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Pirin Down-Regulates the EAF2/U19 Protein and Alleviates its Growth Inhibition in Prostate Cancer Cells

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Abstract

BACKGROUND—The tumor suppressor ELL associated factor 2 (EAF2/U19) has been reported to induce apoptosis of LNCaP cells and suppress AT6.1 xenograft prostate tumor growth. EAF2/U19 expression level is down-regulated in advanced human prostate cancer. EAF2/U19 is also a putative transcription factor with a transactivation domain and capability of sequence-specific DNA binding. Identification of binding partners and regulators of EAF2/U19 is essential to understand its function in regulating apoptosis/survival of prostate cancer cells.

METHODS—Through a yeast two-hybrid screening system, we identified Pirin as a binding partner of EAF2. We further determined the interaction between epitope-tagged EAF2/U19 and Pirin by co-immunoprecipitation in mammalian cells. The effect of Pirin on EAF2/U19 inhibition of LNCaP growth was assayed by colony formation.

RESULTS—Pirin co-immunoprecipitated with EAF2/U19 and the overexpressed Pirin decreased the expression level of EAF2/U19 protein in prostate cancer cell lines LNCaP and PC3. Furthermore, overexpression of EAF2/U19 suppressed LNCaP colony formation, and co-expression of Pirin significantly blocked the growth inhibition induced by EAF2/U19 overexpression.

CONCLUSION—Pirin is a newly identified binding partner of EAF2/U19 capable of down-regulating EAF2/U19 protein and alleviating its inhibition of prostate cancer cell survival/proliferation. Pirin may play an important role involved in EAF2/U19 function as an androgen-responsive gene and tumor repressor.

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The authors have nothing to disclose.

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Keywords

EAF2/U19; Pirin; prostate cancer

INTRODUCTION

EAF2/U19 is a tumor suppressor initially identified as a novel androgen-responsive gene in the prostate [1,2]. EAF2/U19 expression is down-regulated in human prostate cancer specimens and cell lines. Overexpression of EAF2/U19 can induce apoptosis in transfected prostate cancer cells and inhibit xenograft tumor growth [2]. Furthermore, EAF2/U19 knockout mice developed prostatic intraepithelial neoplasia, B-cell lymphoma, hepatocellular carcinoma, and lung adenocarcinoma [3]. Although EAF2/U19 plays an important role as a tumor suppressor, the mechanism of its action remains poorly understood.

EAF2/U19 was also identified as an Eleven-nineteen lysine-rich leukemia (ELL)-associated in factor 2 (EAF2) [4]. ELL was initially identified as a fusion partner of myeloid/lymphoid or mixed-lineage leukemia gene (MLL) in a recurrent chromosomal translocation in acute myeloid leukemia [5,6]. ELL is a RNA polymerase 2 transcriptional elongation factor and a component in super elongation complexes [7,8]. EAF2/U19, along with its homolog EAF1, can also modulate transcriptional elongation [4,9,10].

Pirin is a highly conserved protein initially identified on the basis of its interaction with NF1/CTF1 transcription/replication factor [11]. It belongs to the functionally diverse Cupin superfamily, which is linked to diverse biological and molecular processes including regulation of transcription, apoptosis, stress response, and enzymatic processes [12]. Pirin has also been found to interact with the proto-oncoprotein Bcl-3 (B-cell lymphoma 3-encoded protein) and modulates the activity of Rel/NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factors [13]. Recent studies showed that Pirin acts as a negative regulator of cellular senescence in melanocytic cells [14]. Also, cytoplasmic localization of Pirin is associated with melanoma progression [15]. In addition, abnormal Pirin expression was observed in several human malignancies. For example, Pirin down-regulation impairs terminal myeloid differentiation and is associated with acute myeloid leukemia [16]. Expression studies showed that Pirin mRNA is abundant in the human prostate [14], suggesting a potential role of Pirin in prostate differentiation and carcinogenesis. However, the function of Pirin in prostate cancer has not been studied.

In an effort to elucidate the mechanism of EAF2/U19 action in prostate cancer cells, we performed a yeast-two-hybrid screening and identified Pirin as a binding partner of EAF2/U19. The interaction between EAF2/U19 and Pirin was further investigated using co-immunoprecipitation, fluorescent microscopy, western blot, and colony formation assay. These studies provided evidence that Pirin is an important binding partner of EAF2/U19 that can alleviate the growth inhibition of EAF2/U19 in prostate cancer cells.

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MATERIALS AND METHODS

Yeast-Two-Hybrid Screening

The N-terminal region of EAF2/U19 (a.a.1–114) was cloned into the pLexA plasmid of the Matchmaker LexA 2-Hybrid system (Clontech, Palo Alto, CA), generating a LexA-DNA-binding domain (BD)-EAF2/U19 (a.a.1–114) fusion protein. The pLexA-EAF2/U19 (a.a.1–114) was transformed into the yeast strain (*Saccharomycs cerevisiae* EGY48) according to the manufacturer's protocol and described previously [17]. The LexA-DNA-BD-EAF2/U19 (a.a.1–114) fusion protein did not exhibit autonomous transcriptional activity and was used as the bait to screen a cDNA library generated from whole prostate glands pooled from 10 Caucasian men (HL4511AK, Clontech), with cDNA coding region fused to the activation domain (AD) of the *E. coli* transcription factor B42. The screening was carried out as described previously [17].

Tissue Culture and Transfection

PC3, LNCaP, COS-1, and 293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). PC3 and LNCaP cells were grown in PRMI 1640 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 2mM L-glutamine. COS-1 and 293T cells were grown in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin and 2mM L-glutamine. Cells were transfected with plasmid DNA using PolyJet™ DNA In Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer's instructions.

Construction of Various Expression Vectors

Various expression vectors with different epitope-tags were constructed using routine molecular cloning techniques. The expression vector of HA tagged Pirin (pHA-Pirin) was generated with pCMV-HA vector and red fluorescent protein (RFP) tagged Pirin was genereated with pDsRed-Express-C1. Both of the vectors were obtained from Clontech (Mountain Veiw, CA). Through PCR, EcoRI, and KpnI sites were added to the 5′ and 3′ ends, respectively, of human Pirin, using the following primers: 5′-ATATCGAATTCGGATGGGGTCCTCCAAGAAAGT-3′ (sense) for HA-Pririn, 5′-ATATCGAATTCGATGGGGTCCTCCAAGAAAGT-3′ (sense) for RFP-Pirin, and 5′-ATATCGGTACCCTAGTTCCCAATCTTTGATTTCC-3′ (anti-sense) for both. The resultant PCR product was cloned into pCMV-HA vector. Vectors were confirmed by sequencing (Macrogen, Seoul, S. Korea). Epitope-tagged EAF/U19 expression vectors were generated previously [18].

Western Blot Analysis

Cultured 293T were lysed in modified radioimmune precipitation assay (RIPA) buffer [50mM Tris (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA (pH 8.0)] with protease inhibitor cocktail (#P8340, Sigma St. Louis, MO) Protein concentration was measured with bicinchoninic acid assay (BCA). Western blot was conducted using antibodies against GFP (#TP401; Chemokine, Houston, TX), HA (#MMS-101P; Covance), and GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA), followed with

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horseradish peroxidase-labeled secondary antibody (sc-2004, sc-2003, Santa Cruz Biotechnology). Signals were visualized using chemiluminescence (ECL Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and were exposed to X-ray film (Fuji film, Stanford, CT).

Immunoprecipitation

COS-1 cells cultured in 10 cm culture dishes were transfected with 5 μg of each plasmid DNA. Cells were washed with PBS 48 hr after transfection, and whole cell lysates were prepared by incubating in NP-40 lysis buffer (50mM Tris–HCl, 150mM NaCl, 1% NP-40, pH 8.0) containing protease inhibitor cocktail (#P8340) for 30 min on ice. The lysates were centrifuged at 12,000*g* for 10 min at 4°C, and the supernatants were collected for immunoprecipitation. After preclearing with normal host IgG, the lysates were immunoprecipitated with 30 μl agarose conjugated with anti-GFP antibody (#D153-8, MBL, Wuborn, MA) overnight at 4°C. The precipitated complexes were washed three times with lysis buffer and boiled for 5 min in SDS sample buffer, followed by immunoblotting with various antibodies as indicated in the figures.

Colony Formation Assay

LNCaP cells, at approximately 80% confluence in 6 well plates, were transfected using PolyJet™ DNA In Vitro Transfection Reagent. Cells in each well were expanded into 3 × 10 cm^2 dishes 24 hr after transfection. Stable transfectants were selected in medium containing 500 μg/ml G418 (Gemini Bio-Products, West Sacramento, CA) for 3 weeks. After 3 weeks, surviving cells were washed once with PBS and stained with 0.5% crystal violet in methanol. Colonies with a diameter larger than 0.5mm were counted. The experiments were reproduced three times.

Immunofluorescent Microscopy

Cells which were seated on the cover glasses or in the 6-well plates were transfected with GFP (GFP-U19/EAF2) or RFP (RFP-Pirin) tagged constructs for 48 hr. Cells were fixed with 2% paraformaldehyde and penetrated with 0.1% Triton X-100 solution, followed by nuclear staining with Hoechst. Slides were observed with a Nikon Eclipse TE2000-U inverted fluorescent microscope (Melville, NY).

RESULTS

Pirin is Identified as a Potential Binding Partner of EAF2/U19 using a Yeast-Two-Hybrid Screening

We used the N-terminal region (a.a.1–114) of EAF2/U19 as bait in our yeast-two-hybrid screening for two reasons. First, EAF2/U19 contains a transactivation domain in its C-terminal region [18], making the full-length EAF2/U19 inadequate as bait in the screening. Second, the N-terminal region of EAF2/U19 is highly conserved and essential for the pro-apoptotic activity of EAF2/U19 [19]. We have identified a total of 13 candidate binding partners of EAF2/U19 (a.a.1–114) (Supplemental Table I). One of the clones isolated from the yeast-two-hybrid screening was Pirin, which is implicated to play an important role in acute myeloid leukemia and abundantly expressed in the prostate [14,16]. Since EAF2/U19

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is also involved in acute myeloid leukemia and both Pirin and EAF2/U19 are expressed in the prostate, we decided to investigate the potential role of Pirin in modulation of EAF2/U19 function in prostate cancer cells.

Pirin Co-Immunoprecipitates with EAF2/U19 in Mammalian Cells

To verify the interaction of Pirin and EAF2/U19 in mammalian cells, COS-1 cells were transiently co-transfected with GFP-tagged EAF2/U19 and HA-tagged Pirin followed with co-immunoprecipitation. As shown in Figure 1, HA-tagged Pirin co-precipitated with GFP-tagged EAF2/U19 by GFP-antibody conjugated agarose in COS-1 cells. However, in the control cells transfected with empty GFP vector, HA-tagged Pirin failed to be detected in the precipitated complex. This result confirmed the interaction between Pirin and EAF2/U19 in transfected mammalian cells.

To further characterize the interaction between Pirin and EAF2/U19, we checked the localization of Pirin and EAF2/U19 in COS-1 cells. With the transient transfection, GFP-tagged EAF2/U19 and RFP-tagged Pirin were predominantly co-localized in the nuclei (Fig. 2). Overexpression of RFP-tagged Pirin did not change the localization and expression pattern of GFP-tagged EAF2/U19 in COS-1 cells, which contrasted the formation of nuclear speckles by EAF2/U19 together with another reported binding partner ELL [18]. Regardless, the predominant co-localization of Pirin and EAF2/U19 was consistent with their co-immunoprecipitation.

Pirin Decreases the Expression Level of EAF2/U19

In LNCaP, PC3, and 293T cells with co-transfection with GFP-tagged EAF2/U19 and RFP-tagged Pirin, the expression level of GFP-tagged EAF2/U19 was significantly decreased compared with cells transfected with empty RFP vector and GFP-tagged EAF2/U19 (Fig. 3). However, the expression of RFP-tagged Pirin was not significantly affected by the co-expression of GFP-tagged EAF2/U19. These results suggested that Pirin affected the expression level and stability of EAF2/U19 in mammalian cells.

To further assess the effect of Pirin regulation of EAF2/U19 expression, we collected the cell lysates after the transfection with HA-tagged EAF2/U19, HA-tagged Pirin, or both vectors and performed Western blot assay. Consistent with the results in Figure 3, the Western blots in Figure 4 showed that the expression level of HA-tagged EAF2/U19 was reduced by the co-expression of HA-tagged Pirin, and the overexpressed EAF2/U19 did not affect the abundance of Pirin in 293T cells. Co-transfected GFP levels verified equal transfection efficiency in all groups (Fig. 4). These results indicated that overexpression of Pirin decreased the expression of EAF2/U19; and since HA-tagged EAF2/U19 was under the CMV promoter, decreased expression of EAF2/U19 is likely due to the protein instability of EAF2/U19 protein induced by the presence of overexpressed Pirin.

Overexpression of Pirin Alleviates the EAF2/U19 Inhibition of LNCaP Colony Formation

As previously reported by our lab, EAF2/U19 has been identified as a tumor suppressor which induces apoptosis of LNCaP cells and suppresses AT6.1 xenograft prostate tumor growth in vivo [2,3]. Colony formation assay was performed to determine whether co-

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transfection of Pirin could modulate the effect of EAF2/U19 on the growth of LNCaP cells. Cells were co-transfected with GFP and RFP, GFP-tagged EAF2/U19 and RFP, RFP-tagged Pirin and GFP, or GFP-tagged EAF2/U19 and RFP-tagged Pirin. Transfection with GFP-EAF2/U19 alone suppressed the colony formation by 55% compared with the control group, which was consistent with the previous report [19]; and co-expression of Pirin with EAF2/U19 significantly enhanced the colony formation ($P<0.05$), although overexpression of Pirin alone did not affect cell growth (Fig. 5).

DISCUSSION

Identification and characterization of binding partners of EAF2/U19 will facilitate studies to define the mechanisms of EAF2/U19 suppression of prostate tumorigenesis. This study identified Pirin as a binding partner of EAF2/U19 capable of modulating the growth suppressive function of EAF2/U19 in prostate cancer cells. Pirin may represent an important factor involved in EAF2/U19 function as an androgen-responsive gene and tumor suppressor.

The N-terminal region of EAF2/U19 is necessary and sufficient to induce apoptosis in prostate cancer cells in vitro [19]. Although the N-terminal region of EAF2/U19 does not contain a transactivation domain, its induction of apoptosis requires the expression of its downstream genes, that is, new protein synthesis, because EAF2/U19 induced apoptosis can be blocked by cycloheximide, a protein synthesis inhibitor [2]. Therefore, the induction of apoptosis by the N-terminal region of EAF2/U19 may involve the recruitment of other transcription factors through protein–protein binding. Pirin may represent one of the EAF2/U19 binding factors capable of modulating transcription activity of EAF2/U19.

Our studies showed that Pirin binds to EAF2/U19 in co-immunoprecipitation assays and that overexpression of pirin downregulates transfected EAF2/U19 protein level as detected by Western blot and fluorescent microscopy. Since Pirin did not affect the protein levels of GFP or RFP and all the transfected genes are under control of the CMV promoter, the effect of Pirin on EAF2/U19 protein level is likely mediated through its effect on EAF2/U19 protein stability. The down-regulation of EAF2/U19 by Pirin (Figs. 3 and 4) contrasts the up-regulation of EAF2/U19 by ELL [18]. These observations suggest that regulation of EAF2/U19 at the protein level by different binding partners may represent an important mechanism by which EAF2/U19 activity is regulated. The mechanism regulating EAF2 protein stability remains unclear. Future studies investigating the mechanism of EAF2/U19 protein stability, particularly in the presence of various binding partners, will be important to understanding the mechanisms regulating EAF2/U19 activity.

Pirin regulation of LNCaP cell survival/proliferation appears to be mediated through EAF2/U19 binding. Overexpression of Pirin alone did not affect colony formation of LNCaP cells (Fig. 5). However, Pirin overexpression alleviated EAF2/U19 repression of LNCaP colony formation. This observation is consistent with down-regulation of EAF2/U19 protein level by Pirin co-transfection. Although our studies suggested EAF2/U19 regulation by Pirin, it is not clear whether EAF2/U19 can modulate Pirin function. Overexpression of EAF2/U19 did not affect the level or subcellular localization of Pirin in co-transfected cells.

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Since Pirin had no significant effect on LNCaP growth, it is difficult to test the effect of EAF2/U19 overexpression on Pirin function in LNCaP growth control. The inability of Pirin overexpression to affect LNCaP colony formation is consistent with the observation that Pirin overexpression did not affect the proliferation of melanoma cells [14].

Our previous studies showed that EAF2/U19 overexpression inhibited cell proliferation and induced apoptosis in stably transfected cells [2]. EAF2/U19 knockout enhanced cell proliferation and induced prostatic intraepithelial neoplasia in the mouse prostate [3]. However, EAF2/U19 knockout had no detectable effect on apoptosis in the mouse prostate. This raises a possibility that EAF2/U19 induction of apoptosis in cultured cells may be a result of high levels of transfected EAF2/U19 expression. It is possible that EAF2/U19 induction of apoptosis in vitro is coupled with its inhibition of cell growth pathways. We were not able to accurately determine the effect of transiently transfected EAF2/U19 on cell death versus proliferation, particular when it was co-transfected with Pirin expression vector. This may be due to the limited transient transfection efficiency, high background caused by transfection reagents, and/or some unknown reasons. Thus, whether EAF2/U19 inhibition of colony formation is mainly mediated by proliferation inhibition or apoptosis induction remains to be further investigated.

Both Pirin and EAF2/U19 have previously been implicated to play important roles in acute myeloid leukemia [4,16]. Pirin is often down-regulated in acute leukemia cells and its expression is thought to be required for terminal myeloid maturation. The finding of Pirin modulation of EAF2/U19 protein level and function in this study suggests that Pirin regulation of terminal myeloid differentiation may be in part mediated through its binding to EAF2/U19.

In conclusion, this study identified Pirin as a novel EAF2/U19 binding partner. Pirin binding to EAF2/U19 can down-regulate the protein level of EAF2/U19 and alleviate its growth suppression of transfected LNCaP cells. Further studies will be required to determine how Pirin downregulates EAF2/U19 protein levels and on the potential role of this interaction in prostate tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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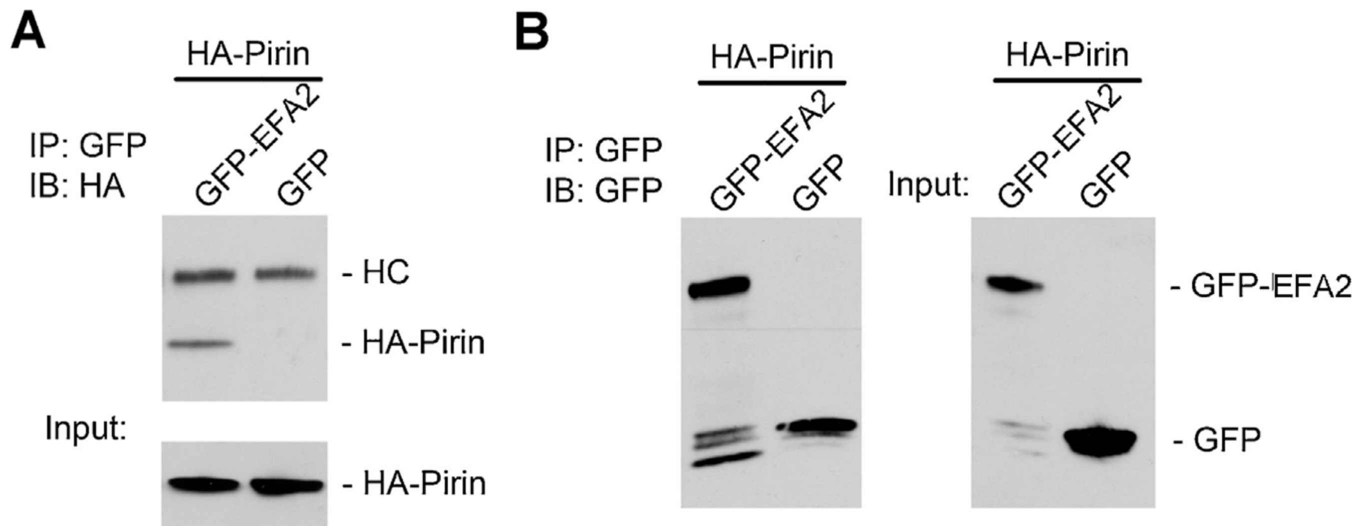

Fig. 1.

EAF2/U19 physically interacts with Pirin. A and B, Co-immunoprecipitation demonstrates a complex formation between EAF2/U19 and Pirin. COS-1 cells were co-transfected with GFP-tagged EAF2/U19 and HA-tagged Pirin or GFP and HA-tagged Pirin as control. Total cell lysates were subjected to immunoprecipitation with agarose conjugated Anti-GFP, and analyzed by immunoblotting with antibody against HA (**A**) and GFP (**B**). Total cell lysates were analyzed by immunoblotting with antibody against HA (A, lower panel) and GFP (B, right panel) as input. IB, immunoblotting; IP, immunoprecipitation.

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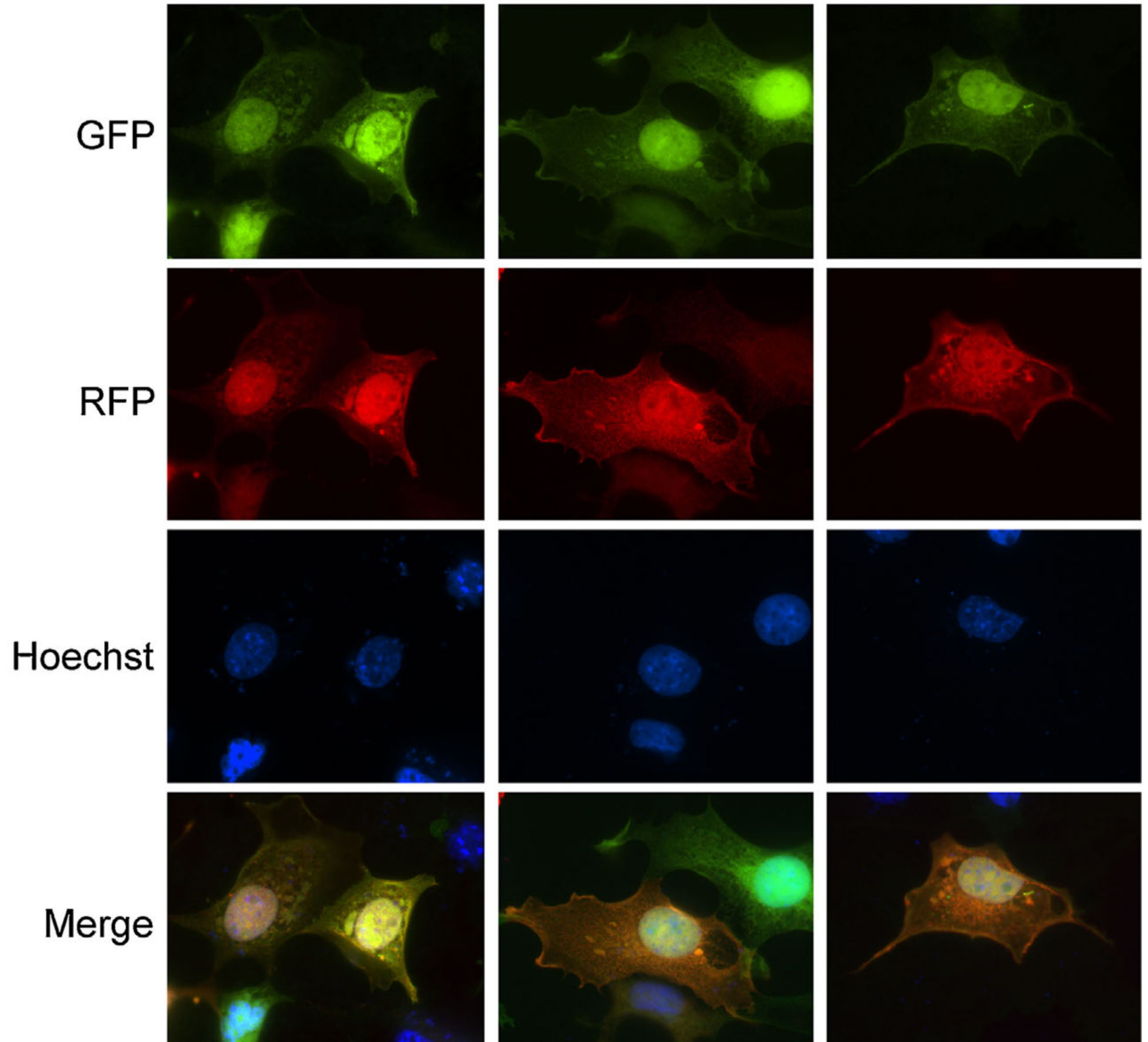

Fig. 2.

Subcellular localization of EAF2/U19 and Pirin overlaps in COS-1 cells. COS-1 cells were co-transfected with GFP or RFP tagged proteins as indicated for 48 hr. Cells were fixed and stained with Hoechst. Images were takenwithNikonT2000 fluorescence microscopy. GFP, green fluorescence protein, as shown in green; RFP, red fluorescence protein, as shown in red; Hochest, as shown in blue; merged signal, as shown in yellow.

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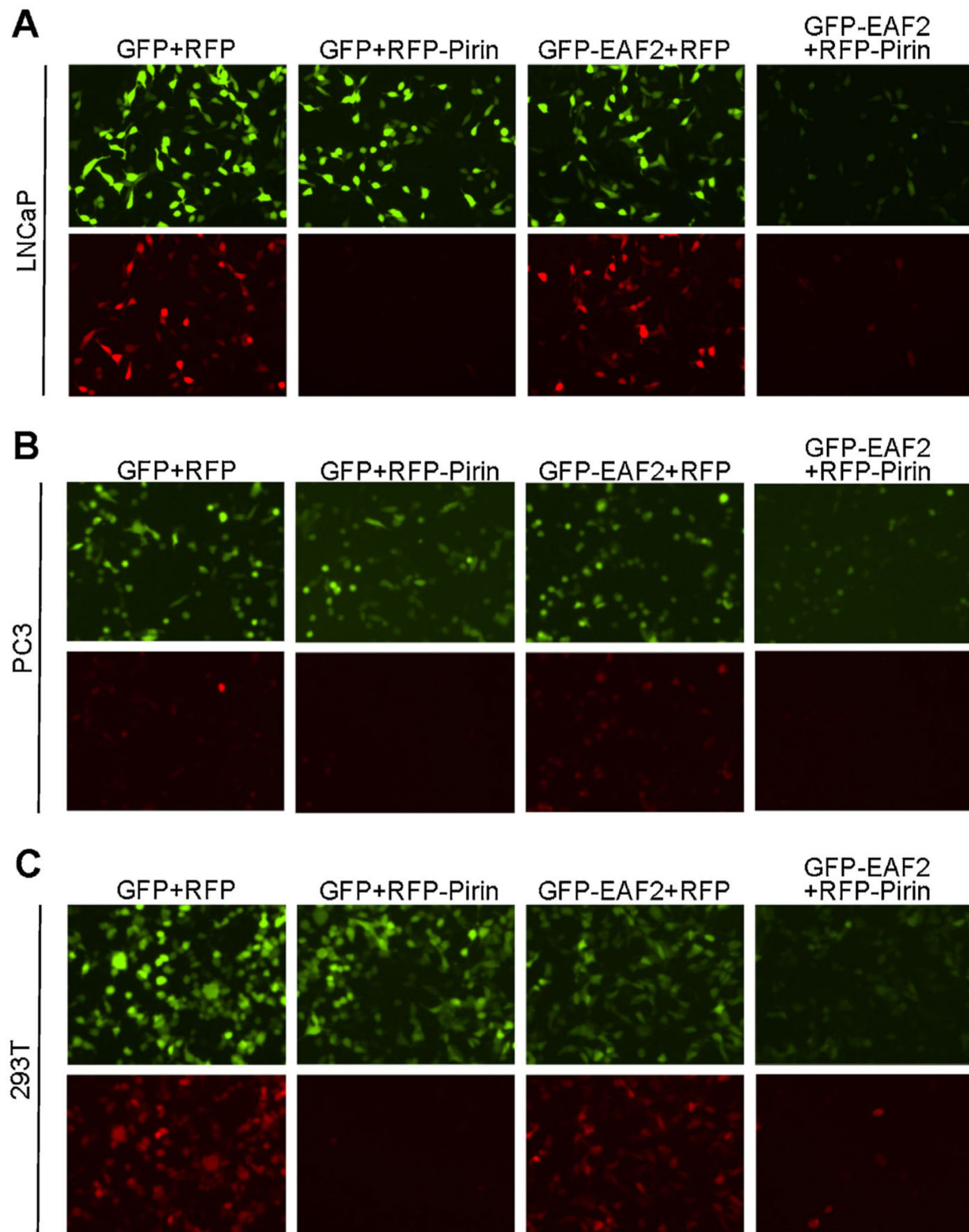

Fig. 3.

Overexpression of Pirin down-regulates the expression level of EAF2/U19. LNCaP (**A**), PC3 (**B**), and 293T (**C**) cells were co-transfected with GFP or RFP tagged proteins asindicatedfor24 hr. All cells were imaged using the same exposure time under a fluorescence microscope. GFP, green fluorescence protein, as shown in green; RFP, red fluorescence protein, as shown in red.

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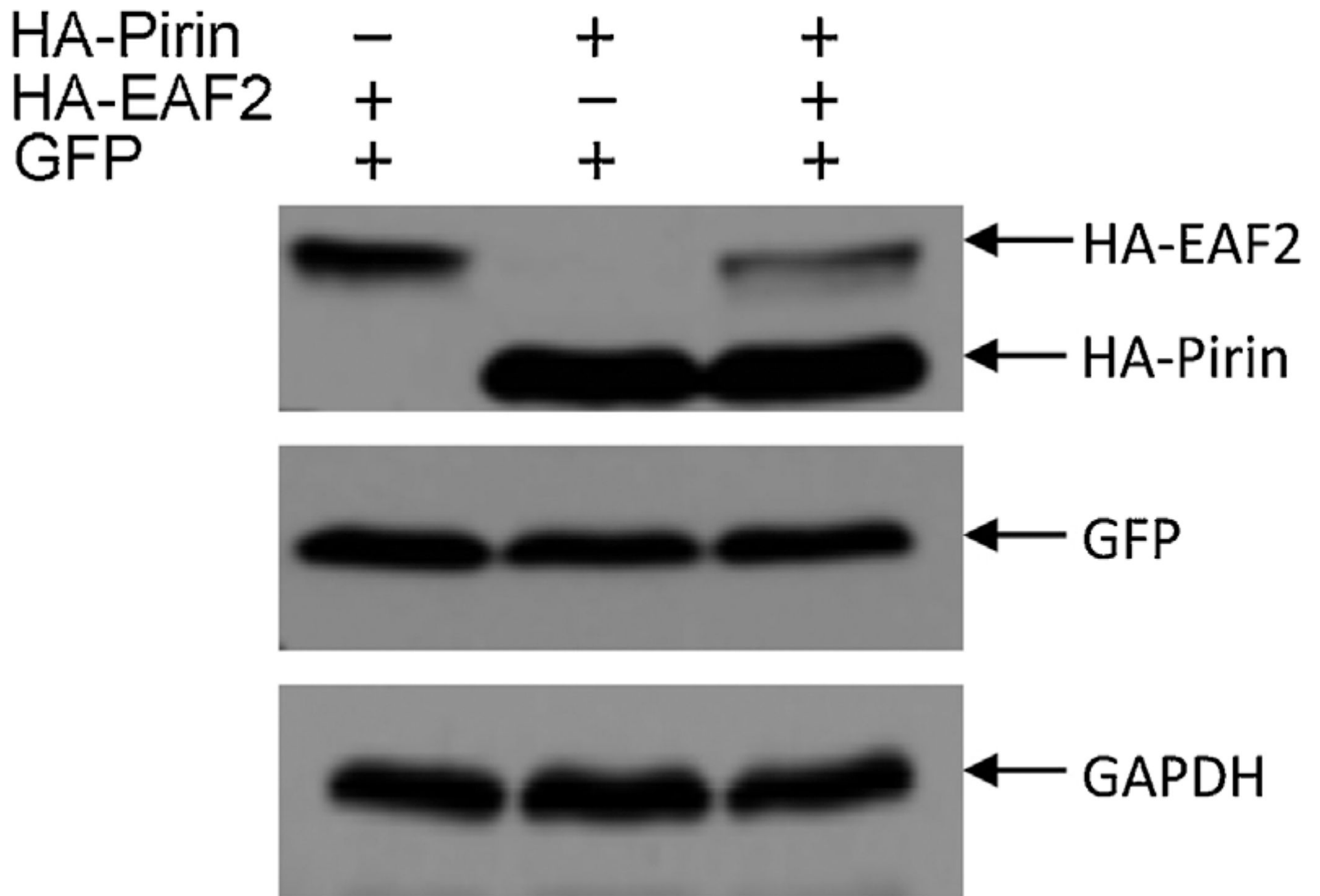

Fig. 4.

Overexpression of Pirin represses HA-tagged EAF2/U19 expression. Western blot analysis of 293T cells co-transfected with HA-tagged Pirin or/and HA-tagged EAF2 vectors as indicated in the figure for 48 hr. Co-transfected GFP expression vector served as control for transfection efficiency. GAPDH severed as loading control.

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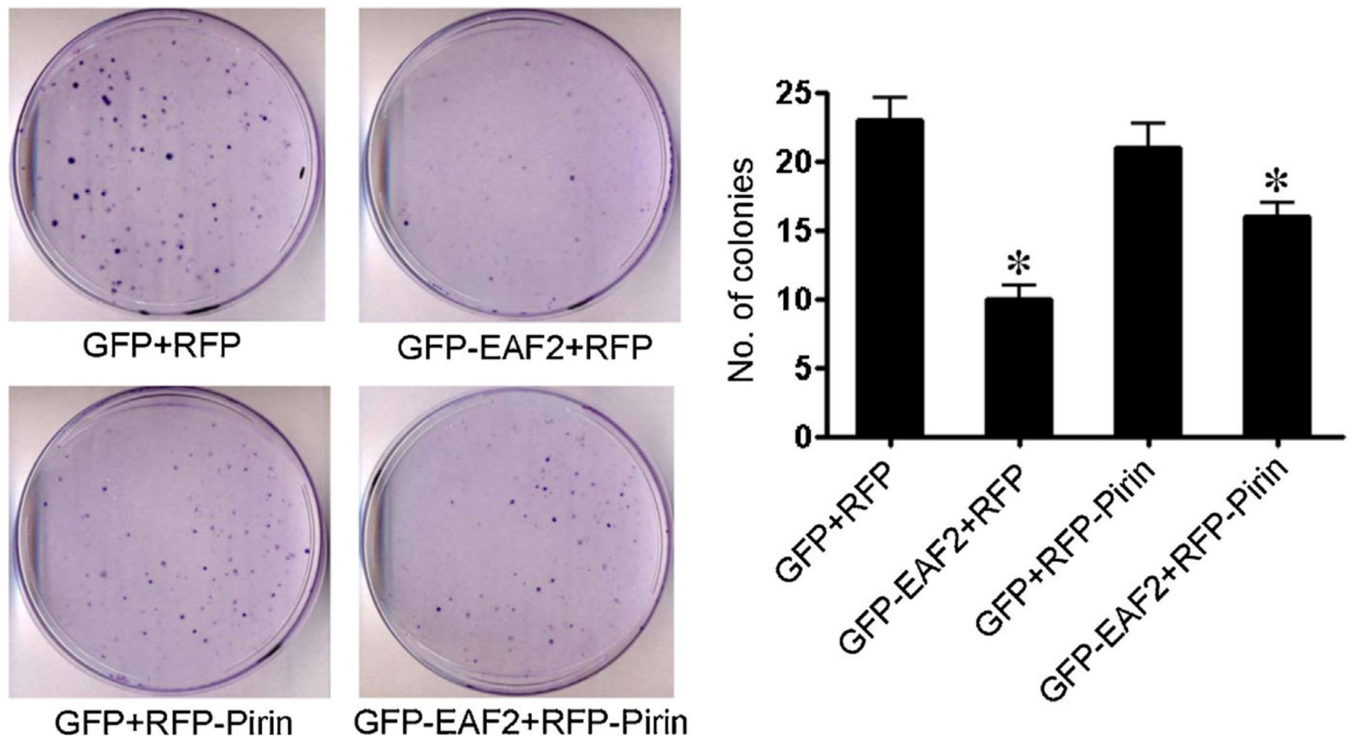

Fig. 5.

Pirin alleviates the growth inhibition of EAF2/U19 in LNCaP cells. Left, LNCaP colonies in 10 cm dishes after transfection and subsequent treatmentwithG418 for additional 21days. Images are representative of 3 separate experiments. Right, Quantification of LNCaP colonies larger than 0.5 mm. Each data point represents mean ± SEM of three independent experiments. $^{*}P < 0.05$.