL cases vs non-ALL controls in the Genome Aggregation Database data set (N = 134 187)<sup>27</sup> and observed a striking 24.0-fold enrichment of damaging variants in ALL ( $P = 2.2 \times 10^{-16}$ ), whereas WT-like variants were equally distributed between ALL and controls (supplemental Figure 2B).

The ETS domain is critical for *ETV6* to suppress transcription.<sup>28</sup> Consistent with this notion, 5 of 6 frameshift variants and all 4 nonsense variants predicted to truncate *ETV6* upstream or within the DNA-binding domain resulted in nearly complete loss of repressor activity. In fact, while examining bone marrow cells from patients carrying nonsense variant R359X, we could not detect the truncated protein (supplemental Figure 3), suggesting that these variants result in loss of function, plausibly through nonsense-mediated decay. Furthermore, of 13 damaging missense variants, all but one (R433H) resided within the ETS domain. For subsequent functional assays, we tested only the 22 damaging variants and excluded any WT-like variants. In keeping with their loss-of-transcription-repressor activity, the 22 damaging variants exhibited a significant decrease in ability to bind to an ETS DNA consensus sequence (Figure 1B; supplemental Figure 4), as assessed using an electrophoretic mobility shift assay. We also examined the subcellular localization of WT and variant *ETV6* proteins. Compared with WT *ETV6*, which

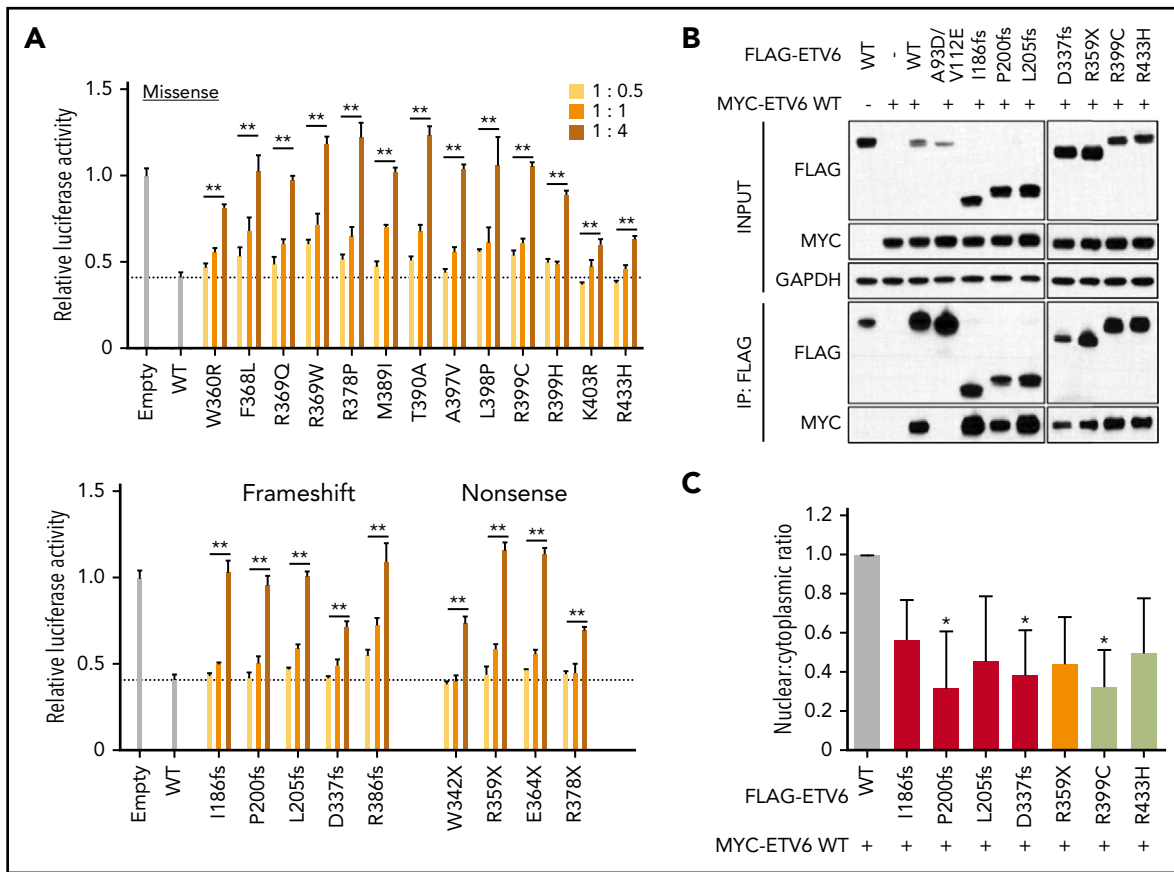


**Figure 1. Functional characterization of germline *ETV6* variants identified in pediatric ALL.** (A) *ETV6* transcription repressor activity was determined using a dual-luciferase reporter assay by cotransfecting an *ETV6* expression construct, *PF4* luciferase reporter construct, and *Renilla* luciferase reporter construct in HEK293T cells. Bars show the mean of triplicate experiments and repeated at least 3 times; error bars represent standard deviation (SD). (B) Electrophoretic mobility shift assays were performed using nuclear extracts from HEK293T cells ectopically expressing WT or variant *ETV6*. Bars show the mean of triplicate experiments; error bars represent SD. (C) Western blotting was performed after subcellular fractionation of HEK293T cells ectopically expressing WT or variant *ETV6*. Bars show the mean of nuclear to cytoplasmic ratio from 3 individual experiments; error bars represent SD. In panels A-C, a 1-way analysis of variance (ANOVA) with multiple comparison was performed to compare each variant with WT ( $*P < .01$ ). (D) Germline *ETV6* variants were classified as damaging or WT-like on the basis of functional characterization as shown in panels A-C.

was sequestered within the nucleus, 21 of 22 damaging variants tested (95%) exhibited a significant loss of nuclear localization, albeit to varying degrees (Figure 1C; supplemental Figure 5). A summary of the functional classification for each variant as either damaging or WT-like is shown in Figure 1D.

Because carriers of *ETV6* variants always have a heterozygous genotype in the germline, we postulated that variant proteins may function in a dominant-negative fashion. To test this hypothesis,

we assessed luciferase activity following cotransfection of constructs encoding WT and increasing amounts of variant *ETV6*. Across all 22 damaging variants, we consistently observed a dose-dependent inhibition of WT *ETV6* activity (Figure 2A), pointing to a potential dominant-negative mechanism of action. In contrast, none of the WT-like variants exhibited such effects (supplemental Figure 6). *ETV6* functions as a homodimer or polymer to efficiently bind ETS consensus sequences.<sup>29,30</sup> Therefore, we next evaluated the ability of deleterious variants to

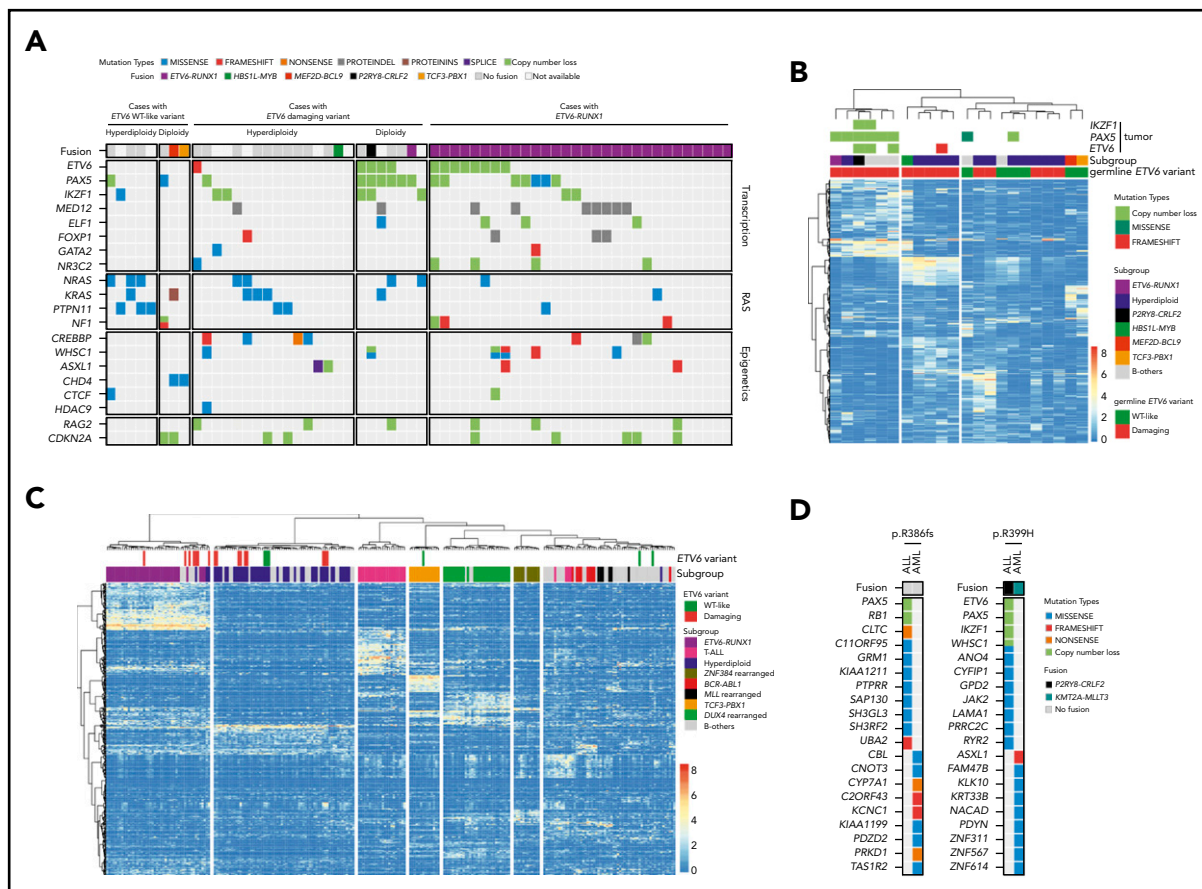


**Figure 2. Dominant-negative effects of variant ETV6 on WT ETV6.** (A) ETV6 transcription repressor activity was measured by cotransfecting HEK293T with constructs encoding WT ETV6, increasing amounts of variant ETV6, and a luciferase reporter. The top panel denotes missense variants, while the bottom panel indicates nonsense or frameshift variants. Bars show the mean of triplicate experiments and repeated at least 3 times; error bars represent SD.  $**P < .01$  in  $\geq 1$  condition. (B) HEK293T cells were cotransfected with MYC-tagged WT and FLAG-tagged variant ETV6 constructs followed by pull-down with anti-FLAG beads for western blotting to examine dimerization. Seven representative variants were chosen for this assay. IP, immunoprecipitation. (C) HEK293T cells were cotransfected with MYC-tagged WT and the same 7 FLAG-tagged variant ETV6 constructs as in panel B followed by subcellular fractionation and western blotting of nuclear and cytoplasmic fractions using an anti-MYC antibody. Red bars indicate frameshift variants, orange bar indicates a nonsense variant, and lime green bars indicate missense variants. Bars represent the nuclear-to-cytoplasmic ratio from triplicate experiments; error bars represent SD.  $*P < .05$ . In panels A and C, a 1-way ANOVA with multiple comparison was performed to compare each variant with WT.

dimerize with WT protein using a coimmunoprecipitation assay, focusing on 7 variants representative of truncating and missense changes. In this assay, all selected variant ETV6 proteins exhibited robust dimerization with WT ETV6, whereas the double mutant A93D/V112E (known to impair polymerization)<sup>31</sup> remained completely uncoupled (Figure 2B). We further asked whether the subcellular localization of WT ETV6 protein would be affected by the presence of mutant ETV6 when both are simultaneously expressed in the same cells. Upon cotransfection, the presence of each of these 7 representative ETV6 variants caused a general reduction in the ratio of nuclear to cytoplasmic WT ETV6 (Figure 2C; supplemental Figure 7). Taken together, these results indicate that damaging ETV6 variants lose their transcriptional repressor function due to impairment of DNA binding and nuclear localization, with minimal effects on dimerization.

Because germline ETV6 variants do not always result in malignancy, with only 25% to 30% of carriers developing leukemia,<sup>32</sup> we hypothesized that additional genomic abnormalities must be acquired somatically to induce leukemia. To identify these cooperating events and exhaustively define the genomic landscape of ALL with germline ETV6 variants, we performed

comprehensive genomic profiling of the leukemia samples from 32 available index cases in whom we identified damaging ( $n = 24$ ) or WT-like ( $N = 8$ ) germline ETV6 variants (supplemental Table 1; 1 case [R386fs] had both an ALL and an AML sample available). Using whole-genome sequencing, we examined genome-wide structural alterations (eg, focal copy-number changes and large chromosomal aberrations) and sequence mutations (single-nucleotide variants and small indels); in parallel, we also identified gene fusions and global expression patterns using whole-transcriptome sequencing (supplemental Tables 3-6). Of 23 cases with damaging ETV6 variants, 16 (70%) showed gross chromosomal gains, consistent with our previous report describing an association of germline ETV6 variants with hyperdiploid ALL<sup>18</sup> (Figure 3A). Along with DNA index, the other clinical features of damaging ETV6 variant carriers can be found in supplemental Table 7. The hyperdiploid cases exhibited recurrent somatic mutations affecting RAS pathway genes, including NRAS, KRAS, and PTPN11, as is commonly seen in this ALL subtype.<sup>33</sup> In contrast, the remaining 7 ETV6 ALL cases had a diploid leukemia genome with a strikingly high prevalence of PAX5 and ETV6 copy-number loss (86% and 57%, respectively). These PAX5 and ETV6 deletions were largely absent in hyperdiploid ALL samples but were frequently observed in ALL



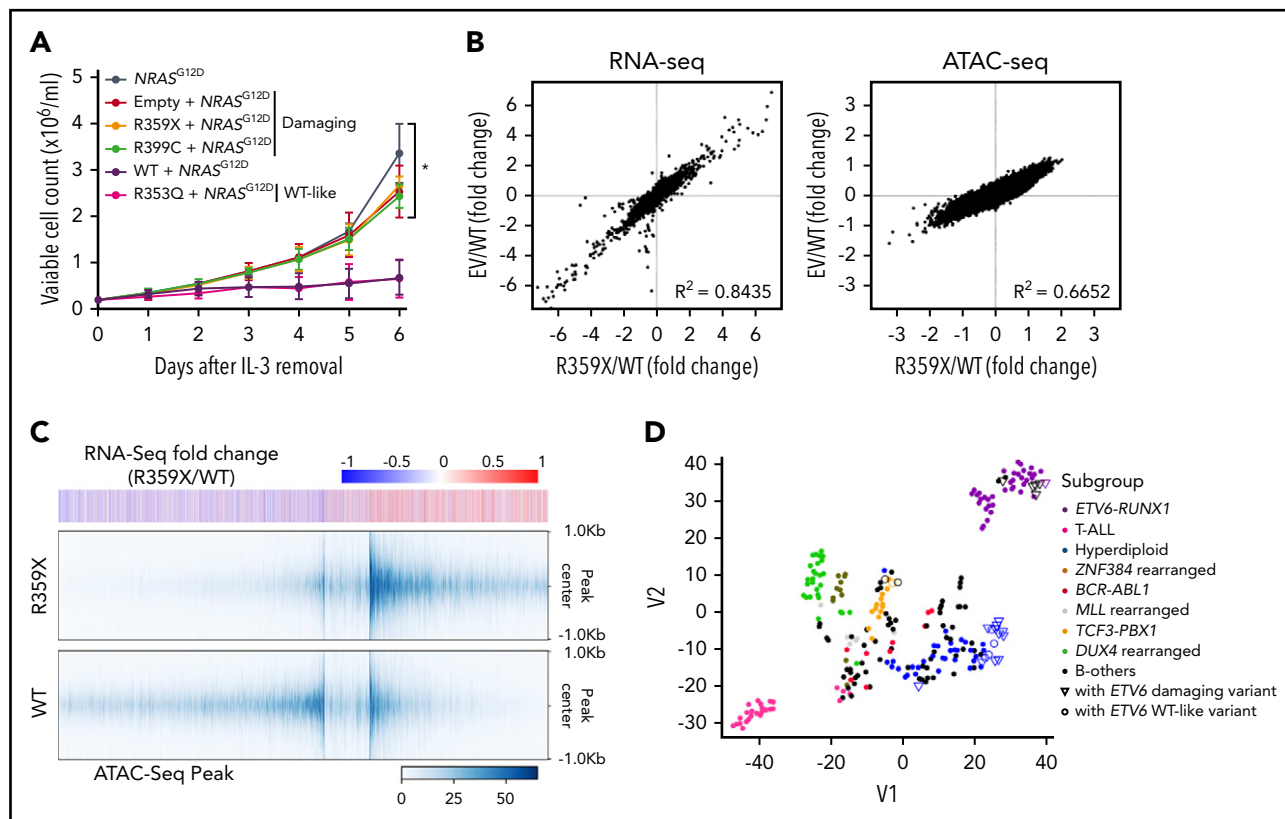
**Figure 3. Comprehensive genomic profiling of ALL harboring germline *ETV6* variants.** (A) Heatmap showing major somatic coding mutations and structural variations identified in cases with a germline *ETV6* variant or *ETV6-RUNX1* fusion. (B-C) Unsupervised hierarchical clustering of global gene expression profile from cases with germline *ETV6* variants (B) or with addition of the 231 pediatric ALL cases of diverse subtypes (C). (D) Somatic genomic clustering features in ALL and AML arising from the same *ETV6* germline variant.

harboring the somatic *ETV6-RUNX1* fusion (Figure 3A). Chromosomal rearrangement (gene fusion) was relatively rare, detected in only 3 of these 23 cases. We did not observe any recurrent genomic features in the 8 cases with WT-like germline *ETV6* variants other than hyperdiploidy in 5 samples. We further performed somatic mutation signature analysis<sup>34,35</sup> of the ALL cases with germline *ETV6* variants. Mutational signatures corresponding to activity of the AID/APOBEC family of cytidine deaminases (SBS2 and SBS13) and defective DNA repair with *BRCA1/2* mutations (SBS3) were identified in 7 and 14 cases, respectively. However, there was no significant difference in mutational signatures between diploid and hyperdiploid subgroups within germline *ETV6* variant-positive ALL cases (supplemental Figure 8).

Similarly, *ETV6* ALL cases also exhibited distinct transcriptomic patterns depending upon the functional effects of germline *ETV6* variants and somatic genomic alterations. Unsupervised hierarchical clustering analysis first separated cases with damaging vs WT-like germline *ETV6* variants, pointing to a global effect of *ETV6* on leukemia gene expression (Figure 3B). However, within cases with damaging *ETV6* variants, the transcriptional profile was largely driven by leukemia ploidy (diploid vs hyperdiploid) or plausibly by other genomic features tracking with ploidy (eg, *ETV6* and/or *PAX5* deletion vs RAS pathway mutations in diploid vs hyperdiploid cases, respectively).<sup>33,36</sup>

When we repeated the clustering analysis using whole-transcriptome sequencing data from 231 pediatric ALL cases representative of diverse subtypes, damaging *ETV6* variant ALL samples with diploid karyotype grouped tightly with *ETV6-RUNX1* cases, whereas *ETV6* ALL with hyperdiploidy shared the expression signature seen in other hyperdiploid cases (Figure 3C). In contrast, 4 of 13 (31%) cases with somatic *ETV6* mutations (3 cases with *ETV6-RUNX1* fusion and 1 case with somatic copy-number loss of *ETV6*) clustered with *ETV6-RUNX1* ALL based on gene expression, while the 9 remaining somatic *ETV6*-mutated cases did not exhibit any characteristic expression profile or distinct pattern of genomic alterations (supplemental Table 2 and supplemental Figure 9). Therefore, unlike germline *ETV6* variants, acquired *ETV6* mutations do not appear to dictate an obvious transcriptomic program or direct the acquisition of specific cooperating alterations in preleukemic cells during the process of malignant transformation.

Notably, in 2 unrelated families, *ETV6* germline variants gave rise to both ALL and/or AML (Figure 3D; supplemental Figure 10), raising the possibility that lineage-specific leukemogenesis is driven by secondary somatic lesions. For example, the patient harboring an *ETV6* R386fs variant first developed ALL and subsequently therapy-related AML. Genomic profiling of the ALL and AML samples from this individual revealed completely different somatic second events, characteristic of lymphoid (*PAX5* deletion)



**Figure 4. Effects of ETV6 variants on oncogenic transformation in vitro.** Ba/F3 cells were transduced with constructs encoding WT ETV6, damaging ETV6 (R359X or R399C), WT-like ETV6 (R353Q), and  $NRAS^{G12D}$ , followed by sorting for cells expressing mCherry and ZsGreen double-positive cells and IL-3 withdrawal 48 hours after transduction. (A) Cytokine-independent cell growth was monitored daily as an indicator of transformation in vitro. Data represent the mean from 3 individual experiments; error bars represent SD. Two-way ANOVA with multiple comparison was performed to compare each variant with WT ( $*P < .05$ ). (B) RNA-seq and (C) ATAC-seq were performed using Ba/F3 cells at 48 hours after IL-3 withdrawal. Cells expressing a damaging ETV6 variant (R359X) exhibited similar RNA-seq and ATAC-seq patterns as cells transduced with empty vector. (B) Differential ATAC-seq peaks in Ba/F3 cells expressing a damaging ETV6 variant (R359X) versus WT ETV6 correspond to upregulated genes identified by RNA-seq (C). (D) t-Distributed stochastic neighbor embedding clustering analysis was performed on the 253 ALL cases as shown in Figure 3C using 94 possible target genes identified by RNA-seq and ATAC-seq of Ba/F3 cells.

and myeloid malignancies (*CBL* mutation), respectively.<sup>36,37</sup> In the second example, 2 individuals from the same family carrying the *ETV6* R399H variant independently developed ALL and AML, respectively. While the ALL genome had a *CRLF2* rearrangement with *ETV6*, *PAX5*, and *IKZF1* deletions, the AML blasts harbored a *KMT2A-MLL3* fusion with an *ASXL1* frameshift mutation. These somatic genomic features are uniquely associated with ALL and AML, respectively, and likely contributed to leukemogenesis in distinct lineages arising from the shared germline *ETV6* variant.

To explore the effects of *ETV6* variants on leukemogenic signaling, we directly compared variant vs WT *ETV6* for their impacts on oncogene-driven transformation using a cytokine-dependent growth assay in the murine hematopoietic progenitor cell line Ba/F3. Given the relatively high frequency of *RAS* mutations in ALL with germline *ETV6* variants, we elected to use the  $NRAS^{G12D}$  mutation as the oncogenic driver in these experiments. We found that coexpression of WT *ETV6* strongly inhibited *RAS*-mediated transformation in this model (ie, interleukin-3 [IL-3]-independent growth), with the WT-like *ETV6* R353Q variant showing similar effects (Figure 4A). However, expression of the damaging variants R359X or R399C consistently failed to suppress in vitro transformation in the presence of  $NRAS^{G12D}$ , suggesting that these variants directly affected a key function of *ETV6* as a tumor suppressor. As a control, we

expressed *ETV6* variants alone and observed that these variants have no transformation capacity in the absence of  $NRAS^{G12D}$  (supplemental Figure 11). In parallel, we performed whole-transcriptome sequencing (RNA-seq) and assay for transposase-accessible chromatin sequencing (ATAC-seq) on these Ba/F3 cells to profile the transcriptional and epigenomic changes induced by WT and variant *ETV6*. Within 48 hours following transduction, there were already significant alterations in the gene expression pattern between cells expressing WT vs variant *ETV6*, whereas the latter were largely indistinguishable from Ba/F3 cells transduced with empty vector (supplemental Figure 12A). These effects were even more pronounced upon cytokine removal (Figure 4B). Interestingly, of 1189 genes differentially expressed, 597 (50.2%) were upregulated in cells expressing variant *ETV6* (supplemental Table 8), consistent with the loss of transcription repressor function. Using ATAC-seq to broadly profile chromatin accessibility as a proxy for epigenomic modifications (eg, chromatin occupancy by transcription factors such as *ETV6*), we observed an overall increase in ATAC-seq peaks in  $NRAS^{G12D}$ -transduced Ba/F3 cells expressing variant compared with WT *ETV6* (5184 upregulated peaks compared with WT, Figure 4B and supplemental Figure 12A). Footprint analysis revealed that *ETV6* and *CEBP* motifs were significantly enriched in the upregulated ATAC-seq peaks (supplemental Figure 12B). Among these 5184 peaks, 3190 (61.5%) loci encompassed a

canonical ETV6-binding motif (supplemental Table 9) (significantly more than what would be expected by chance,  $P = 7.44 \times 10^{-137}$ ). Of the 668 genes with potential loss of ETV6 binding (as evidenced by presence of ATAC-seq peaks and ETV6 binding motifs), 107 showed concordant upregulation in expression in ETV6 variant cells (significantly more than by chance,  $P = 5.40 \times 10^{-50}$ ), suggesting a loss or reduction of ETV6 binding at these targets and, consequently, relief of ETV6-mediated transcriptional repression (Figure 4C).

Comparing ATAC-seq and whole-transcriptome sequencing data and mapping to homologous human genes, we identified 94 putative ETV6 target genes (based on upregulation of expression, increase in chromatin openness, and the presence of an ETV6 binding motif) (supplemental Table 10). These genes are most likely to be transcriptionally regulated by ETV6, with expression altered in cells harboring variant ETV6. Gene Ontology analysis revealed significant enrichment of genes involved in innate immune response and leukocyte migration in inflammatory response (supplemental Table 11). Interestingly, we found that these 94 genes showed significant overlap with ETV6 targets identified using chromatin immunoprecipitation sequencing in ALL cell lines (12 out of 94 genes,  $P = .038$  by Fisher's exact test) (supplemental Table 10).<sup>38</sup> This supports the notion that these genes may be direct transcriptional targets of ETV6. Strikingly, t-distributed stochastic neighbor embedding clustering analysis<sup>39</sup> of the transcriptome of 253 primary ALL tumors on the basis of these 94 genes recapitulated the transcriptional relationship of germline ETV6 variant-associated ALL with other subtypes as determined by transcriptome seq (Figure 4D). This finding strongly suggests that these 94 genes are essential ETV6 targets, potentially linking their deregulation to the pathogenesis of germline ETV6 variant ALL. Finally, a comprehensive summary of the functional consequences of each germline ETV6 variant identified in this study can be found in Figure 5.

## Discussion

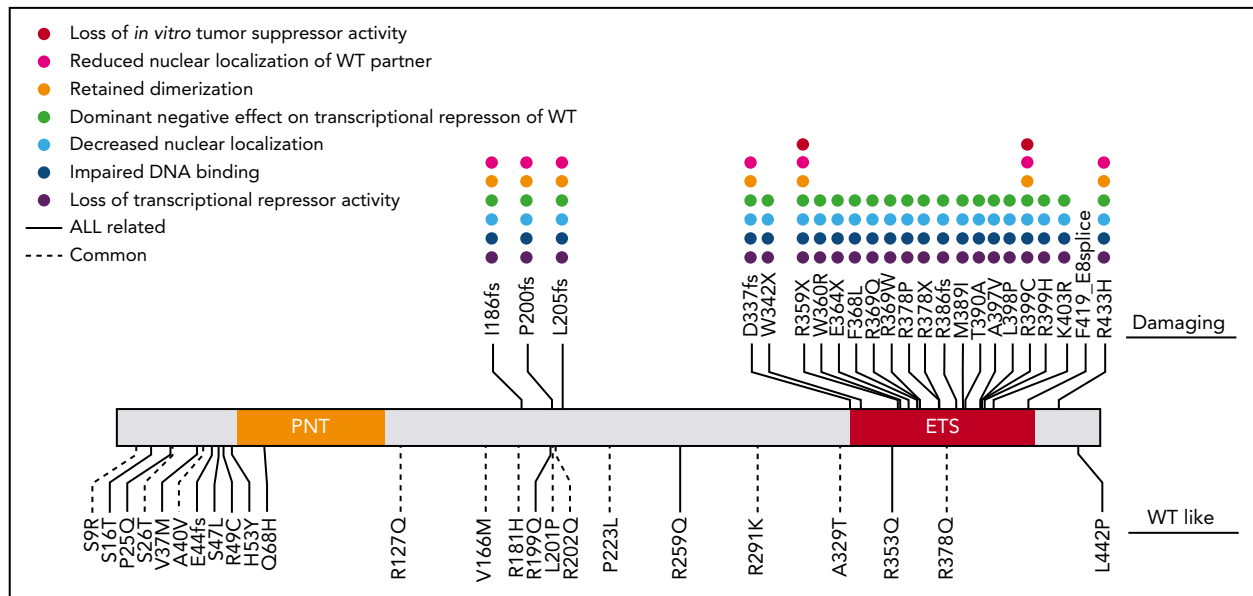
In this study, we comprehensively characterized the functional impacts of a large panel of germline ALL risk variants in ETV6, profiled the somatic genomic landscape of ALL arising from this unique genetic predisposition, and explored how these germline and somatic genetic variations interact to enable transformation and alter the epigenomic and transcriptional landscape of leukemia cells. Through these studies, we classified 22 of 34 ETV6 variants as damaging based on the degree of reduction in transcription repressor activity, all of which also lost the ability to bind ETV6 target DNA sequences and properly localize to the nucleus. Nearly all of the 22 damaging variants resided within, or caused truncation of, the ETS DNA-binding domain (Figure 1), and they maintained their ability to dimerize with WT ETV6, leading to cytoplasmic sequestration and impaired repressor activity of the WT protein.

Compared with the damaging germline variants, most WT-like variants were missense and located outside the ETS DNA-binding domain. Despite their normal transcription repressor activity, it remains possible that at least some of these WT-like variants have functional consequences. For example, the PNT domain facilitates homo-oligomerization and formation of corepressor complexes, and the central domain has been implicated

in binding of corepressors.<sup>29,40-43</sup> Therefore, ETV6 variants in or near these domains could disrupt protein-protein interactions and thus alter the binding of corepressor complexes to specific targets. Such effects might not be observed in a heterologous ectopic expression system using a single target promoter.

Intriguingly, 2 of the 12 WT-like variants demonstrated enhanced repressor activity compared with WT (E44fs and L442P). Close examination of E44fs sequence revealed 2 possible protein products: (1) it encodes an early termination at amino acid residue 44 of the canonical reading frame, which results in a truncated ETV6 missing most of the functional domains; or (2) it uses the +1 reading frame and an alternative start codon, which gives rise to a shortened ETV6 protein that loses the first 45 amino acids at the N-terminal portion but retains the PNT, linker, and ETS domains (supplemental Figure 13). The latter is similar to a previously reported ETV6 isoform (TEL-M43), which lacks the first 42 amino acids, but exerted enhanced repressor activity compared with WT ETV6 protein due to altered nuclear export.<sup>44</sup> To test this directly, we transfected 293T cells with constructs encoding WT ETV6 or the TEL-M43 or E44fs variants and then performed western blots to evaluate protein expression. A shortened ETV6 protein was indeed detected in cells expressing the E44fs variant, with the same molecular weight as TEL-M43 (supplemental Figure 13). While evaluation of endogenous protein expression driven by the E44fs variant in patient samples or knockin cells would be ideal to confirm this finding and rule out the possibility of nonsense-mediated decay, our data nevertheless suggest that the E44fs variant encodes a shortened E44fs protein that is highly analogous to the TEL-M43 isoform and exerts a similar mechanism of enhanced repression. By contrast, the L442P variant is located in close proximity to the C-terminal helix that directly interacts with the ETS domain, which potentially blocks ETV6 DNA binding with inhibitory effects.<sup>30,45</sup> We postulate that the L442P variant introduces changes to this highly conserved leucine residue and interferes with this autoinhibitory function and thus enhances repressor activity.

Given the consistent damaging effects of ETV6 variants on the activity of this critical hematopoietic transcription factor, we hypothesized that ALL arising from this predisposition syndrome would have a unique global transcriptional landscape. This notion is partly supported by our whole-transcriptome sequencing-based clustering analysis in which ALL cases with damaging ETV6 variants were clearly separated from those with WT-like variants. The impact of these germline variants is profound at a genome-wide level, and the preservation of such effects in leukemia cells suggest their importance in leukemogenesis. On the other hand, within ETV6 ALL, the transcriptional program was also strongly influenced by secondary somatic abnormalities, with an especially striking separation of hyperdiploid vs diploid ALL cases. In contrast to hyperdiploid cases with RAS pathway mutations, diploid ALL cases with germline ETV6 variants exhibited highly recurrent focal deletion of ETV6 and PAX5 (and to a lesser degree IKZF1). This suggests that even with the same underlying genetic predisposition factor, preleukemic cells can invoke a few restricted but distinctive pathways as major cooperating events during leukemogenesis. Because hyperdiploidy can arise in utero, this early and large-scale genomic abnormality could efficiently promote leukemogenesis, coupled with germline ETV6 defects, with a small



**Figure 5. Summary of in vitro functional characterization of *ETV6* variants.** A variety of in vitro assays were performed to comprehensively characterize the impacts of germline *ETV6* variants. Dot colors represent different assays; blanks denote that the variant was not tested in the assay. The top panel includes “damaging” variants as determined by impairment of transcriptional repression in luciferase reporter assays. The bottom panel includes WT-like variants, which showed no impairment of transcriptional repression.

number of additional somatic hits (eg, *RAS* mutations). In contrast, diploid *ETV6* ALL may take a narrower path to leukemia, requiring loss of several hematopoietic transcription factors (eg, *ETV6*, *PAX5*, and *IKZF1*) that would result in disruption of normal B-cell differentiation. This is further demonstrated by our discovery of distinct lymphoid- and myeloid-related somatic genetic events in the same individual (*ETV6* R386fs) with both ALL and AML, and in 2 members of the same family (*ETV6* R399H), one with ALL and the other with AML, respectively. While germline *ETV6* variants might set the stage for leukemogenesis, the development of leukemia subtypes of differing cellular origin likely results from the acquisition of specific second somatic hits (eg, *PAX5* deletion in ALL or *AXSL1* frameshift in AML) and the hematopoietic compartment in which they arise. Functional validation of these somatic genomic features in appropriate leukemia model systems is warranted in future studies to determine their cooperativity with germline *ETV6* variants.

The shared transcriptional landscape between ALL with germline *ETV6* variants and those with the somatic *ETV6*-*RUNX1* fusion is highly reminiscent of the recently described “*ETV6*-*RUNX1*-like” ALL subtype.<sup>46,47</sup> These notable similarities beg the question as to whether all or a proportion of *ETV6*-*RUNX1*-like cases arise in individuals harboring damaging germline (or somatic) *ETV6* variants. Also of note is a high frequency of somatic copy-number loss of *ETV6* in *ETV6*-*RUNX1* ALL and germline *ETV6* variant ALL, arguing that the deletion of the WT *ETV6* allele is required for development of leukemia in some, but not all, cases. This raises the possibility of complex mechanisms by which *ETV6* is involved in leukemogenesis, similar to the multifaceted roles of the major tumor suppressor p53, which can promote tumorigenesis by loss of tumor suppressor function or conversely by acquisition of dominant-negative or even “gain-of-function” effects.<sup>48</sup> Knockin of variant *ETV6* in murine or other models is needed to more definitively dissect the leukemogenic properties associated with specific germline *ETV6* variants.

Testing *NRAS*<sup>G12D</sup>-mediated transformation in vitro, we demonstrated that damaging *ETV6* variants lack the tumor suppressor activity of WT protein and significantly alter the transcriptional and epigenomic programs. Our sequencing analyses identified 94 potential *ETV6* target genes that were upregulated in cells expressing the damaging *ETV6* R359X variant. Interestingly, expression profile analysis using this gene set recapitulated the clustering of germline *ETV6* variant ALL cases with other ALL subtypes, as observed by whole-transcriptome analysis, pointing to an essential role for these putative *ETV6* targets in the global transcriptional landscape. Comparing our gene set with *ETV6* and/or *ETV6*-*RUNX1* targets reported previously,<sup>25,49,50</sup> we observed a number of overlapping genes, including *CLIC5*, *LRP8*, *SLC7A11*, *CHST12*, and *IL21R* (supplemental Figure 14). One of the most consistently upregulated genes in *ETV6* mutant ALL is *CLIC5*, the gene encoding chloride intracellular channel 5. *CLIC5* was recently reported as a direct target of *ETV6* and is overexpressed in *ETV6*-*RUNX1* pediatric ALL cases.<sup>25</sup> Overexpression of *CLIC5* in that study revealed its ability to suppress lysosomal-mediated apoptosis induced by hydrogen peroxide. This represents a potential mechanism by which variants in *ETV6* could lead to *CLIC5*-mediated resistance to oxidative stress and accumulation of DNA damage. Given these intriguing findings, there is a pressing need to elucidate the molecular mechanisms by which *ETV6* and its downstream target genes contribute to normal and malignant hematopoiesis.

In summary, we provide a comprehensive functional characterization of ALL risk variants in *ETV6* that result in profound alterations in biochemical, transcriptional, and epigenomic functions of *ETV6*. The functional data gathered in this study will be helpful to improve upon clinical classification of germline *ETV6* variants. Genomic profiling of ALL arising from this rare genetic syndrome highlights remarkable similarities in the effects of damaging germline and somatic *ETV6* aberrations on the transcriptional landscape of leukemic blasts, implying intricate



interactions between the host and leukemia genomes in ALL pathogenesis.

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## Footnotes

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The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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