HHS Public Access

Author manuscript

Exp Cell Res. Author manuscript; available in PMC 2021 November 15.

Published in final edited form as:

Exp Cell Res. 2020 November 15; 396(2): 112318. doi:10.1016/j.yexcr.2020.112318.

Role of RIN1 on telomerase activity driven by EGF-Ras mediated signaling in breast cancer

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Abstract

Epidermal growth factor (EGF)-receptor regulates several downstream signaling pathways upon EGF stimulation that involves cell proliferation, migration and invasion. Internalized EGF-receptor is either recycled or degraded, which fate is regulated in part by Ras interference 1 (RIN1). In this study, we tested the hypothesis that RIN1, a Ras effector protein and Rab5 guanine nucleotide exchange factor, controls several signaling molecules leading to the modulation of the telomerase activity; thus, allowing proper cell proliferation. We report that expression of RIN1 completely blocked proliferation of MCF-12A and MCF-7 cells, while partially inhibited proliferation of MDA-MB-231 cells upon EGF stimulation. Furthermore, expression of the C-terminal region of RIN1 selectively plays a critical role in the inhibition of the proliferation of MDA-MB-231 cells. However, this inhibitory effect was specifically affected by the independent expression of RIN1:Vsp9 and RIN1:RA domains. Additionally, endogenous level of expression of RIN1 was decreased in metastatic MDA-MB-231 cells as compared with non-tumorigenic MCF-12A cells. We observed that expression of RIN1:R94A mutant blocked the proliferation of MDA-MB-231 cells, while expression of RIN1:Y561F and RIN1:R629A mutants completely reversed the inhibitory effect of RIN1:WT. Consistent with our observations, we found that expression of RIN1:WT in MDA-MB-231 cells diminished both protein kinase B (AKT) and extracellular-

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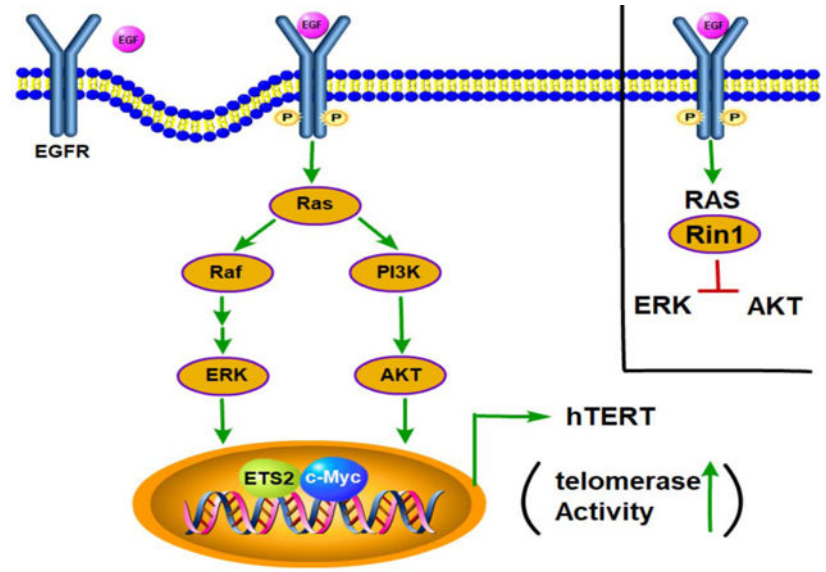
Conflicts of Interest
The authors declare that they have no conflicts of interest.

signal-regulated kinase 1/2 (ERK1/2) activities while p38 mitogen-activated protein kinases (p38MAPK) and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) were unaffected, but it produced downregulation of cellular-myelocytomatosis (c-Myc), erythroblast transformation specific (Ets2) and signal transducer and activator of transcription 3 (Stat3) activities. Inversely, expression of high-mobility group box 1 (HMBG1) was inhibited whereas expression of forkhead box transcription factor 1 (FOXO1) was increased in cells expressing RIN1. Interestingly, expression of RIN1 blocked telomerase activity and human telomerase reverse transcriptase (hTERT) expression, which correlated with the downregulations of c-Myc, Ets-2 and Stat3 activation. Taken together these findings indicate that RIN1 is a critical player in the modulation of the telomerase activity as well as hTERT expression in MDA-MB-231 cells upon EGF stimulation.

RIN1-hTERT signaling connection—Upon EGF stimulation, activated EGF-receptor mediated the activation of both Ras-PI3K-AKT and Ras-ERK pathways, which in turn phosphorylates the ETS2 and c-Myc, resulting in upregulation the expression of hTERT as well as telomerase activity. RIN1 expression will allow an significance decrease of phosphorylation of AKT and ERK by affecting the interaction of Ras with Raf. This inhibitory effect of RIN1 will downregulate the hTERT expression and telomerase activity.



INTRODUCTION

Ras interference 1 (RIN1) was originally identified as a Ras effector protein and it was found to bind GTP-Ras, Bcr-Abl, and 14–3-3 through several functional domains [1–3]. Numerous studies have shown that through its interaction with Abl tyrosine kinase, RIN1 mediates actin cytoskeleton remodeling associated with migration and adhesion of epithelial cells [4]. Other studies suggested that RIN1 is an exchange factor for small GTPase Rab5, which overexpression stimulates EGF-mediated endocytosis and it also inhibits the activation of extracellular-signal-regulated kinase 1/2 (ERK1/2) [5]. The RIN family now has at least three members, all of which have guanine nucleotide exchange factor (GEF) domain for Rab5 activation (also known as Vps9 domain), Ras association (RA) domain,

Proline rich (PR) domain and SH2 domain [5–7]. More importantly, RIN1 is also recruited to the epidermal growth factor (EGF)-receptor via its SH2 domain and down-regulates EGF-induced signal transduction [8, 9] then consequently diminished cellular proliferation [5].

It is also well-documented that EGF-receptor plays an essential role in cell proliferation, survival, and migration [10]. EGF stimulation induced EGF-receptor dimerization and trans-autophosphorylated followed a rapidly internalized by clathrin coated pits [11]. Then, EGF-receptor is sorted through early endosomes, transported to and degraded within multivesicular bodies (MVB) and lysosomes [12]. This internalization event is contemplated to be a vital cellular approach for signal attenuation [13]. Specifically, this signaling event driven by EGF is arranged by a subsequent phosphorylation of several downstream effector proteins which are involved in Ras/ Mitogenic Activated Protein (MAP) kinase and Ras/ phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathways [14]. Additionally, the transcriptional activation of growth-related genes such as cellular-myelocytomatosis (c-Myc), erythroblast transformation specific (Ets), class O of forkhead box transcription factors (FOXOs), cellular-FBJ murine osteosarcoma (c-fos), and high-mobility group box (HMGB) proteins are involved in the EGF driven cell signaling pathways [15–18]. EGF activates the transcriptional ability of c-Myc via phosphorylation, which is the target of MAP kinase [19]. Furthermore, the Ets family is the major target of MAP kinase signaling [20]. FOXO transcription factors have diverse cellular functions including proliferation, and the PI-3kinase/AKT pathway, downstream effector molecules of EGF-receptor, are major regulators of FOXO activity [15]. HMGB proteins are ubiquitous, highly conserved [21] and its overexpression has been reported in a variety of human cancers, including breast cancer [22]. Finally, EGF-stimulation produce reactive oxygen species (ROS) and may function as secondary messengers to control various signaling cascades, including p38 mitogen-activated protein kinases (p38MAPK) and the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) [23]. Alterations in receptor-mediated trafficking as well as attenuation of ligand-driven intracellular signals have been associated with carcinogenesis [14, 24].

To clarify the molecular mechanism of growth factor-dependent RIN1 regulation, we examined the effect of EGF on several signaling molecules activities in breast cancer cell lines expressing RIN1 constructs and analyzed the signal transduction pathway involved in telomerase activation. Identification of key elements of RIN1 protein is critical to understand the dynamic of EGF driven cellular proliferation. The presence of several domains (i.e., Vps9 and RA) suggest that RIN1 targets a network of EGF-dependent signaling molecules. In this study, we identified specific RIN1 domains and selective key point mutants on RIN1 that allow us to delineate a novel signaling transduction leading to the inhibition of telomerase activity in breast cancer cells. We found that RIN1 expression inhibits EGF stimulated telomerase through a selective modulation of signal transduction pathway in which c-Myc, Ets2 and FOXO1 factors seems to be involved.

MATERIAL AND METHODS

Cell Culture and Materials.

MCF7 (ATCC CRL-3435), MCF-12A (ATCC CRL-10782) and MBA-MD-231 (ATCC CRM-HTB-26) cells were obtained from ATCC and they grow in appropriate medium as indicated by ATCC Cell Biology Collection (Manassas, VA). Antibodies (i.e., p44/42 (ERK1/2), phospho(p)-p44/42, Akt1, pAkt1, p38MAPK, p-p38MAPK, JNK, p-JNK, c-Myc, Ets2, p-Ets2, FOXO1, Stat3, p-Stat3, HMGB1, hTERT and GAPDH) were purchased from Cell Signaling Technology (Beverly, MA). RIN1 antibodies were from Abcam Inc. and secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Epidermal growth factor (EGF) was purchased from Cell Signaling Technology (Beverly, MA). Other chemicals were obtained from Sigma unless otherwise stated. MBA-MD-231 cells were utilized in all experiments, except indicated.

Construction of recombinant pMX-puro retroviruses and cell lines.

cDNAs of green fluorescent protein (GFP), RIN1 constructs were sub-cloned into the pMX-puro vector as previously described [8]. Transfection of 80% confluent PhoA cells was performed using Fugene (Roche) and the virus harvested after 48 h post-transfection. Cell lines were infected with retrovirus encoding GFP and RIN1 and selected after 72 h with 4 μg/ml puromycin as previously described [8].

Western Blotting analysis.

Cells were cultured in plates in growth medium and then serum-starved for 16 h. After starvation, cells were washed with HBSS-BSA, pH 7.0 and then allowed to bind EGF at 4°C for 90 min, washed again with cold HBSS-BSA, and then after which the uptake was done at 37°C in a CO_2 incubator at the noted time. After incubation, cells were washed again with cold HBSS-BSA, and then lysed in ice-cold lysis buffer. The lysates were clarified by centrifugation and subjected to SDS-PAGE and analyzed by Western blotting using the specific antibodies. Relative Units of proteins were determined by densitometry using the ratio of phosphor-protein to total protein or GAPDH, and RIN1 and to GAPDH, respectively.

Cell Proliferation Assay.

Cells expressing RIN1 constructs or GFP alone were seeded in 24-well plates at a density of 0.1×10^6 cells/well in 500 ul medium and incubated overnight. Cells were then serum starved for 16 h followed by treatment serum and phenol red free DMEM with or without EGF for 24 h. After, the MTT solution (5 mg/ml) was added to the plates and the cells incubated at 37°C for 4 h. The formazan, derived from MTT by living cells, was dissolved in 10% SDS (150 μl per well), and the absorbance was measured at 490 nm. All MTT experiments were performed in triplicate and repeated at least 3 times.

RNA preparations and RT-PCR.

Total RNA was extracted from breast tumor and normal breast cell lines using TRIzol® reagent (Invitrogen, Life Technologies) following the manufacturer's protocol. Isolated RNA

was then used to synthesize cDNA using an iScript cDNA synthesis kit (Biorad). Real time PCR was performed using the iCycler™ PCR platform (Biorad). Thermal cycling conditions were as follows: an initial incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72° C for 30 s. Followed by a final cycle of 95°C for 1 min, 55 °C for 30 sec and 95°C for 30 s. IQ SYBR Green Supermix (Biorad) was used in accordance with the manufacturer's instructions. The primers used were: RIN1 5'-GGCAGCAGAGGAGTAGCTTGA and 5'-GCTTGCTGGCGCTAAAAGG; hTERT 5′-ATGCGACAGTTCGTGGCTCA-3′ and 5′-ATCCCCTGGCACTGGACGTA-3′; GAPDH 5'-CATTGCCGACAGGATGCA and 5'-CGCTCAGGAGGAGCAATGAT. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method [25]. Mean CT of triplicate measures was computed for each sample. Sample mean CT of GAPDH (internal control) was subtracted from the sample mean CT of the respective gene of interest (ΔCT). The ΔCT of the sample with no treatment was selected calibrator and subtracted from the mean ΔCT of each experimental sample (ΔΔCT). $2^{-\Delta\Delta CT}$ yields fold change in gene expression of the gene of interest normalized to the internal control gene expression.

qTRAP ASSAY.

qTRAP is a real-time PCR-based method that measures the ability of telomerase to add telomeric repeats to a substrate. The real-time PCR-based version of the TRAP assay allows the estimation of telomerase activity in real time via fluorescence measurements. The qTRAP assay was modified from a conventional TRAP assay for use on the Rotor-Gene 6000 system (Qiagen) as described previously [26]. Briefly, cells expressing GFP or RIN:WT were treated with EGF, and samples were lysed in 0.5% (v/v) CHAPS buffer (pH 7.5) supplemented with 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, and 10% glycerol for 30 min on ice. Following lysis, the samples were centrifuged for 20 min at 12,000 x *g* at 4 °C to remove cell debris. The telomerase reaction was carried out in 1X TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.1 mg/ml BSA) and 50 uM each of the four dNTPs and 80 ng/ul TS primer (5′-AAT CCG TCG AGC AGA GTT-3′) and 2 ug (protein amount) of cell lysate in a total volume of 10 ul for 30 min at 30 °C and was stopped by incubation at 94 °C for 10 min. The qTRAP was subsequently carried out by adding 10 ul of the following 2 X PCR mixture (2X TRAP buffer, 1 mg/ul BSA, 40 ng/ul ACX primer (5′-GCG CGG CTT ACC CTT ACC CTTACC CTA ACC-3′), 15% glycerol, 1:10,000 SYBR Green, 0.08 unit/ul Taq polymerase). The PCR conditions used were as follows: 10-min incubation at 94 °C and 40 cycles of PCR at 94 °C for 30 s and 60 °C for 90 s. All of the samples were quantified using the Rotor-Gene quantification software and then compared with the standard curve generated using 293T or TSA positive control. Telomerase activity in cell lines or samples was calculated based on the threshold cycle (C_t). All samples were run in triplicate, including positive and negative controls.

Statistical analysis.

All experiments were done in duplicates and they were repeated at least three times. Values are represented as the standard error of the mean (SEM.) of triplicates and the statistical significance was analyzed by one-way ANOVA or two-tail Student's test. Results with $*P<.05$ and $**P<.01$ were considered as statistically significant.

RESULTS

Expression of RIN1 expression in several breast epithelial cell lines

Cancerous and normal human breast cells (i.e., epithelial spontaneous immortalization and non-tumorigenic MCF-12A, tumorigenic MCF-7, and metastatic MDA-MB-231 cells) have been used on cell culture models based on their unique cellular behavior. We decided to examine the level of expression of RIN1 in three these cell lines that have a distinctive and selective gene expression and mutations [27]. GAPDH, a housekeeping gene, was used as loading control in Western blotting analysis. In Fig. 1A, we showed that RIN1, at the protein level, is highly expressed in MCF-12A cells while in both MDA-MB-231 and MCF-7 cell lines, expression of RIN1 was significantly reduced. We next examined whether RIN1 protein levels in these cells reflected the level of RIN1 mRNA. For that, RIN1 mRNA transcript levels from these three cell lines were quantified by RT-PCR. The normalized results indicated consistent reduction level of RIN1 mRNA in both MDA-MB-231 and MCF-7 cell lines as compared with the levels of RIN1 mRNA observed in MCF-12A cells (Fig. 1B).

Selective effect of RIN1 expression on EGF-driven proliferation in several breast epithelial cell lines

Based on these observations, we examined the effect of the expression of RIN1 on the proliferation of these cells upon EGF-stimulation. GFP-control cells or RIN1 expressing cells were starved in serum-free media for 24 h, cultured in the presence or absence of EGF, and then the MTT assay was performed as described in Material and Methods. As expected, the proliferation of MCF-12A, MCF-7, MDA-MB-231 cells were stimulated by the addition of EGF. Specifically, the addition of EGF significantly increased proliferation of MDA-MB-231 cells (72 ± 4 %) as compared with not-EGF treated MDA-MB-231 cells, while in MCF-7 cells, the addition of EGF increased its proliferation by 50 ± 6 % as compared with not-EGF treated MCF-7 cells. In contrast, the addition of EGF only increased proliferation of MCF-12A cells by 22 ± 6 % as compared with not-EGF treated MCF-12A cells (Fig. 2A-C).

We also observed a differential inhibitory effect on cell proliferation, when RIN1 was expressed in these cell lines. In MCF-12A and MCF-7 cell lines, RIN1 expression completed blocked the EGF proliferative effect, while the addition of EGF to MDA-MB-231 cells expressing RIN1 poorly increase proliferation as compare with GFP-MDA-MB-231 cells (Fig. 2A-C). Particularly, we observed 48 ± 5 % reduction on the proliferative effect of EGF on MDA-MB-231 cells expressing RIN1. We also examined the effect of depleting RIN1 from MDA-MB-231 cells, raising the possibility that silencing of RIN1 might promote the cell growth. Indeed, MDA-MB-231 cells stably transduced with RIN1 RNAi had an increased capacity for growth, compared to GFP-control MDA-MB-231 cells (compare proliferation in RNAi control treated-cells 100 ± 5 % vs proliferation in RIN1 RNAi treated-cells 135 ± 7 %). Thus, RIN1 exhibited differential effect on EGF-driven cell growth that was dependent upon cell characteristic and type.

Rin1-C terminal region blocks proliferation of MDA-MB-231 cells

As we have observed a differential inhibitory effect when RIN1 is expressed in cell proliferation upon EGF stimulation (Fig. 2), we prepared several RIN1 constructs (Fig. 3A) to analyze the RIN1 requirements for such inhibition of proliferation of MDA-MB-231 cells. RIN1 protein contains an SH2 (Src homology 2) domain, a proline-rich (PR) domain, a Vps9 domain, and a Ras association domain (RA) [1, 28]. The removal of the C-terminal of RIN1 (i.e., RIN1:ΔC; deletion the C-terminal region containing Vps9 and RA domains) partially decreased proliferation of MDA-MB-231 cells upon EGF stimulation. In contrast, the removal of the N-terminal of RIN1 (i.e., RIN1:ΔN; deletion the SH2 and PR domains) strongly decreased the proliferation of MDA-MB-231 cells upon EGF stimulation (Fig. 3B). Interestingly, the inhibitory effect of RIN1:ΔN mutant was stronger than the inhibition observed with RIN1:WT [compare cell proliferation in RIN1:ΔN mutant cells (1.00 ± 0.02) and RIN1:WT cells (1.32 ± 0.04) with GFP-control (1.00 ± 0.05)]. These results suggest that the C-terminal region of RIN1 plays a critical role in inhibiting the proliferation of MDA-MB-231 cells.

Since the C-terminal region of RIN1 contains the Vps9 and RA domains, which regulate Rab5 activity and interact with Ras proteins, we decided to evaluate whether these domains could independently affect the proliferation of MDA-MB-231 cells. As shown in Fig. 3B, expression of each domain blocked the proliferation of MDA-MB-231 cells. The inhibitory effect of each domain on proliferation of MDA-MB-231 cells was as effective as the inhibition observed in cells expressing RIN1:WT [compare cell proliferation of RIN1:Vps9 deletion mutant (1.37 ± 0.04) and RIN1:RA deletion mutant (1.23 ± 0.05) cells with RIN1:WT cells (1.32 ± 0.04)], but it was not as efficient as the inhibitory effect of RIN1:ΔN mutant on the proliferation of MDA-MB-231 cells [compare cell proliferation of RIN1:Vps9 deletion mutant cells (1.37 ± 0.04) and RIN1:RA deletion mutant cells with cell proliferation of RIN1: ΔN mutant (1.00 ±0.02)]. These results suggest that RIN1 Vps9 and RIN1: RA domains work together toward decreasing proliferation of MDA-MB-231 cells upon EGF stimulation.

To get a complete picture of the potential role of each domain of RIN1, we prepared several RIN1 point mutants in the context of the full length of RIN1 protein (see Fig. 3A). In order to investigate the importance of the RIN1:SH2 due its significance in RIN1 targeting EGF-receptor [29], we analyzed the effect of the expression of RIN1-SH2:R94A mutant on cell proliferation. This point mutant in RIN1:SH2 domain was chosen because of their similarity to residue in Src-SH2 [30] decreased the binding to the EGFR in pull-down assays [9]. In Fig. 3C, we observed that expression of RIN1-SH2:R94A mutant completely blocked the proliferation of MDA-MB-231 cells upon EGF stimulation.

We then examined the importance of the RIN1:RA domain due its role in the binding of the GTP- bound Ras. Specifically, we analyzed the effect of the expression of RIN1-RA:R629A mutant on cell proliferation. This point mutant in RIN1:RA domain was chosen because of their similarity to residue in RalGDS [31], and it decreased (80±5 % inhibition) the binding to the Ras:G12V mutant in pull-down assay (Barbieri personal communication). Interestingly, we observed that expression of RIN1-RA:R629A mutant completely reversed proliferation of MDA-MB-231 cells as compared with RIN1:WT cells upon EGF

stimulation (Fig. 3C). More importantly, the RIN1-RA:R629A mutant increased cellular proliferation as effectively as the proliferation observed in GFP-control upon EGF stimulation.

Finally, we tested the importance of the RIN1-Vps9 domain due its role in the activation of Rab5 [5]. Specifically, we analyzed the effect of the expression of RIN1-Vps9:Y561F mutant on cell proliferation. This point mutant in RIN1:VPS9 domain was chosen because of their similarity to residues in Rabex-5 [32] as well as the poorly interaction of the Rab5:S34N mutant, and also because failed to activate Rab5:WT [33]. To our surprise, we observed that expression of the RIN1-Vps9:Y561F mutant not only reversed proliferation of MDA-MB-231 cells as compared with RIN1:WT cells upon EGF stimulation (Fig. 3C), but also stimulated two fold cellular proliferation as compared with GFP-control cells. These results demonstrate that both Vps9 and RA domains of RIN1 together play a critical role in the EGF-driven cellular proliferation.

Differential attenuation of EGF-induced signaling by RIN1expression in MDA-MB-231 cells

Our observations (see Fig. 2) have shown that incubating EGF with MDA-MB-231 cells strongly promotes cell growth in cells expressing GFP but not in cells expressing RIN1. This activity could be linked to either the interaction of RIN1 (via the SH2 domain) with EGF-receptor, activation of Rab5 or the ability of RIN1 to interact with Ras GTPases, which in turn will enhance the EGF-receptor internalization and its down-regulations of EGF-induced signaling [34]. Furthermore, it was showed that expression of RIN1:WT blocked the ERK1/2 phosphorylation upon addition of EGF [1, 5]. Therefore, to identify the intracellular signal transduction that modulates the transcription of several growth-regulating genes by the expression of RIN1, MDA-MB-231 cells expressing GFP (Control cells) or RIN1 were serum starved for 16 h, incubated with EGF, washed, lysed followed by Western blotting analysis was carried with specific antibodies as described in Material and Methods.

As expected, we found a significant inhibition (82±6 % inhibition) of the activation of ERK1/2 activity in MDA-MB-231 cells expressing RIN1:WT upon EGF stimulation (Fig. 4A). Moreover, we found that the activation of AKT1 was also strongly blocked (>95 % decrease) in MDA-MB-231 cells expressing RIN1:WT as compared with GFP-MDA-MB-231 cells upon EGF stimulation. (Fig. 4B). Furthermore, we observed that the expression of RIN1:WT did not affect the stimulation by EGF of p38MAPK and JNK activities (Fig. 4C and D) as compared with GFP-control MDA-MB-231 cells. In addition, expression of RIN1:ΔN deletion mutant potentiated the inhibitory effect, but not the expression of the RIN1:ΔC deletion mutant on the activation of both ERK1/2 and AKT1 activity upon EGF stimulation. Interestingly, we also observed a strong activation of phosphorylation of both ERK/1/2 and AKT1 activity in MDA-MB-231 cells expressing RIN1-Vps9:Y561F (ERK1/2: 57±6 % increase, AKT1: 65±5 % increase) and RIN1-RA:R629A (ERK1/2: 45±6 % increase, AKT1: 35±5 % increase) mutants, but not in MDA-MB-231 cells expressing RIN1-SH2:R94A (ERK1/2: 87±6 % decrease, AKT1: 93±5 % decrease) mutant, as compared with GFP-control MDA-MB-231 cells upon EGF stimulation.

We then decided to investigate the effect of the expression of RIN1:WT in MDA-MB-231 cells upon EGF stimulation on other downstream signaling transduction molecules of growth-related genes such as c-Myc. As expected, the addition of EGF increased the phosphorylation of c-Myc in GFP-control MDA-MB-231 cells. However, we observed a significant inhibition of the level of phosphorylation of c-Myc in MDA-MB-231 cells expressing RIN1:WT upon the addition of EGF (Fig. 4E). To our surprise, we found that the expression of c-Myc was also decreased (~20 % of inhibition) as compared with the expression of GAPDH (data not shown). Moreover, phosphorylation of Ets2 was also decreased in cells expressing RIN1:WT as compared with GFP-control MDA-MB-231 cells while the expression of Ets2 was not affected (Fig. 4F). Similarly, RIN1 also blocked phosphorylation of Stat3 (Fig. 4G). Expression of HMGB1 was also blocked in MDA-MB-231 cells expressing RIN1 upon EGF stimulation (Fig. 4H). In contrast, we observed that expression of FOXO1 was upregulated in MDA-MB-231 cells expressing RIN1:WT upon EGF stimulation (Fig. 4I). Furthermore, we also found that the level of FOXO1 mRNA transcript correlate with the protein levels observed when of RIN1:WT was expressed (data not shown). Taken together, these data revealed a distinctive effect of RIN1:WT on several signaling pathways leading to proliferation of MDA-MB-231 cells and it also demonstrated the significance of the RIN1 C-terminal region in this differential regulation of EGF-dependent intracellular signaling.

RIN1 down-regulates telomerase through direct decrease of hTERT transcription

Telomerase is a regulated enzyme and its activity is tightly associated with cell proliferation [35]. Telomerase activity has been detected in reproductive organs, embryonal tissues, stem cells and some rapidly regenerating tissues, however, it is observed in more typical of malignant tumors [36]. Taken together our observations, that the expression of RIN1, but not silencing of RIN1, decreases proliferation of MDA-MB-231 cells, jointly with the differential effect of RIN1 on signaling pathways modulated by EGF, we decided to examine whether the expression and activity of telomerase activity of driven by EGF was affected by the expression of RIN1 in MDA-MB-231 cell lines.

To determine whether RIN1 expression was linked to telomerase activity driven by EGF, MDA-MB-231 cells expressing RIN1:WT were incubated in the absence or in the presence of EGF, respectively. As control, we prepared MDA-MB-231 cells expressing GFP protein alone. Then, the standard TRAP assay combined with RT-PCR was performed as described in material and methods, which allows to obtain quantitative results [37]. In Fig. 5A, we showed that addition EGF increased telomerase activity in control GFP-expressing cells. In contrast, in cells expressing RIN1:WT, we found a significant inhibition (52 ± 5% inhibition) of the telomerase activity as compared with GFP-control MDA-MB-231 cells upon addition of EGF. To get a better understanding of the role of RIN1 on the human telomerase reverse transcriptase (hTERT) expression in MDA-MB-231 cells, we performed quantitative RT-PCR to determine whether or not this inhibition of the hTERT activity was due to the down-regulation of hTERT mRNA expression. We showed that addition of EGF to GFP-control MDA-MB-231 cells increase expression of both hTERT mRNA (2.6 fold increase) (Fig. 5B) and proteins (2.2 fold increase) (Fig. 5C) while the expression of RIN1:WT significantly blocked expressions hTERT mRNA (46 ± 6% inhibition) and protein

(35 ± 6% inhibition) as compared with GFP-control cells stimulated by EGF (Fig. 5B and C). These observations suggest that the RIN1 expression decreases hTERT mRNA expression via transcriptional down-regulation.

DISCUSSION

Our results define a novel role for RIN1 in regulating EGF-receptor signaling leading the inhibition of proliferation and telomerase activity. Our hypothesis was built around the concept of RIN1 as a unique modifier of EGF stimulated signal transduction. RIN1 interacts with the active form of Ras as well as the activated EGF-receptor tail, which in turn will activate Rab5. Expression of Rab5 increased cell proliferation while expression of RIN1 blocked cell proliferation in several types of cell lines. However, RIN1 overexpression has been associated with progression in some type of cancers but, in breast cancer cells, RIN1 silencing may contribute to breast cancer progression.

We questioned whether RIN1 could affect the telomerase activity and cell proliferation EGF-dependent on metastatic, no-metastatic and normal breast cell lines. Strong evidences show that telomerase activity, which is down-regulated by cellular conditions that block cell proliferation, is closely associated with cell proliferation [38]. In fact, some growth factors (i.e., EGF, insulin-like growth factor 1 (IGF-1)) regulate telomerase activity, suggesting the involvement of signal molecules (i.e., RIN1) in telomerase regulation. This led us to overexpress and silence RIN1 to better understand key molecular requirements of RIN1 and determine whether the negative effect of RIN1 on the telomerase activity is due to a selective outcome on EGF-receptor signaling.

We observed that although endogenous RIN1 is present in all examined cell lines, it is highly expressed in the non-tumorigenic MFC-12A cells, as compared with tumorigenic/metastatic cell lines (i.e., MBA-MD-231 cells). Furthermore, the level of RIN1 expression in MCF-10A as well as in normal human mammary epithelial cells (HMECs) was also comparable with those RIN1 level observed in MCF-12A cells (Barbieri personal communication).

As expected, the addition of EGF enhanced cell growth was tested in all cell lines, but the EGF proliferative effect was differential. These observations suggest a potential altered and/or distinctive cellular pathways controlling cell proliferation in MBA-MD-231 cells.

Previous studies have shown that the SH2 domain of RIN1 interacts with the EGF-receptor [9]. In this report, we show that RIN1:ΔN, a mutant lacking the both SH_2 and PR domains, retains the ability to strongly block proliferation of MBA-MD-231 cells. Furthermore, RIN1-SH2:R94A mutant, a point mutant on the SH_2, fails to bind to the EGF-receptor [9], but retains the capacity to reduce the cell proliferation upon EGF stimulation. In fact, our observations reveal that two selective RIN1 mutants (i.e., RIN1-Vps9:Y561 mutant, which decreases the activation of Rab5, and RIN1-RA:R629A, which diminishes the binding of Ras in the GTP-bound form [31] completely reversed the inhibitory effect to RIN1:WT. Supporting this view, it is reasonable to hypothesize that mechanistically, the C-terminus region of RIN1 could have a critical and dual effect on the regulation of cell proliferation by

suppressing EGF-receptor signaling via the RIN1:RA domain, which targets the active form of Ras and by accelerating the EGF-receptor internalization through the RIN1:Vsp9 domain, which activate Rab5. These independent approaches carried out with RIN1 mutants provided further evidence that the association of RIN1 with Rab5 and Ras are essential to regulate cell proliferation. Additionally, expression of either RIN1-SH2:R94A mutant or RIN1:ΔN showed a more robust inhibitory effect on the activation of ERK1/2 upon EGF stimulation as compared with RIN1:WT, suggesting the idea that the interaction is not absolutely required for such inhibitory effect. RIN1-Vps9:Y561F and RIN1-RA:R629A completely reversed the inhibitory effect on the activation of ERK1/2 and AKT1. These observations also reinforce the concept that both Ras and Rab5 play a key role in this signaling pathway.

As a component of the signal transduction machinery, Ras and BRAF play a key role during EGF-receptor mediated intracellular signaling. Both genes are mutated in MBA-MD-231 cells, but not in MCF-12A cells [27], which may suggest a potential effect on the accelerated proliferation of MBA-MD-231 cells upon EGF situation. Specifically, the Ras:G13D mutant has been described in MBA-MD-231 cells and it is considered to be defective in GAP-mediated GTP hydrolysis, which results in the accumulation of constitutively GTP-bound RAS in cells [39]. On the other hand, the BRAF:G646V mutant has been associated with induction of p-MEK and p-ERK as well as inhibition of levels of RAS-GTP. However, this BARF mutant is not active as the BRAF:V600E mutant, which significantly enhanced both MEK and ERK phosphorylation [40]. In addition, TP53 gene is also mutated and highly expressed in this cell line, but its role together with high levels of phospholipase D (PLD) activity, seem to provide a survival signal in these cells when deprived of serum growth factors [41]. Thus, the presence of these two mutants in MBA-MD-231 cells could help us to explain the partial inhibitory effect of RIN1:WT or RIN1:ΔN deletion mutant on the ERK1/2 activity.

Remarkably, RIN1:WT expression did not block the activities of p38-MAPK and JNK, respectively. Consistent with these observations, RIN1 expression failed to affect these signaling pathways in HL60 and K562 cell lines [42]. This peculiarity of RIN1 to selectively affect the EGF-stimulation signaling could be explained, at least in part, by the fact that upon addition of EGF, cells respond with a strong effect on the reactive oxygen species (ROS) signaling. It may also function as secondary messengers to control various signaling cascades [23], including p38-MAPK, JNK and AKT pathways. Further analysis of the down-stream signaling molecules in RIN1 expressing cells showed a clear down regulation of c-Myc, HMBG1 and phosphorylation of Ets-2 proteins. These key molecules are master regulators in a number of cellular pathways and are found at elevated levels in most cancers [43]. Consistent with this observation, knockdown of HMGB1 inhibited telomerase activity and cell proliferation. There is evidence that c-Myc may increase hTERT expression since the hTERT promoter contains binding sites for c-Myc and Ets-2 proteins as well as they form a complex [44, 45]. Therefore, it is tempting to speculate that, at least in part, diminishing both phosphorylation of c-Myc and Ets-2 as well as expression of both HMGB1 and hTERT by RIN1 expression could be a possible candidate mechanism that could trigger a decrease of telomerase inhibitory pathway. Unexpectedly, RIN1 expression also increased the expression of FOXO1. This is particularly interesting because FOXO1 may work as a putative tumor suppressor, but a multitude of cellular processes can critically be affected by

FOXO1 function [46]. Our data showing that JNK activity is not inhibited by the expression of RIN1 in MBA-MD-231 cells is supported by the fact that the oxidative stress and the JNK activity seem to play a key role in up-regulating the expression of FOXO1 [47]. Furthermore, it seems that FOXO1 is a direct target of JNK activity [48]. Thus, further experiments are required to determine how this potential role of JNK signaling toward the increase of expression and changes in the cellular distribution of FOXO1 in cells expressing RIN1:WT.

Based upon these data, a model for the role of RIN1 in down-regulating telomerase activity during EGF stimulation can be envisioned through the direct inactivation of hTERT transcription in which the Ras/ERK pathway is down-regulated while the JNK is unaffected. Therefore, the decrease of expression of both c-Myc and HMBG1 as well as an increase of the expression of FOXO1 in MDA MB-231 cells expressing RIN1 could be a potential mechanism to trigger inhibition of cell proliferation and telomerase inhibitory pathway through a proper membrane trafficking and EGF-receptor signaling upon EGF stimulation.

ACKNOWLEDGMENTS

This work was partially supported by the National Institutes of Health grant SC1DK084343 (to MAB) and by the U.S. *Department of Defense grant* W911NF-13-0167 (to MAB).

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Highlights

- Overexpression of RIN1 blocked telomerase activity as well as hTERT expression
- RIN1 blocked both AKT1 and ERK1/2 activities without affecting p38MAPK and JNK
- RIN1 produced downregulation of c-Myc, Ets2 and Stat3 activities
- RIN1 inhibited expression of HMBG1 without affecting FOXO 1 expression

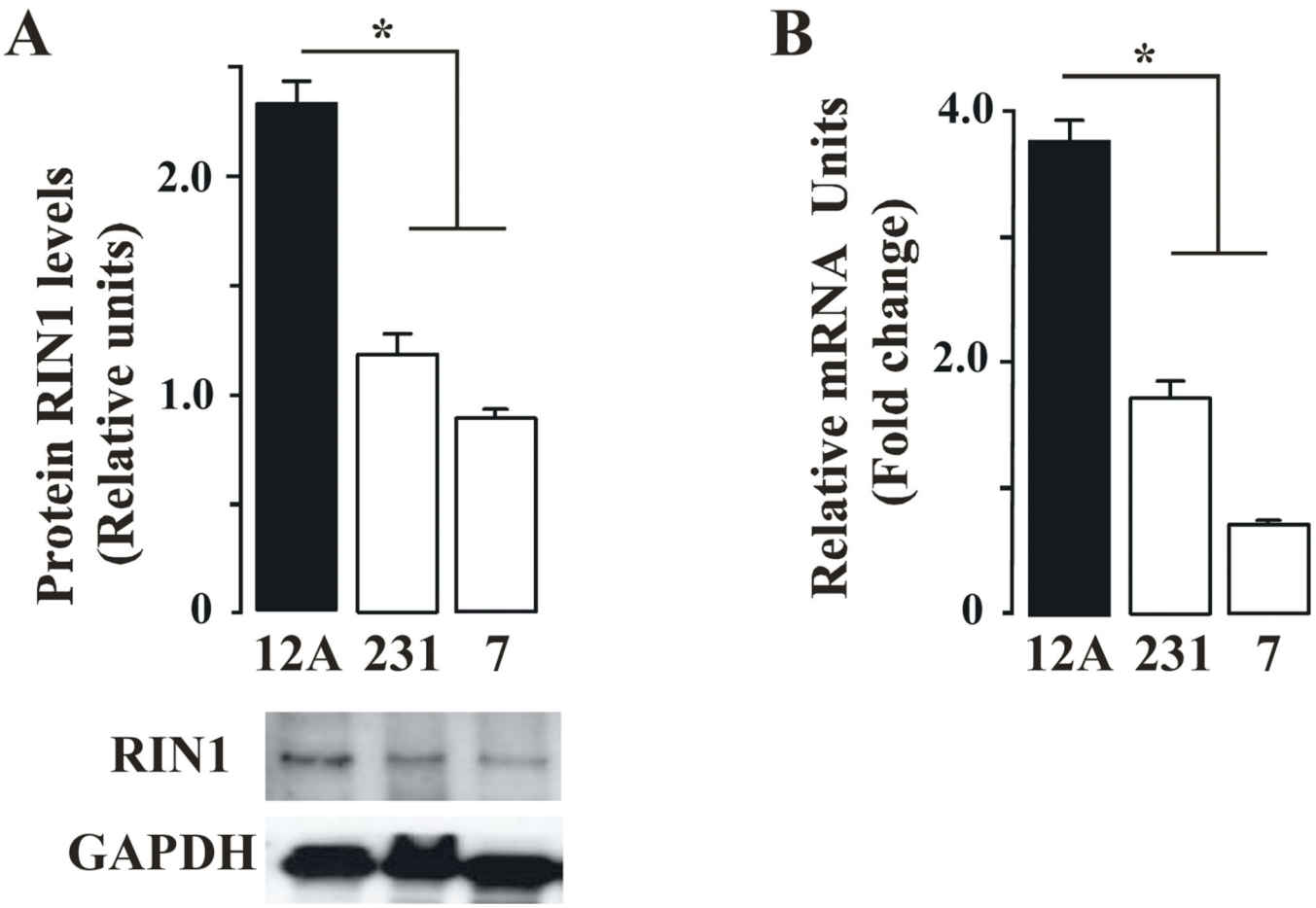

Figure 1. Expression of RIN1 in breast cancer cell lines.

Cells (i.e., MCF-12A(12A), MCF-7(7) and MBA-MD-231(231)) were analyzed for RIN1 expression. (A) Cells were serum-starved for 16 h, washed with cold HBSS-BSA, lysed in lysis buffer and subsequently RIN1 and GAPDH proteins were evaluated by Western blotting with anti-RIN1 and anti-GAPDH antibodies and densitometry. The intensity of the signal for targeted protein were normalized to GAPDH (loading control). Inset: It represents one of three independent experiments with similar results. Data represents the mean ± SEM of three independent experiments. * $P<.05$ according to two-tailed Student's test as compared to MCF-12A cells. (B) Cells were prepared and harvested as indicated above, and RT-qPCR assays were performed to examine the mRNA hTERT levels, normalized with GAPDH mRNA level, and evaluated by $2^{-\Delta\Delta CT}$ method. Data reported as mean ± SEM of three independent experiments. *$P<.05$ according to two-tailed Student's test as compared to MCF-12A cells.

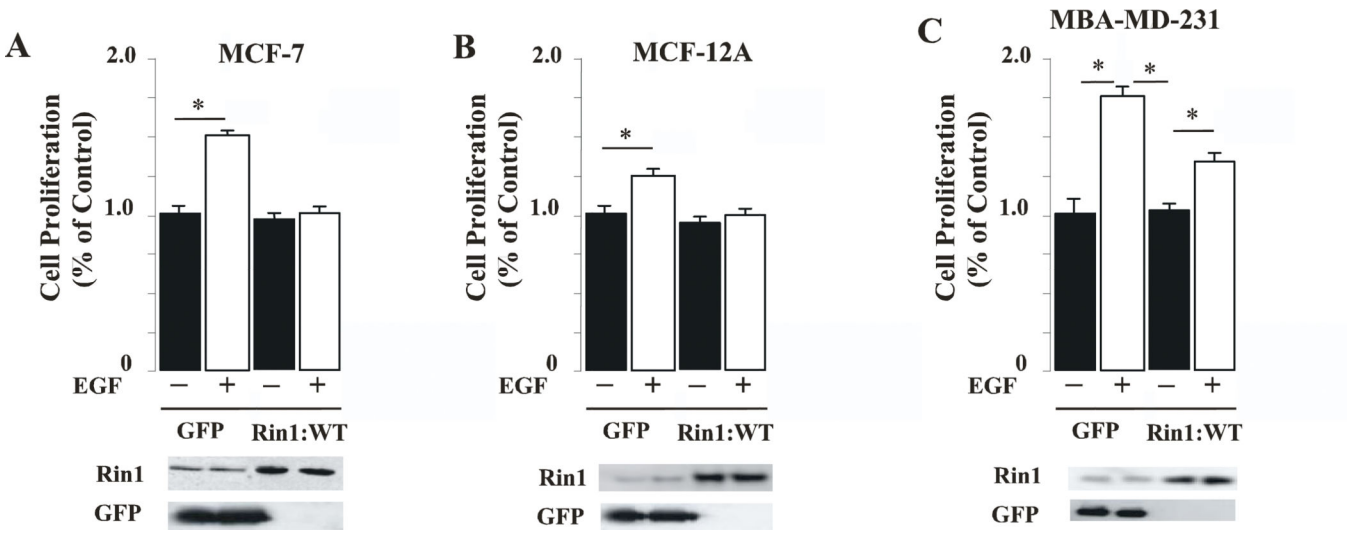

Figure 2. Effect of the expression of RIN1 on the EGF-dependent proliferation in breast cancer cell lines.

MCF-7 (A), MCF-12A (B), and MBA-MD-231 (C) cells were serum-starved for 16 h, washed with cold HBSS-BSA and afterwards incubated in the absence or in the presence of EGF-1 (100 ng/ml) at 4°C for 90 min. Cells were then washed with cold HBSS-BSA and allowed to proliferate for 24 in DMEM-BSA media. Proliferative levels were assessed by the addition of MTT reagent, and changes in absorbance were evaluated at a wavelength of 490 nm. Cell proliferation represents proliferation levels (expressed as % of control) relative to control (proliferation of GFP-cells in the absence of EGF). Data represents the mean ± SEM of three independent experiments. *P<.05 according to two-tailed Student's test as compared to GFP-control in the absence of EGF for each cell line.

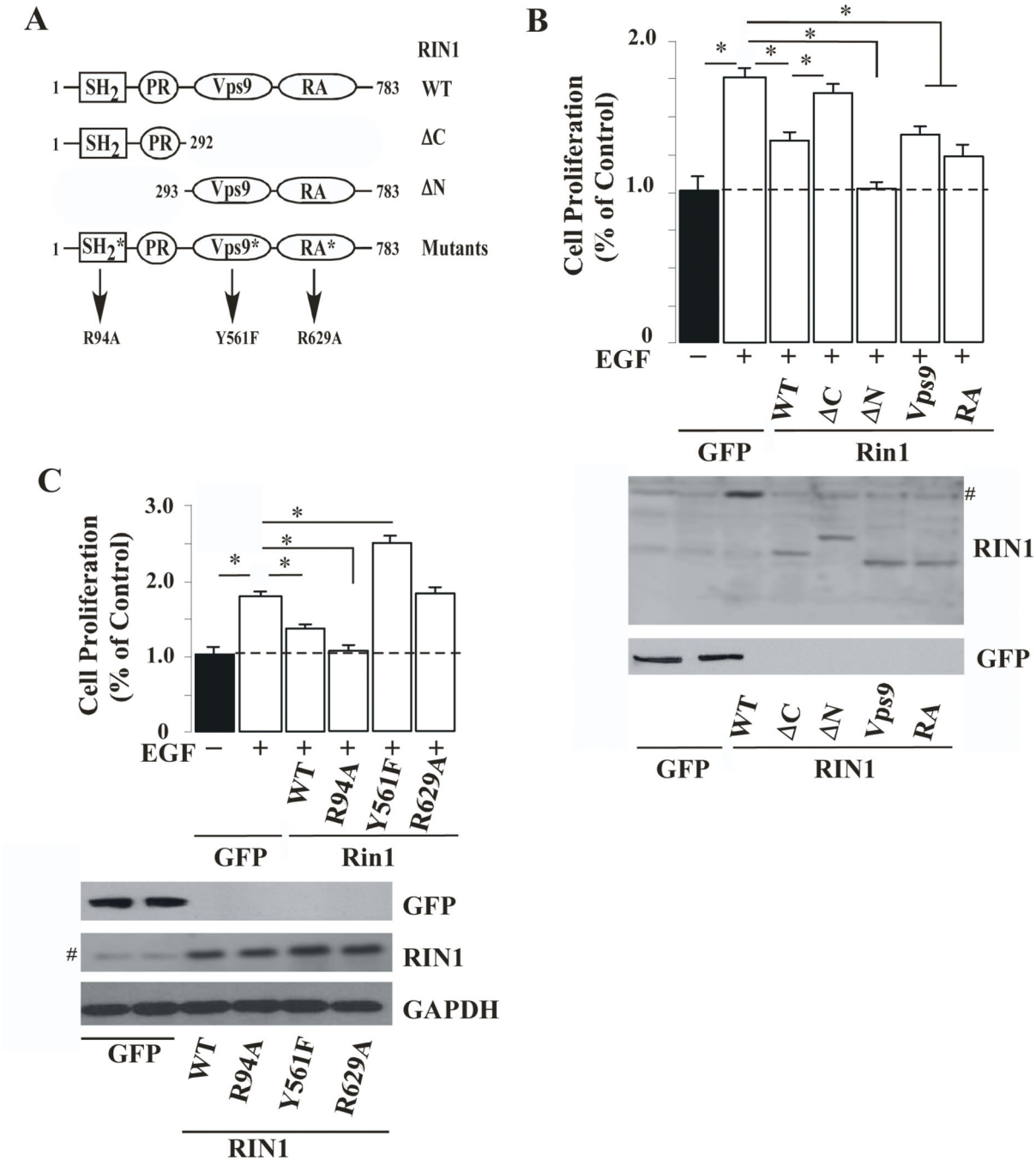

Figure 3. Effect of RIN1 constructs on the EGF-dependent proliferation in MBA-MD-231 cells.

(A) Scheme of RIN1 constructs used in this study. SH2:Src Homology 2 domain, Vps9:Vacuolar sorting protein 9 domain, PR:Proline Rich domain, RA:Ras Association domain; aa, amino acids; Asterisk (*) denotes selective mutation in each domain. (B-C) MBA-MD-231 expressing either GFP (Control) or RIN1 constructs [i.e., RIN1:WT (WT), RIN1:ΔN, RIN1: ΔC, RIN1:Vps9, RIN1:RA, RIN1:R94A, RIN1:Y561F or RIN1:R629A] were serum-starved for 16 h, washed with cold HBSS-BSA and after that incubated in the absence or in the presence of EGF (100 ng/ml) at 4°C for 90 min. Cells were afterward

washed with cold HBSS-BSA and allowed to proliferate for 24 h in DMEM-BSA media. Proliferative levels were assessed by the addition of MTT reagent, and changes in absorbance were evaluated at a wavelength of 490 nm. Cell proliferation represents proliferation levels (expressed as % of control) relative to control (proliferation of GFP-cells in the absence of EGF). Inset: it shows the levels of expression of GFP, GAPDH and RIN1 constructs. Data represents the mean ± SEM of three independent experiments. *P<.05 according to two-tailed Student's test as compared to GFP-control in the absence of EGF for each cell line.

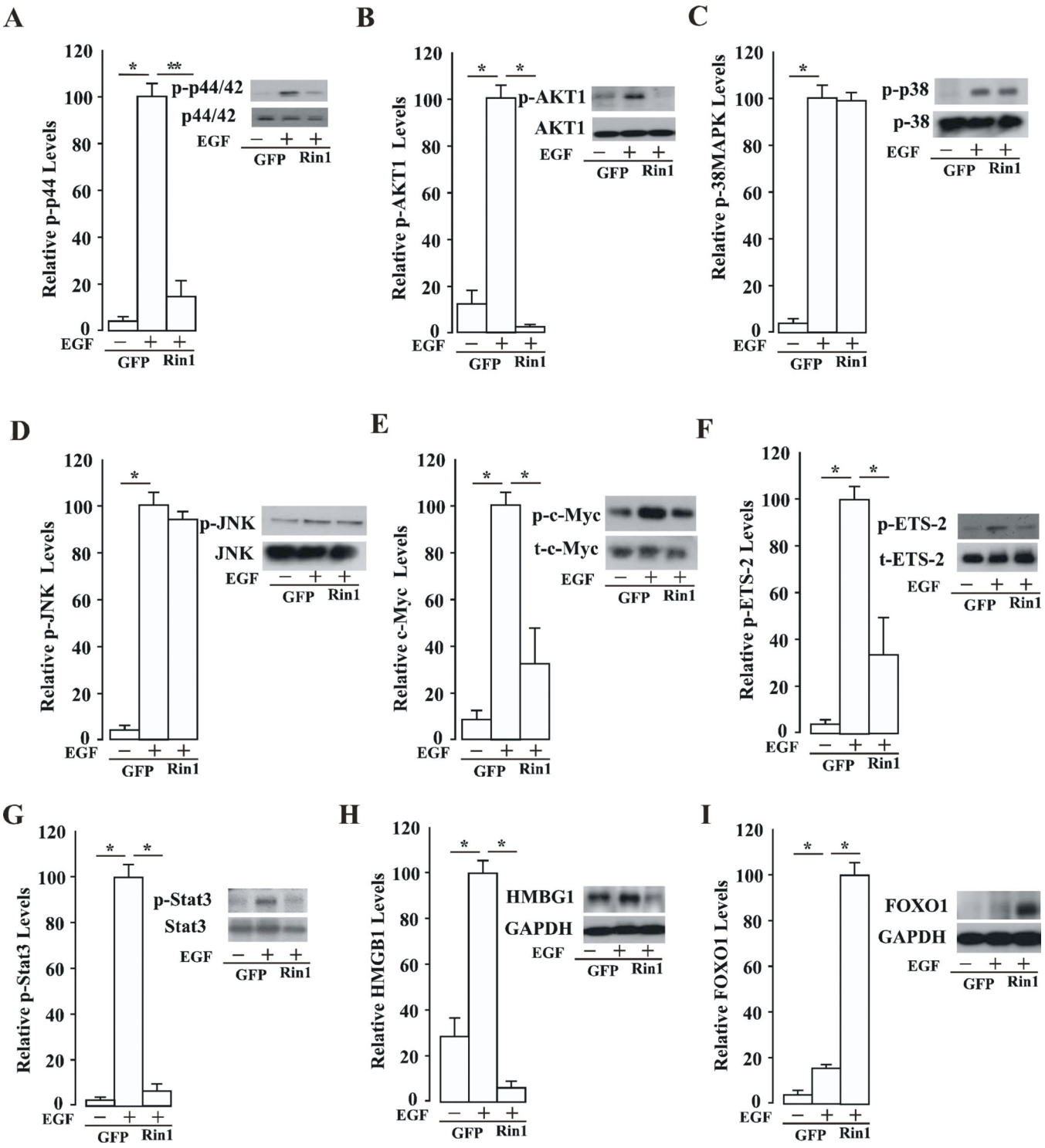

Figure 4. Effect of RIN1 constructs on the EGF-dependent signaling in MBA-MD-231 cells.

MBA-MD-231 expressing either GFP (Control) or RIN1:WT (Rin1) were serum-starved for 16 h, washed with cold HBSS-BSA and then incubated in the absence or in the presence of EGF(100 ng/ml) at 4°C for 90 min. Cells were after that washed with cold HBSS-BSA and incubated for 5 minutes at 37°C. After incubation, cells were washed with HBSS-BSA and

lysed in lysis buffer as described in Material and Methods. The cell proteins were evaluated by Western blotting (WB) with (A) phospho-(p)p44/42(ERK1/2) and total (t)-p44/42(ERK1/2, (B) p-AKT1 and t-AKT1, (C) p-p38MAPK (p-p38) and t-p38MAPK (t-p38), (D) p-JNK and t-JNK, (E) p-c-Myc and t-c-Myc, (F) p-Ets2 and t-Ets2, (G) p-Stat3 and t-Stat3, (H) HMGB1 and GAPDH, and (I) FOXO1 and GAPDH, antibodies and quantified by densitometry. The intensity of the signal for targeted total-protein or targeted phosphor-protein were normalized to that from either GAPDH or total expressed protein as indicated in each case (loading control). Inset: It represents one of three independent experiments with similar results. Data represents the mean ± SEM of three independent experiments. *$P<.05$ according to two-tailed Student's test as compared to GFP control in the presence of EGF (Relative levels =100), except in the case of FOXO1 that it was compared to RIN1 in the presence of EGF (Relative levels = 100).

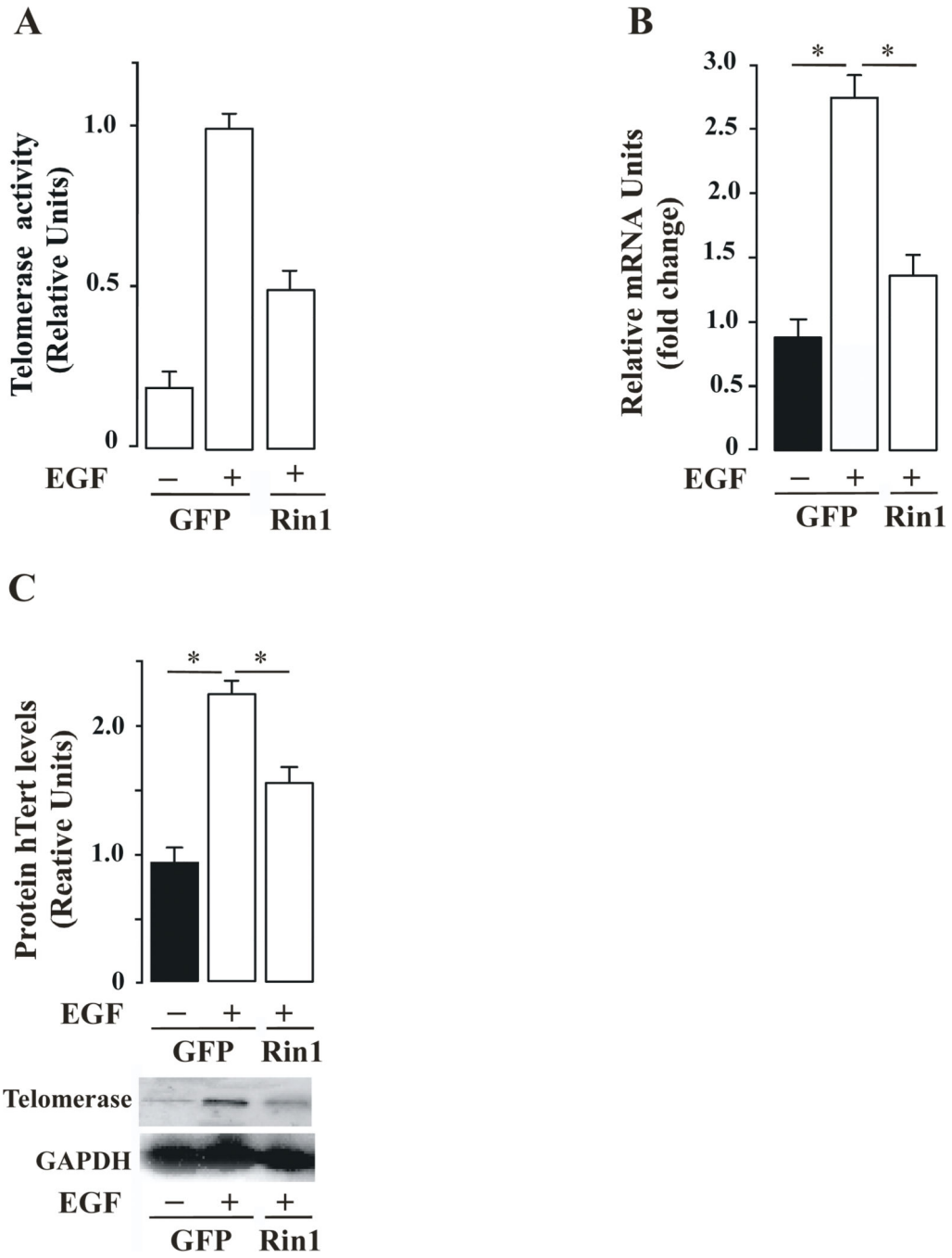

Figure 5. Effect of the expression of RIN1 on activity and expression levels of Telomerase.

MBA-MD-231 cells expressing GFP (Control), or RIN1:WT (Rin1) were starved in serum-free media for 24 h, then incubated with 100 ng/ml EGF for 24 h. Cells were then harvested and prepare for either TRAP-combined RT-qPCR assay, RT-qPCR assay or Western blotting analysis to measure telomerase activity (A), level of expression of either mRNA hTERT (B) or hTERT proteins (C) as described in Material and Methods. GAPDH was used as an internal standard. Internal control for telomerase activity was used as described in Material

and Methods. Data represents the mean ± SEM of three independent experiments. *P<.05 according to two-tailed Student's test as compared to GFP-control.