Identification of Novel Post-transcriptional Targets of the BCR/ABL Oncoprotein by

Ribonomics: Requirement of E2F3 for BCR/ABL Leukemogenesis

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Running Title: Ribonomics and E2F3 in blast crisis CML

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ABSTRACT

Several RNA binding proteins (RBPs) have been implicated in the progression of chronic myelogenous leukemia (CML) from the indolent chronic phase to the aggressively fatal blast crisis. In the latter phase, expression and function of specific RBPs are aberrantly regulated at transcriptional or post-translational levels by the constitutive kinase activity of the BCR/ABL oncoprotein. As a result, altered expression/function of RBPs leads to increased resistance to apoptotic stimuli, enhanced survival, growth advantage and differentiation arrest of CD34+ progenitors from CML blast crisis patients. Here, we identified the mRNAs bound to the hnRNP A1, hnRNP E2, hnRNP K, and La/SSB RBPs in BCR/ABL-transformed myeloid cells. Interestingly, we found that the mRNA encoding the transcription factor E2F3 associates to hnRNP A1 through a conserved binding site located in the E2F3 3’UTR. E2F3 levels were upregulated in CML-BCCD34+ in a BCR/ABL-kinase- and hnRNP A1 shuttling-dependent manner. Moreover, by using shRNA-mediated E2F3 knock-down and BCR/ABL-transduced lineage-negative bone marrow cells from E2F3+/+ and E2F3−/− mice we show that E2F3 expression is important for BCR/ABL clonogenic activity and in vivo leukemogenic potential. Thus, the complexity of the mRNA:RBP network, together with the discovery of E2F3 as an hnRNP A1-regulated factor, outlines the relevant role played by RBPs in post-transcriptional regulation of CML development and progression.
INTRODUCTION

CML, a clonal myeloproliferative disorder of the pluripotent hematopoietic stem cell, is clinically characterized by a chronic phase (CML-CP) that, if untreated, progresses into a rapidly fatal blast crisis (CML-BC). Responsible for CML induction and maintenance is the BCR/ABL oncoprotein, product of the Philadelphia chromosome (Ph1) translocation t(9;22)(q34;q11). Although the mechanisms responsible for disease progression remain poorly understood, increased BCR/ABL expression in CML-BC significantly contributes to the phenotype of leukemic progenitors. Enhanced and unrestrained BCR/ABL kinase activity alters processing, export and translation of specific mRNAs, thereby controlling survival and differentiation of myeloid progenitors. In fact, aberrant expression of various RNA binding proteins (RBPs), which bind mRNA in a sequence-specific manner, is among the changes found in primary blasts from CML-BC patients and in BCR/ABL-transformed murine myeloid progenitors. Different BCR/ABL-dependent mechanisms alter RBP expression/function, some of which involve PI-3K/Akt-, ERK- or PKC-mediated phosphorylation events leading to enhanced gene transcription (e.g. hnRNP-K) or increased protein stability (e.g. TLS/FUS, hnRNP-A1, hnRNP-E2 and La/SSB). Conversely, expression of the RNA binding protein CUGBP1 inversely correlates with BCR/ABL activity and diminishes in CML-BC compared to CML-CP. Altered expression of these RBPs contributes to differentiation arrest and resistance to apoptosis of CML-BC progenitor cells, and results in either loss-of-function of tumor suppressors (e.g. C/EBPα and PP2A), increased expression of proteins favoring BCR/ABL leukemogenesis (e.g. MYC, Bcl-XL, SET and MDM2) or altered expression of differentiation regulatory factors (e.g. C/EBPβ, IL-3R and G-CSFR). Moreover, molecular and/or pharmacologic interference with the expression/activity of these RBPs antagonizes BCR/ABL oncogenic
potential both in vitro and in vivo by inhibiting survival, restoring differentiation, and/or impairing proliferation of BCR/ABL+ hematopoietic progenitors\textsuperscript{6-9,11-14}. Thus, increased BCR/ABL activity post-transcriptionally controls expression of factors essential for the establishment and/or maintenance of a CML-BC phenotype.

Here we employed RIP (RNA Immuno-Precipitation)-Chip assays\textsuperscript{15} to characterize the mRNAs associated with those RNA-binding proteins (hnRNP-A1, E2, K and La/SSB) whose expression is altered in CML-BC\textsuperscript{5}. Among the identified RBP-associated mRNAs, several encode important factors whose gain- or loss-of-function has been associated with altered proliferation, survival and/or differentiation of hematopoietic cells, or with malignant transformation\textsuperscript{16-27}. Interestingly, expression of the transcription factor and regulator of proliferation E2F3\textsuperscript{28} is higher in CD34\textsuperscript{+} bone marrow (BM) progenitors from CML-BC than CML-CP patients and is regulated by BCR/ABL through hnRNP-A1. Furthermore, the clonogenic potential of primary mouse BCR/ABL\textsuperscript{+} lineage negative (Lin\textsuperscript{-}) progenitors and their ability to induce leukemia in mice is markedly impaired in BCR/ABL\textsuperscript{+} E2F3\textsuperscript{-/-} myeloid progenitors and upon shRNA-mediated downregulation of E2F3 expression. Thus, aberrant RBP expression plays an essential role in post-transcriptional control of BCR/ABL leukemogenesis.

**MATERIALS AND METHODS**

**Cell cultures and primary cells.** The IL-3-dependent 32Dcl3, 32D-BCR/ABL, K562, and TonB210.1 cells were maintained in culture in IMDM, 10% FBS, and 2mM L-glutamine (Gibco). Where indicated, 10% WEHI-conditioned medium was used as source of mIL-3. 32D-BCR/ABL- and K562-derivative lines were generated by retroviral infection followed by sorting of GFP\textsuperscript{+} (green fluorescent protein) cells or antibiotic-mediated selection\textsuperscript{6,9,10,12}. Frozen samples
of CD34+ bone marrow cells from healthy donors (NBM) were purchased from Cincinnati Children’s Hospital (Cincinnati, OH). Frozen samples of mononuclear cells from BM of unidentifiable CML-CP and CML-BC patients were Ficoll-separated and directly lysed in Laemmli buffer (2x10^5 cells/20 µl) for Western blot analysis or used for isolation of the CD34+ fraction (CD34-MultiSort kit; Miltenyi Biotec). Prior to use in different assays, CD34+ progenitors from healthy donors and CML patients were kept overnight in IMDM containing 30% FBS, 2 mM glutamine, rhIL-3 (20 ng/m), rhIL-6 (20 ng/m), rhFlt-3 ligand (100 ng/m) and rhKL (100 ng/ml) (Stem Cell Technologies Inc.). All studies with human specimens were performed with approval of The Ohio State University (OSU) IRB. Murine hematopoietic BM cells from femurs of wild-type and E2F3-/- mice underwent lineage negative (Lin-) magnetic-activated depletion (MACS, Miltenyi Biotech) and were grown for 2 days in IMDM containing mKL (10 ng/ml), mIL-3 (2 ng/ml), IL-6 (1.2 ng/ml), mFlt3-ligand (5ng/ml), and mGM-CSF (5ng/ml) prior to infection with MigRI-BCR/ABL (W. Pear, UPENN, Philadelphia, PA) or MigR1 vector.

Plasmids. pSuper.retro-E2F3a and pSuper.retro-E2F3ab: The shRNA retroviral constructs for E2F3a and E2F3ab were obtained by subcloning the E2F3a-specific (5’-CCCTTCCACCAGCTGCTGTG-3’) or the common E2F3ab (5’-GGGCAAGAGACTGGAAGAGCTGCA-3’) sequences, targeting mE2F3 mRNA (NM_010093) at nucleotides 578-597 and 836-854, respectively, into the pSUPER.retro.neo+GFP vector (OligoEngine Inc.). pSuper.retro-hnRNP-A1 and pSuper.retro-hnRNP-E2: To generate shRNAs targeting hnRNP A1 and hnRNP E2, the hnRNP A1 (5’-AGCAGAGATGGCTAGTG-3’) and hnRNP E2 (5’-GGGAATTTGCTGAGAGAA-3’) sequences were subcloned into pSUPER.retro.neo+GFP. The MigR1-A1-HA, MigR1-hnRNP-K-HA, pMSCV-Flag-hnRNP-E2 and pSUPER.retro.HNRPK were previously described6,9,10,12,14.
RNA immunoprecipitation, microarray hybridization, statistical and bioinformatics analysis, and PCR-mediated validation of microarray data. $10^8$ parental and hnRNP-A1-HA-, Flag-hnRNP-E2-, and hnRNP-K-HA-expressing K562 cells were washed with ice-cold PBS, collected by low-speed centrifugation, lysed in ~2 pellet/volumes of 10 mM HEPES [pH 7.0], 100 mM KCl, 1 mM MgCl$_2$, 1 mM DTT, 0.5% Nonidet P-40, 100 U/ml RNase-OUT (Invitrogen), 0.2 mM PMSF, 1 mg/ml pepstatin A, 5 mg/ml bestatin and 20 mg/ml leupeptin, and clarified (100,000xg, 2.5h). To isolate hnRNP-A1-associated mRNAs, RNP-containing particles were purified from the S100-fraction by centrifugation (300,000xg, 3h) and resuspended in 1 ml of 50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM MgCl$_2$, 0.05% NP-40, 100 U/ml RNase-OUT, 0.2 mM PMSF and 20 mM EDTA. Immunoprecipitation (IP) and extraction of the hnRNP-A1-, hnRNP-K-, hnRNP-E2- and La/SSB-bound mRNAs was performed as described$^{15}$. Antibodies used for IPs were: monoclonal anti-hnRNP-A1$^{29}$, anti-hLa Go-serum$^{30}$, anti-Flag (clone M2, Sigma) and anti-HA (Covance). For hnRNP-A1, mRNA was extracted from HA-hnRNP-A1-expressing K562 cells using anti-hnRNP-A1 and anti-HA antibodies to isolate mRNAs associated to endogenous and exogenous hnRNP-A1, respectively. mRNAs isolated with anti-HA antibody from lysates of parental K562 cells served as negative controls. Signals were compared to those obtained by using mRNA from mRNP-enriched lysates (input) of HA-hnRNP-A1-expressing K562 cells. For the isolation of hnRNP-K- and hnRNP-E2-bound mRNAs, mRNA was obtained from anti-HA and anti-Flag IPs from HA-hnRNP-K- and Flag-hnRNP-E2-expressing K562 cells, respectively. Total RNA from the K562-derivative cell lines (input) was used as positive control, whereas anti-Flag or anti-HA IPed mRNAs from parental K562 served as negative controls. To isolate La/SSB-associated mRNA, anti-hLa IP from parental K562 lysates were used as source of mRNA. As a control, mRNA was isolated from IPs
with non-related isotype-matched antibody and K562 cell lysates. Immunoprecipitated RNA (1.0 µg) was processed for labeling and hybridization with HG-U133AB (hnRNP-A1-associated mRNAs) or HG-U95 chips (hnRNP-E2-, hnRNP-K-, and La-associated mRNAs) by the University of Pennsylvania (UPENN) Microarray facility and OSU Comprehensive Cancer Center (CCC) Microarray Facility. Arrays were scanned using the Affymetrix Agilent scanner, and the signal calculated for any given gene was the average of triplicate cDNA spots. Raw intensity files were processed using MicroArray Suite (MAS), version 5.0 (Affymetrix). Negative values with hybridization intensities below the background signal were excluded, and remaining signals were normalized after background subtraction using global median normalization (www.bioconductor.org). For each experiment, profiles were first statistically analyzed (pairwise comparison with a p value <0.05) in Microsoft Excel using as baseline the mRNA profile of HA-IP mRNA from parental K562 cells (which do not express any HA-tagged protein), and were subsequently compared to the input mRNP-enriched (or total) RNA profile. Comparison files for each experiment were then sorted to include mRNAs that were designated ‘present’ in the RBP-associated fraction >10-fold over the negative control and >5-fold over the input. Resulting gene data lists were uploaded to the WebGestalt data mining system (http://genereg.ornl.gov/webgestalt/) and analyzed for potential roles in cell growth, survival and differentiation and in cancer development/progression31. The obtained lists of genes where further analyzed a) for the presence in their UTRs of putative binding sites for the RBP to which they were found associated [hnRNP-A1 (UAGGGA/U)29, hnRNP-K [UC3-4(U/A)(U/A)]32, hnRNP-E2 (C3-5UC3-5)33 or La/SSB (GGGACCCU)12,34 ] and b) through PubMed for selection, based on published literature, of candidate genes with strong potential to be regulators of BCR/ABL leukemogenesis. Selected lists of the hnRNP-A1-, hnRNP-E2-, hnRNP-K- and
La/SSB-associated mRNAs (20 mRNAs for each RBP studied) are depicted in Fig. 1. It is noteworthy to mention that, due to the nature of this assay which strictly depends on the sensitivity of the antibody used in the IP, this is a qualitative assay rather than a quantitative microarray-based expression analysis. Because these RIP-Chip assays are aimed at identifying the mRNA subset associated to a specific RBP, the experiment was performed one time for each RBP and it was internally controlled by hybridization of arrays with mRNA immunoprecipitated with a non-specific antibody (negative control) and with the RNP-enriched lysate (positive control) as described above. Thus, the statistical analysis performed between the three data sets of each experiment will not provide a gene expression signature, but will rather allow for identification of mRNAs, which need to be singularly validated by RT-PCR-mediated IP-RNA analysis.

For validation, selected mRNAs were subjected to IP and RT-PCR amplification; 1.0µg of RNA from mRNP-enriched lysates, anti-hnRNP-A1-associated mRNA, and anti-Flag (or HA)-bound mRNA was treated with DNase I and reverse-transcribed using 200U MMLV-RT. cDNAs were used in RT-PCR and/or SYBR-green real-time PCR analyses. Primers were: Cyclin D2: 5’-GGTGCAGAAGGACATCCCAAC-3’ and 5’-GTGTCGTTGTTAATGACAGAC-3’; HOXB7: 5’-CTCAAGGAAACTGACCCCAAA-3’ and 5’-CGGCGCTCTGCTCTCTCTT-3’; SET: 5’-GAGGTCAAGATGATGCCAAA-3’ and 5’-TCAAGATGAAATCTTTGGAGAG-3’; SNF2β: 5’-ATGTCACCTCCGACACCACC-3’ and 5’-TCACTCTCTTGGCTGCACT-3’; ILF3: 5’-ATGCTTCAATGGAATTTTG-3’ and 5’-CAGCCATCAGATCCCGAG-3’; Tra2β: 5’-ATGACGACGGCTTGACCAAGCA-3’ and 5’-ACGCGGCGGTATATAAGCTT-3’; E2F3a: 5’-CAGGGACCCTCCAAGACGAC-3’ and 5’-AGCATGCCGCCATCTCAGA-3’; E2F3b: 5’-CTGTCGTAATGCCCTTTACA-3’ and 5’-GCTTGAACACTGCGGGCCAGCAT-3’.

REMSA and UV-crosslinking. 20 µg protein lysates were incubated (30 min., RT) with a 32P-labeled oligoribonucleotide (25x10^3 cpm) corresponding to nucleotides 2686-2709 (5’-
AGACCUCUAGGGGAGAAGACAUCG-3') of the hE2F3 mRNA 3'UTR containing the putative hnRNP-A1 binding site\(^{29}\) (underlined). Where indicated, oligoribonucleotide containing a duplicate consensus hnRNP-A1 binding site (5'-AGACCUCUAGGGGAGAAGACAUCG-3')\(^{29}\) was used as a competitor. hnRNP-A1 immunodepletion (ID) was performed by incubation of lysates (1 mg) with anti-hnRNP-A1-coated protein G beads. After IP, equal amounts of ID-supernatant and total lysate were used in REMSA. Binding reactions were incubated with 10 mg/mL heparin (20 min.) and loaded onto 5% PAGE for REMSA or UV-crosslinked and fractionated on SDS-PAGE\(^{10,12}\).

**Western blot analysis.** \(10^7\) cells were lysed in 100\(\mu\)l RIPA buffer and processed as described\(^{14}\). The antibodies used were: anti-phosphotyrosine (4G10), anti-SMAD4 (Upstate Cell Signaling Solutions); anti-STAT3 (Cell Signaling Technology, Inc.); anti-GRB2, anti-SIPA-1, anti-hLa (BD Transduction Laboratories); anti-FoxO1A, anti-FLAG(M2) (Sigma), anti-DOCK4 (Novus Biologicals); anti-hPML (PG-M3), anti-SNF2b(BRG-1), anti-E2F3(C-18), anti-MCL-1, anti-SET (Santa Cruz Biotechnology, Inc.); anti-PKC\(\beta\) (GeneTex, Inc.); anti-PI3KR3, anti-LATS1 (Abgent); anti-SOCS1 (Abcam, Inc.); anti-Tra2\(\beta\) (J.L. Manley, Columbia University, New York, NY)\(^{35}\); anti-HA (Covance); anti-hnRNP-A1 and anti-hnRNP-K (G. Dreyfuss, UPENN, PA)\(^{36,37}\); anti-hnRNP-E2 (R. Andino, University of California, CA)\(^{38}\); and anti-PPP2R3A (B.E. Wadzinski, Vanderbilt University, TN)\(^{39}\).

**Clonogenic assays.** Methylcellulose colony assays were performed by plating in 0.9% MethoCult (H4230, Stem Cell Technologies Inc.) in the presence or absence of mIL-3 (2.0 ng/ml), \(10^3\) 32D-BCR/ABL, E2F3a-, and E2F3ab-shRNA-expressing 32-BCR/ABL cells or \(10^4\) Lin\(^-\) BM cells from E2F3\(^{+/+}\) and E2F3\(^{-/-}\) C57BL/6J mice transduced with p210\(^{BCR/ABL}\) or the MigR1 vector. Colonies were scored after 7 (cell lines) and 15 (primary cells) days.
**Leukemogenesis in mice.** To determine the effect of E2F3 downregulation on *in vivo* leukemic cell-growth, parental and E2F3ab-shRNA-expressing 32D-BCR/ABL cells were injected (5x10^6 cells/mouse) subcutaneously into 5-week-old SCID mice (Taconic) and tumor growth was monitored daily for 15 days. To evaluate the importance of E2F3 expression for BCR/ABL leukemogenesis, two different approaches were used. First, 6-week-old SCID mice (n=2 per group) were intravenously-injected with 5x10^5 p210^{BCR/ABL}-expressing Lin^− E2F3^{+/+} or E2F3^{−/−} BM cells. BCR/ABL retroviral-transduction and transplantation assays were performed in the presence of recombinant murine KL (10 ng/ml), IL-3 (2 ng/ml), IL-6 (1.2 ng/ml), Flt3-ligand (5ng/ml), and GM-CSF (5ng/ml). Second, 6-week old SCID mice (n=13 per group) were intravenously-injected with 5x10^5 32D-BCR/ABL cells transduced with pSuper.retro-E2F3ab or empty vector. Mice were monitored daily for signs of general illness. BCR/ABL mRNA transcripts were detected by nested RT-PCR as described\(^{14}\). Mice injected with BCR/ABL-transformed wild type or null E2F3 BM cells were sacrificed at 15 weeks post-injection and organs were analyzed for signs of leukemia by gross visualization and histological examination. Formalin-fixed tissue sections from spleen, liver, and BM were hematoxylin/eosin-stained. E2F3^{−/−} C57BL/6J mice were maintained and genotyped as described\(^{40}\). Conversely, mice (n=3 per group) injected with wild type or E2F3 shRNA-expressing 32D-BCR/ABL cells were sacrificed at 3.5 weeks post-injection and analyzed in the same manner. The remaining mice were used for survival studies. All *in vitro* and *in vivo* animal experiments were done with OSU-ILACUC approval.
RESULTS

Identification of the hnRNP-A1, hnRNP-E2, hnRNP-K and La/SSB-associated mRNAs in BCR/ABL+ hematopoietic cells. To characterize the BCR/ABL-regulated post-transcriptional network controlling the phenotype of CML-BC progenitors, cytoplasmic mRNAs specifically associated with the BCR/ABL-regulated hnRNP-A1, hnRNP-E2, hnRNP-K and La/SSB were analyzed by oligonucleotide array hybridization using mRNA present in the anti-hnRNP-A1, -hnRNP-E2, -hnRNP-K or -La/SSB immunoprecipitates of CML-BC-derived K562 cells. To identify the mRNAs bound to hnRNP-A1, immunoprecipitates from the ribonucleoprotein (RNP)-enriched lysates of parental and MigR1-hnRNP-A1-HA-transduced K562 cells were used as source of mRNAs. RNA isolated from anti-HA or anti-Flag immunoprecipitates of parental K562 and mRNP-enriched K562 cytoplasmic lysates served as controls. Note that levels of mRNA immunoprecipitated with the anti-HA antibody were significantly lower than those isolated with the anti-hnRNP A1 antibody. Accordingly, expression of HA-hnRNP-A1 did not significantly increased total hnRNP A1 levels (not shown). Of the 33,000 total genes present on the HG-U133A microarray, 4,235 genes (~12.8 %) were detected in the mRNP-enriched K562-HA-hnRNP-A1 lysate (input). Among these 4,235 mRNAs, we found that 144 (~3.4 % of the total mRNP-associated mRNAs) were specifically bound to both endogenous hnRNP-A1 and ectopic HA-hnRNP-A1 and were also significantly enriched in the IPs compared to the mRNP-bound mRNA (input) (Fig. 1A, left). To ensure that the newly identified mRNAs were indeed bound to hnRNP A1, the hnRNP-A1-IP mRNA profile was assessed by using two different antibodies: one against the hnRNP-A1 protein (9H10) and the other against the HA-tagged hnRNP-A1 (anti-HA). Both profiles were first statistically (pairwise comparison with a p value <0.05) analyzed using as baseline the mRNA profile of HA-IP mRNA from parental K562 cells
(which do not express any HA-tagged protein), and were subsequently compared to the input mRNP-enriched RNA profile. Thus, a mRNA was considered truly associated to hnRNP A1 if it was meeting the following criteria: 1) it was present, regardless of its levels, in both anti-HA and anti-hnRNP-A1 immunoprecipitates from K562-HA-A1 cells; 2) its levels were at least 5 fold greater in the anti-hnRNP-A1 IP from K562-HA-A1 cells than in the anti-HA IP from parental K562 (negative control); and 3) its levels were at least 5-fold increased in the anti-hnRNP-A1 IP compared to the those revealed by the hybridization with the K562-HA-A1 RNP-enriched lysate (input/total).

Similarly, analyses of the microarray data performed with mRNAs isolated by anti-Flag, anti-HA or anti-hLa immunoprecipitations from cytoplasmic lysates of MigR1-Flag-hnRNP-E2-, MigR1-hnRNP-K-HA- or parental K562 cells, respectively, revealed that: a) 222 mRNAs, approximately 1.5% of the total K562 mRNAs (25.3% of the total number of mRNAs present on the HG-U95A) were enriched in the Flag-hnRNP-E2 immunoprecipitates (>10-fold over the negative control and >5-fold over the input) (Fig. 1B); b) 353 mRNAs, approximately 2.4% of the total K562 mRNAs, were enriched in the HA-hnRNP-K immunoprecipitates (>10-fold over the negative control and >5-fold over the input) (Fig. 1C); and c) 957 mRNAs, approximately 6.6% of the total K562 mRNAs were enriched in the La/SSB immunoprecipitates (>10-fold over the negative control and >5-fold over the input) (Fig. 1D). However, out of the 222 mRNAs bound to hnRNP-E2, only 56 (0.4%) were specifically bound and not enriched in the immunoprecipitates of the other poly(rC)-binding protein hnRNP-K (not shown). As a negative control for the hnRNP-E2 IP-RNA microarray hybridization, we used mRNA non-specifically bound to the anti-Flag antibody used in IP with lysates of parental K562 cells. Likewise, the mRNA non-specifically interacting with the anti-HA antibody used in IP with lysates of parental
K562 cells was used as a negative control for the hnRNP-K and La/SSB microarray experiments. Note that the mRNAs present in a similar amount in the negative control (e.g. anti-Flag in K562 cell lysate) and the specific IP (e.g. anti-Flag in Flag-hnRNP-E2-expressing K562 cell lysate) or those mRNAs present in equal amount in the specific IP and input (cytoplasmic mRNA or mRNA isolated from mRNP-enriched lysate) were not considered as specifically bound to the RBP. Interestingly, functional clustering and literature mining (see Methods section) of the hnRNP-A1- as well as hnRNP-E2-, hnRNP-K- and La/SSB-associated mRNAs revealed that a discrete number encode for factors that are genetically altered or aberrantly expressed in various types of cancers, including myeloid leukemias (Fig. 1, right panels).

Validation of the RIP-Chip data and expression analysis of the hnRNP-A1, hnRNP-E2, hnRNP-K and La-associated mRNAs. Sequence analysis of the human and mouse RBP-associated mRNAs (Fig. 1, right panels) revealed the presence of single or multiple conserved hnRNP-A1, hnRNP-K, hnRNP-E2 and La/SSB binding sites located in their 3’ or 5’ UTR (not shown). For example, the UAGGG(A/U)³⁹ hnRNP-A1 binding site was present as single element or multiple repeats in all human and mouse 3’UTRs of the hnRNP-A1-associated mRNAs (not shown). Notably, seven of these putative hnRNP-A1-associated mRNAs (Tra2beta, HOXB7, Cyclin D2, E2F3, ILF3, SNF2beta and SET) may play a relevant role in BCR/ABL leukemogenesis¹⁴,¹⁹-²¹,³⁵,⁴⁰-⁴³. The association of these mRNAs with hnRNP-A1 was validated by RT-PCR on mRNA isolated from the anti-hnRNP-A1-immunoprecipitated cytoplasmic K562 lysates. As expected, human cyclin D2, HOXB7, SET, SNF2β, ILF3, TRA2β, E2F3a or E2F3b mRNAs were detectable in both total and hnRNP-A1-enriched mRNA fractions but not in the anti-Flag immunoprecipitates, used as negative control (Fig. 2A). Importantly, high levels of E2F3a, E2F3b, SET, and SNF2β mRNA transcripts were clearly detectable in association with
hnRNP-A1 and similar to those present in the mRNP-enriched mRNA fraction (Fig. 2A). As we recently described for the hnRNP-A1-associated SET mRNA, E2F3, SNF2β and Tra2β protein (Fig. 2B, left) as well as ILF3 and HOXB7 mRNA (Fig. 2B, right) levels were higher in BCR/ABL-transformed than in parental 32Dcl3 myeloid precursors and correlated with those of hnRNP-A1 and BCR/ABL (not shown). Similarly, E2F3 and SNF2β were upregulated in the doxycycline-treated (2 µg/ml; 3 days) BCR/ABL-inducible TonB210.1 lymphoid precursors (Fig. 2B, left). In addition, treatment (24h, 2 µM) with the ABL tyrosine kinase inhibitor imatinib mesylate markedly impaired E2F3, SNF2β and Tra2β protein and ILF3 and HOXB7 mRNA expression in 32D-BCR/ABL and K562 cells (Fig. 2B), indicating that increased expression of those factors is BCR/ABL kinase-dependent. Consistent with the modulation of hnRNP-A1-regulated SET expression in CML-CPCD34+ and CML-BCCD34+ BM cells, SNF2β, E2F3 and Tra2β protein levels (no antibodies are available for ILF3 and HOXB7) correlated with BCR/ABL activity and hnRNP-A1 expression, and were higher in myeloid CML-BC than CML-CP BM mononuclear cells from paired patient samples (Fig. 2C). Furthermore, shRNA-mediated downregulation of hnRNP-A1 resulted in decreased SET, SNF2β and E2F3 protein expression (Fig. 2D). Thus, as we previously demonstrated for SET, BCR/ABL and hnRNP A1 activities may also account for E2F3, SNF2β and Tra2β upregulation in CML-BC.

By contrast, BCR/ABL kinase-dependent regulation of hnRNP-E2, hnRNP-K and La/SSB expression did not always result in altered expression of factors encoded by the associated mRNAs (Fig. 3). In fact, Western blot analyses showed that, among the proteins encoded by mRNAs identified as associated to hnRNP-E2 (STAT3, PI3KR3, PML, PKCβ1, PPP2R3A, and FOXO1A), only PML (promyelocytic leukemia gene) and FOXO1A (forkhead box O1A) levels were markedly upregulated in 32D-BCR/ABL (compared to parental cells) and inhibited by
imatinib treatment in both 32D-BCR/ABL and K562 cells (Fig. 3A). Conversely, expression of STAT3 and PI3KR3 remained unchanged upon imatinib treatment (Fig. 3), suggesting that hnRNP-E2-dependent translational regulation is not the primary mechanism regulating the expression of these factors in BCR/ABL+ cells. Moreover, levels of PKCβ1 (protein kinase C beta 1), which potentially undergoes regulation by hnRNP-E2 and hnRNP-K, were regulated by BCR/ABL activity in 32D-BCR/ABL but not in K562 cells (Fig. 3A). A similar incongruence was also observed when we assessed BCR/ABL-dependent expression of the factors whose mRNA was found specifically bound to the translational regulators hnRNP-K and La/SSB (Fig. 3). Indeed, only LATS1 (large tumor suppressor homolog 1), SIPA1 (signal-induced proliferation-associated gene), SMAD4, FOXO1A and MCL1 (myeloid cell leukemia sequence 1) expression was markedly increased in 32D-BCR/ABL cells and downregulated by imatinib treatment (Fig. 3A), suggesting that their expression may depend on translational regulation by the hnRNP-E2, hnRNP-K and/or La/SSB. Accordingly, shRNA-mediated hnRNP-E2 downregulation impaired FOXO1A but not PKCβ1, LATS1, and STAT3 expression in 32D-BCR/ABL cells (Fig. 3B, top). Likewise, hnRNP-K-shRNA expression resulted in decreased LATS1, SIPA1 and PKCβ1 but not DOCK4 expression (Fig. 3B bottom).

hnRNP-A1 interacts with the 3’UTR of E2F3 mRNA and its nucleocytoplasmic shuttling activity is required for E2F3 expression. Because the human and mouse 3’ UTRs of E2F3 mRNA transcripts (E2F3a and E2F3b) contain a conserved putative hnRNP-A1 consensus binding sequence (Fig. 4A), a ribo-oligonucleotide spanning nucleotides 2686-2709 of hE2F3 mRNA [E2F3(A1) rODN] was used in REMSA and UV-crosslinking to assess interaction with hnRNP-A1. A single RNA:protein complex was detected by REMSA in K562 and 32D-BCR/ABL lysates (Fig. 4B, lanes 2 and 4), whereas a faster migrating complex was detected in
parental 32Dc13 myeloid precursors (Fig. 4B, lane 1). Moreover, imatinib mesylate markedly reduced formation of this RNA:protein complex in 32D-BCR/ABL and K562 cells (Fig. 4B, lanes 3 and 5), suggesting that expression and/or binding of the E2F3 mRNA-interacting RBP is BCR/ABL kinase-dependent. To assess binding specificity, competition assays were performed with a synthetic rODN (hnRNP-A1 rODN) previously described to interact with hnRNP-A1 and sharing only the UAGGGA hnRNP-A1-binding site with the E2F3(A1) rODN (Fig. 4A). Addition of 100x hnRNP-A1 rODN specifically inhibited the RBP:E2F3(A1) complex formation in 32D-BCR/ABL and K562 lysates (Fig. 4B, lanes 7 and 8) but not in parental cells (Fig. 4B, lane 6), suggesting that hnRNP-A1 might be the RBP interacting with E2F3 mRNA. To formally demonstrate the hnRNP-A1:E2F3 mRNA interaction, 32D-BCR/ABL and K562 cell lysates were immunodepleted of hnRNP-A1 (see inset in Fig. 4B) and equal amounts of total (Fig. 4B, lanes 1-3) and hnRNP-A1-immunodepleted (Fig. 4B, lanes 9-11) lysates were used in REMSA with the E2F3(A1) rODN. Consistent with the presence of hnRNP-A1 in the RBP:E2F3(A1) complex, hnRNP-A1-immunodepletion impaired RNA:protein complex formation in BCR/ABL-expressing (Fig. 4B lanes 10 and 11) but not in parental (Fig. 4B, lane 9) cell lysates. The hnRNP-A1:E2F3 mRNA interaction was further confirmed by UV cross-linking in which a 32D-BCR/ABL and K562 protein of ~34 kDa corresponding to the hnRNP-A1 molecular weight interacts with E2F3(A1) rODN (Fig. 4B, right panel). Notably, the 32Dc13 protein interacting with the E2F3(A1) rODN has an apparent MW of ~25 kDa and does not require the UAGGGA element for binding RNA (see * in Fig. 4B, right panel).

To determine whether increased E2F3 expression in BCR/ABL+ cells requires hnRNP-A1 mRNA-export activity, E2F3 protein levels were evaluated in IL-3-cultured 32D-BCR/ABL-NLS-A1-HA cells, which express a nucleus-localized and shuttling-deficient hnRNP-A1 mutant.
(NLS-A1-HA) that binds mRNA and competes with wild-type hnRNP-A1 for nucleocytoplasmic export. Consistent with the ability of hnRNP-A1 to bind E2F3 mRNA, expression of the dominant negative NLS-A1-HA mutant resulted in decreased E2F3 levels (Fig. 4C). Thus, BCR/ABL-induced E2F3 expression requires hnRNP-A1 mRNA-export activity. Accordingly, imatinib treatment strongly inhibited E2F3 mRNA expression (inset Fig. 4C).

**In vitro and in vivo requirement of E2F3 expression for BCR/ABL leukemogenesis.** The E2F3 gene encodes both E2F3a, which is regulated by cell growth, and E2F3b, which is equally expressed in quiescent and proliferating cells. Similar to BCR/ABL and hnRNP-A1, E2F3a expression is higher in CD34+ BM cells from CML-BC (CML-BC\(^{CD34^+}\)) (n=3) than CML-CP (CML-CP\(^{CD34^+}\)) (n=3) patients (Fig. 5A, lanes 2, 3, 6-9). Interestingly, expression of E2F3a (slower migrating band) is higher than that of E2F3b (faster migrating band) in CML-BC\(^{CD34^+}\). Likewise, upregulation of E2F3a but not of E2F3b is evident in CML-CP\(^{CD34^+}\) when compared to CD34+ BM progenitors from a healthy donor (NBM\(^{CD34^+}\)) (Fig. 5A, lanes 1, 2, 4-7). By contrast, E2F3b is slightly higher in CML-CP\(^{CD34^+}\) than CML-BC\(^{CD34^+}\) (Fig. 5A, lanes 2 and 3). Additionally, E2F3a and E2F3b expression is more pronounced in the more immature CD34+ CML-BC BM progenitors than in the CD34- fraction (Fig. 5B), which includes more committed hematopoietic precursors and post-mitotic mononuclear cells that express low levels of the BCR/ABL oncoprotein. Consistent with the fact that the hnRNP-A1-interacting site is conserved in both E2F3a and E2F3b mRNAs, imatinib treatment (2 \(\mu\)M; 0-72h) markedly reduced E2F3a/b protein levels in CML-BC\(^{CD34^+}\) progenitors (n=2) (Fig. 5B, lanes 3-5 and 8).

To determine whether E2F3 plays an important role in the regulation of BCR/ABL-dependent proliferation and survival, we interfered with E2F3a and E2F3ab expression by retrovirally infecting 32D-BCR/ABL cells with the pSuper.retro-E2F3a and pSuper.retro-E2F3ab shRNA
constructs carrying a short hairpin RNA (shRNA) corresponding to nucleotides 578-597 (E2F3a) and 836-854 (E2F3ab) of the mouse E2F3 mRNA (NM_010093), respectively. In 32D-BCR/ABL cells, shRNA-mediated downregulation of E2F3a and E2F3ab (inset in Fig. 5C) did not significantly affect IL-3-dependent proliferation (not shown). By contrast, the growth factor-independent methylcellulose colony-forming ability of 32D-BCR/ABL cells was significantly reduced ($P<0.001$; t-test) by 60-70% and >90% in E2F3a-shRNA- and E2F3ab-shRNA-expressing, respectively, but not in vector-transduced cells (Fig. 5C). Accordingly, shRNA-mediated downregulation of both E2F3a and E2F3b expression exerted a profound effect on the ability of 32D-BCR/ABL cells to grow and form tumors in vivo. In fact, while SCID mice injected (5x$10^6$ cells/mouse, n=5 mice per group) subcutaneously with vector-transduced 32D-BCR/ABL cells formed tumors (incidence: 5/5) in 6-7 days, tumors in mice injected with E2F3ab shRNA-expressing cells were palpable (incidence: 5/5) only after 9-10 days post-injection (Fig. 5D). At 15 days post injection, all mice injected with E2F3ab shRNA-expressing cells developed tumors that weighed ~80% less than those formed by injection of vector-transduced 32D-BCR/ABL cells (Fig. 5D).

Because both E2F3a and E2F3b activities appear important for BCR/ABL leukemogenesis, albeit to different extents, the Lin$^-$ fractions of bone marrow cells (BMC) from E2F3$^{+/+}$ and E2F3$^{-/-}$ C57BL/6J mice$^{21,40}$ were retrovirally transduced with either MigR1-p210-BCR/ABL or MigR1 vector. Thereafter, GFP-sorted E2F3$^{+/+}$ and E2F3$^{-/-}$ p210-BCR/ABL-expressing Lin$^-$ BMCs were used to assess BCR/ABL-driven colony formation (Fig. 6A). Similar to the effects of E2F3ab shRNA, the clonogenic potential of GFP$^+$-BCR/ABL$^+$-Lin$^+$E2F3$^{+/+}$ BMC cultured in the absence of cytokines was significantly ($P<0.001$; t-test) lower than GFP$^+$-BCR/ABL$^+$-Lin$^-$ E2F3$^{+/+}$ BMCs (average: 64% reduction) (Fig. 6A, lanes 3 and 4). As expected, MigR1-
transduced GFP⁺-Lin⁺E2F3⁺/⁺ and GFP⁺-Lin⁺E2F3⁻/⁻ BMCs did not form colonies in the absence of cytokines (Fig. 6A, lanes 5 and 6). However, in the presence of IL-3, MigR1-transduced GFP⁺-Lin⁺E2F3⁻/⁻ BMCs showed a slightly decreased colony formation when compared to MigR1-GFP⁺-Lin⁺E2F3⁺/⁺ BMC (average: 25% reduction) (Fig. 6A, lanes 1 and 2), consistent with the role of E2F3 as regulator of cell growth and the ability of other E2F proteins to compensate for loss of E2F3 expression⁴⁶.

To determine whether lack of E2F3 activity impairs the ability of BCR/ABL to induce an acute leukemia-like process, retroviral-transduction/transplantation assays were performed with GFP⁺-BCR/ABL⁺-Lin⁺E2F3⁺/⁺ and GFP⁺-BCR/ABL⁺-Lin⁻E2F3⁻/⁻ BMC. After BCR/ABL-transduction, E2F3⁻/⁻ and E2F3⁺/⁺ GFP⁺-BCR/ABL⁺-Lin⁻ BMCs were intravenously injected into SCID mice (5x10⁵ cells/mouse, n=2 mice per group). At 12 weeks post-injection, mice transplanted with GFP⁺-BCR/ABL⁺-Lin⁺E2F3⁺/⁺ BMC, but not those transplanted with equal numbers of GFP⁺-BCR/ABL⁺-Lin⁻E2F3⁻/⁻ BMC, showed initial signs of general disease (rough coat and delayed response to external stimuli) and the presence in PB of circulating BCR/ABL⁺ cells as determined by nested RT-PCR (Fig. 6B). Because all mice that received p210-BCR/ABL E2F3⁺/⁺ BMCs appeared severely lethargic at 15 weeks post-transplant, cell-injected and age-matched mice were sacrificed and various organs evaluated by visual inspection and light microscopy. GFP⁺-BCR/ABL⁺-Lin⁺E2F3⁺/⁺ BMC-transplanted mice showed modest to massive splenomegaly, whereas morphology of spleens from GFP⁺-BCR/ABL⁺-Lin⁻E2F3⁻/⁻ BMC-injected mice resembled that of control age-matched mice (Fig. 6C). Hematoxylin/eosin-stained sections of spleen, BM and liver of E2F3⁺/⁺-BCR/ABL⁺-mice showed extensive infiltration of blasts typical of an overt acute leukemia-like process (Fig. 6D). Conversely, histopathology of organs from the GFP⁺-BCR/ABL⁺-Lin⁻E2F3⁻/⁻ BMC-injected group was similar to that of age-
matched controls (Fig 6D). In fact, BM from p210-BCR/ABL\(^+\)-E2F3\(^+\) and age-matched mice showed similar marrow cellularity with presence of myeloid cells at different stages of differentiation (Fig. 6D) and normal spleen and liver parenchymal architecture (Fig. 6D). In similar experiments, SCID mice were intravenously injected with vector- or E2F3ab shRNA-expressing 32D-BCR/ABL cells (10\(^5\) cells/mouse; n=13 mice per group) (inset in Fig. 5C). Age matched mice were used as controls. At 3 weeks post-injection, three mice per control group and 12 of 13 experimental mice were analyzed by nested RT-PCR for presence of circulating BCR/ABL\(^+\) cells. As expected, PB from mice receiving vector-transduced BCR/ABL (wild type) cells were all BCR/ABL\(^+\), whereas BCR/ABL transcripts were detectable in only 9 of 12 mice injected with E2F3ab shRNA-expressing 32D-BCR/ABL (shE2F3) cells (Fig. 6E). Note that because the 13\(^{th}\) shE2F3 mouse appeared severely sick, we did not withdraw blood from this animal that died 3.5 days later from an acute leukemia-like process (not shown). At 3.5 weeks post-transplant, 3 mice per group were sacrificed and subjected to histopathologic examination. Interestingly, all three shE2F3 mice (2 were BCR/ABL\(^+\) and 1 BCR/ABL\(^-\) at week 1 post-injection) showed normal spleen size (Fig. 6F), bone marrow cellularity and spleen architecture (Fig. 6G), similar to that of age-matched mice (control). By contrast, massive splenomegaly (average spleen weight was 300% increased if compared to that of spleens from control or shE2F3 mice) and substantial myeloid infiltration of spleen and bone marrow were observed in mice transplanted with vector-transduced 32D-BCR/ABL cells (wild type) Fig. 6F and 6G). Furthermore, at 3.75 weeks post-transplant, 80% of shE2F3 mice were alive whereas all wild type BCR/ABL\(^+\) mice were dead (Fig. 6H). Ultimately, all shE2F3 mice died of a BCR/ABL\(^+\) acute leukemia-like process within 4.5 weeks post-transplant (Fig. 6H). Furthermore, real-time PCR revealed no difference in E2F3 mRNA expression in spleens of wild type and shE2F3 mice.
(not shown), suggesting that the significantly delayed (median survival wild type vs. shEF3 $P<0.0001$, logrank test) leukemic process in the shE2F3 mice might result from expansion of a 32D-BCR/ABL cell population expressing normal E2F3 levels and/or a negative selection process against 32D-BCR/ABL cells expressing low E2F3 levels that, as shown in Fig. 5C and 6A, have impaired clonogenic potential.

**DISCUSSION**

Increased BCR/ABL expression/activity, as observed in CML-BC, induces pathways that drive myeloid progenitors towards a phenotype typical of malignant blasts\(^1\). A likely mechanism also involves the control of mRNA metabolism via altered expression/function of the RNA binding proteins hnRNP-A1, hnRNP-E2, hnRNP-K and La/SSB\(^5\). Some of these mRNAs (e.g. SET, C/EBP\(\alpha\), MYC and MDM2), which are targets of the post-transcriptional and/or translational regulatory activities of these RBPs, are essential for the BCR/ABL-dependent phenotype of CML-BC progenitors\(^8-10,12,14\). Here, microarray-mediated RIP-Chip analysis\(^47\) was used to screen for mRNA subsets specifically associated with and potentially regulated by hnRNP-A1, hnRNP-E2, hnRNP-K or La/SSB in BCR/ABL-transformed human leukemic cells. Global characterization and functional analysis (Ribonomics\(^15,48\)) of RNAs associated to RBPs involved in human diseases is not unprecedented. For example, the Fragile-X syndrome results from altered translation of a specific subset of mRNAs due to absence of the RNA binding protein FMR\(\alpha\)\(^49\). Interestingly, functional clustering and literature mining of mRNAs associated to hnRNP-A1, hnRNP-E2, hnRNP-K and La/SSB revealed that some of them (e.g. SET\(^14\), Tra2\(\beta\)\(^35\), cyclin D\(2\)\(^20,41\), E2F3\(^21,40\), HOXB7\(^19\), MYC\(^9,50,51\), PML\(^52\), MCL1\(^16,53\), MLL2\(^54\), MDM2\(^12,55\) and the SNF2\(\beta\)/BRG1\(^42,43\)) are key players not only in the determination of cell fate, but also in
tumor (leukemia included) development, maintenance and progression. Although it is still an open question whether all these mRNAs are truly post-transcriptionally/translationally-regulated by hnRNP-A1, hnRNP-E2, hnRNP-K or La/SSB, several lines of evidence support the specificity of RBP:mRNA binding and the accuracy of our RIP-Chip data. First, IP-RNA/RT-PCR-mediated validation of the hnRNP-A1 microarray data demonstrated that all the analyzed mRNAs (Fig. 2) were bound to hnRNP-A1. Secondly, computational analysis revealed the presence of conserved single or multiple binding sites for hnRNP-A1 (UAGGGA/U)\textsuperscript{29}, hnRNP-K \(\text{[UC}_{3-4}(U/A)(U/A)]\textsuperscript{32}\), hnRNP-E2 \(\text{(C}_{3-5}\text{UC}_{3-5})\textsuperscript{33}\) or La/SSB \(\text{GGGACCU}12,34\) within the 5’ and/or 3’ UTRs of all the RBP-associated mRNAs analyzed (not shown). Third, shRNA-mediated hnRNP-A1, hnRNP-E2 and hnRNP-K downregulation resulted in decreased expression of some but not all of the factors encoded by the RBP-bound mRNAs, suggesting that some of these mRNAs, despite their interaction with a specific RBP, are not regulated by its activity. Conversely, we demonstrated that these RBPs do play a crucial role in regulating the expression of other mRNAs (e.g. FOXO1A, E2F3 and SIPA1) in BCR/ABL-expressing cells. Indeed, some of these RBP-associated mRNAs (see tables in Fig. 1) have been described as associated to and regulated by one of these RBPs. For instance, hnRNP-A1 pre-mRNA associates to hnRNP-A1 protein\textsuperscript{56}; MYC expression is regulated by hnRNP-K\textsuperscript{9,57}; and MDM2 is translationally regulated by La/SSB\textsuperscript{12}. Notably, \textit{CEBPA} mRNA was not found among the hnRNP-E2-immunoprecipitated mRNAs because it is transcriptionally inhibited in K562 cells\textsuperscript{10}. Finally, seventeen of the hnRNP-E2-associated mRNAs were also present in a previous hnRNP-E2 RIP-Chip study\textsuperscript{33}. Moreover, consistent with the notion that the poly(rC)-binding hnRNP-K and hnRNP-E2 proteins bind RNA in a sequence specific manner\textsuperscript{58} and may simultaneously control mRNA
34% of the hnRNP-E2-associated mRNAs (74 mRNAs) were also bound to hnRNP-K (not shown).

While fewer mRNAs were in complex with each hnRNP, 967 mRNAs were found associated to La/SSB in K562 cells. Although non-specific interactions cannot be excluded, our data are in line with the reported broader role of La/SSB in the regulation of mRNA metabolism\textsuperscript{12,59,60}. Conversely, hnRNP-A1 primarily regulates mRNA processing and nuclear export, whereas hnRNP-E2 and hnRNP-K are mainly regulators of mRNA translation\textsuperscript{5,58,61}.

Expression analyses in BCR/ABL cell lines and/or primary blasts from CML-CP and CML-BC patients (Fig. 2 and 3) indicated that levels of proteins encoded by several but not all of the hnRNP-A1-, hnRNP-E2-, hnRNP-K- and La-associated mRNAs parallel those of the RBPs, which are induced by BCR/ABL and downregulated by imatinib\textsuperscript{6,9,10,12,14}. Thus, BCR/ABL may utilize these RBPs to directly control at the post-transcriptional and/or translational level expression of several of these factors, as reported for C/EBP\textgreek{a}, MYC, MDM2 and SET\textsuperscript{5}.

PPP2R3A mRNA, encoding one of the regulatory subunits of the protein phosphatase 2A (PP2A), was found in complex with hnRNP-E2 and La/SSB (Fig. 1). Accordingly, its expression was downregulated in BCR/ABL\textsuperscript{+} compared to parental cells (Fig. 3). Because the PPP2R3A 5'UTR (BC065531) contains multiple upstream open reading frames, it is conceivable that PPP2R3A undergoes hnRNP-E2- and/or La-dependent translational control. In agreement, we previously reported that BCR/ABL, to exert its leukemogenic potential, suppresses PP2A activity through a hnRNP-A1-SET-dependent mechanism\textsuperscript{14}. In fact, molecular (SET shRNA-mediated) or pharmacologic (forskolin, 1,9-dideoxy-forskolin or FTY720) reactivation of PP2A impairs clonogenic potential, restores differentiation and suppresses \textit{in vivo} leukemogenesis of wild-type and T315I BCR/ABL\textsuperscript{+} primary CML-BC and Ph1 ALL blasts\textsuperscript{14,62}. 

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Like SET, E2F3a and E2F3b mRNA transcripts interact with hnRNP-A1 through a binding site present in the 3’UTR. Consistent with the effect of BCR/ABL on hnRNP-A1 expression⁶,¹⁴, E2F3 mRNA and/or protein levels are higher in CML-BC⁷⁺ than CML-CP⁷⁺ patient-derived BM cells, in BCR/ABL-expressing cell lines compared to non-transformed cells, and decrease upon inhibition of BCR/ABL kinase activity. Interestingly, E2F3a rather than E2F3b seems to play a major role in controlling BCR/ABL oncogenic activity (Fig. 5) even if both transcripts bind hnRNP-A1. Furthermore, their expression is required for in vivo BCR/ABL leukemogenesis, as demonstrated by transplantation assays with BCR/ABL⁺ primary cells and cell lines in which the E2F3 gene was knocked-out or its expression was knocked-down by a specific shRNA.

E2F3 is not the only member of the E2F family of transcription factors with a potential role in BCR/ABL leukemogenesis. Indeed, in vitro studies suggest that E2F1 might have an important role in cell cycle regulation of BCR/ABL-transformed cell lines⁶³,⁶⁴. However, genetic loss of E2F1 and E2F2 expression does not impair but rather favors expansion of BCR/ABL-transduced BM mouse progenitors injected into lethally-irradiated recipients⁶⁵, indicating that BCR/ABL is capable of restoring S-phase progression in replication-impaired E2f1⁻/⁻ Lin⁻ BM progenitors. Conversely, the importance of E2F3a overexpression in regulation of the CML-BC⁷⁺ phenotype is corroborated by the following evidence: a) enhanced proliferation appears to be a characteristic of CML-BC⁷⁺ but not CML-CP⁷⁺ blasts and, most likely, depends on increased BCR/ABL levels¹); and b) the E2F3 proliferation-promoting effect relies on E2F3a but not E2F3b expression⁴⁴,⁶⁶, which is also present in quiescent cells⁴⁴,⁶⁶. Moreover, while cell-growth is only slightly inhibited in E2F3a/b-null cells, proliferation is totally impaired in E2F1,
E2F2 and E2F3 triple knock-out MEF cells and it can be partially-to-fully rescued by singular or combined expression of E2F3a and E2F3b\(^4\).

Thus, it is possible that the growth advantage of CML-BC\(^{CD34+}\) progenitors may, at least in part, depend on high levels of BCR/ABL which post-transcriptionally induces E2F3a upregulation through hnRNP-A1. In support of this hypothesis, cytoplasmic E2F3 mRNA levels were upregulated in a BCR/ABL kinase-dependent manner, and expression of the dominant-negative NLS-A1-HA\(^6\) hnRNP-A1, which impairs hnRNP-A1-dependent mRNA nuclear-export\(^6\), as well as shRNA-mediated hnRNP-A1 knock-down, inhibited E2F3 expression.

In summary, we show here that BCR/ABL has a profound effect on the expression of genes which are aberrantly post-transcriptionally-regulated through the activity of RNA binding proteins. Because altered mRNA metabolism is not limited to BCR/ABL-driven leukemias, unraveling the post-transcriptional/translational networks that aberrantly affect gene expression in different types of cancers will enhance our understanding of the mechanisms controlling cancer development and progression and may also provide novel targets (e.g. E2F3) for therapeutic intervention. Indeed, our recent discovery of the BCR/ABL-hnRNP-A1-SET-PP2A interplay represents the best example of how studying mRNA metabolism in leukemia could lead to potential new therapeutic approaches\(^5\).
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Figure legends

Figure 1. **RIP-Chip analysis of the hnRNP-A1-, hnRNP-E2-, hnRNP-K- and La/SSB-associated mRNAs in CML-BC-derived Ph1(+) K562 cells.** (left) Venn diagram of the RNAs associated with (A) hnRNP-A1, (B) hnRNP-E2, (C) hnRNP-K, and (D) La/SSB within the cytoplasm of Ph1(+) K562 cells. The squares depict the genes available on the indicated Affymetrix chips. The grey ellipses represent the genes detected in lysate input. The white ellipses indicate the genes significantly enriched in the hnRNP-A1-, hnRNP-E2-, hnRNP-K- and La/SSB-immunoprecipitates and not present in immunoprecipitates with a non-related antibody. (right) Tables show the hnRNP-A1-, hnRNP-E2-, hnRNP-K- and La-associated mRNAs with a reported relevance in cancer. mRNAs are represented as fold increase versus negative control (anti-Flag or anti-HA) and fold increase versus total input RNA.

Figure 2. **Validation and expression analysis of the hnRNP-A1-associated mRNAs in BCR/ABL cell lines and patient-derived CML bone marrow cells.** (A) RT-PCR for cyclin D2, HOXB7, SET, SNF2β, ILF3, Tra2β, E2F3a, and E2F3b on hnRNP-A1-immunoprecipitated mRNA from K562-A1-HA cytoplasmic lysates. hnRNP-A1 RNP complexes isolated from K562 cytoplasmic fractions immunoprecipitated with an anti-hnRNP-A1 antibody (9H10, lane 3) or an unrelated isotype-matched anti-FLAG antibody (clone M2, lane 2). mRNA extracted from mRNP-enriched lysates was used as a positive control (lane 1). (B) Western blots show E2F3, SNF2β, and Tra2β expression in untreated and imatinib-treated K562 cells, doxycycline-induced and not-induced TonB2.10 cells, and in parental and untreated or imatinib-treated BCR/ABL-expressing 32Dcl3 cells. GRB2 levels were detected as a control for equal loading. (Right) Real-time PCR shows expression of ILF3 and HOXB7 mRNA in untreated and imatinib-treated BCR/ABL-expressing myeloid cells and in the BaF3-derived BCR/ABL-inducible TonB2.10 lymphoid cell line. GAPDH was used for normalization. (C) SNF2β, E2F3, Tra2β and GRB2 protein levels and BCR/ABL phosphorylation in mononuclear bone marrow cells from two CML patients in chronic phase (CML-CP) and after blastic transformation (CML-BC). (D) Effect of shRNA-mediated hnRNP-A1 downmodulation on hnRNP-A1, SET, SNF2β and E2F3 protein levels.

Figure 3. **Expression analysis of the hnRNP-K-, hnRNP-E2- and La-associated mRNA products in BCR/ABL* cells.** (A) Western blots show levels of the candidate hnRNP-E2-, hnRNP-K and La/SSB-associated mRNA products in 32Dcl3 (lane 1) and in untreated and imatinib-treated 32D-BCR/ABL (lanes 2 and 3) and K562 (lanes 3 and 5) cells. Levels of La/SSB, hnRNP-E2, hnRNP-K, BCR/ABL (anti-phosphotyrosine) and GRB2 were detected as controls. (B) Western blots show expression of hnRNP-E2, FOXO1A, PKC1β and STAT3, and of hnRNP-K, LATS1, SIPA1, PKCb1 and DOCK4 in hnRNP-E2 (top) shRNA- and hnRNP-K (bottom) shRNA-transduced 32D-BCR/ABL, respectively. Expression in parental and BCR/ABL-expressing 32Dcl3 cells served as controls.

Figure 5. *E2F3 is a bona fide target of BCR/ABL tyrosine kinase activity.* (A) Western blot shows E2F3a (top arrow) and E2F3b (bottom arrow) protein expression in CD34+ bone marrow cells from normal donor (NBM), chronic phase (CML-CP) and blast crisis (CML-BC) patients (n=3 per group). (B) E2F3 protein levels in the CML-BC (n=2) CD34+ and in untreated or imatinib-treated (for the indicated times) CD34+ bone marrow cell fractions. GRB2 was used as a control for equal loading. (*): indicate a non specific band detected by a new lot of the E2F3 antibody. (C): Growth factor-independent methylcellulose colony formation of 32D-BCR/ABL cells transduced with the empty pSUPER.retro.neo+GFP retrovirus (black), or with retroviruses carrying shRNA for E2F3a (yellow) or E2F3a/b (red). Inset: Western blot shows shRNA-mediated E2F3a/b downregulation. (D) Analysis of tumors from SCID mice subcutaneously injected with vector- (black) or E2F3a/b shRNA-transduced (red) 32D-BCR/ABL cells.

Figure 6. *In vitro and in vivo requirement of E2F3 for BCR/ABL leukemogenesis.* (A) IL3-dependent and -independent methylcellulose colony forming ability of primary lineage-negative (Lin-) E2F3+/- and E2F3-/- C56BL/6J bone marrow cells (BMC) retrovirally transduced with either the empty MigR1 or MiGR1-p210BCR/ABL retrovirus and GFP-sorted (P<0.001; t-test). (B) Nested RT-PCR for p210-BCR/ABL in peripheral blood isolated at 12 weeks post-transplant from SCID mice (#1 and #2) injected with E2F3+/- and E2F3-/- Lin- BMC. Sensitivity of the system was assessed by detecting p210-BCR/ABL transcripts in K562:32Dcl3 cells used at 1:10⁶ ratio. (C) Visual analysis of spleens isolated from controls, E2F3+/- or E2F3-/- BCR/ABL-transduced GFP+ -BCR/ABL+Lin- SCID mice. (D) Hematoxylin/eosin-stained of bone marrow (BM), spleen and liver tissue sections from controls, E2F3+/- or E2F3-/- BCR/ABL-transduced GFP+ -BCR/ABL+Lin- SCID mice (#1 and #2) sacrificed at 15 weeks post-transplant. (E) Representative nested RT-PCR for p210-BCR/ABL in peripheral blood isolated at 3 week post-transplant from SCID mice either not-injected (control; n=3) or injected with vector-transduced (wild type; n=3) or shRNA E2F3ab-transduced (shE2F3; n=12) 32D-BCR/ABL cells. (F) Visual analysis and average weight of spleens isolated from controls, wild type and shE2F3 mice at 3.5...
weeks post-transplant. (G) Representative hematoxylin/eosin staining of bone marrow (BM) and spleen tissue sections from controls, wild type and shE2F3 mice at 3.5 weeks post-transplant. (H) Survival of SCID mice (n=13 per group) i.v. injected with vector- (blue line) and E2F3ab shRNA-transduced (red line) 32D-BCR/ABL cells. Estimated probabilities for survival were calculated using the Kaplan-Meier method and the log-rank test evaluated the differences among survival distributions (P=0.0001). Images were taken with a Zeiss Axioskope 2 Plus and a 40x/0.75 (BM) or a 25X/0.75 (liver and spleen) NA objective, with a Canon Powershot A70 (Canon, Lake Success, NY) and Canon Capture software.
Figure 1.
Figure 2.

A.

B.

C.

D.
Figure 3.

A.

B.
Figure 4.

A

5'-AGACCUUAGGGUAGGGACAUCC-3'

hnRNP A1 rODN

hnRNP A1 Consensus Binding Site

2066 - GAGGCCCUAGGAGGAAAGACAUCC - 2089 - Mouse E2F3 3'UTR (XM_127250)
2066 - AGACCCUCUAGGAGGAAAGACAUCC - 2700 - Human E2F3 3'UTR (NM_001949) E2F3 (A1) rODN

B

RNA probe: E2F3 (A1)

REMSA

C

hnRNP A1: E2F3 RNA complex

IMatinib [2μM; 24h]

hnRNP A1 depleted

hnRNP A1

UV crosslinking

E2F3

GRB2

IL-3-stimulated

1 2 3

32Dc13 32D-BCR/ABL 32D-BCR/ABL NLS-A1-HA

K562

+ IMatinib

kDa 50 37 25

kDa 37 25
Figure 5.

A

CD34+

NBM #1  CML-CP #1  CML-BC #1  NBM #2  CML-CP #2  CML-BC #2  CML-BC #3  CML-BC #4  CML-BC #5

E2F3

Grib2

B

CML-BC#4  CML-BC#5

CD34+  CD34+

E2F3  Grib2

Imatinib (2 µM)

time [hours]

C

P<0.001

32D-BCR/ABL

E2F3α

E2F3α and Grib2

D

P<0.01

32D-BCR/ABL

E2F3 α/b

E2F3 α/b and Grib2
Figure 6.
Identification of novel post-transcriptional targets of the BCR/ABL oncoprotein by ribonomics: requirement of E2F3 for BCR/ABL leukemogenesis

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