Biochemical and Biophysical Research Communications 425 (2012) 711–716

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Sepantronium Bromide (YM155) induces disruption of the ILF3/p54^{nrb} complex, which is required for survivin expression

Tomohiro Yamauchi *, Naoto Nakamura, Masashi Hiramoto, Masatoshi Yuri, Hiroyuki Yokota, Masanori Naitou, Masahiro Takeuchi, Kentaro Yamanaka, Aya Kita, Takahito Nakahara, Isao Kinoyama, Akira Matsuhisa, Naoki Kaneko, Hiroshi Koutoku, Masao Sasamata, Masato Kobori, Masao Katou, Shuichi Tawara, Shigeki Kawabata, Kiyoshi Furuichi

Drug Discovery Research, Astellas Pharma, Inc., Tsukuba-shi, Ibaraki, Japan

ARTICLE INFO

Article history:
Received 18 July 2012
Available online 27 July 2012

Keywords:
YM155
survivin expression
p54^{nrb}
ILF3

ABSTRACT

YM155, a small-molecule survivin suppressant, specifically binds to the transcription factor ILF3, which regulates the expression of survivin [1]. In this experiment we have demonstrated that p54^{nrb} binds to the *survivin* promoter and regulates survivin expression. p54^{nrb} forms a complex with ILF3, which directly binds to YM155. YM155 induces disruption of the ILF3/p54^{nrb} complex, which results in a different subcellular localization between ILF3 and p54^{nrb}. Thus, identification of molecular targets of YM155 in suppression of the survivin pathway, might lead to development of its use as a novel potential target in cancers.

1. Introduction

Survivin is a member of the inhibitor of apoptosis proteins (IAP) family, which has been implicated in controlling cancer progression and drug resistance [2,3]. YM155 is a small-molecule survivin suppressant [4,5]. YM155 exhibited pronounced anticancer activity in pre-clinical models and modest evidence of antitumor activity in phase I and II trials of heavily pretreated cancer patients [6,7]. The mode of action of YM155 has been demonstrated to be mediated through a 2 Kb promoter region of the *survivin* gene. An exact molecular pathway by which YM155 suppresses survivin transcription has been unknown up to now.

We recently identified that one of the direct molecular targets of YM155 is the transcription factor interleukin enhancer-binding factor 3 (ILF3), which regulates survivin expression. The ILF3 family proteins consist of several different but closely related proteins generated through alternate splicing [8–11]. ILF3 is predominantly localized in the nucleus [9], and interacts with ILF2, and with PRMT1, the predominant protein-arginine methyltransferase in cells [12].

p54^{nrb}, which has high affinity for RNA, ssDNA, and dsDNA [13,14], and is involved in transcription, RNA export, and splicing. Recently, it has been reported that p54^{nrb} regulates the activity of transcription factors such as AR, SOCS3, SOX9, and CREB [15–18].

In this experiment we have found that the ILF3/p54^{nrb} complex, as well as E2F1 and E2F2, binds to the *survivin* promoter and regulates the expression of survivin. We then determined that YM155 dissociates the ILF3/p54^{nrb} complex, resulting in a different subcellular localization between ILF3 and p54^{nrb}.

2. Materials and methods

2.1. Culture and transfection of PC-3 cells, HEK293 cells, siRNA, and YM155

PC-3 cells and HEK293 cells were cultured in RPMI1640 or DMEM, respectively, at 37 °C and in 5% CO_2. Both mediums were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Cells were transfected with DNA plasmids using Lipofectamine 2000 (Invitrogen), and with siRNA (10 nM) using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturers' instructions. The siRNA was designed in accordance with the parameters of Tuschl and colleagues for human *p54^{nrb}*; (AACACUGGGCCAAGUCUGGCUCGUU), *ILF3*; (GCGGAU CCGACUACAACUACG), *survivin*; (CAGGCAGCGUACAUACGGUUC). YM155 were synthesized by Astellas Pharma Inc.

2.2. Purification of survivin promoter sequence-specific DNA binding proteins

PC-3 cells were extracted with NETN buffer (20 mM Tris–HCl at pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, and

* Corresponding author.
E-mail address: tomohiro.yamauchi@astellas.com (T. Yamauchi).

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http://dx.doi.org/10.1016/j.bbrc.2012.07.103

protease inhibitor cocktail (Roche)) for 15 min at 4 °C, and lysates were collected by centrifugation at 15,000*g* for 10 min. The extracts were incubated with a biotinylated *survivin* promoter sequence fragment, SurP; (−44 to −168), purified by SoftLink avidin resins (Promega), and analyzed by mass spectrometry.

2.3. Real-time RT-PCR

The preparation of total RNA from siRNA-transfected PC-3 was performed using an RNeasy kit (QIAGEN) and synthesis of the cDNA was performed by using ThermoScript RT-PCR system (Invitrogen) in accordance with the manufacturer's instructions. Primer sequences were designed using Primer Express 1.0 software (Applied Biosystems) (Supplementary Table). Quantitative PCR was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with Power SYBR Green PCR master mix (Applied Biosystems).

2.4. Co-immunoprecipitation

HEK293 cells expressing FLAG-ILF3 were extracted with NETN buffer for 15 min at 4 °C and lysates were collected by centrifugation at 15,000*g* for 10 min. The ILF3 complex was immunoprecipitated from the extracts by incubating with M2 anti-FLAG agarose (Sigma) for 12 h with rotation at 4 °C. After three washes with HBS-EP buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and protease inhibitor cocktail), the bound proteins were eluted from M2 agarose by incubation for 30 min with 0.25 mg/mL FLAG peptide (Sigma) in the same buffer. The immunocomplexes were analyzed by SDS–PAGE, followed by Western blotting using anti-p54^{nrb} (A300-587A, Bethyl Laboratories); anti-ILF3 (abcam); anti-Ku80 (abcam); anti-Ku70 (abcam); anti-DNA-PK (Calbiochem); anti-ILF2 (Aviva systems biology); anti-PRMT1 (Upstate); anti-PP1 (Santa Cruz Biotechnology); anti-EWS (Santa Cruz Biotechnology); anti-Nucleolin (abcam); anti-PARP-1 (Santa Cruz Biotechnology); anti-TCF4 (Lifespan biosciences); anti-H-Ras (Santa Cruz Biotechnology); anti-E2F-2 (Santa Cruz Biotechnology); anti-E2F-1 (Santa Cruz Biotechnology); anti-HDAC1 (Santa Cruz Biotechnology); and anti-beta-Action (abcam).

2.5. Immunocytochemistry

Immunocytochemistry and imaging were performed essentially as described [19]. Cells were grown in collagen-coated glasses, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. After incubation for 60 min with 10% Block-Ace (DS Pharma Biomedical) in PBS, cells were incubated for 1 h with the following primary antibodies: anti-ILF3 (Sigma); anti- p54^{nrb} (Bethyl Laboratories); anti-PRMT1 (Upstate); and anti-E2F-1 (Santa Cruz Biotechnology). The cells were washed, incubated with Alexa Fluor 488-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies (Invitrogen). DNA was labeled with VECTASHIELD Mounting Medium with PI (Vector Laboratories).

2.6. Imaging

Confocal images were obtained using an LSM510 (Zeiss, Germany) laser scanning microscope. To minimize overlapping signals, images were obtained by sequential excitation at 488/543 nm to detect Alexa Fluor 488 and 546, respectively, and emission signals were detected at 505–530 nm for Alexa Fluor 488 and >560 nm for Alexa Fluor 546. Images were processed using graphics editing software, Photoshop, ver. 8.0 for Windows.

2.7. EMSA

HEK293 cells were transfected with the FLAG-tagged WT ILF3 expression plasmid, and whole-cell lysates were used for co-immunoprecipitation with anti-FLAG antibody. The eluted ILF3/

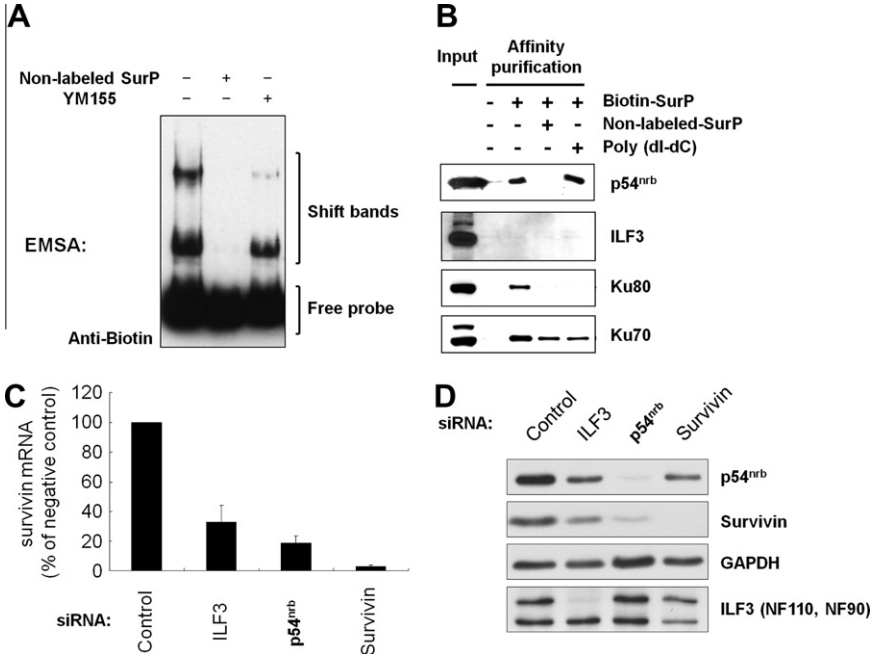

Fig. 1. p54^{nrb} specifically binds to the *survivin* promoter and regulates survivin expression. (A) Electrophoretic mobility shift assay (EMSA). Nuclear extract from PC-3 cells was incubated with a biotinylated *survivin* promoter fragment (−168 to −44; SurP) (50 fmol), ± non-labeled SurP (13.2 pmol), ± YM155 (0.5 mM). (B) Affinity purification of *survivin* promoter sequence-specific DNA binding proteins. PC-3 lysates were incubated with a biotinylated SurP, purified by avidin resins, and the purified protein fraction was analyzed by Western blotting to detect the indicated proteins. (C) The mRNA levels of *survivin*. PC-3 cells were treated with the indicated siRNA. The mRNA levels of *survivin* gene were measured using real-time RT-PCR ($n = 3$). Values are mean ± SD. (D) The protein levels of survivin. PC-3 cells were treated with the indicated siRNA, and whole-cell lysates were used for Western blotting to detect the indicated proteins.

p54^{nrb} complexes were incubated with a biotinylated *survivin* promoter oligonucleotide, followed by EMSA using LightShift Chemiluminescent EMSA Kit (Pierce) in accordance with the manufacturer's instructions. The *survivin* promoter sequences were designed for SurP; (−44 to −168), for #1; (−185 to −142), for #2; (−149 to −110), for #3; (−113 to −69), and for #4; (−74 to −35).

3. Results

3.1. p54^{nrb} is a positive regulator of survivin expression that specifically binds to the survivin promoter

To examine transcription factors bound to the *survivin* promoter directly, EMSA analysis was conducted with PC-3 cell extract and biotinylated oligodeoxynucleotides containing the *survivin* promoter. Two specific gel shift bands that disappeared with excess non-labeled probes were found (Fig. 1A). This result indicated that proteins binding to the *survivin* promoter sequence existed in the PC-3 cells, and the binding was inhibited with YM155. To identify these proteins, affinity purification of DNA binding proteins was used with the *survivin* promoter DNA fragment, followed by protein identification with MS. Among the identified proteins, one promising protein was nominated, p54^{nrb}, which was confirmed by Western blotting to bind to the *survivin* promoter, compared with other DNA-binding factors Ku80 and Ku70 (Fig. 1B). Excess non-labeled SurP probes competed with the biotinylated probes for binding of p54^{nrb}, whereas prevention of non-specific DNA-binding by poly(dI-dC) did not affect the binding level of p54^{nrb} to the biotinylated probes (Fig. 1B). These results suggest that the transcription factor p54^{nrb} specifically binds to the *survivin* promoter.

To investigate the role of p54^{nrb} in the regulation of survivin expression, *survivin* mRNA and protein were examined in siRNA-treated PC-3 cells. It was found that knockdown of p54^{nrb} reduced expression of survivin at the levels of both mRNA and protein (Fig. 1C and D). These results indicate that p54^{nrb} regulates survivin expression.

3.2. p54^{nrb} forms a complex with ILF3, which is bound to the survivin promoter

To test the association between p54^{nrb} and ILF3, we conducted co-immunoprecipitation assays with 293 cells expressing FLAG-tagged ILF3 and two deletion mutants of ILF3. We showed that ILF3 formed a complex with endogenous p54^{nrb} as well as with ILF2 and PRMT1, known to be binding partners of ILF3 (Fig. 2A). We also found that ILF3 associated with p54^{nrb} in an RNA-dependent manner, and both the N-terminal and C-terminal region of ILF3 were responsible for binding to p54^{nrb}, whereas ILF3 interacted with both ILF2 and PRMT1 in an RNA-independent manner, and either N-terminal or C-terminal region of ILF3 was responsible for binding to ILF2 or PRMT1, respectively (Fig. 2A). These results

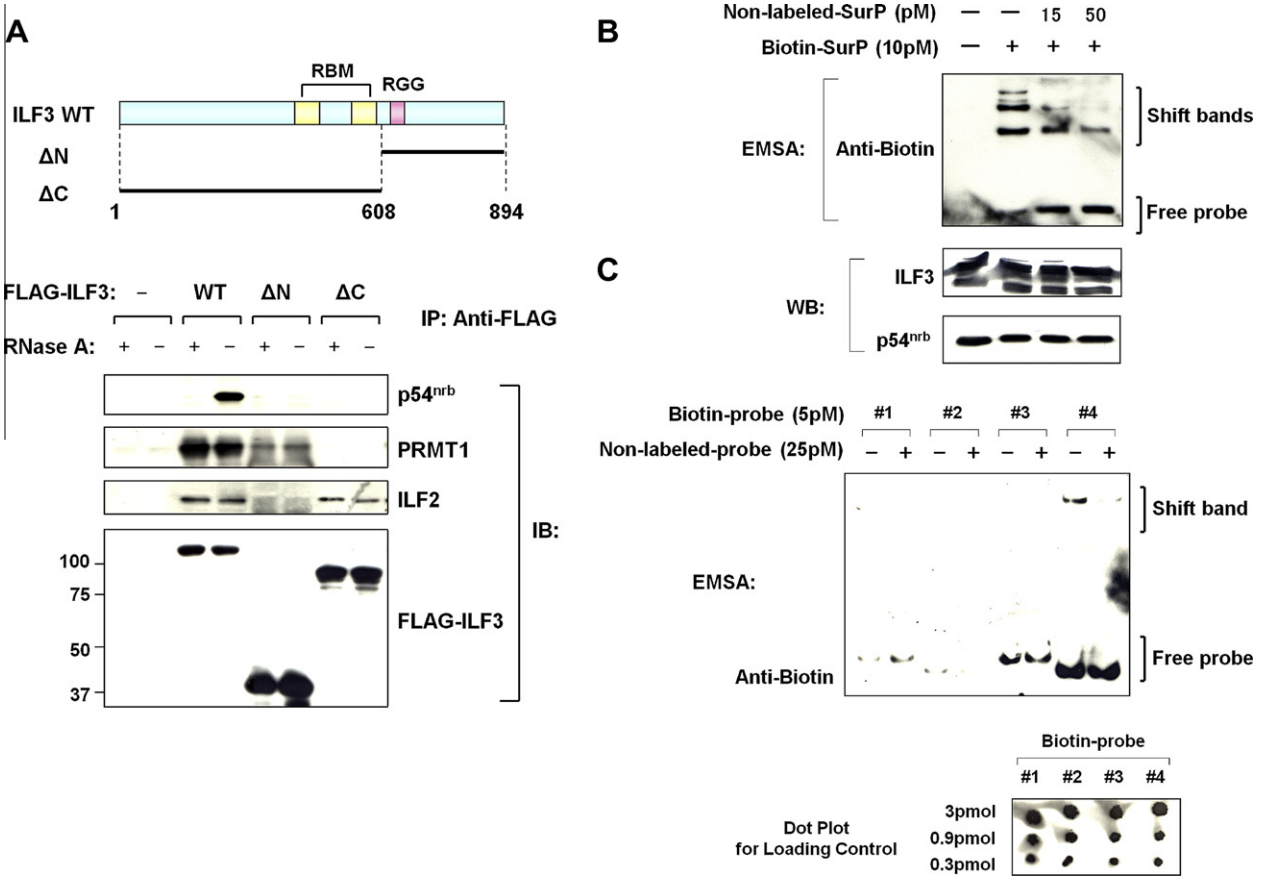

Fig. 2. p54^{nrb} physically interacts with ILF3 through RNA. (A) HEK293 cells were transfected with the FLAG-tagged WT, ΔN, or ΔC ILF3 expression plasmid, and whole-cell lysates were used for co-immunoprecipitation with anti-FLAG antibody, followed by Western blotting to detect the indicated proteins. (B) ILF3/p54^{nrb} complex binds to the *survivin* promoter directly. HEK293 cells were transfected with the FLAG-tagged WT ILF3 expression plasmid, and whole-cell lysates were used for co-immunoprecipitation with anti-FLAG antibody. The precipitated protein complexes containing ILF3 and p54^{nrb} were used for EMSA and Western blotting. (C) ILF3/p54^{nrb} complex binds to the specific *survivin* promoter directly. HEK293 cells were transfected with the FLAG-tagged WT ILF3 expression plasmid, and whole-cell lysates were used for co-immunoprecipitation with anti-FLAG antibody. The precipitated protein complexes containing ILF3 and p54^{nrb} were used for EMSA. Dot plots as a loading control are shown below the figure.

show that p54^{nrb} interacts with ILF3 through cellular RNA, which may be the reason that the region of ILF3 that binds with p54^{nrb} is different from those of ILF2 and PRMT1.

To examine whether ILF3/p54^{nrb} complex binds to the *survivin* promoter, EMSA analyses were conducted using ILF3/p54^{nrb} complex protein that was purified with anti-FLAG antibody from 293 cells overexpressing FLAG-ILF3. Specific gel shift bands were found that disappeared with excess non-labeled SurP probe (Fig. 2B). To determine the binding site for ILF3/p54^{nrb} complex in detail, four fragments from SurP sequence were used (Supplementary Fig. 1). ILF3/p54^{nrb} complex could only interact with the probe #4 (−74 to −35) including p54^{nrb} binding half-site (Fig. 3C) [14]. These results indicate that ILF3/p54^{nrb} complex binds to that specific region of the *survivin* promoter.

3.3. YM155 induces disruption of ILF3/p54^{nrb} complex and translocation of ILF3, not p54^{nrb}, from the nucleoplasm to the nucleolus

To examine how YM155 treatment affected ILF3/p54^{nrb} complex, the binding between p54^{nrb} and other proteins in the complex was studied after YM155 treatment. YM155 treatment induced dissociation of p54^{nr} from ILF3/p54^{nrb} complex in a time- and concentration-dependent manner (Fig. 3A and B). This result suggests that YM155 attenuates the association of ILF3 with p54^{nrb}.

To examine whether the p54^{nrb} dissociated from ILF3 complex was translocated to the nucleolus by the YM155 treatment as ILF3, the change in the subcellular localizations of ILF3 and p54^{nrb} were analyzed. The localization of p54^{nrb} was found unaffected by YM155 treatment (Fig. 3C). On the other hand, it can be seen that ILF3 translocates from the nucleoplasm to the nucleolus after YM155 treatment (Fig. 3D). These results are consistent with the dissociation of ILF3/p54^{nrb} complex by YM155 treatment, and suggest that YM155 treatment disrupts the ILF3/p54^{nrb} complex, resulting in the different subcellular localizations between ILF3 and p54^{nrb}.

3.4. ILF3/p54^{nrb} complex associates with both E2F1 and E2F2, which are the key regulators of survivin expression

It was previously reported that E2F activators (E2F1, E2F2, and E2F3) can bind to the *survivin* promoter and induce *survivin* transcription [20]. In the present study, whether ILF3/p54^{nrb} complex interacts with E2F activators was next examined. Fig. 4A shows that E2F1 and E2F2 were co-immunoprecipitated with FLAG-ILF3, but not with either TCF4 or H-Ras, which are also regulators of survivin expression. To further characterize the association between E2F1/2 and ILF3/p54^{nrb} complex, co-immunoprecipitation assays were conducted after RNA digestion. It was also found that ILF3/p54^{nrb} associated with E2F1 and E2F2 in an RNA-dependent manner (Fig. 4B), and translocation of E2F1 with YM155 or p54^{nrb} were not observed (data not shown). These results suggest that ILF3/p54^{nrb} complex regulates survivin expression with E2F1 and E2F2 coordinately.

It was also found that ILF3/p54^{nrb} complex contained other nuclear proteins, which were purified from 293 cells overexpressing FLAG-ILF3, identified by MS analysis, and confirmed to be components of the ILF3/p54^{nrb} complex by Western blotting (Fig. 4B). These results demonstrated that ILF2 (NF45), PRMT1, and Nucleolin were RNA-independent binders to ILF3, whereas p54^{nrb}, YY1, E2F1, E2F2, HDAC1, Ku80, PP1, PARP-1, and Ews were RNA-depen-

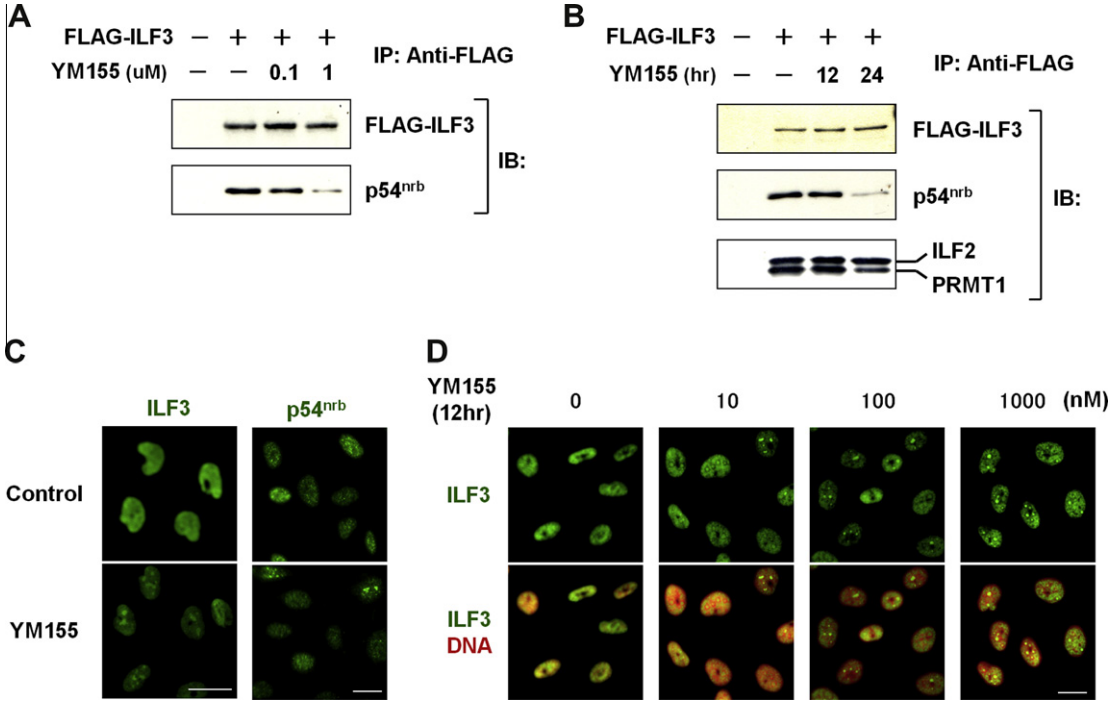

Fig. 3. YM155 induces disruption of ILF3/p54^{nrb} complex. (A and B) HEK293 cells were transfected with the FLAG-tagged WT ILF3 expression plasmid, and treated with the indicated concentration of YM155 for 12 h (A) or with YM155 (0.1 μM) for the indicated time (B). Whole-cell lysates were immunoprecipitated with anti-FLAG antibody, followed by Western blotting to detect the indicated proteins. (C) YM155 induces ILF3 translocation from the nucleoplasm to the nucleolus, not p54^{nrb}. PC-3 cells were treated with the indicated concentration of YM155 for 12 h, fixed and subjected to indirect immunofluorescent staining with anti-ILF3 (green) and visualized using confocal microscopy. Scale bars: 10 μm. (D) YM155 induces the translocation of ILF3 from the nucleoplasm to the nucleolus in a time-dependent manner. PC-3 cells were treated with the YM155, fixed, and subjected to indirect immunofluorescent staining with the indicated antibodies (green) and visualized using confocal microscopy. DNA was visualized by staining with propidium iodide (red). Scale bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

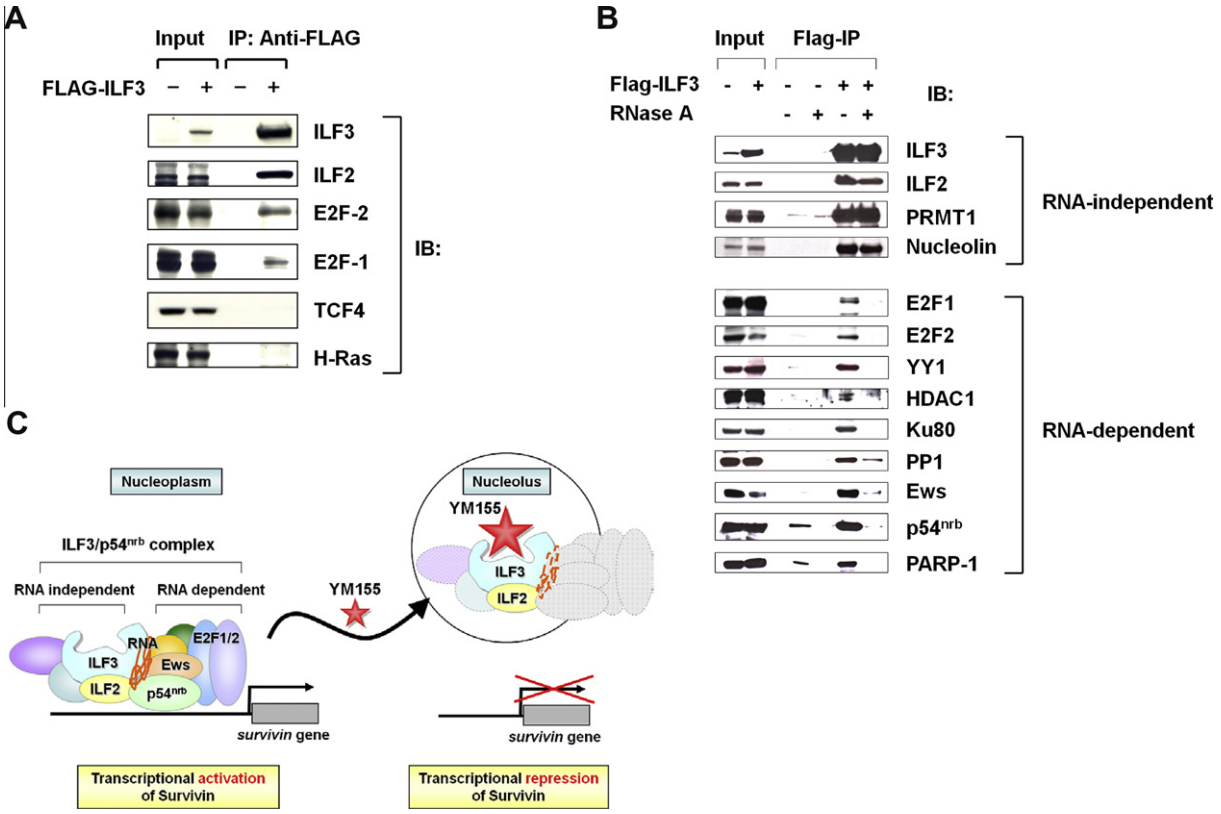

Fig. 4. ILF3/p54^{nrb} complex binds to both E2F1 and E2F2 through RNA. (A and B) HEK293 cells were transfected with the FLAG-tagged WT ILF3 expression plasmid, and whole-cell lysates were used for co-immunoprecipitation with anti-FLAG antibody, followed by Western blotting to detect the indicated proteins. (C) Potential roles of the ILF3/p54^{nrb} complex in survivin transcription, which is regulated by YM155.

dent binders. We also tried to confirm the translocation of Ews with YM155 treatment. The results showed that Ews was dissociated from ILF3 as was p54^{nrb} (Supplementary Figs. 3 and 4), and suggest that ILF3/p54^{nrb} complex interplays dynamically among proteins and RNAs, and reveals a unique regulatory machinery in regulating *survivin* gene expression.

4. Discussion

Results of the present study, shows four main findings First, p54^{nrb} specifically binds to the *survivin* promoter and positively regulates survivin expression. Second, p54^{nrb} associates with ILF3, the direct target of YM155, and ILF3/p54^{nrb} complex binds to the *survivin* promoter. Third, YM155 treatment causes the dissociation of p54^{nrb} from ILF3, which results in different subcellular localizations of ILF3 and p54^{nrb}, which in turn results in survivin downregulation. Fourth, ILF3/p54^{nrb} complex interacts with several nuclear proteins that induce survivin transcription, such as E2F1 and E2F2. Thus, ILF3/p54^{nrb} complex can be a key target that connects anticancer activity of YM155 and survivin expression, as illustrated in Fig. 4C.

Analysis of the ILF3/p54^{nrb} complex has revealed that ILF3/p54^{nrb} complex is formed by protein–protein interactions and is bridged through cellular RNAs. p54^{nrb} and ILF3 are typical double-stranded RNA (dsRNA) binding proteins (DRBPs). They have dsRNA binding domains, which are known to stabilize homo- or heterotypic protein–protein interactions between different DRBPs and often subserve a regulatory function between these proteins [21,22]. Consistent with this, the ILF3/p54^{nrb} complex is bridged through not-yet-defined cellular dsRNA components. It has recently been reported that regulatory ncRNAs can act *in cis*, at the site of transcription, or *in trans*, by regulating gene expression at the transcription site [23–25]. The data of the present study support the existence of functional RNAs in the ILF3/p54^{nrb} complex and therefore require further studies of ILF3/p54^{nrb} complex-bound RNA for resolution of the regulation of survivin expression by YM155.

ILF3/p54^{nrb} complex consists of RNA-independent parts (ILF2, PRMT1, and Nucleolin) and RNA-dependent parts (p54^{nrb}, Ews, YY1, E2F1, E2F2, HDAC1, Ku80, PP1, and PARP-1). ILF2 is known to be a core binder of ILF3, and translocates from nucleoplasm to nucleolus with ILF3 by YM155 treatment. On the other hand, YM155 does not change the localization of p54^{nrb}, which binds to ILF3 through RNA. We speculate that YM155 may compete with RNA in the ILF3/p54^{nrb} complex, though the exact mechanism of the disruption of ILF3/p54^{nrb} complex by YM155 remains to be determined.

PRMTs are recruited to promoters to control gene expression by the methylation of histones and components of the transcription machinery [26]. The recruitment of PRMT1 by YY1 results in the local methylation of histone H4 Arg_3 [27–29]. This modification is critical for subsequent histone acetylation and further transcriptional activation events [30]. We found that ILF3/p54^{nrb} complex is associated with PRMT1 and YY1, suggesting that ILF3/p54^{nrb} complex may recruit PRMT1 and YY1 to the *survivin* promoter and regulate gene expression.

ILF3/p54^{nrb} complex includes an oncogenesis-related molecule, Ews, which is fused to the oncogenic protein in Ewing's sarcoma family tumors, and clinical trials for sarcoma subtypes that include Ews are currently underway to investigate tumor response [31]. Ews was dissociated from the ILF3/p54^{nrb} complex by YM155

(Supplementary Figs. 3 and 4). The findings of the present study indicate that ILF3/p54^{nrb} complex is an oncogenic complex and suggests that YM155 could be an effective chemotherapeutic option for cancer treatment. Further characterization of the ILF3/p54^{nrb} complex might improve our understanding of the regulation of survivin expression, as well as further development of YM155 for cancer therapy.

Acknowledgments

We are grateful to members of our laboratories at Astellas Pharma for their stimulating discussions, and to Ms. M. Mito and Ms. N. Kamata for her help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.103.

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