

LEF-1 activates the transcription of E2F1

Fangfang Zhou¹, Long Zhang¹, Kai Gong, Guangyuan Lu, Baiyang Sheng, Aijun Wang, Nanming Zhao, Xiufang Zhang, Yandao Gong^{*}

State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

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Abstract

LEF-1 and E2F are both transcription factors involved in cell proliferation, differentiation and apoptosis. The present study shows for the first time that LEF-1 associates with E2F1 and further β -catenin independently activates the E2F-responsive reporter gene by attenuating the interaction between E2F1 and Histone deacetylase 1 (HDAC1), which indicates that LEF-1, except for its function in Wnt signaling, may play a distinct role via activating the transcription of E2F1.

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Lymphoid enhancer factor 1 (LEF-1) is a member of the high mobility group (HMG) box family and context dependently participates in the regulation of the T cell receptor (TCR) α enhancer [1,2]. Together with the dependence on other factor-binding sites in regulation of gene expression and DNA bending induced by its HMG domain, LEF-1 is suggested as an “architectural” transcription factor, facilitating the assembly of a high-order multiprotein enhancer complex together with other lymphoid-specific proteins such as Ets-1, PEBP2- α , and ATF/CREB [3,4]. To date, LEF-1 has only been reported to activate gene transcription in the condition of binding with ALY and β -catenin [5,6]. In addition, LEF-1 is the most downstream factor in Wnt signaling. It interacts with β -catenin, and thus forms the ternary complex with DNA, transducing Wnt signals transmitted by β -catenin to a transcriptional response [7,8].

E2F family plays a crucial role in regulation of cell-cycle progression, differentiation and apoptosis. It advances G1-S transition mediated by pRB, a retinoblastoma tumor

suppressor protein. In quiescent cells, the members of pRB family bind to E2F heterodimer and then E2F-targeted genes are repressed. However, high-phosphorylated pRB dissociates from E2F and then activates the E2F-targeted genes, allowing the expression of many genes required for cell-cycle progression and S phase entry [9,10].

It has been reported that E2F1 regulates transcription as well as associates with many other cofactors such as HDAC1 [11], poly(ADP-ribose) polymerase-1 (PARP-1) [12], CBP [13], C/EBP β , and CREB-binding protein/P300 [14]. In this context, we discovered for the first time that LEF-1 activated E2F-luciferase with or without E2F1 overexpression. Moreover, LEF-1 interacted with E2F1 and attenuated the association between E2F1 and HDAC1, which indicated that LEF-1 activated E2F-luciferase possibly by competing with HDAC1 and derepressing the transcriptional inhibition of E2F1 mediated by HDAC1. The present work implied that LEF-1 might participate in E2F-regulated events such as differentiation, proliferation and apoptosis via activating the transcription of E2F1.

Materials and methods

Reagent and plasmids. Polyclonal β -catenin antibody (#9582S) and LEF-1 antibody (#2230) were purchased from Cell Signaling Technology,

^{*} Corresponding author. Fax: +86 10 62794214.

E-mail address: gongyd@tsinghua.edu.cn (Y. Gong).

¹ These authors contributed equally to this work.

Inc (USA). E2F1 antibody (sc-251) was purchased from Santa Cruz Biotechnology, Inc (USA). siRNA against E2F1 β -catenin and LEF-1 were purchased from Santa Cruz Biotechnology, Inc (USA). LEF-1, TopFlash-luciferase, Dishevelled2 (Dvl2), β -catenin were from Prof. Zhijie Chang of Tsinghua University. E2F-luciferase, GSK3 β , Axin1, TCF4 were kindly provided by Prof. Yeguang Chen. E2F1 plasmid was kindly provided by Dr. Michael D. Cole of Princeton University.

Cell culture. HEK293T and COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone) and incubated at 37 °C in a humidified atmosphere of 5% CO₂.

Luciferase reporter assay. HEK293T and COS7 cells were transfected with various plasmids as indicated in the figures. Thirty-six hours after transfection, cells were harvested and the luciferase activities were measured by a luminometer (Berthold Technologies) using Luciferase Reporter Gene Assay Kit of Beyotime (China). All of the experiments for luciferase assay were performed by co-transfection of Renilla (20 ng) as an internal control. Each experiment was performed in triplicate, and the data represent means \pm SD of three independent experiments after normalized to Renilla activity.

Transfection, immunoprecipitation, and immunoblotting. HEK293T and COS7 cells were transiently transfected using Lipofectamine (Invitrogen). At 40 h post-transfection, the cells were lysed with 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Sigma) for 30 min at 4 °C. After centrifugation at 12,000g for 15 min, the lysates were immunoprecipitated with specific antibody and protein A-Sepharose (Zymed Laboratories Inc.) for 3 h at 4 °C. Thereafter, the precipitants were washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95 °C and analyzed by SDS-PAGE. Immunoblotting was performed with specific primary antibody and secondary anti-mouse or anti-rabbit antibodies that were conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were visualized by chemiluminescence.

Real-time reverse transcription (RT)-PCR. Total RNA was isolated using Trizol (Roche, Switzerland) reagent and the genomic DNA was removed by DNase (Takara, Japan). Two microgram RNA was reverse-transcribed at 42 °C for 45 min in a 20 μ l reaction mixture using the Reverse Transcription System (Promega, USA). Sequences for the forward primer and reverse primer are as follows: 5'-GAAGAAGACCGG TTGTCACC-3' (E2F1 forward), 5'-GAAATCCAGAGGGGTCAA GTC-3' (E2F1 reverse); 5'-GGGTATGGAATCCTGTGGC-3' (α -actin forward), 5'-AGTCCGCCTAGAAGCACTTG-3' (α -actin reverse). The reaction system and procession was employed as previously described [15].

Results

LEF-1 specifically activates E2F-responsive reporter gene

Co-transfected E2F-luciferase with Wnt signaling components as indicated in Fig. 1A showed that LEF-1 dramatically activated the expression of E2F-luciferase whereas the other factors such as β -catenin, TCF4 and GSK3 β showed obvious inhibition, and Axin1, Dvl2 had no effect on E2F-luciferase activation (Fig. 1A). Similar results were obtained in COS7 cells. This was supported by the real-time RT-PCR analysis. The auto-regulated effect of E2F1, which stimulates its own activity directly through the presence of binding sites in the promoter, revealed that the mRNA level of E2F1 was increased by LEF-1 in vivo (Fig. 1B) [16]. Using Wnt-responsive TopFlash-luciferase reporter gene, we verified the effect of Wnt signaling components and further revealed that Wnt signaling was not elevated by E2F1, which suggested that LEF-1 specifically activated E2F-responsive reporter gene (Fig. 1C).

LEF-1 activates E2F-responsive reporter gene in a β -catenin-independent manner

E2F-luciferase reporter gene assay showed that LEF-1 dose-dependently activated E2F-responsive reporter gene and further cooperated with E2F1 in activation (Fig. 2A). Consistent result was obtained by knocking down endogenous LEF-1 or E2F1 in Fig. 2B that both LEF-1 siRNA and E2F1 siRNA down-regulated the expression of E2F-luciferase, whereas β -catenin siRNA slightly up-regulated it (Fig. 2B). Notably, knocking down endogenous E2F1 by siRNA not only down-regulated the activity of E2F-luciferase induced by E2F1, but also suppressed the activity induced by LEF-1 (Fig. 2C). Western

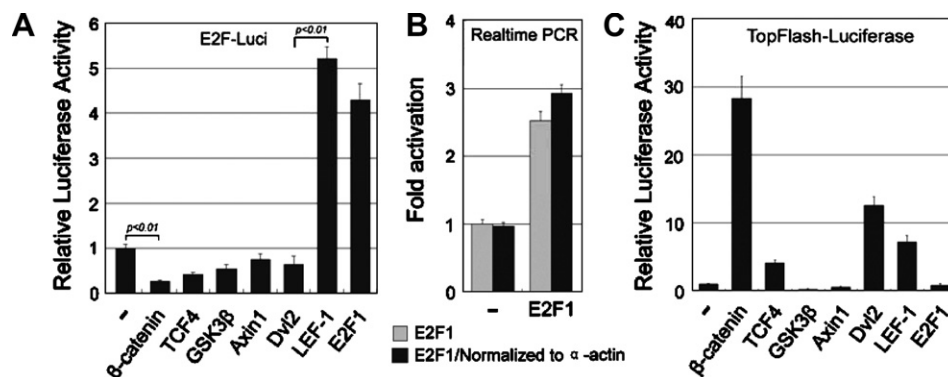


Fig. 1. Activation of E2F-luciferase reporter gene by LEF-1. (A) E2F-luciferase reporter plasmid (0.2 μ g) or (C) TopFlash-luciferase reporter plasmid (0.2 μ g) were cotransfected with various constructs encoded β -catenin (0.1 μ g), TCF4 (0.1 μ g), GSK3 β (0.1 μ g), Axin1 (0.1 μ g), Dvl2 (0.1 μ g), LEF-1 (0.1 μ g) and E2F1 (0.1 μ g) in HEK293T cells as indicated. Transfected cells were lysed for luciferase assay at 36 h post-transfection. All experiments for luciferase assay were performed by cotransfection of Renilla (20 ng) as an internal control. (B) Quantitative real-time RT-PCR was used to detect the expression of the endogenous E2F1 gene expression. HEK293T cells transfected with LEF-1 or with control of the empty expression vector were employed. Both non-normalized (grey) and via α -actin normalized mRNA values (black) were depicted. The amount of mRNA within the control cells was set as one. The deviation from the mean of two independent experiments is indicated.

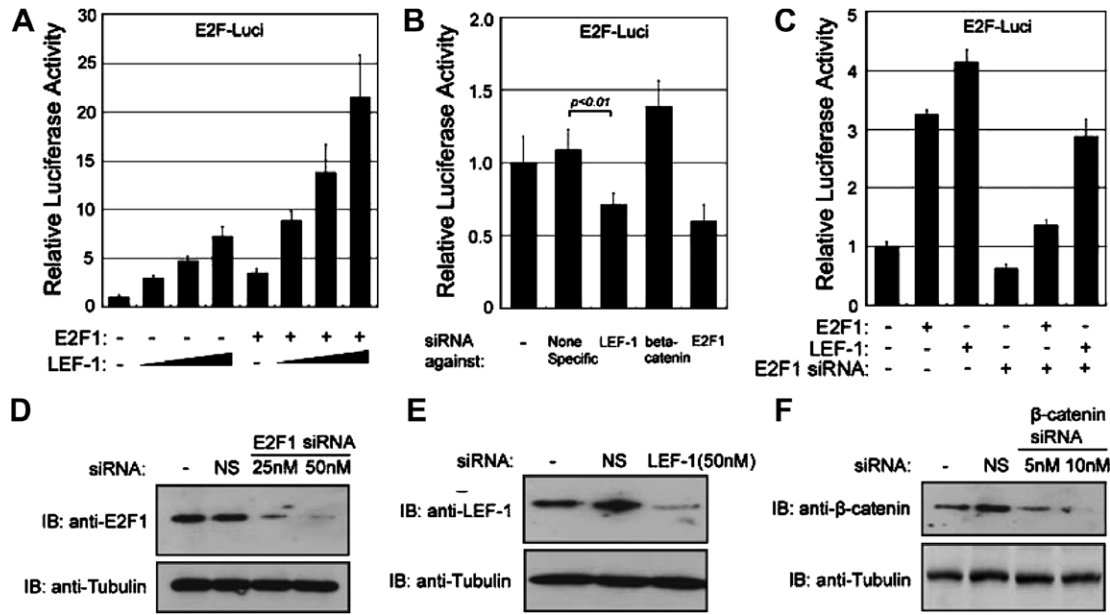


Fig. 2. LEF-1 activates E2F-luciferase in an E2F1-dependent β -catenin and independent manner. (A) E2F-luciferase reporter plasmid (0.2 μ g) was co-transfected with E2F1 (0.1 μ g) and LEF-1 (0.1, 0.2, and 0.3 μ g) constructs in HEK293T cells as indicated. The transfected cells were lysed for luciferase assay at 36 h post-transfection. (B) E2F-luciferase reporter plasmid (0.2 μ g) was co-transfected with siRNA (50 nM) against LEF-1, E2F1, β -catenin, and none specific in HEK293T cell as indicated. Transfected cells were lysed for luciferase assay at 36 h post-transfection. (C) E2F-luciferase reporter plasmid (0.2 μ g) was co-transfected with constructs encoded E2F1 (0.1 μ g) LEF-1 (0.1 μ g) siRNA (50 nM) against E2F1 in HEK293 T cells as indicated. Transfected cells were lysed for luciferase assay at 36 h post-transfection. (D–F) HEK293T cells expressing siRNA against E2F1 (D), LEF-1 (E), and β -catenin (F) were tested for the expression by anti-E2F1, anti-LEF-1, and anti- β -catenin antibodies via western blotting. Tubulin was detected as loading control (lower panels).

blotting showed the inhibitory effect of siRNA against E2F1, LEF-1, and β -catenin by specific antibody, recognizing endogenous E2F1, LEF-1, or β -catenin (Fig. 2 D–F). All these results indicated that the activation of E2F-responsive reporter gene by LEF-1 independent of β -catenin, would partially, if not entirely, relate with E2F1.

LEF-1 associates with E2F1 in vivo

Subsequent immunoprecipitation between LEF-1 and E2F1 supported the result above. As shown in Fig. 3A, immunoprecipitated E2F1 was only detected in samples

with overexpressed HA-LEF-1. Consistently, LEF-1 protein could be only detected in samples immunoprecipitated with E2F1 antibody, not in samples immunoprecipitated with pre-immune serum. These results indicated that LEF-1 and E2F1 associated in vivo.

LEF-1 attenuates the association of HDAC1 to E2F1

Previous reports have demonstrated that pRB could recruit histone deacetylase 1(HDAC1) to E2F1 and that pRB cooperated with HDAC1 to repress the E2F1-regulated promoter of the gene encoding the cell-cycle protein cyclin E [11]. Here, a deacetylase inhibitor, trichostatin A (TSA) was used as indicated in Fig. 4A. We found that TSA obviously up-regulated the expression of E2F-luciferase, particularly when activated by LEF-1 or E2F1. This result demonstrated that HDAC1 may be involved in LEF-1-mediated activation of E2F-luciferase. To verify this hypothesis, we performed immunoprecipitation assay which identified the association between HDAC1 and E2F1. As shown in Fig. 4B, overexpressed LEF-1 attenuated the association between HDAC1 and E2F1, whereas down-regulating endogenous LEF-1 by siRNA strengthened it (Fig. 4B).

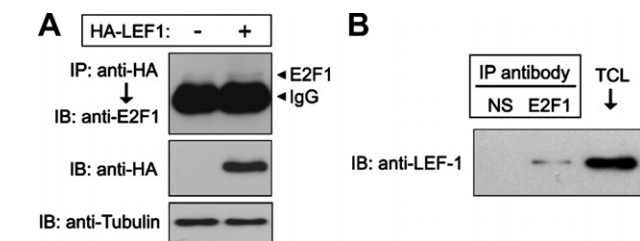


Fig. 3. Association between LEF-1 and E2F1 in vivo. (A) HEK293T cells were transfected with HA-tagged LEF-1 (3 μ g) as indicated. At 40 h post-transfection, cells were harvested for anti-HA immunoprecipitation (IP). LEF-1-associated E2F1 was revealed by anti-E2F1 immunoblotting (upper panel). The protein expression was confirmed by anti-HA or anti-LEF-1 immunoblotting (IB) with the total cell lysates (middle and lower panels). (B) HEK293T cells were harvested for anti-E2F1 or control anti-none specific (NS) immunoprecipitation (IP). E2F1-associated LEF-1 was revealed by anti-LEF-1 immunoblotting. “TCL” represented “total cell lysates” as control (upper panel).

Discussion

The present study showed for the first time the activation of E2F-luciferase by LEF-1 and association between

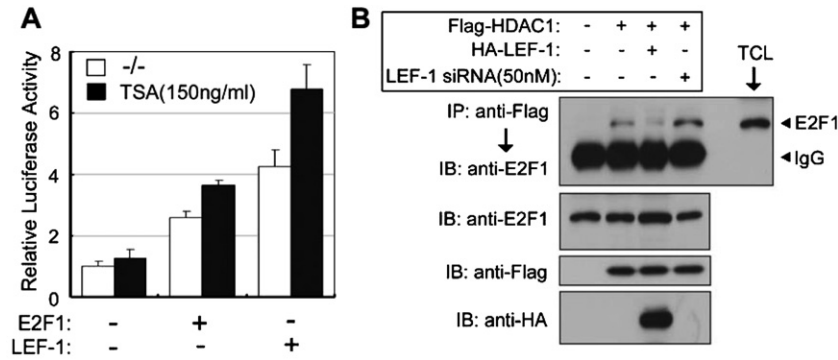


Fig. 4. LEF-1 derepresses E2F1-targeted genes by interfering with HDAC1 association. (A) E2F-luciferase reporter plasmid (0.2 μ g) was co-transfected with E2F1 (0.1 μ g) and LEF-1 (0.1 μ g) constructs in HEK293T cell as indicated. At 28 h post-transfection, cells were treated with deacetylase inhibitor trichostatin A (TSA) using the drug at 150 ng/ml for 4 h as indicated. Cells were then lysed for luciferase assay. (B) HEK293T cells were transfected with Flag-tagged HDAC1 (3 μ g), HA-LEF-1 (3 μ g) and LEF-1 siRNA (50 nM) as indicated. At 40 h post-transfection, cells were harvested for anti-Flag immunoprecipitation (IP). HDAC1-associated endogenous E2F1 was revealed by anti-E2F1 immunoblotting (upper panel). The protein expression was confirmed by anti-E2F1, anti-Flag, or anti-HA immunoblotting (IB) with total cell lysates (middle and lower panels). “TCL” represented “total cell lysates” as control (upper panel).

E2F1 and LEF-1. Both E2F1 and LEF-1 have been reported to be associated with cell proliferation and apoptosis. However, no reports on crosstalk between E2F and LEF-1 could be found in the literature. The Pearson correlation coefficient (PCC) indicates if two proteins are transcriptionally correlated or anti-correlated, where 1 for perfectly correlated, 0 for no correlation, and $PCC = -1$ for perfectly anti-correlated. We looked into the mRNA expression profile of the protein LEF-1 and E2F1 in different human tissues [17] and found they were apparently correlated ($PCC = 0.27$). Furthermore, our luciferase reporter assay revealed the activation of E2F-luciferase by LEF-1 in a dose-dependent manner and following quantitative real-time RT-PCR supported the transcriptional activation of E2F1 by LEF-1 *in vivo*. Other factors involved in Wnt signaling such as β -catenin, TCF4, GSK3 β , Axin1, Dvl2 could not activate E2F-luciferase and E2F1 could not reversely activate Wnt signaling indicated that the activation of E2F-luciferase by LEF-1 was specific. Previous reports demonstrated that the transcriptional activation of LEF-1 depends on its associated protein such as β -catenin and ALY. However, in our study, siRNA-targeted β -catenin and E2F1 showed that the activation of E2F1 transcription by LEF-1 was β -catenin-independent and possibly E2F1-dependent. Subsequent immunoprecipitation between E2F1 and LEF-1 supported the role of E2F1 in this activation. To date, no reports announced that LEF-1 recognized E2F-binding site. We also screened the binding site of E2F1 via TFSEARCH: Searching Transcription Factor Binding Sites (ver 1.3) and no sites bound by LEF-1 were found. A recent report discovered that C/EBP β activated E2F-regulated genes *in vivo* via recruitment of the coactivator CREB-binding protein/P300 [14]. Therefore, LEF-1 may recruit some other coactivator to E2F1 or interfere with the corepressor and then activate the transcription of E2F1. Supporting this notion is the TSA (HDAC inhibitor) treatment and the immunoprecipitation between HDAC1 and E2F1, which showed the

activation of E2F-luciferase by LEF-1 was up-regulated by TSA and LEF-1 attenuated the repression of E2F transcription by HDAC1. However, LEF-1 activated E2F-luciferase much more than E2F1 itself did, which suggested there might be some other manners that regulated this activation.

LEF-1 and TCF4 belong to HMG box family and have a virtually identical DNA-binding domain and a β -catenin interaction domain [18]. In the regulation of Wnt signaling, they function similarly. However, in this finding, TCF4, different with LEF-1, could not activate E2F-luciferase, which suggested that the activation was not dependent on HMG domain.

In light of observations mentioned above, it is likely that LEF-1 associated with E2F1 and activated the transcription of E2F1 and E2F1-targeted genes by interfering with HDAC1 binding. In addition, lots of reports discovered that both LEF-1 and E2F1 involved in cell proliferation and apoptosis. All these suggested that LEF-1 might participate in E2F1-regulated cell cycle and apoptosis. Our results described a new activity of LEF-1 and a new coactivator for E2F1. Clearly, further studies are required to ascertain the key roles of this activation in the regulation of E2F1-dependent function.

Acknowledgments

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