

# Predicting interactome network perturbations in human cancer: application to gene fusions in acute lymphoblastic leukemia

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**ABSTRACT** Genomic variations such as point mutations and gene fusions are directly or indirectly associated with human diseases. They are recognized as diagnostic, prognostic markers and therapeutic targets. However, predicting the functional effect of these genetic alterations beyond affected genes and their products is challenging because diseased phenotypes are likely dependent of complex molecular interaction networks. Using as models three different chromosomal translocations—*ETV6-RUNX1* (*TEL-AML1*), *BCR-ABL1*, and *TCF3-PBX1* (*E2A-PBX1*)—frequently found in precursor-B-cell acute lymphoblastic leukemia (preB-ALL), we develop an approach to extract perturbed molecular interactions from gene expression changes. We show that the MYC and JunD transcriptional circuits are specifically deregulated after *ETV6-RUNX1* and *TCF3-PBX1* gene fusions, respectively. We also identified the bulk mRNA NXF1-dependent machinery as a direct target for the *TCF3-PBX1* fusion protein. Through a novel approach combining gene expression and interactome data analysis, we provide new insight into *TCF3-PBX1* and *ETV6-RUNX1* acute lymphoblastic leukemia.

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## INTRODUCTION

The development of every cancer is characterized by frequent genomic aberrations. Investigations focused on specific human neoplasms have identified numerous sequence variants in which mutations are implicated in oncogenesis. These human cancer genes are listed in the Cancer Genome Project database, with genes encoding protein kinase and transcriptional regulation domains highly represented (Futreal et al., 2004). Characterization of the bio-

logical properties of some mutated genes, such as the breakpoint cluster region-v-Abelson murine leukemia viral oncogene homologue 1 (*BCR-ABL1*), has led to the development of successful targeted therapies (Lynch et al., 2004; Gazdar, 2009; Quintas-Cardama and Cortes, 2009; Agrawal et al., 2010; Kaulfuss et al., 2013). The most prevalent category among the known cancer genes are chromosomal translocations, often involving immunoglobulin, T-cell receptor, and transcription factor genes (Futreal et al., 2004). Although these rearrangements represent important diagnostic markers that are used to define cancer subtypes (Mitelman et al., 2004; Maher et al., 2009), their molecular interactions and the pathways affected by the result of gene fusions are poorly characterized.

Genes and their products do not act in isolation but as part of complex molecular networks in which most genes play their roles through several molecular functions or interactions. The changes induced by gene fusions and other genetic alterations, as well as modifications of expression levels, do not lead to a complete loss of the gene products and are thus very likely to alter the different interactions of the same gene or protein in distinct fashions (Zhong et al., 2009). Classically, genome-wide transcriptomic studies have been

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Abbreviations used:

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used to identify genes or gene expression signatures in order to characterize and classify cancer types or subtypes (Golub *et al.*, 1999; Andersson *et al.*, 2005; Gandemer *et al.*, 2007; Den Boer *et al.*, 2009; Li *et al.*, 2009; Fuka *et al.*, 2011). Although very useful to identify oncogenes and for diagnostic purposes, these methods are limited in their ability to understand the underlying molecular biology, as they are focused on genes, transcripts, and proteins, neglecting the interactions between them.

In this study, we propose a strategy that uses gene expression profiles to identify genes, molecular interactions, and pathways that are important in a specific genetic alteration. We use as models two chromosomal translocations found in precursor-B-cell acute lymphoblastic leukemia (preB-ALL) and involving key specific transcription factors regulating hematopoietic development: 1) the Ets transcription factor variant 6 (ETV6)–runt-related transcription factor 1 (RUNX1) fusion (also known as TEL-AML1) and 2) the transcription factor 3 (TCF3)–pre-B-cell leukemia homeobox 1 (PBX1) fusion (also known as E2A-PBX1; Okuda *et al.*, 1996; Zhou *et al.*, 2012; Tjichon *et al.*, 2013). These chromosomal rearrangements alone are insufficient for leukemogenesis but may support leukemia when additional molecular perturbations are present (Andreasson *et al.*, 2001; Seto, 2010). We thus extracted perturbed molecular interactions and showed that MYC and JunD interactomes are specifically deregulated after ETV6-RUNX1 and TCF3-PBX1 gene fusions, respectively. Furthermore, we demonstrated that the TCF3-PBX1 fusion could impair the normal mRNA export machinery.

## RESULTS

### Predicting perturbed interactions linked to gene fusions

To predict perturbed molecular interactions specifically linked to ETV6-RUNX1, TCF3-PBX1, and BCR-ABL1 gene fusions, we used the human B-cell interactome (HBCI; Lefebvre, 2007, 2010) and expression data sets from two microarray series (Den Boer *et al.*, 2009; Mullighan *et al.*, 2009), including 24 samples with BCR-ABL1 fusion, 77 with ETV6-RUNX1 fusion, 16 with TCF3-PBX1 fusion, and 248 samples with other different genetic subtypes. Expression data were first normalized by frozen robust multiarray analysis (fRMA; McCall and Irizarry, 2011). For each interaction in HBCI, we computed the difference between the correlation of expression profiles in a group of samples exhibiting a genotype of interest and in the control samples (groups of samples with other genotypes). Because interacting genes/proteins are likely to be involved in similar biological processes and are likely coexpressed (Ge *et al.*, 2001), we selected interactions with significant differences of correlation as deregulated (corrected  $p < 0.05$ ; Figure 1A).

We detected 2550 perturbed interactions (~4.5% of interactions in the HBCI, involving 664 human genes) and 3334 (~5.8% of the HBCI, involving 1022 human genes) in the ETV6-RUNX1 and TCF3-PBX1 ALL samples, respectively (Supplemental Tables S1 and S2). We found only 74 (0.13%) overlapping interactions between ETV6-RUNX1 and TCF3-PBX1 ALL samples, showing the specificity of the method (Figure 1B). For BCR-ABL1 genotype, which does not involve direct translocation of a transcription factor–coding gene, we detected only 10 (~0.018%) potentially perturbed interactions (Supplemental Table S3). Our next analyses thus will compare perturbed networks for ETV6-RUNX1 and TCF3-PBX1 fusions.

We ranked proteins/genes according to the number of perturbed interactions, and identified MYC (~46% of HBCI) as the most perturbed in the ETV6-RUNX1 subtype of preB-ALL. To confirm the direct link between MYC network alteration and the presence of ETV6-RUNX1 fusion protein, we used HEK293 cells stably expressing ETV6-RUNX1 and control cells expressing similar amounts of

MYC (Figure 2A). We performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) in cells expressing the ETV6-RUNX1 fusion protein to detect the MYC-binding sites at a genome scale. We identified 557 MYC target genes in both cell lines (Figure 2B and Supplemental Table S4, HEK293 +ETV6-RUNX1 anti MYC and HEK293 anti MYC), representing 19% of MYC target genes reported in the human B-cell interactome (Lefebvre, 2007, 2010). As predicted, this experiment showed a high modification of MYC targets in the presence of ETV6-RUNX1 fusion, with ~88% (489 of 557) of the targets being different between the two cell lines. Among these, 52% were also identified as MYC–perturbed interactions by our method (Figure 2C and Supplemental Table S1), further supporting the use of differences of correlation between expression profiles to predict perturbed interactions.

### Topological analysis of the perturbed networks

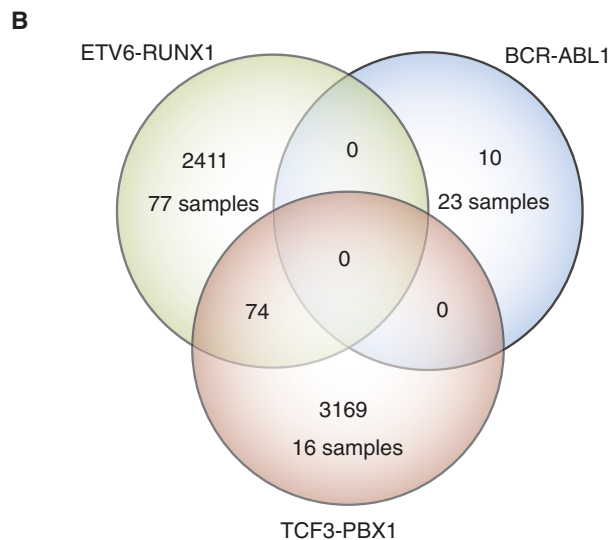
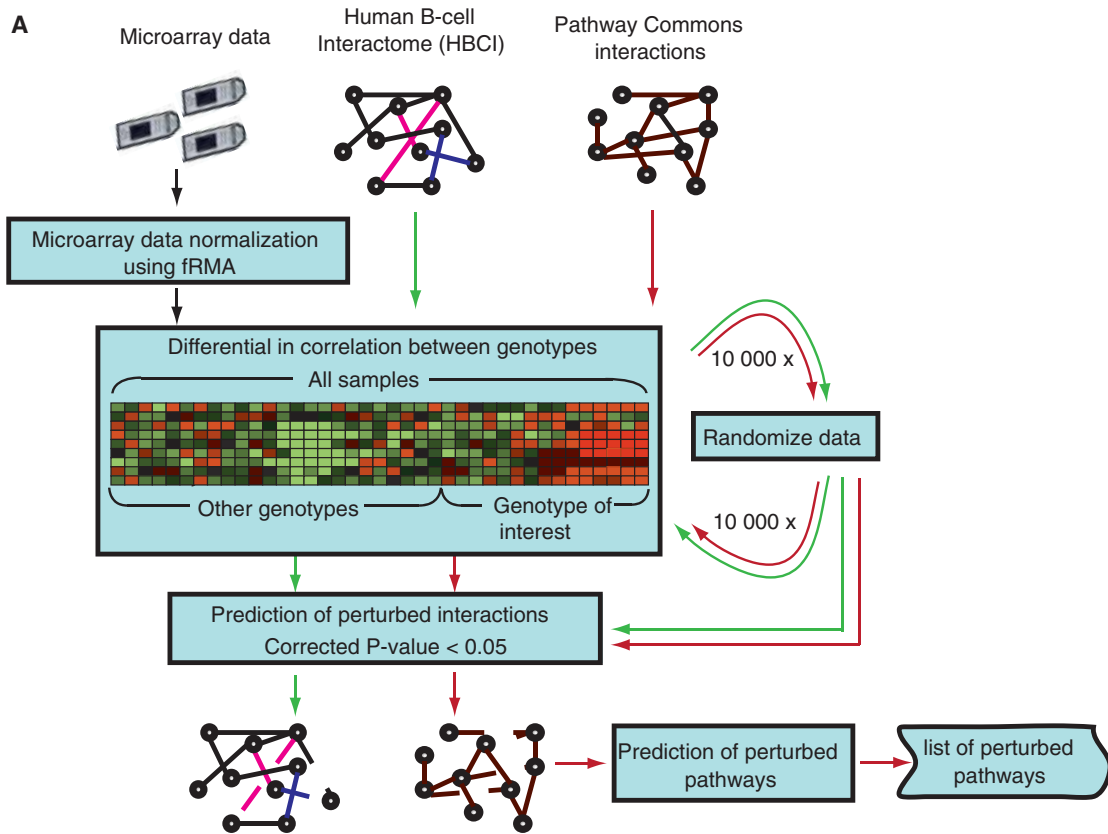
To determine whether the structure of the network is modified after ETV6-RUNX1 or TCF3-PBX1 fusions, we analyzed network topology perturbations using three metrics: characteristic path length (cpl), edge betweenness centrality (ebc), and edge-clustering coefficient (ecc).

We sequentially removed edges corresponding to perturbed interactions by decreasing order of significance, calculated the cpl, average ebc, and average ecc of the resulting network at each step, and compared these metrics to those obtained by removing random edges (Figure 3, red lines). For ETV6-RUNX1 fusion, we observed a significant increase of cpl and ebc, whereas ecc decreased, indicating that edge perturbations in ETV6-RUNX1 fusion leads to a less compact network but with a globally higher, more evenly distributed communication potential and a lower local connectivity on high-degree nodes than expected at random (Figure 3, compare green to red lines). In the case of TCF3-PBX1 fusion, on the contrary, the perturbed network becomes more compact, with a slightly lower communication potential and local connectivity than expected at random (Figure 3, compare blue and red lines). We also compared ebc, ecc, and the edge shortest path length (espl) of the network composed of perturbed edges with the rest of the network (the network of not-perturbed edges). The espl was computed as the mean of all shortest path lengths between the vertices of an edge and all other vertices in the network. It appeared that perturbed edges after ETV6-RUNX1 fusion are characterized by significantly higher ebc, higher ecc, and lower espl than other edges in the network (Table 1A). Similar local metrics for the TCF3-PBX1 fusion showed minor changes (Table 1B).

Taken together, our network topology analysis suggests that ETV6-RUNX1 chromosomal translocation may lead to disruption of molecular interactions important for B-cell communication circuits, whereas TCF3-PBX1 fusion only slightly modifies the structure of the network.

### Specific deregulation of transcription factor networks

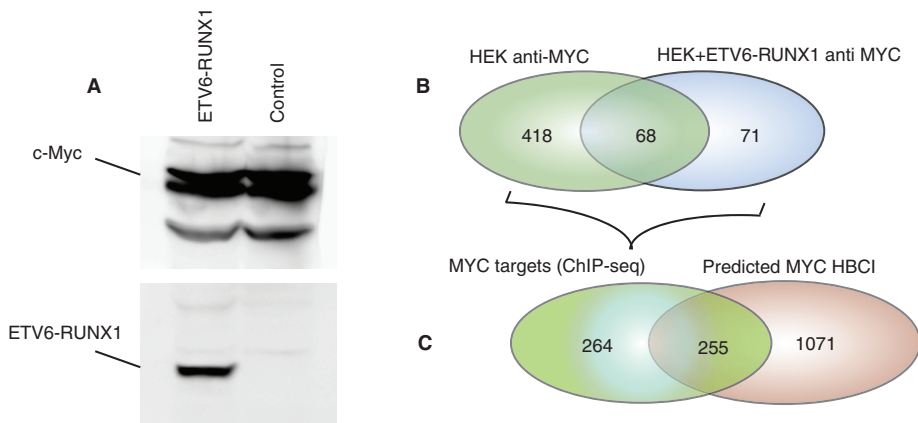
We ranked nodes based on the proportion of their perturbed interactions in HBCI, and highlighted the 10 most deregulated nodes in ETV6-RUNX1 and TCF3-PBX1 fusion subtypes of ALL. It appeared that, for both ALL subtypes, top deregulated nodes correspond to diverse transcription regulators (Figure 4, A and B). This result suggests that ETV6-RUNX1 and TCF3-PBX1 fusions support oncogenesis mostly by specifically deregulating other transcriptional regulators. We therefore analyzed the interaction networks of transcription factors (TFs) that have at least one interaction predicted as deregulated and categorized the TFs according to the number of perturbed interactions and the published classification of human TFs



**FIGURE 1:** Prediction of perturbed interactions. (A) Flowchart of the method. Arrows show the flow of data analysis: black for microarrays, and green and red for HBCI and Pathway commons interactome, respectively. For each interaction in the B-cell or pathways interactome, we computed the differential in correlation between genotypes. Significance of the difference in correlation is estimated from randomized data. Interactions with corrected  $p < 0.05$  are predicted as perturbed. Dashed lines represent perturbed interactions. (B) Venn diagram representing the number of detected perturbed interactions (DPIs) in the B-cell interactome for ETV6-RUNX1, BCR-ABL1, and TCF3-PBX1.

(Wingender *et al.*, 2013). We found that two classes of transcription factors, basic helix-loop-helix (bHLH) and leucine zipper (bZIP), account for the majority of perturbed interactions for ETV6-RUNX1

and TCF3-PBX1 fusions, respectively (Figure 5, A and B, red arrows). In particular, the majority of bZIP members of the activating protein-1 (AP-1) complexes, including JunD, JunB, c-Jun, c-Fos,



**FIGURE 2:** ETV6-RUNX1 expression perturbs MYC binding to its targets. (A) HEK 293T expressing V5-ETV6-RUNX1 and control cells were subjected to Western blot analysis using anti-MYC and anti-V5 antibodies. (B) Chromatin immunoprecipitation was performed using an anti-MYC antibody, followed by massively parallel sequencing of isolated DNA fragments. Venn diagrams indicate the comparison of MYC target genes identified in HEK vs. HEK+ETV6-RUNX1 cell lines. (C) Venn diagrams showing a comparison between the numbers of perturbed MYC targets identified by ChIP-seq and those predicted by computing the differences of correlation between expression profiles.

Fra-1 (FOSL1), FosB, ATF2, ATF3, CREB1, and CREBL2, seems affected after TCF3-PBX1 fusion (Figures 4B and 5B).

To validate our analysis experimentally, we investigated TCF3-PBX1-induced transcriptional regulation of the Jun/AP-1 pathway. HEK293 cells were cotransfected with a luciferase reporter construct harboring AP-1-binding sites (Samuel *et al.*, 2008) and increasing amounts of expression vectors for TCF3-PBX1, TCF3, or PBX1 transcription factor. As shown on Figure 4C, AP-1 transactivation is significantly increased in the presence of the oncogenic fusion TCF3-PBX1, in accordance with previous reports that demonstrated functional interplay between TCF3-PBX1 and the CREB-binding protein (CBP) and p300 transcriptional coactivators (LeBrun, 2003; Bayly *et al.*, 2004; Denis *et al.*, 2012; Hyndman *et al.*, 2012) and that CBP/p300 and AP-1 factors have many partners in common (Denis *et al.*, 2012).

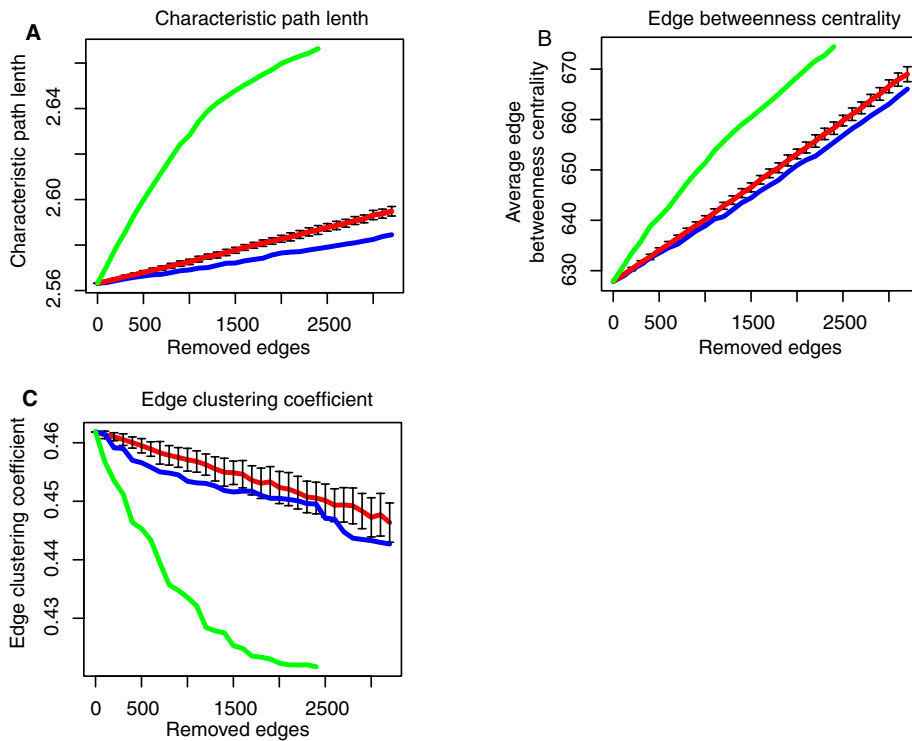
Among the bHLH TFs, the proto-oncogene MYC was the top-ranked gene, with ~46% of HBCI perturbed interactions (Figure 5A). By comparison, only a small minority (1%) of MYC interactions is perturbed in TCF3-PBX1-associated ALL samples, suggesting a specific interplay between MYC and ETV6-RUNX1 transcriptional regulation. This is also illustrated by the network of MYC transcriptional regulators, showing that the majority of protein-DNA interactions (such as STAT5 and the MYC promoter; Denis *et al.*, 2012; Ott *et al.*, 2012; Mangolini *et al.*, 2013) and protein-protein interactions (such as SP1 and MYC coregulation of some target genes; Zhang *et al.*, 2012; Wang *et al.*, 2013, 2014) are specifically deregulated in ETV6-RUNX1 compared with TCF3-PBX1 ALL subtypes (Figure 6). To further validate MYC deregulation in ETV6-RUNX1 compared with TCF3-PBX1 subtypes of ALL, we analyzed the relative expression of MYC and its targets involved in cell cycle regulation, including cyclin-dependent kinase inhibitors (CKIs), which are known targets repressed by the MYC/Miz1 complex (Figure 6C and Supplemental Table S5). Among these key cell cycle regulators, TP53, MYC, and CDKN3 were relatively underexpressed, whereas CDKN1A (p21WAF1/CIP1) expression was unchanged in ETV6-RUNX1 samples compared with other groups of samples. As recently reported for the MDM2 promoter (Kaindl *et al.*, 2014), this result indicates a p53-independent mode of CDKN1A promoter

regulation in ETV6-RUNX1 ALL samples. We thus hypothesized that the ETV6-RUNX1 fusion could compete with the formation of MYC/Miz1 complex or the binding to its down-regulated targets, including the CDKN1A promoter. To test the latter hypothesis, we analyzed the CDKN1A promoter sequence (-499 to +100 base pairs around the start site) using the eukaryotic promoter database (EPD; epd.vital-it.ch) and the TF-search program (Heinemeyer *et al.*, 1998) and identified two putative RUNX1-binding sites. The ETV6-RUNX1 chromosomal translocation conserves the RUNX1 DNA-binding domain (Supplemental Figure 1A), suggesting that any putative RUNX1-binding site would also be a potential target sequence for ETV6-RUNX1 fusion protein, as previously demonstrated (Wotton *et al.*, 2008; Krapf *et al.*, 2010; Kaindl *et al.*, 2014). We therefore performed ChIP assays using leukemic REH cells (or REH cells silenced in ETV6-RUNX1 expression by short hairpin RNA [shRNA; REH-G1]; Fuka *et al.*, 2012;

Supplemental Figure 1B) and a specific antibody to ETV6 transcription factor. We specifically amplified a genomic CDKN1A promoter fragment encompassing both putative RUNX1-binding sites, indicating that ETV6-RUNX1 fusion protein directly binds the CDKN1A promoter (Supplemental Figure S1C). In a transcriptional reporter assay, we also demonstrated that the activation of the CDKN1A promoter by a phorbol ester (PMA; Zeng and el-Deiry, 1996) could be inhibited by overexpression of ETV6-RUNX1 fusion protein (Supplemental Figure 1D), similar to the previously reported repressor effect of MYC on CDKN1A promoter (Seoane *et al.*, 2002; Wu *et al.*, 2003). Finally, using HEK293 cells stably expressing ETV6-RUNX1, we compared MYC and ETV6-RUNX1 ChIP-seq data sets and showed that both transcription regulators could target similar genes (Supplemental Table S9). Taken together, these results support the idea that the ETV6-RUNX1 fusion protein might interplay with the MYC transcriptional network.

### Perturbations in signaling pathways

To explore signaling pathways potentially disturbed after ETV6-RUNX1 and TCF3-PBX1 gene fusions, we adopted two strategies. First, we performed a gene enrichment pathway analysis using the 664 or 1024 human genes involved in ETV6-RUNX1 or TCF3-PBX1 perturbed interactions, respectively. As stated earlier, this data set is restricted to genes involved in B-cell interactions reported in the HBCI database. We performed a functional enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang *et al.*, 2009). We linked perturbed interactions in ETV6-RUNX1 or TCF3-PBX1 ALL samples to similar signaling pathways, including pathways in cancer, T- and B-cell receptor signaling, Toll-like and growth factor signaling, mitogen-activated protein kinase signaling, and cell cycle and cell adhesion (Figure 7, A and D). However, the proportions of perturbed interactions (Figure 7, compare B and E) and the pathway subnetwork profiles (Figure 7, compare C and F) are very different. Of interest, pathways involved in B-cell migration (chemokine receptor signaling) and cell adhesion were specific to the ETV6-RUNX1 fusion, consistent with previous studies showing that ALL cells, including REH, are highly motile and capable of rapid migration within lymphoid tissues (Makrynikola



**FIGURE 3:** Topological analysis of the perturbed BCI network. We show the evolution of three network metrics while removing predicted perturbed interactions in their order of significance (highest difference of correlation first) for *ETV6-RUNX1* and *TCF3-PBX1* fusions. Abscissa, number of removed edges (perturbed interactions); ordinate, values of the metric. Green, blue, and red curves represents the distributions of the values of the metric removing edges sequentially in *ETV6-RUNX1*, *TCF3-PBX1*, and randomly (100 random iterations), respectively. Vertical bars, SEs of random iterations. (A) Characteristic path length (cpl). (B) Edge-betweenness centrality (ebc). (C) Edge-clustering coefficient (ecc).

*et al.*, 1994; Gandemer *et al.*, 2007). Alternatively, we predicted NF- $\kappa$ B pathway deregulation for *TCF3-PBX1*, in accordance with the significant proportion of perturbed interactions involving NF- $\kappa$ B RELA (p65) and REL subunits (16 and 14% of HBCI reported interactions, respectively).

Metrics	Perturbed edges	Other edges	<i>p</i>
A. <i>ETV6-RUNX1</i>			
Edge shortest path length (espl)	2.084	2.288	<2.22E-16
Edge betweenness (ebc)	1838	572	<2.22E-16
Edge clustering coefficient	0.683	0.451	<2.22E-16
B. <i>TCF3-PBX1</i>			
Edge shortest path length (espl)	2.259	2.280	3.5219E-13
Edge betweenness (ebc)	614	628	1.859E-12
Edge clustering coefficient (ecc)	0.457	0.462	0.4265

Difference of the means (*p*) is assessed through a Mann–Whitney *U* test.

**TABLE 1:** Comparison of local metrics between perturbed edges and the rest of the network for (A) *ETV6-RUNX1* and (B) *TCF3-PBX1* fusion.

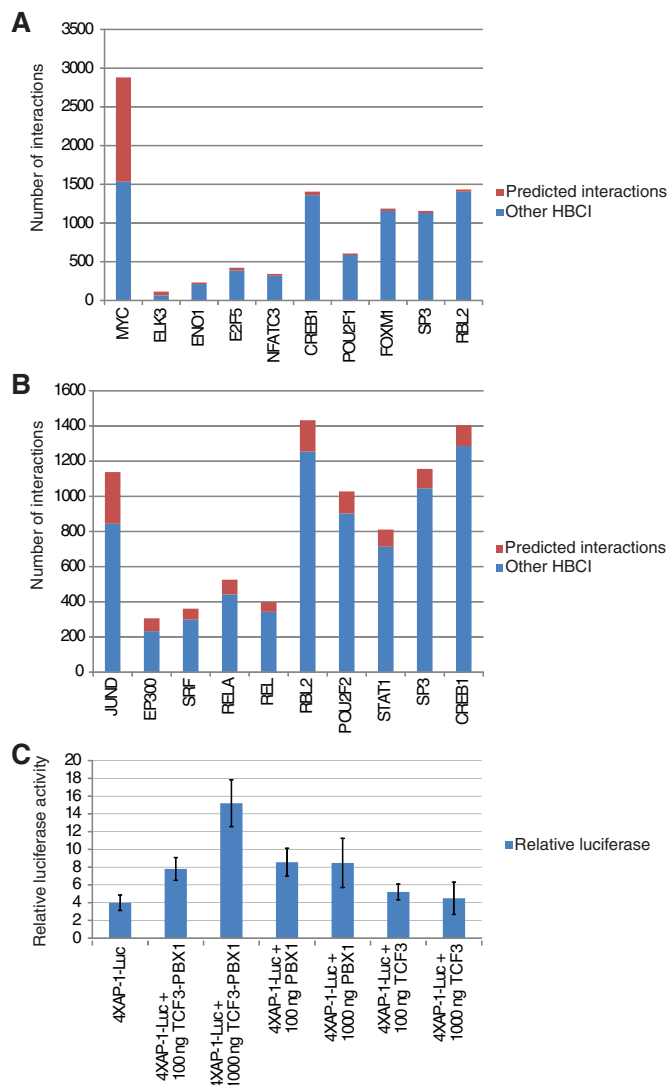
Next we used Pathway commons ([www.pathwaycommons.org](http://www.pathwaycommons.org)), which is a collection of pathways from multiple sources and organisms. Compared to the cell-context HBCI database, we reasoned that Pathway Commons might allow us to uncover unexpected novel functions for *TCF3-PBX1* or *ETV6-RUNX1* fusion proteins. We thus considered Pathway Commons as a single network and predicted disrupted interactions in the same way as for the HBCI. This analysis revealed 61 and 45 perturbed pathways for *ETV6-RUNX1* and *TCF3-PBX1* fusions, respectively (Supplemental Tables S6 and S7). Confirming our foregoing results, several pathways linked to MYC transcription factor were predicted as perturbed by the *ETV6-RUNX1* fusion. Of interest, we highlighted a potential deregulation of pathways linked to RNA transport machinery after *TCF3-PBX1* (Table 2). Perturbed interactions involve several proteins important for RNA processing, including mRNA export proteins such as the eukaryotic translation factor 4A3 (eIF4A3), the nuclear pore complex (NCP/NUP), and the nuclear export receptor NXF1/TAP (Siddiqui and Borden, 2012; Supplemental Table S8). Eukaryotic mRNA is exported from the nucleus either by the bulk export NXF1-dependent pathway or via more specialized factors such as the chromosome region maintenance 1 (CRM1, also called exportin-1 [XPO1]; Hutten and Kehlenbach, 2007; Siddiqui and Borden, 2012). Because most *TCF3-PBX1* perturbed

interactions involved the NXF1 rather than the CRM1 pathway, we analyzed potential interaction between *TCF3-PBX1* fusion protein and NXF1 by examining the subcellular localization of both proteins. We showed that both NXF1 and *TCF3-PBX1* colocalize in the nucleoplasm (Supplemental Figure S2A), indicating functional interplay. To test whether *TCF3-PBX1* fusion protein could interfere with RNA localization, we visualized RNA molecules in cells transfected with the *TCF3-PBX1* fusion and observed colocalization between *TC3-PBX1* and RNA molecules, and, most important, RNA was delocalized from the nucleoli to the nucleoplasm (Supplemental Figure S2B). Together these results suggest a potential deregulating role of *TCF3-PBX1* in the mRNA export machinery.

## DISCUSSION

As genome-wide expression profiling and interactomic data accumulate and are stored in public databases, the integration to drive interpretation of genotype–phenotype relationships and identify genes and pathways associated with specific diseases remains challenging. Several approaches have been conducted on cancer samples to identify tumor markers and gene expression signatures and to classify cancer types or subtypes. However, functional perturbations arising from expression changes are rarely interpreted in the context of molecular network perturbations, which may be sensitive to subtle transcriptional changes.

In this study, we integrated data from gene expression in B-cell ALL subtypes, molecular interaction networks from the human B-cell interactome, and Pathway Commons databases to provide novel



**FIGURE 4:** Deregulation of transcription factor interactions. (A) *ETV6-RUNX1*. (B) *TCF3-PBX1*. Ten most perturbed nodes, that is, genes/proteins showing the highest number of perturbed interactions. Colors: red bars (pred\_inter) represent perturbed interactions, blue bars (other HBCI) represent other interactions in the HBCI database. (C) Transactivation of an AP-1 reporter by TCF3-PBX1. HEK293 cells were transfected with the reporter plasmid p4XAP1-luc and indicated amounts of effector plasmids (pFlag-TCF3-PBX1, pFlag-TCF3, pFlag-PBX1). Firefly luciferase data were normalized to *Renilla* luciferase counts, and data are reported as mean and SE of three independent experiments in triplicate.

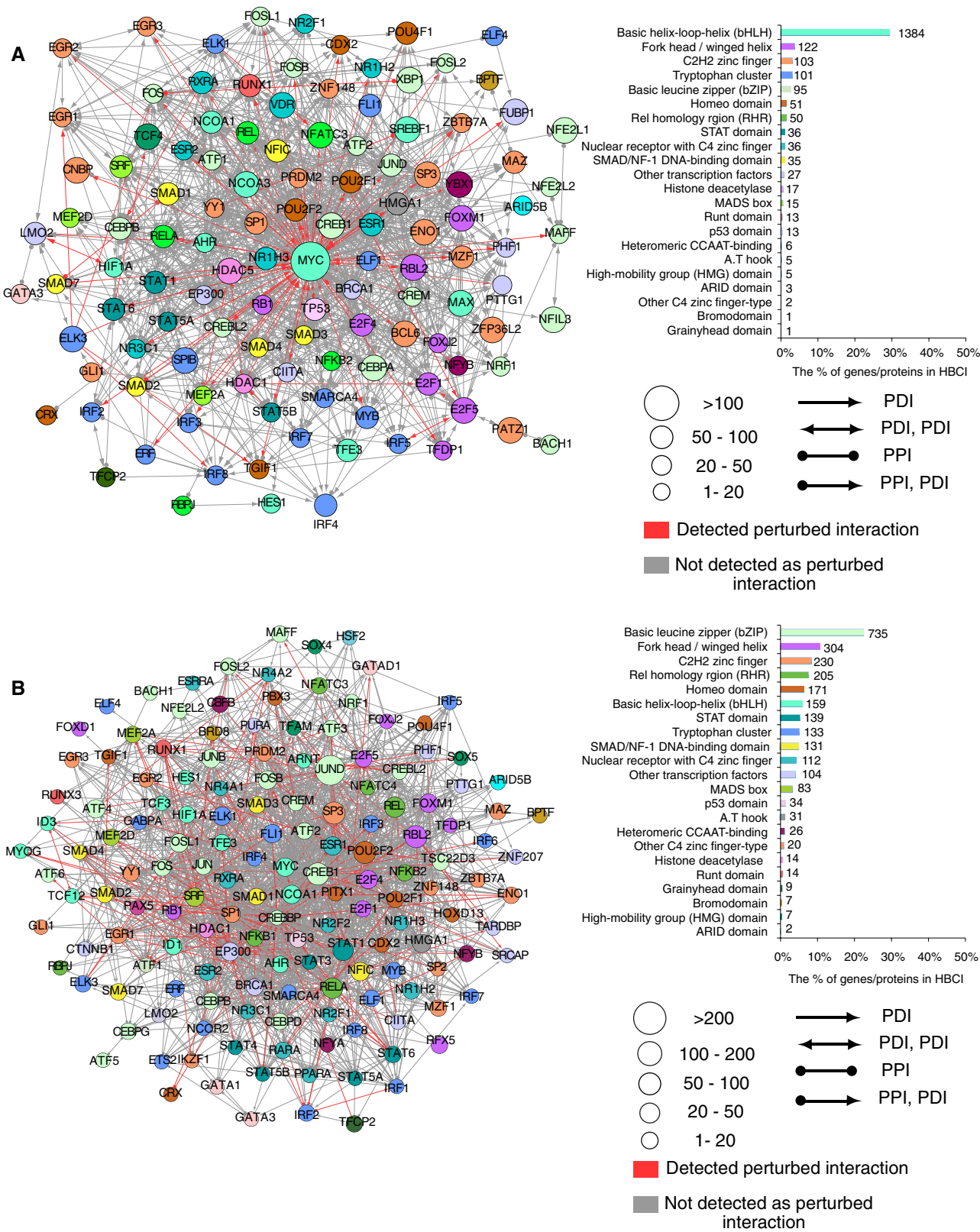
hypotheses about deregulated molecular interactions and pathways. We detected 0.018, 4.5, and 5.8% of perturbed interactions in the human B-cell interactome after chromosomal translocations of *BCR-ABL1*, *ETV6-RUNX1*, and *TCF3-PBX1* fusions in ALL, respectively. Potential perturbed interactions were ranked according to the magnitude of change in gene expression for a pair of interacting partners (Supplemental Tables S1–S3).

The relatively low number of specific perturbed interactions for *BCR-ABL* subtype (Supplemental Table S3) is unexpected and implies that, for most pairs of interactions, 1) gene expression profiles are too different between *BCR-ABL1* ALL samples, consistent with the fact that several breakpoints on chromosomes 9 (for the *ABL* gene) and 22 (for the *BCR* gene) may generate kinases with different

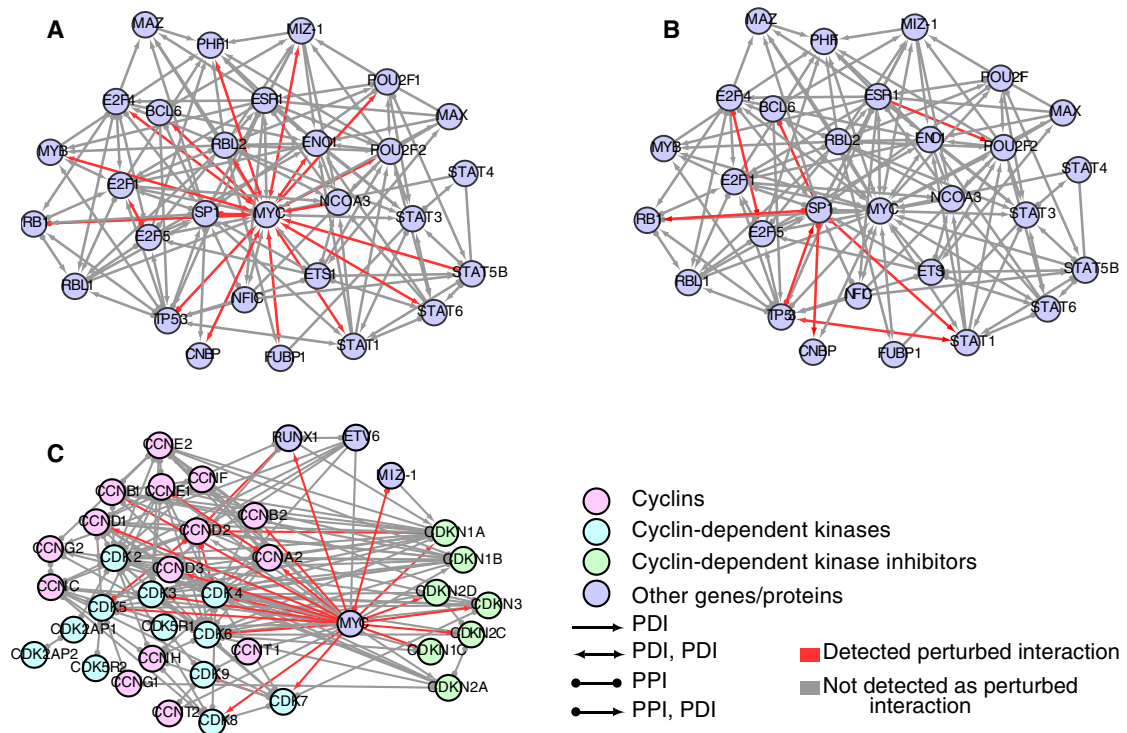
outcome on the downstream signaling transduction molecules (Pendergast *et al.*, 1993; Skorski *et al.*, 1995; Ren, 2005); and 2) the common changes are not observable in *BCR-ABL1* ALL samples and may have been missed. Those unidentified changes found in all subtypes could define the major networks implicated in *BCR-ABL1*, *ETV6-RUNX1*, and *TCF3-PBX1* leukemia subtypes.

Although we showed that our method is useful in the identification of previously unknown mutant-specific deregulated biological processes, this strategy, like any other system biology model that predicts perturbations, presents some limitations: 1) To predict interactome network perturbations, we calculated the difference of correlation between expression profiles of two genes coding for proteins involved in a protein–protein or protein–DNA interaction. High-throughput data concerning other variables that may influence an interaction, such as mutations in coding sequences, proteins localization and translocation, protein modifications (phosphorylation, acetylation, glycosylation, etc.), and mRNA processing (transport, degradation, stability, etc.), were not included. 2) We applied our methodology to the analysis of *ETV6-RUNX1* or *TCF3-PBX1* chromosomal rearrangements restricted to precursor-B-cell leukemia, whereas we interrogated a mature-B-cell interactome data set (HBCI), which is an interaction network assembled from a collection of 254 B-cell gene expression profiles representing 24 distinct phenotypes of normal and diseased B cells (Lefebvre *et al.*, 2010). To the best of our knowledge, similar cell-context interactomes for all stages of B-cell development, including precursor B cells, are not yet available. Some important interactions specific for precursor B cells may be missed and some irrelevant interactions may be included in our analysis. 3) The accuracy of our predictions depends on the technical quality of transcriptome and interactome data sets. In our study, transcriptome data sets were from published microarray hybridization data (Den Boer *et al.*, 2009; Mullighan *et al.*, 2009). High-throughput RNA sequencing should provide more precise measurement of gene expression levels and increase the accuracy of our predictions (Wang *et al.*, 2009). In addition, the human B-cell interaction data were obtained by either reverse engineering of transcriptome data or literature curation of interactions. For technical reasons, both methods capture a number of false-positive and false-negative interactions and do not give a complete view of interactomes (Cusick *et al.*, 2009; Dreze *et al.*, 2010; Lefebvre *et al.*, 2010; Yu *et al.*, 2011; Tsang *et al.*, 2014). To summarize, our strategy, like other systems biology predicting models, will improve over time as more accurate cell-specific interactome and transcriptome data are available. In all cases, biological validations are necessary to confirm perturbations of interactome networks in cancer subtypes of interest.

The identification of the MYC network as specifically deregulated after *ETV6-RUNX1* fusion could not be anticipated. Of interest, we did not observe dramatic changes in MYC transcript expression levels in *ETV6-RUNX1* compared with other subtype of B-ALL, and at the protein level, we show that ectopic expression of *ETV6-RUNX1* does not affect MYC expression. We thus speculate that the deregulation of MYC network may be attributed to functional interplay between MYC and *ETV6-RUNX1* transcriptional activities. MYC forms highly stable heterodimers with MYC interacting factor X (Max) through their respective basic helix-loop-helix leucine zipper (bHLHZ) domains, which specifically bind the E-box (5'-CACGTG-3') DNA sequences (Nair and Burley, 2003) and recruit different cofactors for transcriptional activation or repression (Conacci-Sorrell *et al.*, 2014; Diolaiti *et al.*, 2014). Our analysis demonstrated that, in *ETV6-RUNX1* samples, MYC/Max interaction was not affected, whereas some MYC interactions with cofactors, such as Miz-1, were



**FIGURE 5:** Network of TFs in (A) *ETV6-RUNX1* and (B) *TCF3-PBX1* fusion. Left, circles represent TFs that have at least one protein–DNA interaction (PDI) predicted as perturbed; colors correspond to the class of transcription factor. Red/gray edges represent the perturbed/not-perturbed interactions, respectively. Right, chart showing the number of perturbed interactions for each transcription factor class according to TF class (Wingender et al., 2013).



**FIGURE 6: MYC regulators and the cell cycle.** Network showing HBCI interactions for transcription factors regulating Myc. Red edges represents interactions detected as perturbed; gray edges, interactions not detected as perturbed. (A) For *ETV6-RUNX1*. (B) For *TCF3-PBX1*. (C) Network showing HBCI interactions for cyclins (CCNs; light magenta), cyclin-dependent kinases (CDK; light blue), and cyclin-dependent kinase inhibitors (CDKs; light green) relative to MYC, *RUNX1*, *ETV6*, and *MIZ-1*.

perturbed (Figure 6, red vs. blue lines). Perturbation of MYC/Miz-1 interaction may suggest that the *ETV6-RUNX1* fusion could preferentially target the repression function of MYC. It is possible that *ETV6-RUNX1* fusion interferes with formation of MYC/Miz-1 complex and recruitment of MYC to target gene transcriptional initiators, as previously reported for the interplay between transforming growth factor- $\beta$ /Smad signaling pathway and MYC/Miz-1 complex to control p15INK4b and p21WAF1 CDK inhibitors (Seoane *et al.*, 2001, 2002, 2004). In addition, *ETV6-RUNX1* may also exert its effect on the MYC network by binding to its target promoters (Supplemental Table S10), as shown here for the *CDKN1A* promoter (Supplemental Figure 1, C and D).

Another important result from this study is the possible involvement of the *TCF3-PBX1* fusion protein in mRNA transport. RNA export is a central process in gene expression regulation and is an exciting new field in cancer biology. Although overexpression of some components of the mRNA export machinery, such as nucleoporins Nup88 and Nup214 (von Lindern *et al.*, 1992; Xu and Powers, 2009; Kohler and Hurt, 2010), CRM1 (Noske *et al.*, 2008), eIF4E (Borden and Culjkovic-Kraljacic, 2010), and GANP, the nuclear adapter for NXF1 (Fujimura *et al.*, 2005), have been associated with other types of cancer, including B lymphomas, our data constitute the first report implicating *TCF3-PBX1* in RNA localization and interaction with an export factor, NXF1 (Supplemental Figure S2). Similar to overexpression of eIF4E being efficiently inhibited by ribavirin in acute myelogenous leukemia (Kentsis *et al.*, 2004), targeting *TCF3-PBX1*/mRNA export pathway interactions could lead to effective ALL therapies.

In conclusion, our study establishes the feasibility of predicting specific perturbations of molecular interactions based on gene

expression profiles from multiple experiments and different biological conditions. Of importance, we show that integration of interactome data with differences of correlation between expression profiles could classify subtypes within the same lineage and provide specific potential targets.

## MATERIALS AND METHODS

### Experimental data

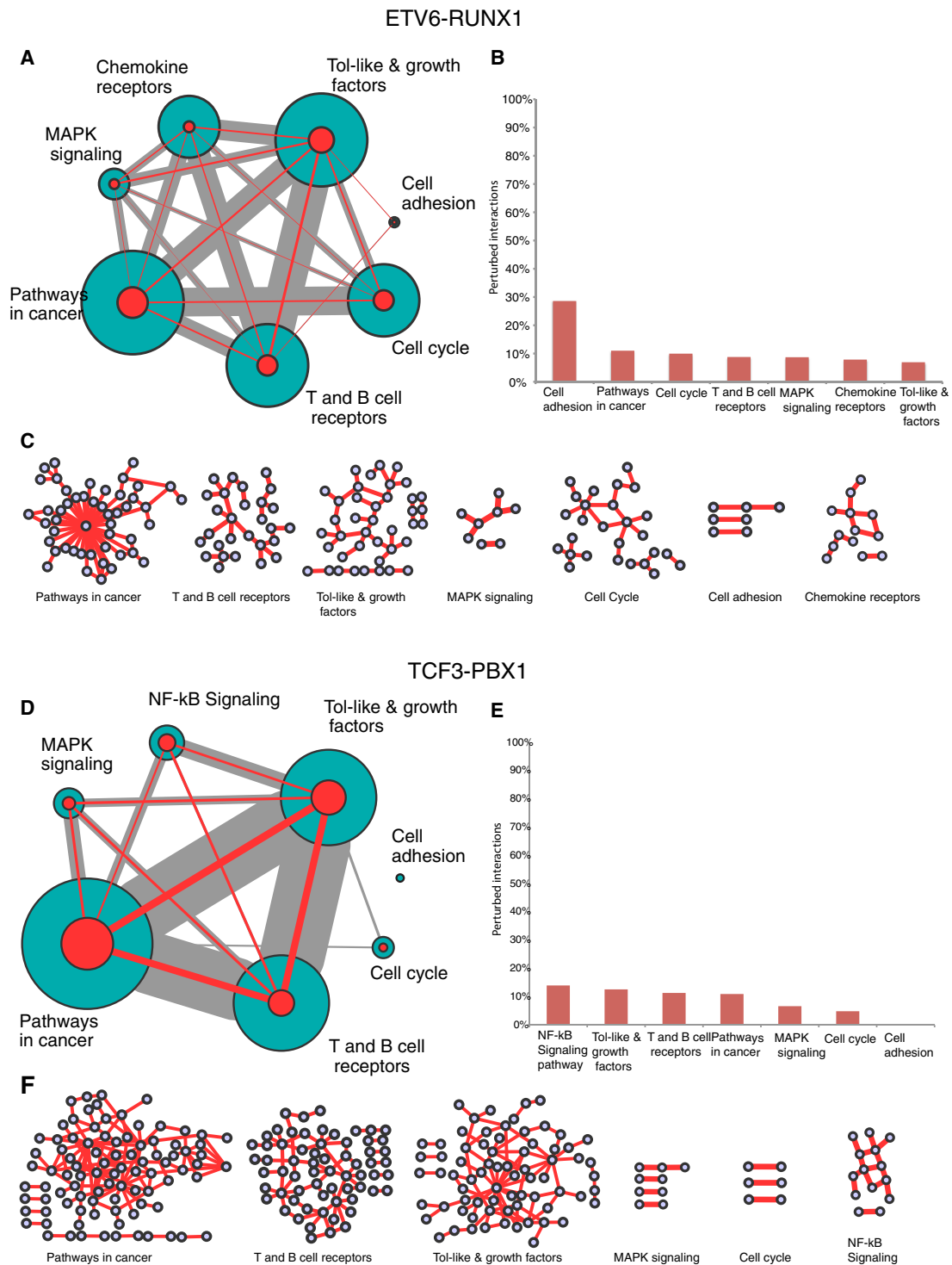
We downloaded from Gene Expression Omnibus (GEO) the Affymetrix HG-U133A expression data sets (GSE13425, GSE12995), comprising 190 and 175 ALL samples, respectively. These data sets contain 24 samples with *BCR-ABL1* fusion, 77 with *ETV6-RUNX1* fusion, 16 with *TCF3-PBX1* fusion, and 248 with various other genetic subtypes (Den Boer *et al.*, 2009; Mullighan *et al.*, 2009)

The list of protein interactions was retrieved from the HBCI (Lefebvre, 2007, 2010), among which were 21,156 protein-protein interactions (PPIs), 41,568 protein-DNA interactions (PDIs), and 1925 transcription factor-modulator interactions (TFMIs). We also analyzed the network composed of known cellular pathways in Pathway Commons (Cerami *et al.*, 2011) to predict affected cellular pathways. The Pathway Commons version of 27 October 2011 used in our study contains 2308 pathways collected from multiple sources (HumanCyc, Reactome, NCI-Pathways Interactions Database, Biocarta, and KEGG; Romero *et al.*, 2005; Matthews *et al.*, 2009; Schaefer *et al.*, 2009; Kanehisa *et al.*, 2012).

### Prediction of disrupted interactions

Inspired by an oncogene prediction method (Mani *et al.*, 2008), we detected changes in correlation of expression between gene pairs





**FIGURE 7:** Pathways enrichment of genes having at least one interaction predicted as perturbed in 1) *ETV6-RUNX1* and 2) *TCF3-PBX1* fusions. (A, D) The size of the circle represents the number of genes involved in the pathway, and the edges size represents the number of shared genes. The size of a green/red circle represents the total number of interactions in HBCI/perturbed interactions between genes involved in the pathway, respectively. The edge size represents the shared interactions (gray for interactions in BCI and red for the perturbed ones) between pathways. (B, E) Proportion of perturbed interactions in each pathway. (C, F) Networks of predicted perturbed interactions in each pathway. Circles represent genes; edges, interactions.

in different groups of patients: gene pairs whose expression correlation values show significantly different values between a test group (e.g., the ALL associated to a particular gene fusion) and a control group of samples (e.g., all other ALL samples).

First, microarray expression profiles are normalized using *fRMA* (McCall and Irizarry, 2011). Second, for each genotype (fusion) and each interaction in the HBCI, we computed the difference of correlations of expression profiles between a genotype of interest (exhibiting

Pathway name	Number of perturbed interactions	Number of interactions in the current pathway	Corrected <i>p</i>
<b>A. ETV6-RUNX1</b>			
Validated targets of c-Myc transcriptional activation	48	489	2.92E-25
Validated targets of c-Myc transcriptional repression	27	272	7.26E-14
Regulation of nuclear SMAD2 3 signaling	41	716	2.56E-13
RNA polymerase II transcription termination	31	576	3.43E-9
Cleavage of growing transcript in the termination region	31	576	3.43E-9
Postelongation processing of the transcript	31	576	3.43E-9
Postelongation processing of intron-containing pre-mRNA	24	406	6.79E-8
mRNA 3'-end processing	24	406	6.79E-8
c-Myc pathway	13	106	1.15E-7
Regulation of nuclear $\beta$ -catenin signaling and target gene transcription	22	362	1.56E-7
<b>B. TCF3-PBX1</b>			
RNA transport	209	1742	5.55E-17
Transport of mature transcript to cytoplasm	138	1004	9.85E-16
Transport of mature mRNA derived from an intron-containing transcript	126	934	8.65E-14
Noncoding RNA metabolism	101	703	1.09E-12
Small nuclear ribonucleoprotein assembly	101	703	1.09E-12
mRNA surveillance pathway	78	595	8.85E-8
Transport of the stem-loop binding protein-dependent mature mRNA	56	378	2.87E-7
Regulation of nuclear SMAD2 3 signaling	86	716	6.49E-7
Transport of mature mRNAs derived from intronless transcripts	57	404	9.90E-7
TNF $\alpha$	244	2784	3.27E-6

Corrected *p* value is computed using Benjamini–Hochberg multtesting correction.

**TABLE 2:** The 10 top perturbed pathways after (A) ETV6-RUNX1 and (B) TCF3-PBX1 gene fusion.

a genotype of interest) and other genotypes. We selected interactions showing significant differences of correlation for both Pearson and Spearman rank correlation coefficients. To detect interactions with significant differences of correlation, we generated 10,000 data sets permuting expression values across the whole table of the original data set. For each random set, we computed the difference of correlation in the same way as for the original data and computed the *p* value and corrected *p* value (using Benjamini–Hochberg multiple testing correction) from the distribution of all differences of correlation values. Interactions with corrected *p* < 0.05 for both Pearson and Spearman correlation measures are predicted as perturbed.

#### Difference of correlation between expression profiles

We computed Spearman's rank correlation ( $\rho$ ; Best and Roberts, 1975) and Pearson's correlation (*r*; Pearson, 1895) using the following formulas:

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}$$

$$r = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2} \sqrt{\sum_{i=1}^n (y_i - \bar{y})^2}}$$

where  $X = (x_1, \dots, x_n)$  and  $Y = (y_1, \dots, y_n)$  are vectors representing expression profiles of two genes/proteins in interaction; *n* is the number of samples;  $i = \{1, \dots, n\}$ ;  $d_i = x_i - y_i$  represents the difference between ranks; and  $\bar{x}$  and  $\bar{y}$  are the sample means of the *X* and *Y* vectors, respectively.

The difference of correlations ( $\Delta_{\text{cor}}$ ) of two genes between a genotype of interest (*gi*) and other genotypes (*og*) was computed as

$$\Delta_{\text{cor}} = \text{cor}(X, Y)_{\text{gi}} - \text{cor}(X, Y)_{\text{og}}$$

where  $\text{cor}(\dots)$  represents the correlation function (Spearman's rank or Pearson's correlation).

## Topological analysis

The characteristic path length (cpl) of a graph  $G$  is the average length of the shortest paths between all distinct pairs of vertices in the graph (Watts and Strogatz, 1998). In a nondirected graph, the characteristic path length  $L(G)$  is computed as follows:

$$L(G) = \frac{1}{|V|(|V|-1)} \sum_{v \in V} \sum_{v' \in V \setminus \{v\}} d(v', v)$$

where  $V$  is the set of vertices and  $d(v, v')$  is distance between vertices  $v$  and  $v'$ , that is, the length of the shortest path joining them.

The edge-betweenness centrality (ebc) or  $B(e)$  is defined as

$$B(e) = \sum_{s \neq v \neq t \in V} \frac{\sigma_{st}(e)}{\sigma_{st}}$$

where  $\sigma_{st}(e)$  is the number of shortest paths between vertex  $s$  and  $t$  that pass through the given edge (Newman, 2010).

The edge clustering coefficient (EC) is computed as the number of triangles to which a given edge belongs, divided by the number of triangles in which the edge may possibly participate at most, given the degrees of the adjacent nodes (Wang *et al.*, 2012):

$$EC(u, w) = \frac{\lambda_G(u, w)}{\min(d(u) - 1, d(w) - 1)}$$

where  $\lambda_G$  denotes the number of triangles that include the edge  $(u, w)$  and  $d(u)$  and  $d(w)$  are degrees of  $u$  and  $w$ , respectively. The  $\min(d(u) - 1, d(w) - 1)$  is the number of triangles in which the edge  $(u, w)$  may possibly participate at most.

## Cell culture and transfection

HeLa and HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin/streptomycin. The same medium was used to culture ETV6-RUNX1 (E/R)-expressing HEK 293T clones generated as previously described (Fuka *et al.*, 2012). The ETV6-RUNX1 (E/R)-positive leukemia cell line REH was cultured in RPMI 1640 supplemented with 10–20% FBS and antibiotics as recommended by the distributor (DSMZ, Braunschweig, Germany). Knockdown of E/R was performed as previously described (Fuka *et al.*, 2012) using validated lentiviral vector encoding a U6 promoter-driven shRNA targeting the E/R fusion sequence. Knockdown of E/R was monitored by quantitative reverse transcription PCR using published primers and probe sets (Fuka *et al.*, 2012), and cells with at least 50% reduction were used in ChIP and Western blot experiments.

## Plasmids

Plasmids pFlag-TCF3, pFlag-PBX1, and pYFP-NXF1 were generated from the corresponding entry clones (human ORFeome 7.1) by LR recombination into pDEST1899 (Flag N-ter vector) or pDEST491 (YFP N-ter vector; gift of James L. Hartley and Dominic Esposito, Protein Expression Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD). TCF3-PBX1 (gift of David P. LeBrun, Queen's University, Kingston, Canada) and ETV6-RUNX1 (gift of Guy Leclerc, University of Miami, Miami, FL) cDNA clones were also subcloned by Gateway technology into pDEST1899 Flag N-ter vector.

## Luciferase reporter assays

HEK293 cells were transfected with 1  $\mu$ g of reporter plasmid (p4XAP1-luc, pkB-luc, CMV-luc, pCDKN1A-luc), different amounts of effector plasmids (pFlag-TCF3, pFlag-PBX1, pFlag-TCF3-PBX1 or pFlag-ETV6-RUNX1, pMX-MYC), and 100 ng of a control *Renilla* luciferase construct using polyethyleneimine (Polysciences Europe,

Eppelheim, Germany) at 3  $\mu$ g/ $\mu$ g of DNA. For *CDKN1A* promoter activation, cells were treated with 100  $\mu$ g/ml of PMA. At 24 h post-transfection, cells were lysed and luciferase activities determined.

## Chromatin immunoprecipitation

We collected 10<sup>7</sup> REH-G1, REH-C, HEK293-E/R-V5, or HEK295-V5 cells, performed DNA–protein cross-linking using 1% formaldehyde for 8 min at room temperature, and stopped the fixation by adding 125 mM glycine for 5 min at room temperature. Cells were collected and lysed using lysis buffers iL1 and iL2 according to the manufacturer's instructions (Diagenode, Liege, Belgium), and chromatin DNA was sheared by sonication for two or three runs of 10–30 cycles (depending on the cell line: two runs of 10 cycles for HEK293 and three runs of 30 cycles for REH cells), using the Bioruptor (Diagenode). DNA–protein complexes were immunoprecipitated overnight using validated specific ChIP antibodies for MYC or ETV6 proteins (Seitz *et al.*, 2011; Torrano *et al.*, 2011; N-262 and N-19, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) and positive and negative control antibodies (histone H3 rabbit and normal rabbit immunoglobulin G). An aliquot (10%) was used for regular PCR amplification using specific primers, human RPL30 Exon 3 (7014; Cell Signaling, Danvers, MA) as positive control for histone H3 immunoprecipitation and *CDKN1A*-specific primers (forward, 5'-ACTGCCCTATTTGGGAC-3'; and reverse, 5'-GATCA-CATACCCTGTTC-3'). The remaining samples (10–20 ng of immunoprecipitated DNA) were used for ChIP-seq library sample preparation and subjected to HiSeq Illumina sequencing according to the manufacturer's instructions (Illumina, San Diego, CA).

The resulting reads were mapped to the human genome (GRCh 37/hg19) using BWA, version 0.6 (Li and Durbin, 2009). We used SWEMBL, version 3.3.1 (ebi.ac.uk/~swilder/SWEMBL/), to identify regions of the genome where multiple reads align (peaks). We adjusted parameters for ChIP-seq and reference (Input) sequence relative to the number of reads in the samples, with a relative gradient of 0.002 ( $R$  parameter). The resulting peaks were submitted to GREAT, version 2.0.2 (McLean *et al.*, 2010), to identify gene targets. We assigned each gene to a "regulatory domain" (Dostie *et al.*, 2006; Lieberman-Aiden *et al.*, 2009; Schoenfelder *et al.*, 2010) of a minimum distance of 5.0 kb upstream and 1.0 kb downstream from its transcription start site. We set the extension of the regulatory domain up to 1000.0 kb in both directions. Then each DNA-binding region was associated with all genes whose regulatory domain it overlaps. Comparison between identified target gene lists and statistical analysis were performed using the R statistical package.

## Immunofluorescence and confocal microscopy

HeLa cells were seeded onto coverslips in a 24-well plate and transfected with 1  $\mu$ g of pFlag-TCF3-PBX1 and/or pYFP-NXF1 plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 24 h post-transfection, cells were washed with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 for 15 min at room temperature, and incubated with anti-flag M2 antibody (Sigma-Aldrich, St. Louis, MO) diluted in immunofluorescence (IF) buffer (5% FBS, 0.1% Tween-20 in PBS) for 1 h at room temperature. After extensive washes, cells were incubated with Alexa 568 secondary antibody diluted in the IF buffer and for 1 h at room temperature. Where indicated, cells were also incubated with the SYTO RNaselect marker (Invitrogen) and mounted with Prolong gold Antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen). Slides were analyzed by confocal microscopy using the Nikon A1R system (Melville, NY) and images processed with Imaris software (Bitplane, Zurich, Switzerland).

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[AQ 2]

**ETOC:**

Genomic variations such as gene fusions are directly or indirectly associated with human diseases. A method is presented combining gene expression and interactome data analyses to identify specific targets in leukemia. The Myc network and the mRNA export machinery are perturbed in ETV6-RUNX1 and TCF3-PBX1 subtypes of leukemia.

# Author Queries

[AQ 1]: Please provide list of abbreviations.

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