BBA - Molecular Cell Research 1865 (2018) 1060–1072

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

# The nonreceptor tyrosine kinase *c*-Abl phosphorylates Runx1 and regulates Runx1-mediated megakaryocyte maturation

Hainan Liu[a], Yan Cui[b], Guang-Fei Wang[a], Qincai Dong[a], Yebao Yao[a], Ping Li[a], Cheng Cao[a,*], Xuan Liu[a,*]

[a] Beijing Institute of Biotechnology, 27 Taiping Rd, Haidian District, Beijing 100850, China
[b] Department of Laboratory Animal Science, Third Military Medical University, Chongqing 400038, China

ARTICLE INFO

*Keywords:*
Abl tyrosine kinase
Phosphotyrosine
Hematopoiesis
Transcription factor
Transcription regulation

ABSTRACT

The transcription factor Runx1 is an essential regulator of definitive hematopoiesis, megakaryocyte (MK) maturation, and lymphocyte differentiation. Runx1 mutations that interfere with its transcriptional activity are often present in leukemia patients. Recent work demonstrated that the transcriptional activity of Runx1 is regulated by kinase-mediated phosphorylation. In this study, we showed that *c*-Abl, but not Arg tyrosine kinase, associated with Runx1 both in cultured cells and in vitro. *c*-Abl-mediated tyrosine phosphorylation in the Runx1 transcription inhibition domain negatively regulated the transcriptional activity of Runx1 and inhibited Runx1-mediated MK maturation. Consistent with these findings, increased numbers of MKs were detected in the spleens and bone marrow of *abl* gene conditional knockout mice. Our findings demonstrate an important role of *c*-Abl kinase in Runx1-mediated MK maturation and platelet formation and provide a potential mechanism of Abl kinase-regulated hematopoiesis.

## 1. Introduction

Runx1 (also known as AML1), which was originally identified at the breakpoint of human t(8;21) chromosome translocation, is a member of the Runt-related transcription factor family (Runx1-Runx3) [1,2]. Runx1 is crucial for definitive hematopoiesis, megakaryocyte (MK) maturation, and lymphocyte differentiation [3–6]. *RUNX1*$^{-/-}$ mice die at 12.5 to 13.5 days postcoitus (dpc), showing severe hemorrhage and complete failure of all definitive hematopoiesis [7,8] because of impeded emergence of the first definitive hematopoietic stem cells (HSCs) from the hemogenic endothelium in the aorto-gonadal-mesonephros (AGM) region [9–12]. Chimeric RUNX1-ETO, RUNX1-EVI1 or ETV6-RUNX1 produced via chromosomal translocation exhibits an impaired transactivation potential, which contributes to a significant proportion of acute leukemia cases.

Over the past 2 decades, Runx1 has been identified as an essential factor in megakaryocytic differentiation. Conditional *Runx1* knockout mice suffer from impaired MK maturation and nonlethal thrombocytopenia [3,5]. In humans, familial platelet disorder with predisposition to AML (FPD/AML) is also caused by RUNX1 haploinsufficiency [13] and is characterized by low MK ploidy levels and defective platelet formation. More recent studies have identified *p19*$^{INK4D}$ and the myosin heavy chain genes *MYL9* and *MYH10* as possible targets of Runx1, which is required for MK polyploidization and endomitosis during MK maturation [14–16].

The transcription factor Runx1 specifically binds to DNA consensus sequences (PuACCPuCA) via a highly conserved N-terminal Runt domain [17]. As a master regulator of hematopoiesis, Runx1 function is tightly controlled through transcriptional control mediated by two promoters, alternative splicing, or posttranslational modifications, including phosphorylation, acetylation, methylation and ubiquitination [18]. Recent studies have shown that Runx1 is phosphorylated by Src family kinases, thereby regulating Runx1 activities related to MK maturation, T cell differentiation and granulopoiesis [19,20]. However, the specific tyrosine kinase that is responsible for Runx1 phosphorylation has not yet been clearly identified.

The nonreceptor protein tyrosine kinases *c*-Abl and Arg (abl-related gene) are ubiquitously expressed in mammalian tissues and share nearly 90% homology in their Src homology 3 (SH3), SH2, and kinase domains, which are highly conserved in Src family kinases [21]. Abl kinases play important roles in regulating cell proliferation, apoptosis, adhesion, cell migration, and stress responses [21,22]. Mice with targeted disruption of the *c-abl* gene are born runted and exhibit abnormal spleen, head, and eye development [23,24]. *c-abl/arg* double-knockout mice die prior to 11 dpc and display defective lymphopoiesis [25]. The constitutively active chimeric Bcr-Abl protein is commonly detected in

* Corresponding authors.
*E-mail addresses:* cao_c@sohu.com (C. Cao), liux931932@163.com (X. Liu).

https://doi.org/10.1016/j.bbamcr.2018.05.001
Received 28 December 2017; Received in revised form 28 April 2018; Accepted 2 May 2018
Available online 04 May 2018
0167-4889/ 

patients with chronic myeloid leukemia (CML) and in some patients with acute lymphoblastic leukemia (ALL) [26–31]. Abl kinases are required for both T cell development and mature T cell function [32,33]. Moreover, *c*-Abl mediates a stage-specific anti-apoptotic response in precursor B cells and is required for efficient V(D)J recombination during B cell development [34]. However, the mechanism by which *c*-Abl regulates lymphocyte development is poorly understood.

In the present study, we showed that *c*-Abl, but not Arg kinase, associated with Runx1 both in cultured cells and in vitro. *c*-Abl-mediated phosphorylation of Runx1 at tyrosines in its inhibitory domain negatively regulated the transcriptional activation activity of Runx1, thereby inhibiting MK maturation. These results demonstrate an important role of *c*-Abl kinase in Runx1-mediated MK maturation and platelet formation and provide a potential mechanism of Abl kinase-regulated hematopoietic cell differentiation.

## 2. Materials and methods

### 2.1. Cell culture and transfections

For cell culture, 293FT cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO), while K562 and MEG-01 cells were grown in Roswell Park Memorial Institute 1640 medium (GIBCO). All media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Transient transfection was performed using the TransIT-X2™ Dynamic Delivery System (Mirus). Cells were treated with murine TPO, murine stem cell factor (SCF) (Peprotech), TPA (Sigma), STI571 (Novartis), or AMN107 (Novartis). K562 cells stably expressing GFP-Runx1 Y4D were generated via plasmid transfection and treatment with 800 μg/ml G418 (Sigma), followed by transfection with Runx1 siRNA.

### 2.2. Vectors and epitope tagging of proteins

HA-, Myc- or GFP-tagged Runx1 and Runx1 mutants were expressed by cloning the genes into pCMV-HA, pCMV-Myc, or pEGFP (Clontech). Flag-tagged *c*-Abl or Arg and *c*-Abl or Arg mutants were cloned into a pcDNA3-based Flag-vector (Invitrogen). GST fusion proteins were generated via expression from pGEX4T-1-based vectors (Amersham Biosciences Biotech, Inc.) in *Escherichia coli* BL21 (DE3).

### 2.3. Immunoprecipitation and immunoblot analysis

Cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A) containing 1% Nonidet P-40. Soluble proteins were immunoprecipitated using anti-Flag (M2, Sigma), anti-HA (E6779, Sigma), anti-Runx1 (ab23980, Abcam), or an anti-mouse IgG antibody (Sigma). An aliquot of the total lysate (5%, v/v) was included as a control. Immunoblotting was performed with horseradish peroxidase (HRP)-conjugated anti-Myc (Sigma), HRP-conjugated anti-Flag, (Sigma), HRP-conjugated anti-HA (Sigma), anti-*c*-Abl (K-12, Santa Cruz), anti-Abl (MABT203, Millipore), anti-β-actin (Sigma), HRP-conjugated anti-pTyr (4G10, Millipore), anti-GST (B-14, Santa Cruz), or anti-Bcr (SC-104, Santa Cruz). The resultant antigen-antibody complexes were visualized via chemiluminescence (ECL system, GE Healthcare). PageRuler Western marker (Thermo) was used as a molecular weight standard.

### 2.4. Protein-binding assays

In GST pull-down experiments, cell lysates were incubated for 2 h at 4 °C with 5 mg of purified GST or GST fusion proteins conjugated with glutathione beads. The adsorbates were then washed with lysis buffer and subjected to SDS-PAGE and immunoblot analysis. An aliquot of the total lysate (5%, v/v) was included as a loading control in SDS-PAGE.

In direct binding assays, immunoprecipitates were separated via SDS-PAGE and then blotted onto nitrocellulose membranes. The membranes were subsequently incubated with purified GST fusion proteins for 2 h at room temperature. The binding of the GST fusion proteins to nitrocellulose was probed using an anti-GST antibody.

### 2.5. In situ proximity ligation assay

The Duolink in situ proximity ligation assay (PLA) (SIGMA-ALDRICH) was applied to detect the interactions between Runx1 and Bcr-Abl in K562 or MEG-01 cells. In brief, cells on glass coverslips were permeabilized with 0.3% Triton X-100 in PBS for 15 min. After blocking with blocking buffer, antibodies against Runx1 (Abcam) and Abl kinase (Millipore) were used according the manufacturer's instructions for PLA. The red fluorescent spots generated from the DNA amplification-based reporter system combined with oligonucleotide-labeled secondary antibodies were detected with a Zeiss LSM 510 Meta confocal microscope.

### 2.6. Kinase assays

Purified GST-Runx1 (2 mg) was incubated with Abl expressed in *E. coli* (Biolabs) in protein kinase buffer for 30 min at 37 °C. The reaction products were analyzed via SDS-PAGE and immunoblotting.

### 2.7. LC-MS/MS analysis

Anti-HA antibody immunoprecipitates prepared from Flag-*c*-Abl- and HA-Runx1-cotransfected 293FT cell lysates were resolved via SDS-PAGE, after which the protein bands were excised. Following adequate digestion with chymotrypsin, LC-electrospray ionization-MS/MS-resolved peptides were analyzed using a Q-TOF2 system (Micromass), and the data were compared against SWISSPROT using the Mascot search engine (http://www.matrixscience.com) for phosphorylation.

### 2.8. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Thermo). The MPL probe employed in this assay contained the reported Runx1-binding sequence [35]. For competition assays, unlabeled oligonucleotides were added to the DNA-binding reaction mixtures.

The sequences of the oligonucleotides were as follows:

5′-CTGGCCCCTGGCCCCAGTGTGGTCTGGATGGGCCCCAGAGGGGCA-3′ and 5′-TGCCCCTCTGGGGCCCATCCAGACCACACTGGGGCCAGGGGCCAG-3′.

The sequences of the unlabeled competitor oligonucleotides with mutations were as follows:

5′-CTGGCCCCTGGCCCCAGTGTTAGCTGGATGGGCCCCAGAGGGGCA-3′ and 5′-TGCCCCTCTGGGGCCCATCCAGCTAACACTGGGGCCAGGGGCCAG-3′.

### 2.9. Chromatin immunoprecipitation assays (ChIP)

K562 cells were cultured with or without 0.1 μM AMN107 for 2 days. The RUNX1 antibody (ab23980) was purchased from Abcam. Chromatin immunoprecipitation (ChIP) was performed with the ChIP-IT High Sensitivity Kit (Active Motif, Catalog No. 53040) as described. Precipitated DNA was quantified using real-time PCR, and the amount of PCR product was evaluated by staining with iTaq™ SYBR Green Supermix (Bio-Rad). The fold-change calculation is based on three formulas: