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Cytoplasmatic Compartmentalization by Bcr-Abl Promotes TET2 Loss-of-Function in Chronic Myeloid Leukemia

Manuela Mancini,[1*] Nevena Veljkovic,[2] Elisa Leo,[1] Michela Aluigi,[1] Enrica Borsi,[1] Chiara Galloni,[1] Ilaria Iacobucci,[1] Enza Barbieri,[3] and Maria Alessandra Santucci[1]

[1] *Dipartimento di Ematologia e Scienze Oncologiche "Lorenzo e Ariosto Seràgnoli," University of Bologna-Medical School, Bologna, Italy*

[2] *Center for the Multidisciplinary Research, Institute of Nuclear Sciences, University of Belgrade, Vinca, Belgrade, Serbia*

[3] *Dipartimento di Scienze Radiologiche e Istopatologiche, Istituto di Radioterapia "Luigi Galvani," University of Bologna-Medical School, Bologna, Italy*

ABSTRACT

The loss-of-function of ten–eleven-translocation (TET) 2, a Fe^{2+}-oxoglutarate-dependent dioxygenase catalyzing 5 methyl cytosine (5mC) conversion into 5-hydroxymethylcytosine (5hmC), contributes to the hematopoietic transformation in vivo. The aim of our study was to elucidate its role in the phenotype of chronic myeloid leukemia (CML), a myeloproliferative disease caused by the Bcr-Abl rearranged gene. We first confirmed TET2 interaction with the Bcr-Abl protein predicted by a Fourier-based bioinformatic method. Such interaction led to TET2 cytoplasmatic compartmentalization in a complex tethered by the fusion protein tyrosine kinase (TK) and encompassing the Forkhead box O3a (FoxO3a) transcription factor. We then focused the impact of TET2 loss-of-function on epigenetic transcriptional regulation of Bcl2-interacting mediator (BIM), a pro-apoptotic protein transcriptionally regulated by FoxO3a. BIM downregulation is a critical component of CML progenitor extended survival and is also involved in the disease resistance to imatinib (IM). Here we reported that TET2 release from Bcr-Abl protein following TK inhibition in response to IM triggers a chain of events including TET2 nuclear translocation, re-activation of its enzymatic function at 5mC and recruitment at the BIM promoter followed by BIM transcriptional induction. 5hmC increment following TET2 re-activation was associated with the reduction of histone H3 tri-methylation at lysine 9 (H3K9me3), which may contribute with DNA de-methylation reported elsewhere to recast a permissive epigenetic "landscape" for FoxO3a transcriptional activity. J. Cell. Biochem. 113: 2765–2774, 2012.

KEY WORDS: CHRONIC MYELOID LEUKEMIA; Bcr-Abl; TET2; BIM; FOXO3A; EPIGENETIC MODIFICATIONS

DNA methylation consists in the addition of a methyl group to the 5th carbon of cytosines (C) within the dinucleotide CpG islands. Such epigenetic modification is critical for transcriptional regulation throughout the development and lifespan. Clustered $5mC_s$ are, in fact, associated with transcriptional silencing of tissue-specific genes and endogenous retrotransposons, X inactivation in females, asymmetric expression of imprinted genes and nuclear reprogramming. Moreover, it may play a role in cancer phenotype. DNA hypermethylation at specific promoter regions drives, in fact, transcriptional repression of tumor suppressor and housekeeping genes [Berdasco and Esteller, 2010]. C methylation is concurrently regulated by two families of enzymes, the DNA methyltransferases (DNMTs] which catalyze the covalent addition of methyl groups, and ten–eleven-translocation (TET) proteins, a family of

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*Correspondence to: Dr. Manuela Mancini, PhD, Dipartimento di Ematologia e Scienze Oncologiche "Lorenzo e Ariosto Seràgnoli," University of Bologna-Medical School, Via Massarenti 9, 40138 Bologna, Italy.
E-mail: mancini_manu@yahoo.com

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Fe (II)-oxoglutarate-dependent dioxygenases which catalyses the conversion of 5 methylcytosine (5mC) to 5 hydroxymethyl cytosine (5hmC) [Tahiliani et al., 2009]. TET hydroxylation facilitates DNA de-methylation either passively (DNMT1 no longer methylates its target C when the complementary strand contains a 5hmC] or actively (5mC conversion to 5hmC reduces the affinity of methyl-CpG binding protein 2 [MeCP2] for methylated CpG dinucleotides hence impairing the recruitment of DNMT1 and other chromatin remodelling proteins) [Valinluck et al., 2004; Valinluck and Sowers, 2007]. In some instances, TET may directly affect the DNA methylation status. This is the case of TET1, the founding member of TET family, which maintains Nanog promoter hypomethylation in embryonic stem cells and promotes region-specific de-methylation in the adult brain [Ito et al., 2010; Guo et al., 2011].

TET alterations have been detected in a variety of myeloid malignancies [Figueroa et al., 2009; Langemeijer et al., 2009]. TET1 was first identified as a fusion partner of the myeloid/lymphoid or mixed-lineage leukemia (MLL) gene in acute myeloid leukemia (AML) carrying t(10;11)(q22;q23) translocation and adult CD10-negative B-cell acute lymphoblastic leukemia (ALL) [Ono et al., 2002; Lorsbach et al., 2003; Burmeister et al., 2009]. Interestingly, the MLL gene encodes a histone methyltransferase (HMT), a component of chromatin epigenetic control involved in the deregulated expression of genes driving leukemic transformation [Wang et al., 2011]. More recently, deletions or mutations of TET2 were detected in patients with primary or secondary AML, myelodisplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and MDS/MPN overlap syndromes including myelomonocytic leukemia (CMML). In many cases TET2 mutations are accompanied by the loss of wild type (wt) allele and in most instances compromise TET2 catalytic activity [Ko et al., 2010; Dahl et al., 2011]. Low levels of 5hmC in a subset of patients with myeloid malignancies carrying the wild-type TET2 suggest that TET2 loss-of-function may occur independently from gene mutations [Ko et al., 2010]. Indeed, in animal models either TET2 loss or TET2 catalytic domain disruption in the hematopoietic compartment results in increased stem cell self renewal and impaired differentiation in vivo [Moran-Crusio et al., 2011; Ko et al., 2011].

In chronic myeloid leukemia (CML) TET2 mutations are rare events associated with the disease progression and likely involved in the loss of imprinting of the insulin-like growth factor 2 gene [IGF2; Randhawa et al., 1998; Makishima et al., 2011; Roche-Lestienne et al., 2011]. Here we proved that TET2 binding with Bcr-Abl protein predicted by a Fourier-based bioinformatic method (which lets identify common repeating pattern[s] involved in protein interaction with putative partners] causes its cytoplasmatic compartmentalization and loss-of-function [Veljkovic et al., 2007]. TET2/Bcr-Abl complex is tethered by the Bcr-Abl protein TK and encompasses FoxO3a, a key transcriptional regulator of BIM expression in Bcr-Abl-expressing cells [Essafi et al., 2005]. Bcr-Abl TK inhibition in response to IM let TET2 release and nuclear import, restored its enzymatic function at 5mC and raised its recruitment at the BIM promoter. Moreover, the complex dissipation allowed FoxO3a nuclear relocation and recruitment at the BIM promoter. The latter event was concurrently promoted by H3K9 de-methylation and drove BIM transcriptional induction addressing Bcr-Abl-expressing cells toward apoptotic death. In conclusion, our findings support that TET2 loss-of-function plays a role in the transcriptional silence of death genes involved in CML phenotype.

MATERIALS AND METHODS

INFORMATIONAL SPECTRUM MODEL (ISM) AND CROSS-SPECTRUM (CS) ANALYSES

Informational spectrum model is a computational tool based on an algorithm which lets the conversion of residues in electron ion interaction potential (EIIP) values. EIIP corresponds to the average energy states of all valence electrons in amino acids calculated by means of a pseudopotential model [Essafi et al., 2005]. We used two ISM softwares modules, the discrete Fourier transformation (DFT) to create a protein informational spectrum, and the fast Fourier transformation (FFT) to explore protein common patterns. CS was obtained from mathematical filtering of common spectral characteristics of two or more protein sequences according to FFT. The first dominant peak in CS indicates the most important common periodicity, a crucial prerequisite for efficient protein interaction. To select potential interactors we scanned the human protein sequence contained in SWISSProt/UniProt for the peak in CS at a given Fourier frequency [Gasteiger et al., 2003]. The gene ontology (GO) terms for every single gene from the CS output list (provided by DAVID bioinformatics tool) were further analyzed and the final selection of putative partner(s) was made by means of visual inspection and previously achieved results [Huang da et al., 2009] (Fig. 1A).

CELLS AND TREATMENTS

Murine pre B cell line BaF3 stably transduced with a Bcr-Abl construct coding for p210 fusion protein was a generous gift of M. Deininger (Division of Hematology and Oncology, Oregon Health and Science University Cancer Center, Portland). Thirty-two D cell clones expressing either the wt or a temperature sensitive (ts) Bcr-Abl construct have been generated according to published methods [Mancini et al., 2007]. Several cell clones (including the 3B one used in the present study) were preliminarily assayed for Bcr-Abl construct integration and p210 TK activity, only apparent at the permissive temperature of 33°C. Cell lines were maintained in RPMI 1640 medium (Life Technologies Ltd, Paisley, UK) additioned with 10% fetal calf serum (FCS, from Gibco), 1% L-glutamine and antibiotics in 5% CO_2 and fully humidified atmosphere at 37°C (parental and wt Bcr-Abl-expressing BaF3 and 32D), 33°C or 39°C (the permissive and non-permissive temperatures for ts p210 Bcr-Abl TK). Parental cell lines and ts Bcr-Abl-expressing 32D cell clones kept at 39°C required 10% WEHI-3 conditioned medium as source of IL-3. One micromolar IM was used to inhibit p210 Bcr-Abl TK activity. Cytofluorimetric analysis of apoptotic cells was performed by the uptake of Annexin V and propidium iodide (PI) (Roche) using a Becton Dickinson FacScan (488 nm excitation wave length and 530 nm bandpass filter wave for fluorescin and >580 nm for PI detection) and dedicated software (Cell Quest from Becton Dickinson). Mononuclear cell fractions (MCFs) from bone marrow samples of 4 CML patients at diagnosis and peripheral blood aphereses of 18 healthy individuals were obtained by centrifugation over Fycoll-Hypaque after informed consent. In the

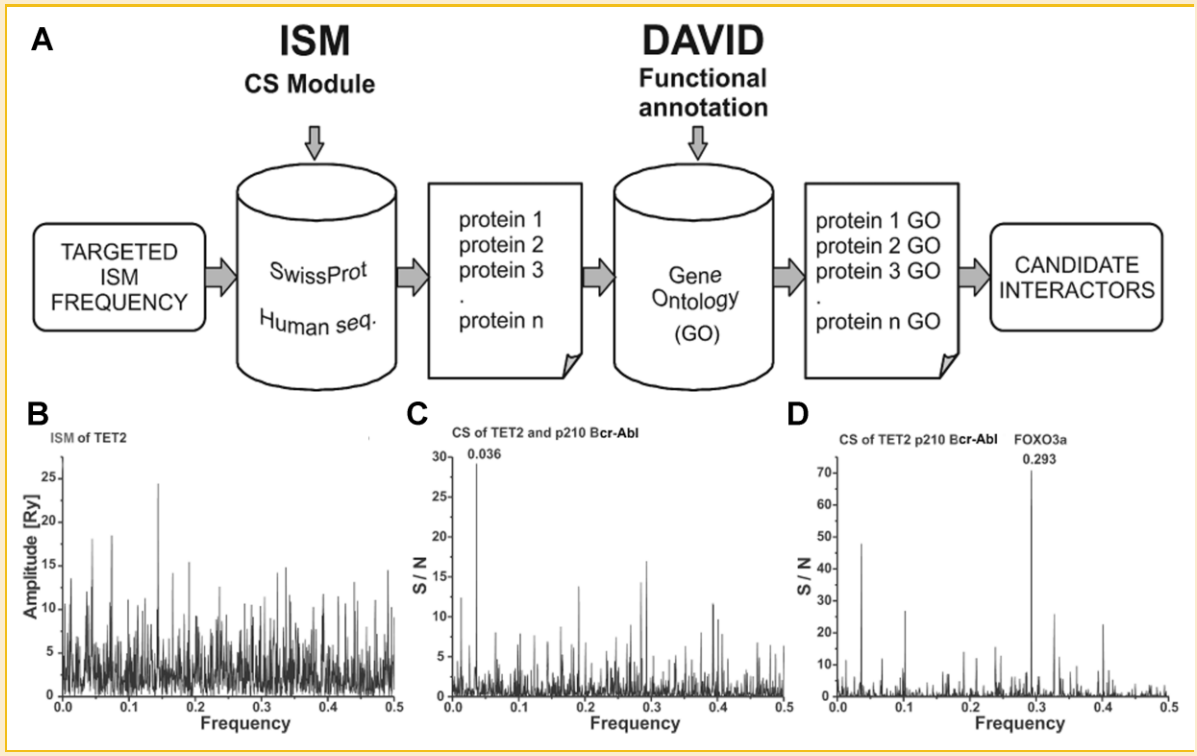


Fig. 1. ISM and CS for the analysis of TET2 interactions with p210 Bcr-Abl and FoxO3a. Sequential steps in the investigation of TET2 candidate interactors (A); ISM frequencies of TET2 (B) and CS between TET2 and p210 Bcr-Abl (C), and TET2, p210 Bcr-Abl and FoxO3a (D). Protein frequencies from the Fourier transform of the sequence of electron–ion interaction potential corresponding to the aminoacid sequence of any protein are put in the abscissa axis. The lowest frequency is 0.0 and the highest is 0.5. The amplitudes, expressed in arbitrary units corresponding to each frequency component in the ISM are put in ordinate axis. The transformation of TET2 sequence into ISM let distinguish the dominant peaks depicting the protein relevant functions (B). CS between TET2 and p210 Bcr-Abl let identify a significant peak at the Fourier frequency 0.036 supporting a direct mutual interaction between the two proteins (C). CS dominant peak at a the Fourier frequency 0.293 supports the participation of FoxO3a to the TET2/p210 Bcr-Abl complex suggested by GO annotations (D).

former group of samples karyotypic and RT-PCR analyses were preliminarily performed to assess the presence of Philadelphia chromosome and Bcr-Abl rearrangement.

PROTEIN ANALYSIS

Western blot (WB) and IP/immunoblotting analyses were performed on whole cell and nuclear lysates according to published methods [Brusa et al., 2005]. Anti-TET2 and anti-beta actin antibodies were purchased from Santa Cruz Biotechnology, anti-FoxO3a, -abl, and -histone H1 antibodies from Upstate Biotechnology and anti-BIM antibody from Oncogene. Beta-actin and histone H1 were used as controls for protein loading and to exclude contamination of nuclear lysates by cytoplasmatic proteins and vice versa. Signal intensities in single blots from three separate experiments were measured by means of ChemiDoc-It instrument equipped with a dedicated software (Launch VisionWorksLS, Euroclone]. The differences among signal intensities in presence of IM were evaluated for statistical significance using paired *t* student test.

RNA ANALYSIS

Total RNA was extracted using a commercial kit (SV total RNA isolation system, Promega) according to manufacturer's instructions. A semi-quantitative PCR strategy was used to quantify BIM expression. It exploited the ratios between BIM and housekeeping beta-2-microglobulin (B2m) amplification signals resolved in 2% agarose and quantified by a GS-700 imagining densitometer (BioRad) equipped with a dedicated software (Molecular Analyst, BioRad). 35 cycles (denaturation 95°C for 30 min; annealing 58°C for 30 min; elongation 72°C for 30 min) were carried using the following primers: 5′-GCCAAGCAACCTTCTGATG-3′ (upper) and 5′-AATACCCACTGGAGGACCG-3′ for BIM and 5′-TGCTTGTCTCACTGACCGGCC-3′ (upper) and 5′-ACATGTCTC-GATCCCAGTAGACGGT-3′ (lower) for B2m amplification.

CHROMATIN IMMUNOPRECIPITATION (CHIP)

Cells were fixed in RPMI at 1% final concentration of formaldehyde. After 10 min incubation at room temperature the reaction was stopped by the addition of 1.25 mM glycine. ChIP was performed using a commercial kit (EpiQuik Chromatin Immunoprecipitation Kit, Epigentek) using anti-FoxO3a, -TET2, -H3K9me3 (Upstate Biotechnology), and anti-5hmC (ZymoResearch) antibodies. After extensive washing DNA was eluted by heating at 65°C for 4 h. Fifty nanograms of DNA was then amplified by PCR (35 cycles: denaturation: 95°C for 30 min, annealing 58°C for 50 min,

elongation 72°C for 50 min). The following specific primers were designed to amplify a 342 bp sequence of murine BIM promoter at region −268 to +90: 5′-ACTCCACAAGCTGGGGAGCTGAT-3′ (upper) and 5′-AGGCCTCTCAGCAGGCTGCAATT-3′ (lower), while the following primers (upper: 5′-ACCTCACGGTGTGCACCTCAGA-3′, lower: 5′-AGGTAGGACGCCGGAGCACA-3′) were used to amplify a 289 bp fragment of human BIM promoter (−607 to −318). PCR conditions were set in order to quantify FoxO3a and TET2 recruitment and H3K9me3 at the BIM promoter relative to the constitutively acetylated promoter of histone H4a (region −40 to +285). Signal intensities and statistical significance of differences were obtained as described in the previous section.

HYDROXYMETHYL-DNA IMMUNOPRECIPITATION (HmeDIP)

The 5hmC content in DNA was quantified by means of a commercial kit (Quest 5-hmC detection Kit™ from Zymo Research) according to manufacturer's instructions. Briefly, samples were sonicated for 30 min (three times) to obtain DNA fragments of about 200–1000 bp. Samples were then modified by a highly specific 5hmC glucosyl-transferase, which tags 5hmC with a glucose moiety, and digested with a glucosyl-5hmC sensitive restriction endonuclease. This enzyme cleaves at cytosine, 5-mC and 5-hmC present in its recognition site but not at glucosylated 5hmC. To identify the 5hmC amount at the BIM promoter 100 ng DNA obtained with the above mentioned procedure were amplified, resolved in 2% agarose and stained with ethidium bromide. Forty PCR cycles (denaturation 95°C for 30 min, annealing 58°C for 35 min, elongation 72°C for 45 min) were carried using the following primers: 5′-ACTCCACAAGCTGGG-GAGCTGAT-3′ (upper) and 5′-TGCCGGGCTCCTGTCTGTGT-3′ (lower) for murine promoter; 5′-ACCTCACGGTGTGCACCTCAGA-3′ (upper) and 5′-CTCTCCGCAGGCTGCAATTGTCT-3′ (lower) for human promoter.

RESULTS

ISM AND CS ANALYSES LET DETECT TET2 AND p210 Bcr-Abl INTERACTION ENCOMPASSING FoxO3a

We first transformed TET2 sequence into ISM to distinguish the dominant peaks which depict the protein relevant functions (Fig. 1B) [Veljkovic et al., 2007]. We then used CS analysis to explore TET2

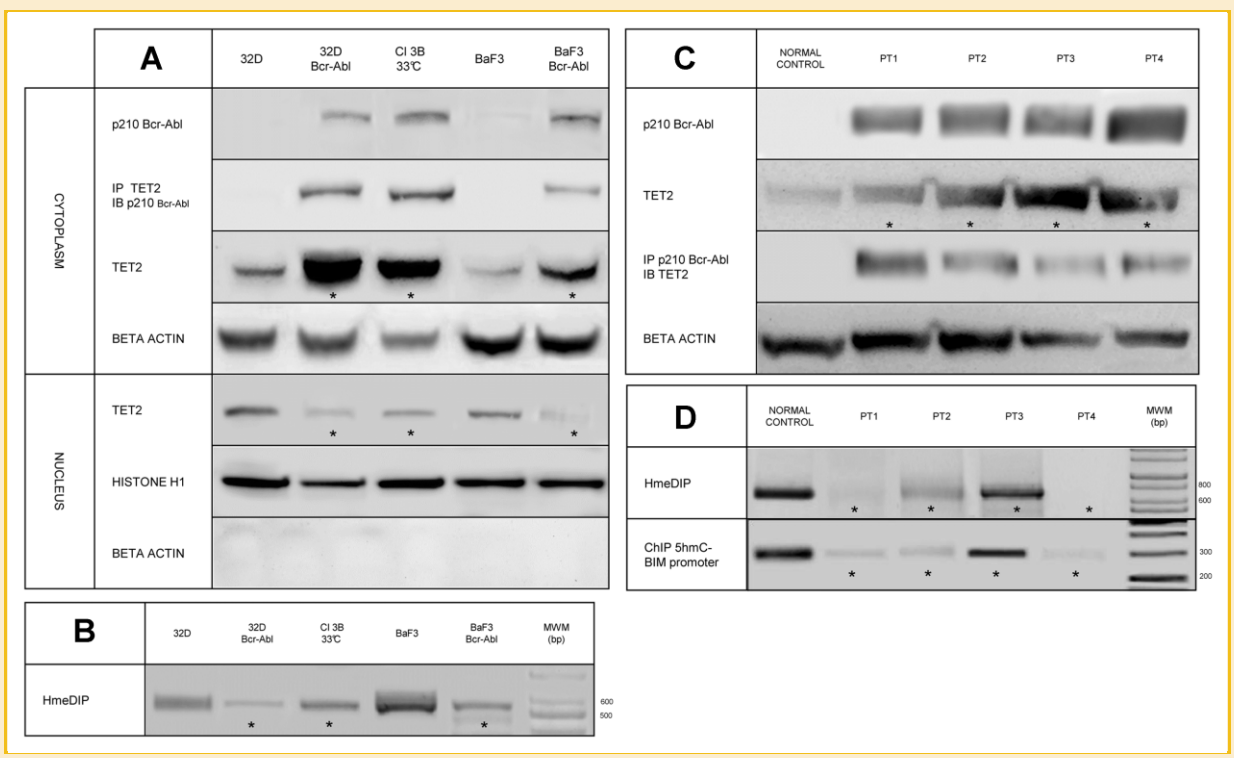


Fig. 2. TET2 expression and interaction with p210 Bcr-Abl protein prevent its nuclear location and enzymatic activity at 5mC. In 32D and BaF3 cell lines transduced with a wt Bcr-Abl construct and in a 32D cell clone transduced with a ts Bcr-Abl construct (Cl 3B 33°C) the interaction with Bcr-Abl protein (p210 Bcr-Abl) promotes TET2 cytoplasmatic compartmentalization hence precluding its nuclear relocation (A) and enzymatic activity at 5mC, assessed by means of PCR amplification of hmeDIP products (B). The results shown in panels A and B have been confirmed in two separate experiments. TET2 cytoplasmatic compartimentalization, its interaction with p210 Bcr-Abl protein and loss-of-function were investigated in MCFs from bone marrow samples of CML patients at clinical diagnosis by means of WB and immunoprecipitation (IP)/immunoblotting (IB) (C), and HmeDIP and PCR amplification of BIM promoter performed on DNA from ChIP products obtained with a 5hmC antibody (D). MWM: molecular weight marker lets identify the PCR amplification products of hmeDIP at 573 bp (mus musculus) and 738 bp (homo sapiens), and BIM promoter amplification signals at 289 bp (homo sapiens). *Statistatically significant differences compared to parental cell lines or normal controls was calculated by paired *t* student test through the comparison of signal intensities in three separate blots. Beta actin and histone H1 were used as controls for cytoplasmatic and nuclear protein loading, respectively. No beta actin signals were apparent in nuclear lysates indicating the lack of cross-contamination between nuclear and cytoplasmatic compartments.

putative interaction with Bcr-Abl protein. CS let identify a significant peak at the Fourier frequency 0.036 supporting a direct mutual interaction between TET2 and Bcr-Abl (Fig. 1C). Since 0.036 is among the dominant TET2 frequencies (see Fig. 1B) such an interaction is expected to affect critical TET2 function(s). Moreover, we confirmed a peak at the Fourier frequency 0.293 whose relevance for p210 Bcr-Abl ligand was previously reported [Mancini et al., 2008]. This spectral characteristic was therefore used to distinguish the putative participants in the Bcr-Abl/TET2 complex. CS analyses across all human protein sequences provided by SWISSProt/UniProt let identify 1,160 proteins with a dominant peak at the Fourier frequency 0.293. Functional G0 annotations obtained by DAVID bioinformatics tool addressed toward the FoxO3a transcription factor as a potential partner of Bcr-Abl protein. Indeed, a CS dominant peak at the Fourier frequency 0.293 supports the participation of FoxO3a in an unique complex tethered in the cytoplasm by Bcr-Abl/TET2 (Fig. 1D).

THE INTERACTION WITH Bcr-Abl PROTEIN INDUCES TET2 CYTOPLASMATIC COMPARTMENTALIZATION AND LOSS-OF-FUNCTION

The central role of Bcr-Abl protein in CML phenotype arises from multiple interactions with cell signaling components [Patel et al., 2011]. In particular, the exclusive cytoplasmatic location of Bcr-Abl protein may interfere with the nuclear translocation of partner proteins hence impairing their function. We first confirmed TET2 interaction with Bcr-Abl advanced by computational analyses in 32D and BaF3 cell lines (Fig. 2A). TET2 higher expression in the cytoplasm of Bcr-Abl-transduced cells and its almost undetectable levels in the nuclear compartment ($P < 0.05$ or less) let assume that Bcr-Abl binding induces TET2 cytoplasmatic compartmentalization hence precluding its enzymatic activity at 5mC (Fig. 2A). Accordingly, hmeDIP revealed a significant reduction of 5hmC levels in 32D and BaF3 cell lines expressing the Bcr-Abl rearranged gene compared to the parental ones ($P < 0.05$ or less; Fig. 2B). Lacking TET2 cytoplasmatic compartmentalization and loss-of-function seen in 32D and BaF3 cells transfected with the empty vector let assume that TET2 cytoplasmatic ligand with Bcr-Abl is not contingent upon complementary events elicited by transfection (data not shown). TET2/Bcr-Abl interaction was further validated in MCFs from bone marrow samples of CML patients at diagnosis. All patients exhibited higher TET2 cytoplasmatic expression ($P < 0.05$ or less) compared to normal control (whole MCF lysates of a pool of peripheral blood apheseres from 18 healthy individuals) and interaction with Bcr-Abl protein (Fig. 2C). Moreover, hmeDIP and PCR amplification of BIM promoter performed on DNA from ChIP products obtained with a 5hmC antibody showed that 5mC prevails over 5hmC in Bcr-Abl-expressing cells ($P < 0.05$ or less; Fig. 2D).

We next confirmed FoxO3a participation in the Bcr-Abl/TET2 complex predicted by CS analyses and G0 annotations (Fig. 3A). FoxO3a/TET2 interaction in parental 32D and BaF3 suggests that the two protein physiological interaction may be enhanced by TET2 increased cytoplasmatic levels associated with Bcr-Abl (Fig. 3A). FoxO3a cytoplasmatic interaction with Bcr-Abl reduced its nuclear import and led to its loss-of-function as proved by the

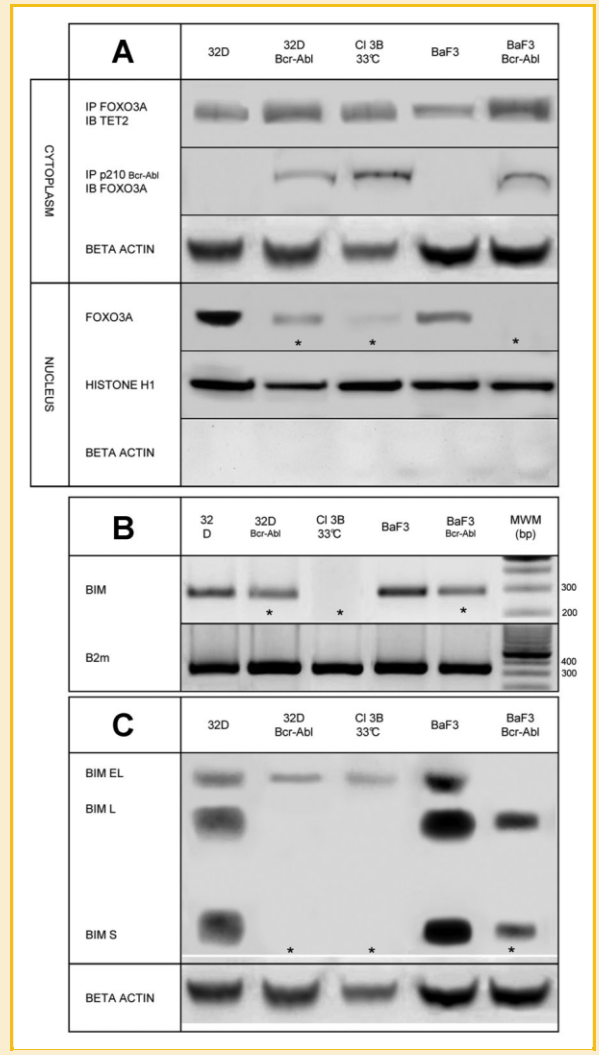


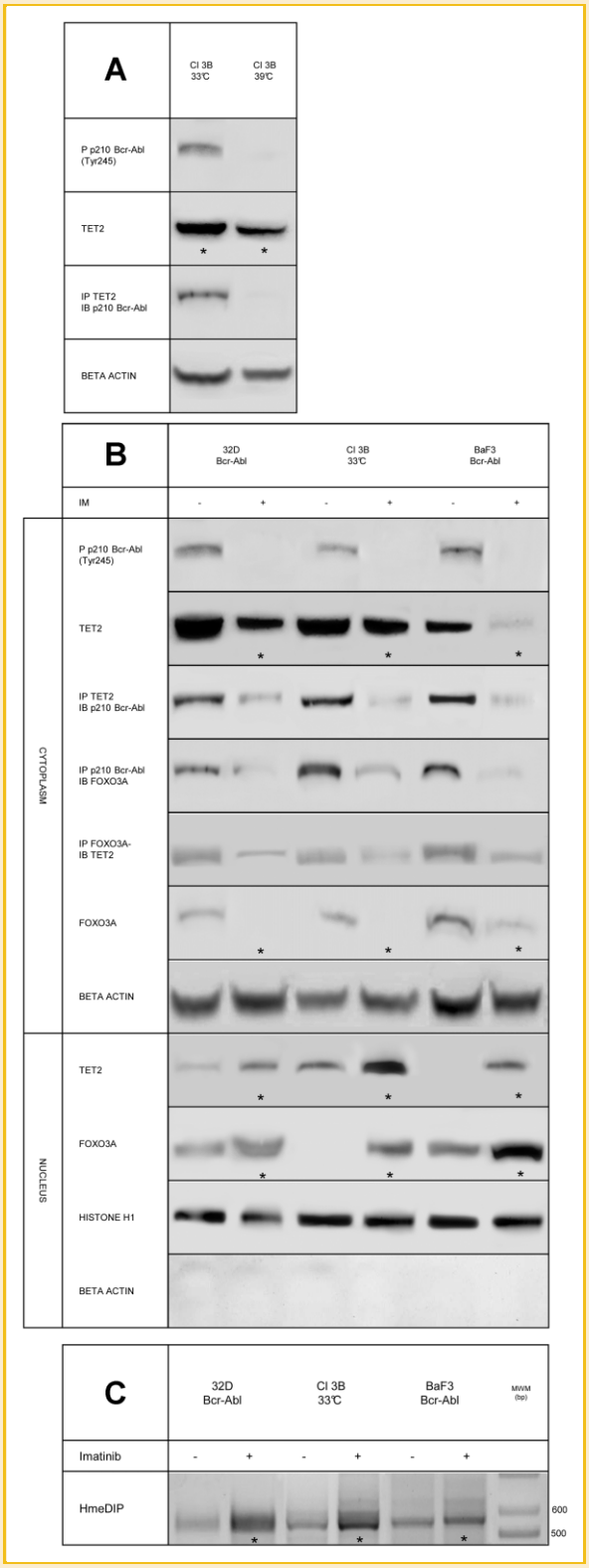
Fig. 3. TET2/p210 Bcr-Abl complex encompasses FoxO3a and leads to reduced FoxO3a nuclear import and transcriptional induction of its target, the pro-apoptotic BIM protein. FoxO3a interaction with p210 Bcr-Abl and its enhanced ligand with TET2 associated with Bcr-Abl expression reduced its nuclear relocation (A) and transcriptional induction of BIM (B) leading to a remarkable reduction of all three BIM isoforms: EL, L, and S (C). B2m was kept as control for transcription rate. MWM let identify BIM amplification products at 274 bp and B2m at 327 bp. the See legend to Figure 2 for further details.

transcriptional down-modulation of BIM, a FoxO3a downstream target involved in CML resistance to apoptotic death ($P < 0.05$ or less; Fig. 3A and B) [Essafi et al., 2005]. Transcriptional down-modulation evoked a significant reduction of all three major BIM isoforms (BIM-EL, BIM-L, and BIM-S] generated by the transcript alternative splicing ($P < 0.05$ or less) in Bcr-Abl-expressing cell lines (Fig. 3C) [Pinon et al., 2008].

Notably, TET2 prominent cytoplasmatic location and interaction with Bcr-Abl in clone 3B was only seen at the permissive temperature of 33°C, suggesting that both events are contingent upon the fusion protein TK activity (Fig. 4A). Indeed, p210 Bcr-Abl TK inhibition in response to IM (1 μM for 24 h) was associated with a significant reduction of TET2 expression and interaction with

Bcr-Abl in the cytoplasm and promoted TET2 nuclear translocation ($P < 0.01$ or less; Fig. 4B). The significant increase of 5hmC at the BIM promoter in response to IM supports that TET2 enzymatic activity is restored following its release from Bcr-Abl and nuclear translocation in response to IM ($P < 0.01$ or less; Fig. 4C). Moreover, Bcr-Abl TK inhibition by IM let FoxO3a disengagement hence promoting its nuclear import ($P < 0.05$ or less; Fig. 4B).

Those results let conclude that: (i) TET2 is a component of a multiprotein complex encompassing FoxO3a and tethered in the cytoplasm by the costitutive TK activity of Bcr-Abl protein; (ii) the interaction with Bcr-Abl TK promotes TET2 and FoxO3a cytoplasmatic compartmentalization and loss-of-function.



FoxO3A AND TET2 RECRUITMENT AT THE BIM PROMOTER ARE COMPONENTS OF BIM TRANSCRIPTIONAL INDUCTION

Bcl2-interacting mediator transcriptional induction is mediated by FoxO3a through its binding to a FOXO consensus site at the BIM promoter preceded by FoxO3a de-phosphorylation at protein kinase B (PKB) target sites and acetylation at lysine residues [Brunet et al., 1999; Essafi et al., 2005; Corrado et al., 2009; Storz, 2011]. PCR amplification of DNA from ChIP products obtained with an anti-FoxO3a ChIP grade antibody showed a significant increment of BIM promoter amplification signals in response to IM (1 μM for 24 h] in Bcr-Abl-expressing cells ($P < 0.01$ or less) (Fig. 5A). Moreover, PCR amplification of DNA from ChIP products obtained with an anti-TET2 ChIP grade antibody showed a time-dependent increase of BIM promoter signals since 4th up to 24th h of exposure to IM ($P < 0.01$ or less) in Bcr-Abl-expressing 32D cells (Fig. 5B). FoxO3a enhanced recruitment at the BIM promoter at 24th h of drug exposure was confirmed in clone 3B kept at 33°C and Bcr-Abl-expressing BaF3 cells (Fig. 5B).

DNA hyper-methylation of BIM promoter is a central component of BIM transcriptional down-modulation in CML cells likely contributing with H3K9me3 in the heterochromatin establishment and gene silencing [Li et al., 2006; San José-Eneriz et al., 2009]. PCR amplification of DNA from ChIP products obtained with an anti-H3K9me3 antibody revealed that H3K9me3 levels at the BIM promoter were significantly reduced following the Brc-Abl TK inhibition by IM (1 μM for 24 h; $P < 0.001$ or less; Fig. 5A). Such epigenetic modification evoked a significant increment of BIM expression ($P < 0.05$ or less) associated with apoptotic death induction (Figs. 4B and 6A,B). Those results let conclude that FoxO3a recruitment at the BIM promoter and transcriptional activation following p210 Bcr-Abl TK inhibition are driven by epigenetic chromatin modifications including 5mC hydroxylation (driven by TET2 recruitment at the chromatin) and H3K9 demethylation. Moreover, they support that FoxO3a recruitment at the BIM promoter, a central component of BIM transcriptional induction in response to IM Bcr-Abl-expressing cells, is promoted

Fig. 4. TET2/FoxO3a/Bcr-Abl cytoplasmatic complex is tethered by the Bcr-Abl protein TK activity. TET2 increased levels and interaction with p210 Bcr-Abl in clone 3B was only apparent at 33°C, the permissive temperature for the fusion protein TK activity (A). Accordingly, p210 Bcr-Abl TK inhibition in response to IM, earnmarked by the protein de-phosphorylation at Tyr^{245}, promoted the reduction of TET2 and FoxO3a cytoplasmatic levels, their binding and interaction with Bcr-Abl, and concurrently upraised TET2 and FoxO3a nuclear expression (B) and TET2 activity at 5mC at the BIM promoter (C). See legend to Figure 2 for details.

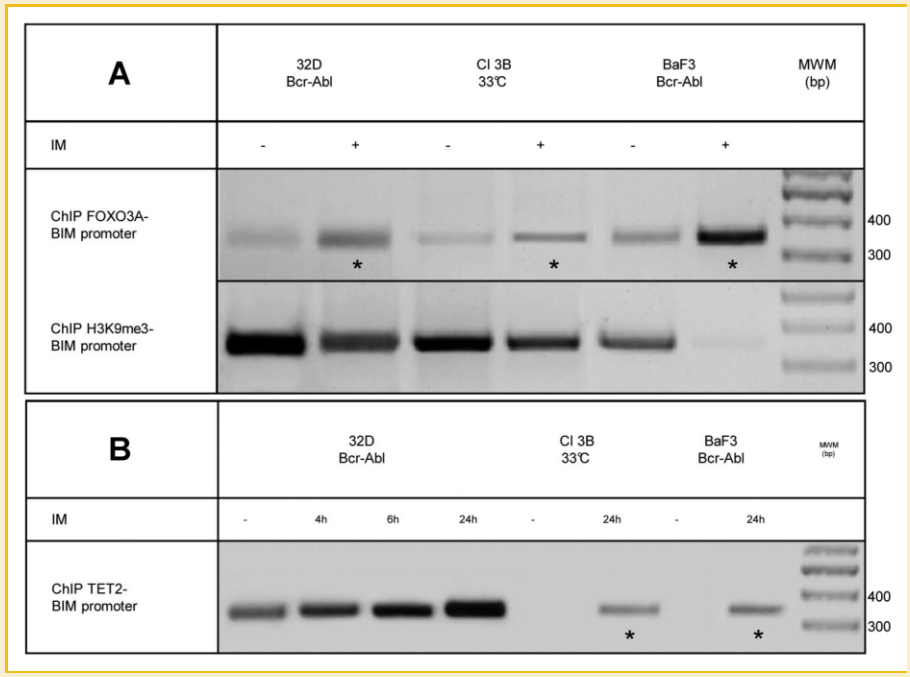


Fig. 5. TET2 recruitment at at the BIM promoter in response to IM associated with FoxO3a recruitment and reduction of H3K9me3. FoxO3a recruitment at the BIM promoter in response to IM (1 μM for 24 h) associated with H3K9me3 reduction was detected by means of PCR amplification of DNA from ChIP products obtained with ChIP grade anti-FoxO3a and anti-H3K9me3 antibodies (A). It was combined with TET2 recruitment at the BIM promoter starting from 4th hour of exposure to IM in Bcr-Abl-expressing cells and culminating at 24th hour in Bcr-Abl-expressing 32D and Ba/F3 cells and in clone 3B kept at 33°C (B). See legend to Figure 3 for details.

by epigenetic events such as 5mC hydroxylation and H3K9me3 reduction.

DISCUSSION

The oncogenic potential of Bcr-Abl rearranged gene of CML arises from multiple interactions of its fusion protein with signals involved in regulated cell proliferation and death [Sharma et al., 2006]. In this context, the Bcr-Abl ability of recasting the epigenetic "landscape" at promoter regions of death genes may contribute to maintain the leukemic phenotype. Aberrant DNA methylation is a peculiar trait of CML more recently associated with the disease prognosis [Jelinek et al., 2011]. DNA methylation is concurrently regulated by DNMTs, which catalyze the covalent addition of methyl groups to 5th carbon of C, and TET proteins, which catalyze the conversion of 5mC to 5hmC. Interestingly, TET2 has a central role in hematopoiesis and participates in the development of myeloproliferative disease. Its loss in mice results, in fact, in the expansion and delayed differentiation of hematopoietic stem cells almost invariably followed by the development of a disease reminiscent of human CMML and TET2 mutations disrupting its enzymatic function are the most frequent genetic lesions in human myeloid malignancies [Ko et al., 2010; Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011]. TET2 mutations in CML are rare events occurring in advanced stages of the disease [Makishima et al., 2011; Roche-Lestienne et al., 2011]. Moreover, recent studies carried in a large cohort of CML patients in chronic phase let conclude that TET2 disruption is not due to mutations of the isocitrate dehydrogenase family IDH1/2 [Figueroa et al., 2010; Makishima et al., 2011; Roche-Lestienne et al., 2011]. Our study supports that TET2 loss-of-function arises from cytoplasmatic compartimentalization by the Bcr-Abl protein predicted by a Fourier-based bioinformatic method (Figs. 1 and 2). TET2/Bcr-Abl complex is tethered by the fusion protein TK activity (Fig. 4A). Accordingly, Bcr-Abl TK inhibition in response to IM was followed by the two proteins dissociation, TET2 nuclear translocation and functional recovery (Fig. 4B and C). We choose BIM, a BH3-only pro-apoptotic protein whose down-regulation has been associated with CML resistance to TK inhibitors, to establish TET2 participation in death gene transcriptional downmodulation associated with Bcr-Abl [Kuribara et al., 2004; Kuroda et al., 2006; Shah et al., 2008; San José-Eneriz et al., 2009]. Previous studies ascribed BIM downmodulation to a transcriptionally inert conformation of its promoter (due to DNA hypermethylation and H3K9 ipoacetylation] hampering FoxO3a recruitment to an upstream region of BIM transcription start site [Mestre-Escorihuela et al., 2007; Bachmann et al., 2010; De Bruyne et al., 2010; Richter-Larrea et al., 2010]. Moreover, BIM promoter DNA methylation status has been involved in BIM down-modulation associated with Bcr-Abl and transcriptional induction in response to IM, supporting that multiple epigenetic events concur to establish a non-permissive chromatin conformation for FoxO3a recruitment at the BIM promoter in Bcr-Abl-expressing cells [San José-Eneriz et al., 2009]. Here we showed that TET2 release from the Bcr-Abl protein and nuclear translocation following the fusion protein TK inhibition by IM is associated with TET2 recruitment at the BIM promoter, 5mC

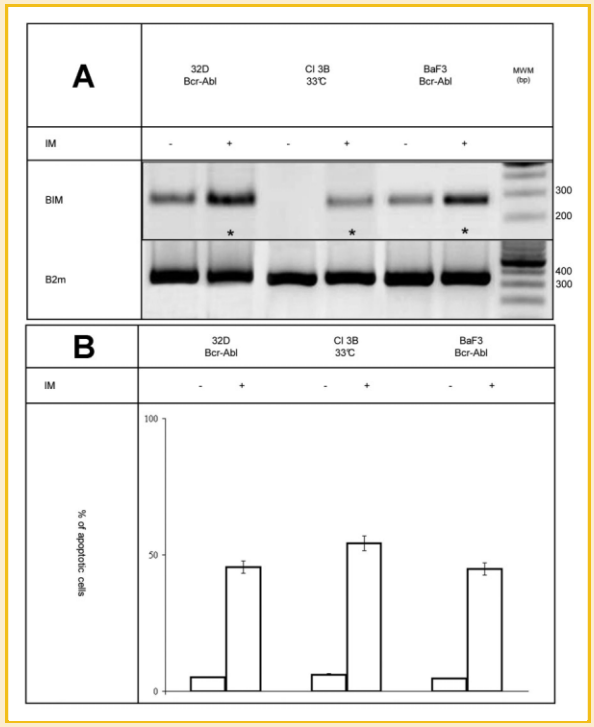


Fig. 6. BIM transcriptional induction in response to IM addresses Bcr-Abl-expressing cells toward apoptotic death. FoxO3a recruitment at the BIM promoter raises the levels of gene transcripts (A) and addresses toward apoptotic death Bcr-Abl-expressing cells (B). The results shown in panel B are the mean values ± standard deviations of three independent experiments. See legend to Figure 3 for details.

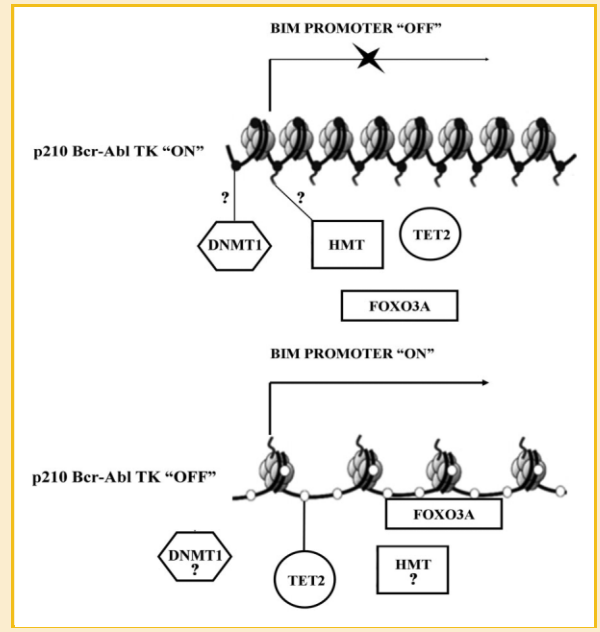


Fig. 7. Epigenetic components of BIM transcriptional down-regulation associated with the TK activity of Bcr-Abl fusion protein. A sequence of epigenetic events may intervene in the establishment of a non-permissive chromatin conformation for the recruitment of FoxO3a transcription factor at the BIM promoter driving BIM transcriptional down-modulation associated with Bcr-Abl. The reduced 5mC hydroxylation proceeding from TET2 loss-of-function is due to its cytoplasmatic compartmentalization in a complex tethered by p210 Bcr-Abl TK and may implement BIM transcriptional silence through the recruitment of DNMT1 and of a still unknown HMT driving, respectively, DNA hyper-methylation and H3K9 tri-methylation. ● Represent methylated-C; ○ represent hydroxymethylated-C; 〉 represent methylated histones.

conversion to 5hmC and FoxO3a recruitment at the BIM promoter (Figs. 4A,C, 5A,B and 6A). H3K9 demethylation contribution to the last event may be driven by the displacement of a histone methyl transferase (HMT) or reactivation a histone demethylase [Yoshimi and Kurokawa, 2011]. Notably, 5mC and H3K9me3 are complementary epigenetic modifications, since both of them provide a recruiting platform for DNMT1 leading to the transcriptional repression of target genes [Berdasco and Esteller, 2010].

A further component of FoxO3a loss-of-function is its cytoplasmatic compartmentalizion in TET2/Bcr-Abl complex (Figs. 1 and 3). Additional events precluding FoxO3a nuclear translocation encompass post-transcriptional modifications and, in particular, PKB-targeted site (Thr^{32} and Ser^{253}) phosphorylation promoting the cytoplasmatic binding with 14-3-3 scaffolding proteins contingent upon the Bcr-Abl TK activity [Brunet et al., 1999; Corrado et al., 2009; Storz, 2011]. Accordingly, FoxO3a nuclear import and chromatin recruitment might be implemented by 14-3-3 binding site targeting [Dong et al., 2008; Mancini et al., 2011].

CONCLUSIONS

Our results support that TET2 is one component of a complex network directing epigenetic modifications which drive BIM down-modulation in CML. TET2 loss-of-function is due to its cytoplasmatic interaction with Bcr-Abl TK and results in impaired 5mC hydroxylation which contributes with DNA and histone hyper-methylation to prevent FoxO3a recruitment at the BIM promoter and BIM transcriptional induction (Fig. 7).

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