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Involvement of RARRES3 in the regulation of Wnt proteins acylation and signaling activities in human breast cancer cells

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The Wnt/β-catenin signaling pathway has emerged as a key regulator of complex biological processes, such as embryonic development, cell proliferation, cell fate decision and tumorigenesis. Recent studies have shown that the deregulation of Wnt/β-catenin signaling is frequently observed and leads to abnormal cell growth in human breast cancer cells. In this study, we identified a novel regulatory mechanism of Wnt/β-catenin signaling through RARRES3 that targets and modulates the acylation status of Wnt proteins and co-receptor low-density lipoprotein receptor-related protein 6, resulting in the suppression of epithelial–mesenchymal transition and cancer stem cell properties. Mutation of the conserved active site residues of RARRES3 indicates that RARRES3 serves as an acyl protein thioesterase that tethers its target proteins and modulates their acylation status. Furthermore, the functions of p53 in cell proliferation and Wnt/β-catenin signaling are significantly associated with the induction of RARRES3. Thus our findings provide a new insight into the molecular link between p53, protein acylation and Wnt/β-catenin signaling whereby RARRES3 plays a pivotal role in modulating the acylation status of signaling proteins.
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Breast cancer is one of the most frequently diagnosed cancers and one of the leading causes of female deaths worldwide.[1] Increasing evidence from basic to clinical studies indicates that most breast cancer tumors contain a minor subpopulation of cells with distinct somatic stem cell-like properties. These cells are referred to as cancer stem cells or tumor-initiating cells (TICs) as they have the ability to self-renew, regenerate all cell types within the tumor mass and seed secondary tumors.[2,3] These cells are enriched in breast cancer patients after common treatments, indicating their intrinsic therapeutic resistance. Recent studies indicated that TIC properties are closely associated with a high expression profile of epithelial–mesenchymal transition (EMT) and the gain of metastatic activity.[4,5] *In vivo* and *in vitro* studies indicate that Wnt/β-catenin signaling activity can introduce metastatic and TIC properties to tumor cells,[6,7] and thereby, through modulating Wnt/β-catenin signaling activities, EMT and TIC properties can be suppressed in breast cancer cells.[8–10] In canonical Wnt/β-catenin signaling, Wnt proteins and co-receptors have been implicated in a wide spectrum of important biological phenomena ranging from early organismal development to diseases by mediating the transcriptional activity of the β-catenin transcription factor.[11–13] Recently, this signaling pathway has also been identified as a key regulatory pathway in tumor development through the modulation of epidermal growth factor receptor (EGFR) transactivation, cell proliferation, and cell metastatic and self-renewal properties.[8–11]

Acylation is a common modification of proteins involved in cellular regulatory pathways and allows proteins to interact with many different substrates.[14] The reversible, covalent attachment of fatty acids to cysteine residues exerts diverse effects on protein stability, function, trafficking and subcellular localization.[15,16] Thus acylation operates as a switch to regulate protein-membrane-binding affinity and the biological activities of proteins. Acyl moieties serve to drive protein substrates into specific membrane domains to facilitate protein interactions or signal transduction.[17–19] Recent studies showed that protein acylation effectively influences the physiological function of canonical Wnt/β-catenin signaling. The fatty acid attachment serves as a secondary modification to affect protein conformation and maintain the stabilities of Wnt proteins and their co-receptors.[20–22] For the lipoprotein receptor-related protein 6 (LRP6), a co-receptor in the canonical Wnt/β-catenin signaling pathway, acylation is required for its exiting from endoplasmic reticulum and

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Abbreviations: RARRES3, retinoic acid receptor responder 3; TICs, tumor-initiating cells; EMT, epithelial–mesenchymal transition; LRP6, lipoprotein receptor-related protein 6; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; EGFR, epidermal growth factor receptor; ABE, acyl biotinyl exchange; sFRP1, secreted Frizzled-related protein 1; Dkk1, dickkopf-related protein 1; JNK, c-Jun N-terminal kinases; 5-FU, 5-fluorouracil; MDA and MCF, control vector transfected MDA-MB 231 and MCF-7 cells; R-MDA and R-MCF, RARRES3-overexpressing cells; shC-MDA and shC-MCF, controlled virus-infected MDA-MB 231 and MCF-7 cell lines; shR-MDA and shR-MCF, RARRES3 knockdown cell lines

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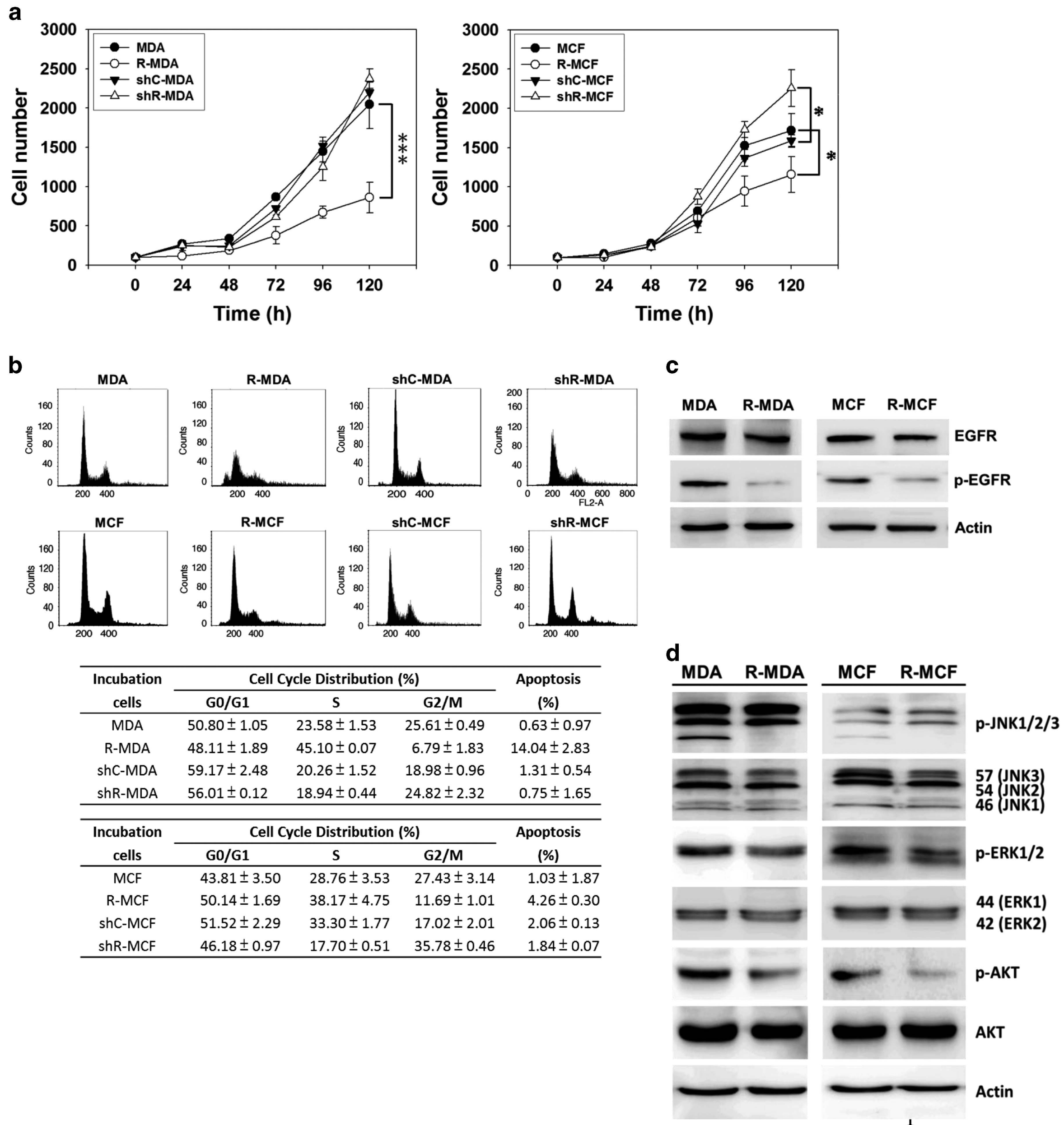
translocating to the plasma membrane (PM).[20] In particular, a dynamic palmitoylation cycle has been shown to regulate the shuttling of small GTPase H-Ras between intracellular compartments and either the PM or synaptic vesicle membranes.[15,23] The dynamic acylation cycle can be regulated by different physiological stimuli that contribute to cellular homeostasis and plasticity.[24,25] Attachment of an acyl moiety by protein acyltransferases results in the shifting of protein substrates from endoplasmic reticulum or Golgi apparatus to the PM. On the contrary, removal of an acyl moiety by acyl protein thioesterase increases the trapping of the protein in intracellular compartments, causing the proteins to possibly be degraded through an ubiquitin–proteasome-dependent pathway.[24,26] It should be noted that, most of the identified acyl protein thioesterases have been shown to display phospholipase activity.[27–29]

Retinoic acid receptor responder 3 (RARRES3), also known as retinoid-inducible gene 1 or tazarotene-induced gene 3, encodes a class II tumor suppressor that belongs to the HREV-107 family, whose members are typically localized in the endoplasmic reticulum or Golgi apparatus. As a p53 target gene, the expression of RARRES3 can be upregulated by various stimuli in normal tissues but is much lower in the corresponding tumor cells.[30–32] RARRES3 has been shown to function as a type I transglutaminase activator that facilitates keratinocyte terminal differentiation and induces cancer cell apoptosis.[33,34] Previous studies indicate that overexpression of RARRES3 suppresses mammalian cell proliferation and promotes cell apoptosis via a posttranslational-dependent manner.[34] Most cell proliferation signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) and the small GTPase H-Ras, have been shown to be suppressed in RARRES3-overexpressing cells.[34,35] In addition, RARRES3 has recently been shown to display phospholipase $PLA_{1/2}$ and acyltransferase activities in *in vivo* and *in vitro* studies.[36,37] Furthermore, previous studies on the tumor-suppressive effect of RARRES3 focused primarily on the characterization of its functional domains and its physiological targets, while few reports have investigated the implications of its enzymatic activity. Because acylation of signaling proteins is important for the maintenance of cell physiological responses, it is assumed that the enzymatic activity of RARRES3 may be closely linked to its tumor-suppressive effect.

RARRES3 has been shown to suppress the main growth-signaling pathways and cell migration activity of tumor cells of various origins, and its function of suppressing breast cancer metastasis by its catalytic activity has also been mentioned.[34,35,38] In addition, the deregulation of Wnt/β-catenin signaling leads to abnormal cell proliferation and metastatic activity, which is frequently found in human breast cancer cells. In the present study, we demonstrate that RARRES3 exerts a remarkable effect on the acylation level of Wnt/β-catenin signaling molecules and therefore inhibits the tumorous growth and stemness properties of breast cancer cells.

Results

RARRES3 suppresses breast cancer cell proliferation and Wnt/β-catenin signaling activity. To study the tumor-suppressive effect of RARRES3, breast cancer cells with RARRES3 stably transfected or knocked down were generated, and the protein expression in these stable cells was verified by western blotting analysis (Supplementary Figure S1). As expected, overexpression of RARRES3 reduced the proliferation of both MDA-MB231 and MCF-7 cells compared with the respective control cells. On the contrary, silencing of RARRES3 markedly increased the proliferation of MCF-7 cells and had a similar, although minor, effect on MDA-MB 231 cells, which express lower levels of endogenous RARRES3 (Figure 1a). Consistent with previous findings, cell cycle distribution analysis showed that RARRES3 shifted the cell population from G2/M phase to S phase, and knockdown of RARRES3 increased the G2/M phase population in both cell lines. Increased apoptosis was also detected in both RARRES3-overexpressing cell lines, particularly in MDA-MB 231 cells, as the sub-G_0 population increased from 0.63 to 14% (Figure 1b).[34,39] We then investigated the effect of overexpressed RARRES3 on cell growth signaling pathways. We found that RARRES3 effectively suppressed EGFR phosphorylation and EGFR-mediated signal transductions, including phosphorylation of Raf/extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3K/Akt, whereas knockdown of RARRES3 increased the activities of these pathways (Figures 1c and d and Supplementary Figure S2). These results suggested that overexpression of RARRES3 reduces the growth signaling of breast cancer cells. To confirm the mechanism of RARRES3-mediated suppressive effect on cell proliferation, the EGFR inhibitor erlotinib was used. Interestingly, we found that erlotinib-treated control cells still proliferated more rapidly than the RARRES3-overexpressing cells (Figure 1e). As Wnt/β-catenin signaling is likely to be involved in cell proliferation and EGFR transactivation in MDA-MB 231 and MCF-7 cells,[8] we also analyzed cell growth in the presence of C-59, which is a Wnt/β-catenin signaling antagonist. The results shown in Figure 1e indicate that, in control cells, C-59 further enhanced the erlotinib-induced growth suppression compared with RARRES3-overexpressing cells. These results suggest that the intracellular level of RARRES3 might correlate to Wnt/β-catenin signaling activity. To determine whether Wnt/β-catenin signaling is involved in the RARRES3-mediated suppression of breast cancer cell growth, the expression levels of Wnt proteins and the co-receptor LRP6 were then examined. We found that the canonical Wnt/β-catenin signaling molecules Wnt1, Wnt3a, Wnt7b, and the co-receptor LRP6 were abundantly present in MDA-MB 231 and MCF-7 cells (Supplementary Figures S3 and S4). The difference in Wnt/β-catenin signaling activities between control and RARRES3-overexpressing cells was further studied. As shown in Figure 1f, in both cell types, overexpression of RARRES3 resulted in an increase in the protein phosphorylation level and simultaneous decrease in total β-catenin. The inhibitory effect of RARRES3 on the transactivation activity of β-catenin was further confirmed using the TOP/FOP Flash luciferase reporter, which is directly



Incubation cells	Cell Cycle Distribution (%) G0/G1	S	G2/M	Apoptosis (%)
MDA	50.80 ± 1.05	23.58 ± 1.53	25.61 ± 0.49	0.63 ± 0.97
R-MDA	48.11 ± 1.89	45.10 ± 0.07	6.79 ± 1.83	14.04 ± 2.83
shC-MDA	59.17 ± 2.48	20.26 ± 1.52	18.98 ± 0.96	1.31 ± 0.54
shR-MDA	56.01 ± 0.12	18.94 ± 0.44	24.82 ± 2.32	0.75 ± 1.65

Incubation cells	Cell Cycle Distribution (%) G0/G1	S	G2/M	Apoptosis (%)
MCF	43.81 ± 3.50	28.76 ± 3.53	27.43 ± 3.14	1.03 ± 1.87
R-MCF	50.14 ± 1.69	38.17 ± 4.75	11.69 ± 1.01	4.26 ± 0.30
shC-MCF	51.52 ± 2.29	33.30 ± 1.77	17.02 ± 2.01	2.06 ± 0.13
shR-MCF	46.18 ± 0.97	17.70 ± 0.51	35.78 ± 0.46	1.84 ± 0.07

Figure 1 RARRES3 expression perturbs cell proliferation and Wnt/β-catenin signaling activity in breast cancer cell lines. (**a**) Overexpression of RARRES3 suppressed breast cancer cell proliferation. Cell growth was analyzed in RARRES3-overexpressing or knockdown cells, and the results are represented as the mean ± S.D., ($n = 3$). MDA, pCR3.1 empty vector-transfected MDA-MB 231 cells; R-MDA, RARRES3-overexpressing MDA-MB 231 cells; shC-MDA, pLKO.1-control vector-transduced MDA-MB 231 cells; shR-MDA, pLKO.1-shRARRES3-transduced MDA-MB 231 cells; MCF, pCR3.1 control vector-transfected MCF-7 cells; R-MCF, RARRES3-overexpressing MCF-7 cells; shC-MCF, pLKO.1-control vector-transduced MCF-7 cells; shR-MCF, pLKO.1-shRARRES3-transduced MCF-7 cells. (**b**) Overexpression or knockdown of RARRES3 influenced the cell cycle distribution and cell apoptosis. Cells with overexpressed or silenced RARRES3 expression were harvested and analyzed for cell cycle distribution and the percentage of apoptosis. The results shown are representative of three independent experiments. (**c**) Overexpression of RARRES3 reduced the phosphorylation level of EGFR in breast cancer cells. Lysates from control or RARRES3-overexpressing cells were extracted, and the total protein and phosphorylation levels of EGFR were examined by immunoblotting. (**d**) Overexpression of RARRES3 suppressed the phosphorylation levels of the downstream signaling targets of EGFR. Lysates from control and RARRES3-overexpressing cells were prepared. The protein levels of total and phosphorylated JNK, ERK, AKT and p38 MAPK were examined. β-Actin served as a loading control. (**e**) RARRES3 overexpression abolishes the cell growth suppression mediated by EGFR and Wnt/β-catenin signaling inhibitors. Cell proliferation was analyzed in control and RARRES3-overexpressing cells treated with erlotinib or C-59. The results represent the mean ± S.D., ($n = 3$). Er, the EGFR tyrosine kinase inhibitor erlotinib; C-59, the Wnt/β-catenin signaling antagonist. (**f**) Overexpression of RARRES3 reduced the expression level of Wnt pathway downstream mediator β-catenin. Cell lysates were extracted, and the total protein and phosphorylation levels of β-catenin were examined by immunoblotting. (**g**) Overexpression of RARRES3 decreased the transactivation activity of the Wnt/β-catenin signaling cascade. The transactivation activity of β-catenin was measured by the TOP/FOP Flash reporter assay. Cell lysates were collected and analyzed for reporter activity in the absence or presence of Wnt agonist. The results represent the mean ± S.D. ($n = 3$). (**h**) RARRES3 overexpression reduces the levels of β-catenin target genes. Lysates from control or RARRES3-overexpressing cells were prepared, and the protein levels of β-catenin target genes cyclin D, Axin 2 and c-myc were examined by immunoblotting

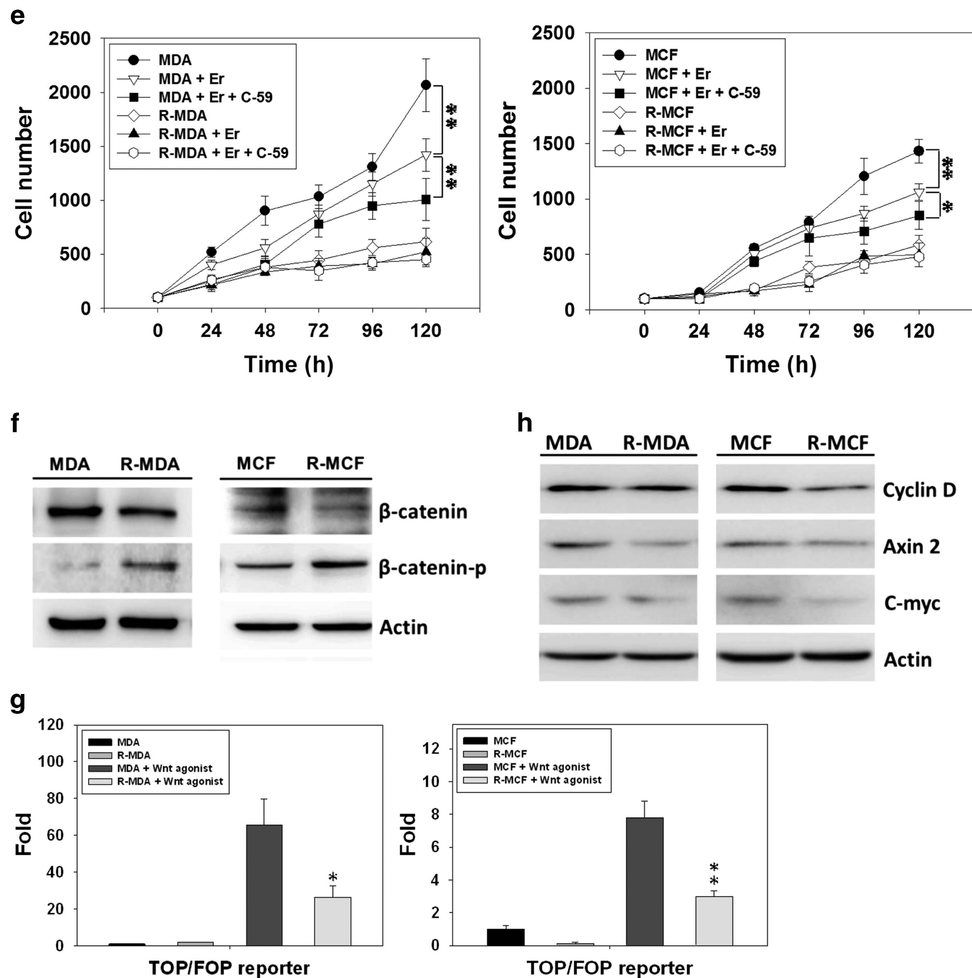


Figure 1 Continued

activated by the TCF/β-catenin transcriptional complex (Figure 1g). Furthermore, overexpression of RARRES3 effectively inhibited the protein expression of β-catenin target genes, including cyclin D, axin 2 and c-Myc. (Figure 1h). Taken together, these results suggest that Wnt/β-catenin signaling is involved in the RARRES3-mediated growth arrest in breast cancer cells.

RARRES3 interacts with and modulates the protein level, acylation level and cellular distribution of Wnt/β-catenin signaling molecules. To show that RARRES3 is involved in the regulation of Wnt/β-catenin signaling cascade, we further analyzed the association of RARRES3 and Wnt/β-catenin signaling proteins. As shown in Figure 2a and Supplementary Figure S5, RARRES3 overexpression enhanced the protein–protein interactions of RARRES3 and Wnt/β-catenin signaling molecules. It should be noted that RARRES3 also reduced the protein levels of Wnt proteins and co-receptor LRP6 in both cell lines (Figure 2b). Because the acylation of juxtamembranous cysteine is required for LRP6 to exit the endoplasmic reticulum and enter the PM, LRP6 cellular localization was further investigated. As shown in Figure 2c, the amounts of LRP6 located in the PM fraction were found to be decreased proportionally with the increased RARRES3 level (the PM fractions in Figure 2c). In cells with the highest level of RARRES3, the amounts of LRP6 in the PM fraction were only 19.4 and 16.5% of control cells for MDA-MB231 and MCF-7 cells, respectively. In contrast, the amounts of LRP6 in the cytoplasm (the post-PM fractions in Figure 2c) were raised approximately two-fold in these cells. These results suggest that the subcellular location of LRP6 is closely related to the presence of RARRES3.

As documented recently, acylation is a major posttranslational modification required for LRP6 to enter the PM and initiate its signaling activity.[20] In addition, previous studies indicate that the subcellular distribution of RARRES3 is diverse in endomembranes, such as the Golgi apparatus and endoplasmic reticulum.[34] Therefore, we assessed whether RARRES3 is colocalized with LRP6 at the endoplasmic reticulum and whether it controls LRP6 protein localization. The results shown in Figures 2d and e compare the locations of LRP6 in control and RARRES3-EGFP-transfected cells. In the control cells, LRP6 was distributed evenly throughout the PM. On the contrary, the LRP6 protein dispersed as broad bands that co-localized with RARRES3 at the endoplasmic reticulum in RARRES3-EGFP-expressing

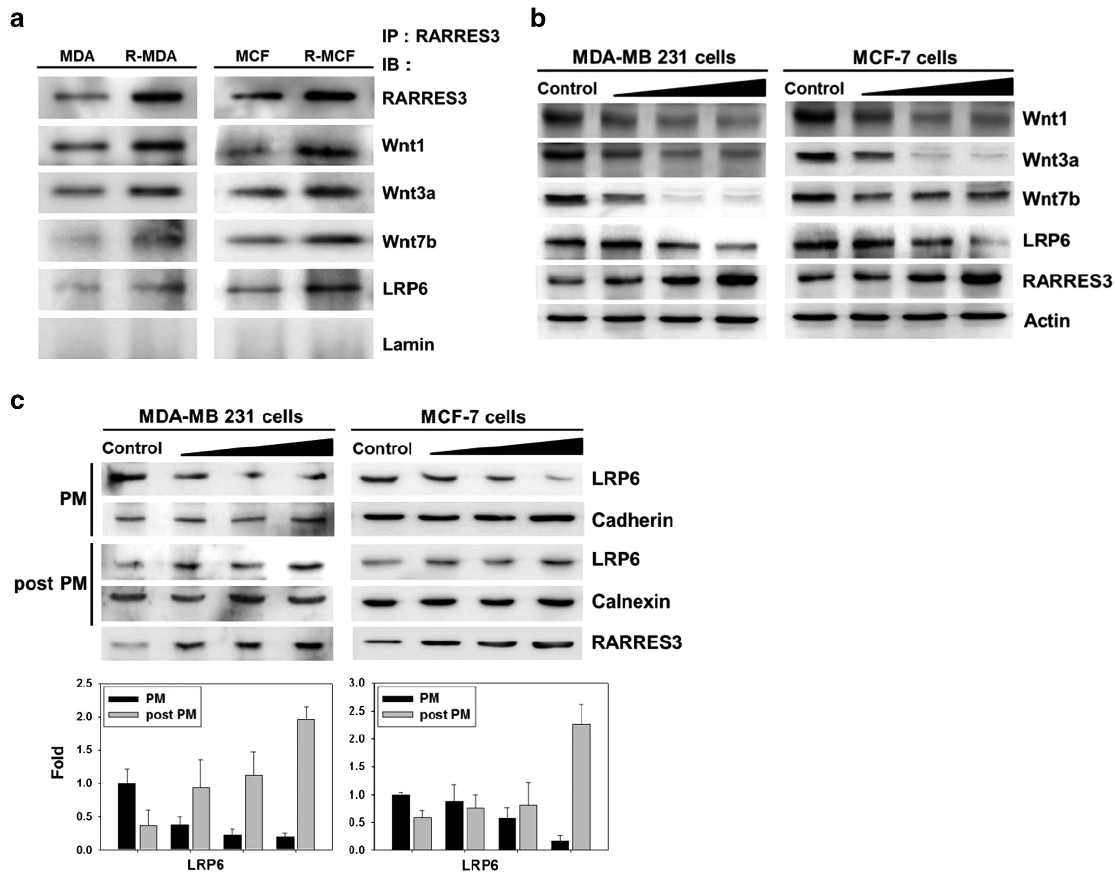
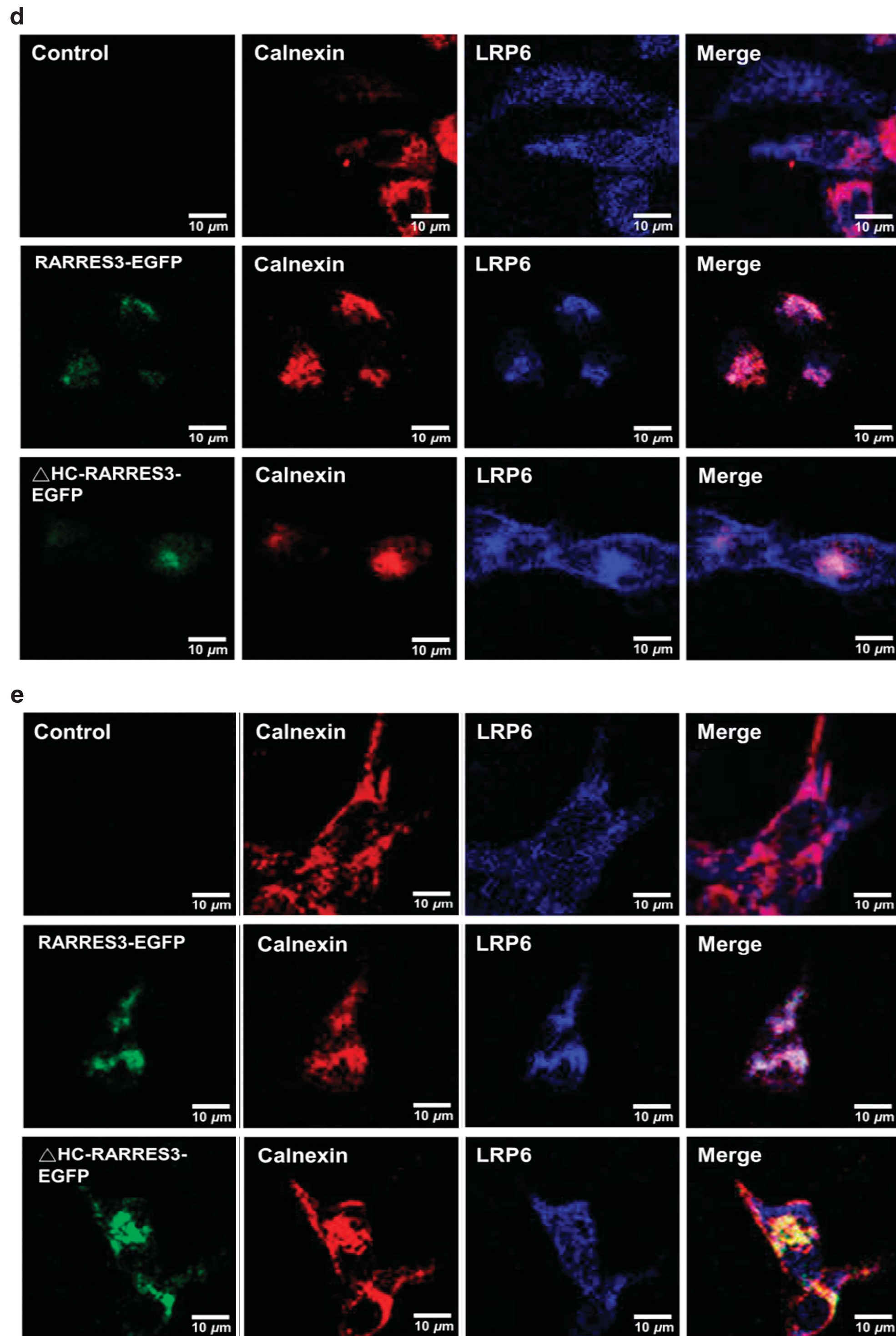


Figure 2 RARRES3 interacts with Wnt/β-catenin signaling molecules and modulates their protein levels, acylation status and subcellular distribution. (**a**) RARRES3 interacted with Wnt proteins and LRP6. RARRES3 was immunoprecipitated from the whole-cell lysates of the indicated cells, and the association of Wnt proteins and LRP6 were determined by immunoblotting. The nuclear protein lamin B served as a negative control. (**b**) Overexpression of RARRES3 reduced the protein levels of Wnt proteins and co-receptor LRP6. The pCR3.1 control vector or pCR3.1-RARRES3 expression vector was transiently transfected into MDA-MB 231 and MCF-7 cells. Forty-eight hours after transfection, the cell lysates from control vector- and RARRES3 expression vector-transfected cells were extracted and analyzed by immunoblotting. (**c**) Distribution of LRP6 in RARRES3-overexpressing cells. The control vector- or RARRES3 expression vector-transfected MDA-MB 231 and MCF-7 cells were subjected to fractionation of the plasma membrane and post-PM after 48 h of transfection. The LRP6, pan-cadherin, calnexin and RARRES3 levels in the PM and post-PM fractions were detected by immunoblotting. Pan-cadherin, a PM marker; calnexin, an endoplasmic reticulum marker. (**d** and **e**) RARRES3 co-localizes with LRP6 and calnexin in MDA-MB 231 cells (**d**) and in MCF-7 cells (**e**). Cells were transfected with the pRARRES3-EGFP or pΔHC-RARRES3-EGFP expression vectors, and after 24 h, the cells were fixed and stained with anti-calnexin (red) and anti-LRP6 (blue). Bars = 10 μm. (**f**) RARRES3 altered the acylation levels of Wnt proteins and co-receptor LRP6. The control vector- and RARRES3 expression vector-transfected cells were lysed and subjected to ABE analysis. The acyl proteins were precipitated and probed with antibodies against Wnt1, Wnt3a, Wnt7b, LRP6 and lamin B. Lamin B, which is an acylated nuclear protein, served as a negative control. (**g**) Double mutation in conserved active site residues impaired RARRES3-mediated protein deacylation. The pCR3.1-ΔHC-RARRES3 expression vector-transfected cell lysates were harvested and examined. The acylation levels of the precipitated proteins were compared with those of the control cells. (**h**) Wnt proteins and co-receptor LRP6 were stabilized in ΔHC-RARRES3-overexpressing cells. The Wnt and co-receptor LRP6 protein levels in the lysates from control vector- or ΔHC-RARRES3 expression vector-transfected cells were analyzed. (**i**) The correlation of trans-activation activity of Wnt/β-catenin signaling cascade and the enzymatic activity of RARRES3. The transcriptional activity of β-catenin was measured in control vector- and ΔHC-RARRES3 expression vector-transfected cells. The reporter activities of these cells were compared in the absence or presence of Wnt agonist. The results represent the mean ± S.D. ($n = 3$)

cells. In addition, RARRES3-mediated restraint of LRP6 was not obvious in cells with H23L and C113S double-mutated RARRES3 (ΔHC-RARRES3). The two highly conserved residues in the active center of proteins of the NlpC/P60 superfamily were shown to be essential.[40,41] These findings indicate that RARRES3 expression influences the subcellular distribution of LRP6 and suggests that it may affect Wnt/β-catenin signaling by influencing protein acylation.

To evaluate the impact of RARRES3 on protein acylation, we analyzed the acylation status of Wnt proteins and LRP6 using the acyl biotinyl exchange (ABE) method. The fatty acid attachment through a thioester linkage on amino-acid residues was replaced by a thiol-specific biotinylation reagent upon concomitant hydroxylamine treatment. The results shown in Figure 2f indicate that RARRES3 overexpression resulted in a reduced basal level of acylation on Wnt proteins and LRP6. In addition, the acylation status of these proteins in ΔHC-RARRES3-overexpressing cells was similar to that of the control cells (Figure 2g). These results reveal that RARRES3 has a functional role in modulating the acylation status of Wnt proteins and LRP6. Additionally, RARRES3-mediated Wnt protein and LRP6 decreases were not obvious in cells overexpressing ΔHC-RARRES3 (Figure 2h, compare to Figure 2b). Although the levels of Wnt7b and LRP6 were



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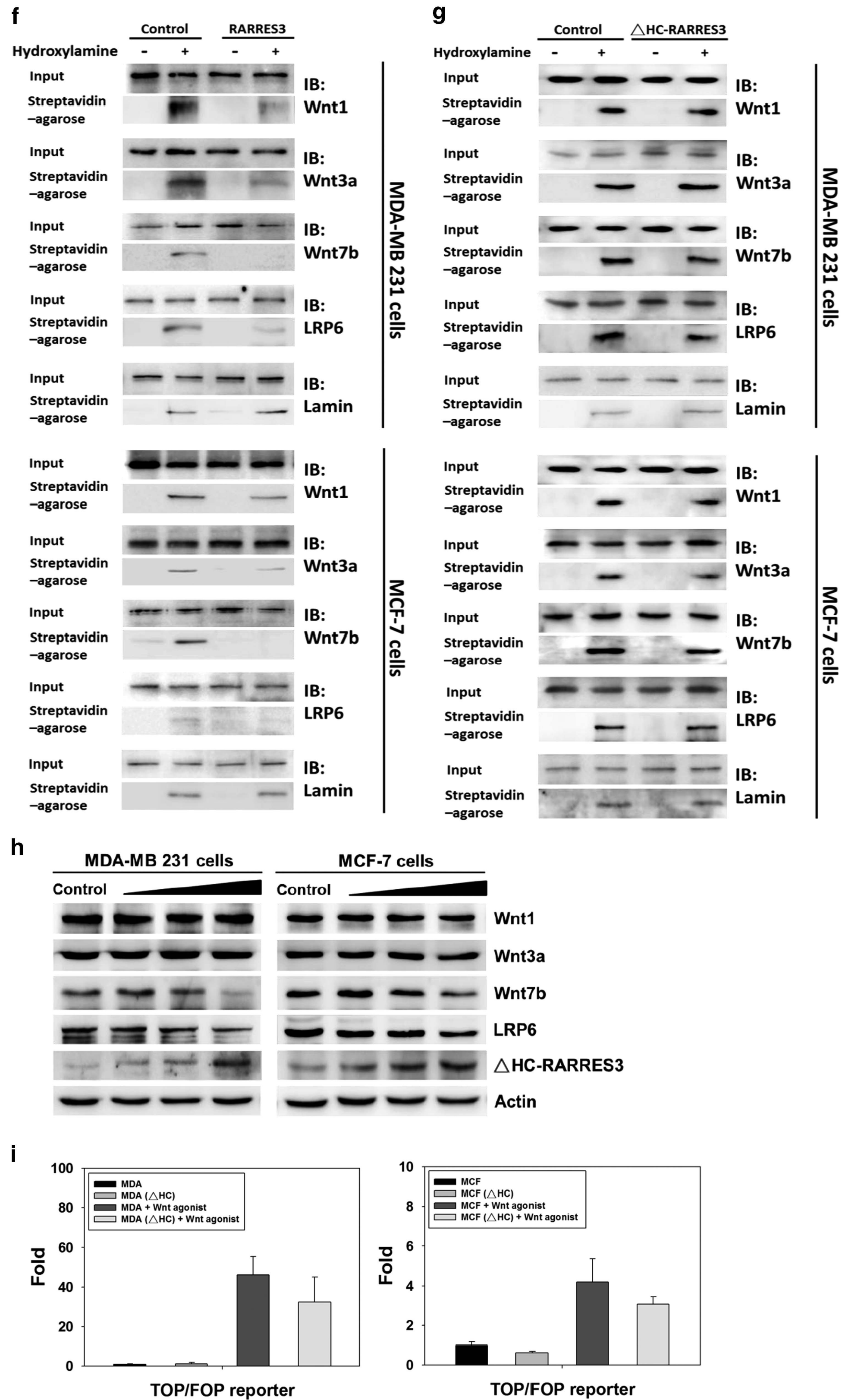
partially decreased, the reduction in TOP/FOP Flash activity was not significant in ΔHC-RARRES3-expressing cells (Figures 2i and g). Our results demonstrate that RARRES3 alters the posttranslational modification of proteins and thereby contributes to the suppression of Wnt/β-catenin signaling activity.

RARRES3 reduces EMT and TIC characteristics in breast cancer cells. It has been reported that Wnt/β-catenin signaling has a crucial role in maintaining the stem-cell-like properties of MDA-MB 231 and MCF-7 cells.[10,42] MDA-MB 231 cells are transformed, fibroblastic, basal-like and highly metastatic, while MCF-7 cells are associated with a more



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differentiated, noninvasive phenotype and form tight cell–cell junctions. To study the function of RARRES3 in EMT and stemness properties, the expression profiles of epithelial and mesenchymal markers in RARRES3-overexpressing cells were first examined. Our results showed that RARRES3-overexpressing cells displayed upregulated E-cadherin, an epithelial marker, and downregulated vimentin and N-cadherin, the mesenchymal markers. The mesenchymal transcriptional factor Slug was also observed and showed a decreased profile in RARRES3 overexpressed MDA-MB 231 cells. On the contrary, knockdown of RARRES3 increased the protein level of mesenchymal markers and decreased the expression of the epithelial marker E-cadherin (Figure 3a and Supplementary Figure S6). Consistent with the changes in the EMT markers, the cell migration and wound-healing abilities were significantly lower in both RARRES3-overexpressing cells (Figures 3b and c, Supplementary Figure S7). These results suggest that RARRES3 reduces EMT properties through upregulating epithelial markers and downregulating mesenchymal markers, which results in diminution of cell motility in both breast cancer cell lines.

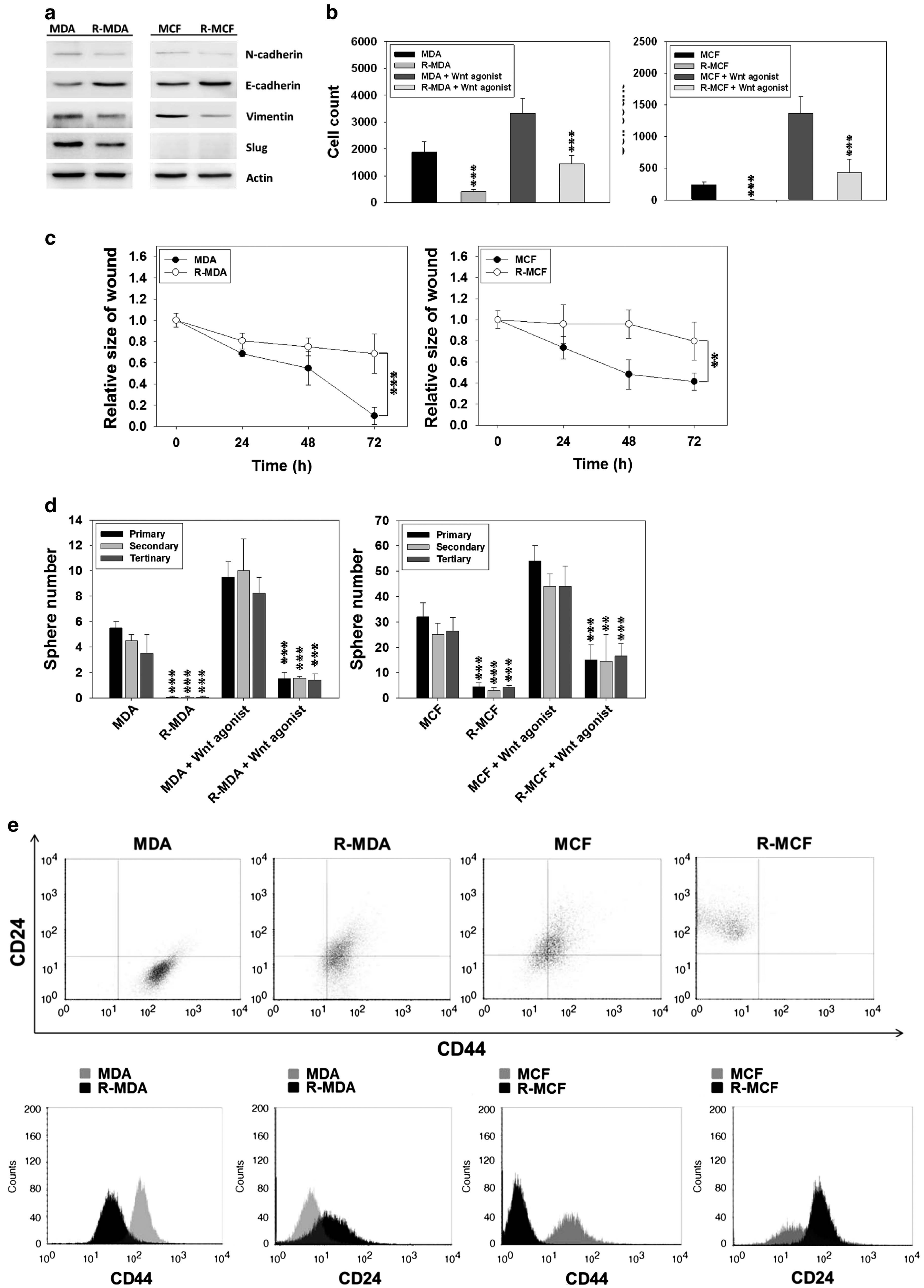
Previous studies indicate that induction of EMT on immortalized human mammary epithelial cells by ectopically expressing transcription factors resulted in an increased ability to form mammospheres and upregulated TIC markers.[43,44] To examine whether RARRES3 influences the expansion of the TICs subpopulation, we compared mammosphere formation and the TICs molecular markers in control and RARRES3-overexpressing cells. TICs are identified as a hyaluronan receptor (CD44)^{hi}/heat stable antigen (CD24)^{lo} subpopulation that is enriched in metastatic breast tumor cells.[45] We found that RARRES3 overexpression led to a more than five-fold reduction in mammosphere formation in the presence or absence of Wnt agonist (Figure 3d) and dramatically elevated the expression level of CD24 in MDA-MB 231 and MCF-7 cells. Furthermore, CD44 expression levels were apparently decreased in these cells (Figure 3e). Our results imply that the intracellular expression level of RARRES3 is critical for the control of EMT properties, cell migration activity, mammosphere formation and stem-cell-like characteristics.

Induction of endogenous RARRES3 regulates cell proliferation and Wnt/β-catenin signaling. To investigate the suppressive effect of endogenous RARRES3 on Wnt/β-catenin signaling and protein acylation, 5-fluorouracil (5-FU) was used to induce the expression of p53, which was shown to activate *RARRES3* gene expression.[31] 5-FU stimulated p53 and RARRES3 protein expression in control and RARRES3-silenced cells, as shown in Figure 4a. The inhibitory effects of endogenous RARRES3 on cancer cell proliferation, Wnt/β-catenin signaling and protein acylation were also examined. Upon 5-FU treatment, cell proliferation was decreased, and knockdown of RARRES3 significantly rescued this suppressive effect (Figure 4b). As shown in Figure 4c, the expression levels of Wnt proteins and co-receptor LRP6 were similarly suppressed in cells with endogenously induced RARRES3, and RARRES3 silencing restored the original levels of these proteins. In addition, the RARRES3-mediated decrease in β-catenin protein expression and transcriptional activity were also reversed in RARRES3-silenced cells (Figures 4d and e). Furthermore, RARRES3-mediated protein deacylation of Wnt proteins and LRP6 was also observed in 5-FU-stimulated cells, and RARRES3 silencing attenuated these deacylation modifications (Figure 4f). These results indicate that p53-mediated upregulation of RARRES3 efficiently suppresses the protein levels of Wnt/β-catenin signaling molecules through modulating their acylation status and thereby altering their signaling activity.

Discussion

RARRES3 has been shown to have a key role in the regulation of tumor cell proliferation, intracellular signaling activity and terminal differentiation of keratinocytes. A key issue regarding RARRES3 is how this protein interplays with different signaling pathways to regulate diverse biological processes. Remarkably, recent studies have shown that RARRES3 functionally displays acyltransferase activity. However, the biological implications of this activity have not been fully understood. In this study, we identified a novel mechanism through which overexpression of RARRES3 inhibits Wnt/β-catenin signaling activation and further mediates the reappearance of epithelial markers in breast cancer cells. Thus the RARRES3-mediated inhibition of Wnt/β-catenin signaling is considered a counteractive system that antagonizes cancer cell growth and stemness properties. Our results also indicate that RARRES3 displays a significant inhibitory effect on many growth-signaling pathways. These observations are consistent with previous findings showing that RARRES3 suppresses cell proliferation and triggers cell apoptosis

Figure 3 RARRES3 alters cellular EMT and TIC properties. (**a**) Overexpression of RARRES3 suppressed the expression of EMT markers. The expression levels of epithelial–mesenchymal-transition morphology-related proteins N- and E-cadherin, vimentin and Slug in control or RARRES3-overexpressing cells were examined by immunoblotting. MDA, pCR3.1 empty vector-transfected MDA-MB 231 cells; R-MDA, RARRES3-overexpressing MDA-MB 231 cells; MCF, pCR3.1 control vector-transfected MCF-7 cells; R-MCF, RARRES3-overexpressing MCF-7 cells. (**b**) RARRES3 overexpression suppressed cell migration activity. The cell migration assays were performed in control and RARRES3-overexpressing cells. The migration activities of these cells were compared in the absence or presence of Wnt agonist. The results represent the means $\pm$ S.D. of the migration levels ($n=3$). (**c**) Overexpression of RARRES3 caused loss of wound-healing ability in breast cancer cells. Confluent monolayers of control or RARRES3-overexpressing cells were scratched, and the relative sizes of the wounds were calculated for nine randomly chosen wound edges after the indicated time. The average size of the wounds was calculated as the mean $\pm$ S.D. ($n=3$). (**d**) RARRES3 overexpression resulted in the reduction of mammosphere formation. Tumorsphere formation of control and RARRES3-overexpressing cells was performed for three passages. The sphere numbers of these cells were compared in the absence or presence of Wnt agonist. Spheres were defined as being $>45\,\mu$m in diameter. The results represent the mean $\pm$ S.D. ($n=3$). (**e**) RARRES3 increased the subpopulation of CD44^{lo}/CD24^{hi} in breast cancer cells. Top panel: flow cytometry-analyzed antigenic phenotypes (CD44/CD24) that are characteristic of control and RARRES3-overexpressing cells. Virtually identical flow cytometry results were obtained from all the analyzed clones. Bottom panel: the expression level of CD44 and CD24 in the control and RARRES3-overexpressing cells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MDA
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E-cadherin
Vimentin
Slug
Actin
b
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R-MDA
MDA + Wnt agonist
R-MDA + Wnt agonist
Cell count
MCF
R-MCF
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R-MCF + Wnt agonist
c
Relative size of wound
Time (h)
d
Primary
Secondary
Tertiary
Sphere number
MDA + Wnt agonist
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CD44
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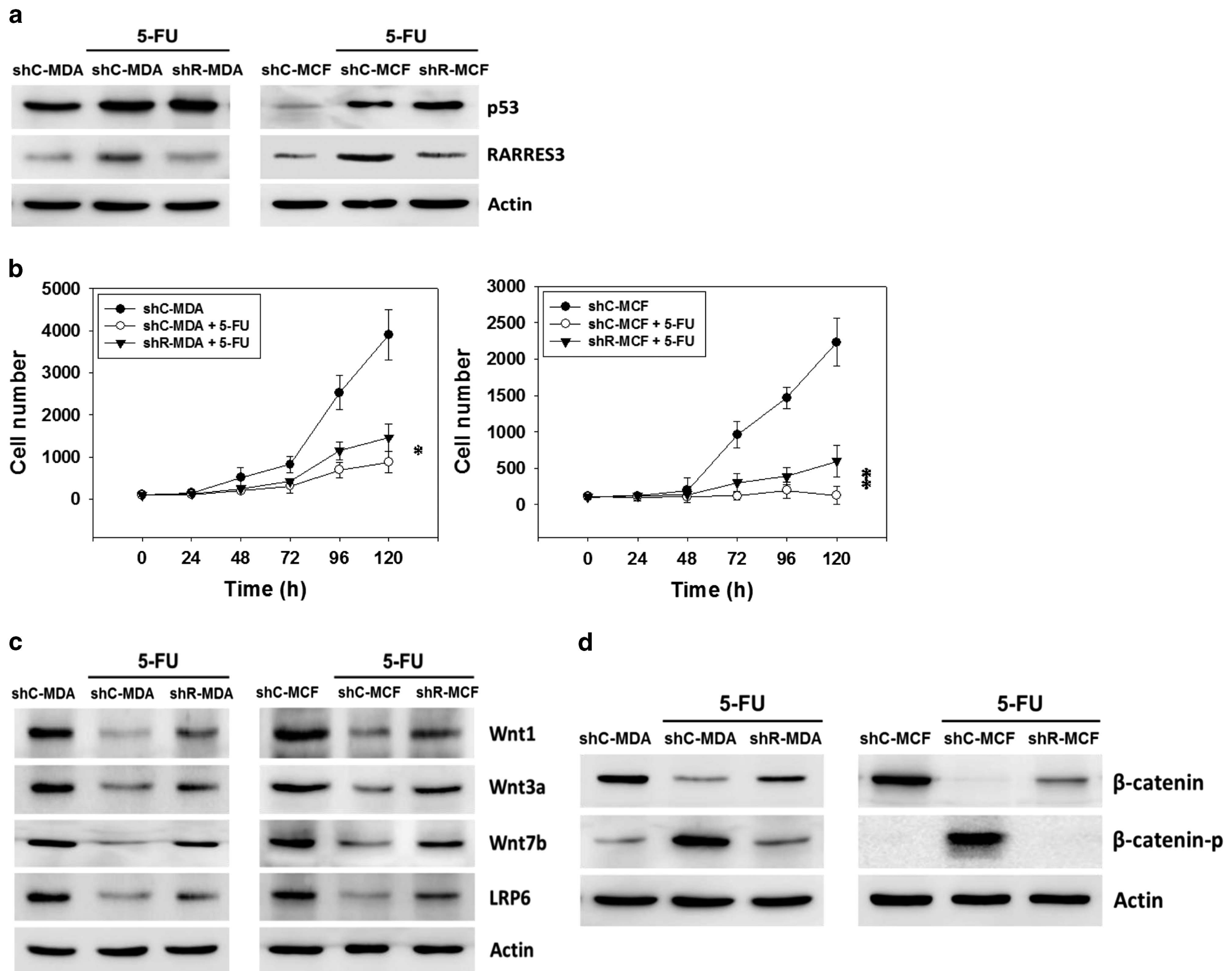

Figure 4 Endogenous RARRES3 modulates breast cancer cell growth and Wnt/β-catenin signaling. (**a**) Induction of RARRES3 in control and RARRES3 knockdown cells. The pLKO.1-shRARRES3- and pLKO.1-control vector-transduced MDA-MB 231 and MCF-7 cells were treated with 5-FU (24 h, 10 μg/ml). The protein levels of p53 and RARRES3 in cells were determined by immunoblotting. shC-MDA, pLKO.1-control vector-transduced MDA-MB 231 cells; shR-MDA, pLKO.1-shRARRES3-transduced MDA-MB 231 cells; shC-MCF, pLKO.1-control vector-transduced MCF-7 cells; shR-MCF, pLKO.1-shRARRES3-transduced MCF-7 cells. (**b**) Knockdown of RARRES3 perturbed the p53-mediated suppression of cell growth. Cell growth was analyzed in p53-mediated, RARRES3-overexpressing and RARRES3 knockdown cells. The results represent the mean ± S.D. ($n=3$). (**c**) RARRES3 knockdown reversed the p53-mediated decrease in Wnt/β-catenin signaling proteins. Cell lysates from control and 5-FU-treated cells were extracted, and the protein levels of Wnt1, Wnt3a, Wnt7b and LRP6 were analyzed by immunoblotting. (**d**) The β-catenin protein level is governed by RARRES3 in p53 upregulated cells. The control and 5-FU-treated cells were lysed, and the total and phosphorylated protein levels of β-catenin were examined by immunoblotting. (**e**) The transactivation activity of β-catenin was suppressed in RARRES3 upregulated cells. The transactivation activity of β-catenin was measured using the TOP/FOP Flash reporter assay. The cell lysates from control, 5-FU-treated and Wnt agonist-treated cells were collected, and the reporter activities in these cells were detected. The results represent the mean ± S.D. ($n=3$). (**f**) Deacylation of Wnt proteins and LRP6 was attenuated in RARRES3 knockdown cells. Lysates from the indicated cell lines were harvested and subjected to ABE analysis. The acyl proteins were precipitated and probed with anti-Wnt1, Wnt3a, Wnt7b and LRP6 antibodies. The acylation levels of these precipitated proteins were compared with those of control cells. (**g**) Proposed model of the p53/RARRES3 axis posttranslationally regulates the canonical Wnt/β-catenin signaling cascade. p53 stimulates the expression of class II tumor-suppressor RARRES3, which induces breast cancer cell apoptosis and cell cycle arrest. In addition, RARRES3 targets and inactivates Wnt/β-catenin signaling molecules via governing their acylation status. Decreased acylation level of these molecules disrupts their protein expression level and subcellular localization, which results in the suppression of Wnt/β-catenin signaling cascade, migration activity and TIC properties in breast cancer cells

by interfering with the c-Jun N-terminal kinases (JNK), ERK and PI3K/Akt pathways.[34,35] However, activation of these pathways is significant in RARRES3-silenced cells (Supplementary Figure S2), suggesting that the RARRES3 expression level is critical for the regulation of cell growth and survival signaling.

Generally, induction of Wnt/β-catenin signaling activity is supported by the downregulation of dickkopf-related protein 1 (Dkk1) or secreted Frizzled-related protein 1 (sFRP1), which have emerged as key regulators of Wnt/β-catenin signaling with the common feature of preventing ligand–receptor interactions.[10,46] Acyl modification of Wnt proteins was also shown to have a crucial role for proteins exiting from the endoplasmic reticulum, secreting to the extracellular domain or binding to the receptor.[21,22] Thus a regulatory mechanism of protein acylation may be necessary for cells to control

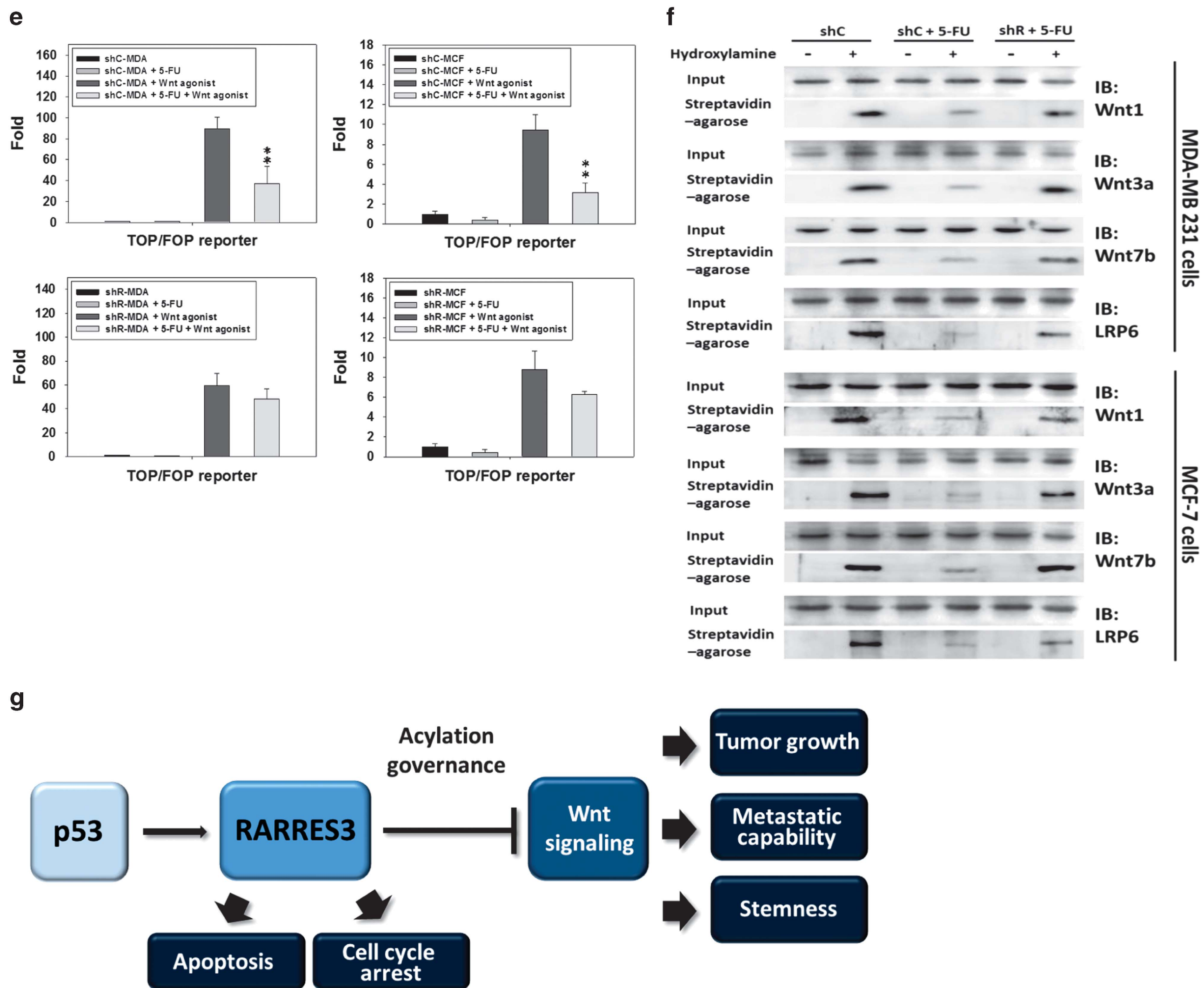

Figure 4 Continued

Wnt/β-catenin signaling activity. In this study, we demonstrate that RARRES3 interacts with and mediates the protein deacylation of Wnt/β-catenin signaling molecules. By using the construct with H23L and C113S double mutation in the conserved active site residues, we show that the enzymatic activity of RARRES3 displays a key role in the induction of protein deacylation. Consistent with our observations, previous studies have shown that acyl-deficient Wnt proteins ineffectively initiate Wnt/β-catenin signaling activity in cultured mammalian cells.[21,22] Furthermore, as in the case of deleting β-catenin in the epidermis or inhibiting the Wnt/β-catenin pathway by overexpressing Dkk1, it converts hair follicles into interfollicular epidermis.[47] The function of modulating Wnt/β-catenin signaling activity may also indicate a notable and distinct role of RARRES3 in the cell fate decision.

Acylation has been shown to act as an activity inductor of diverse signaling molecules to promote biological responses. In the present study, the influences of RARRES3 on protein acylation have been demonstrated. Our results provide evidence that correlate the enzymatic activity with the tumor-suppressive effect of RARRES3 in breast cancer cells. The co-localization of LRP6 and RARRES3 in the endoplasmic reticulum, which is the primary acylation locus of Wnt/β-catenin signaling molecules, implies that RARRES3 might compete with or adjust protein acyltransferases, which are involved in the regulation of Wnt/β-catenin signaling. Consistently, previous studies have shown that acylation is required for Wnt proteins to be transported to the PM.[20,22] As RARRES3 extensively suppresses diverse growth signaling in many tumor cells,[34,35] it is likely that RARRES3 exerts its protein deacylation role on other growth-signaling pathways. For example, it has been reported that RARRES3 co-localizes with and restrains H-Ras in the Golgi apparatus.[34] Notably, the protein acylation promotion of H-Ras out of intracellular compartments has also been indicated.[48] Thus it is expected that RARRES3 would promote protein deacylation of H-Ras in a similar manner as for Wnt/β-catenin signaling molecules. To prove this notion, we overexpressed RARRES3 and demonstrated the decrease in the H-Ras protein and protein acylation levels in breast cancer cells

(Supplementary Figures S8A and B). Consistently, the reduction of the total protein and acylated protein levels was attenuated in ΔHC-RARRES3-expressing cells (Supplementary Figures S8C and D). Thus our results demonstrate that RARRES3 suppresses H-Ras through modulating its protein acylation status. Consequently, the functional role of RARRES3 in mediating protein deacylation is evidently involved in the regulation of growth and survival signaling.

A number of developmental signaling pathways, including Notch, PI3K/Akt, Wnt/β-catenin, Hedgehog and p53,[49–51] have been shown to have central roles in mammary tumorigenesis and stem cell biology. Activation of the PI3K/Akt pathway has emerged as a central feature of EMT.[52] Similarly, ERK-MAPK, H-Ras and Wnt/β-catenin signaling activities have been implicated to be involved in EMT and TIC properties.[53,54] In the present study, we found that RARRES3 suppresses H-ras, Akt, ERK and Wnt/β-catenin signaling, which indicates its role in controlling malignant properties. As a type II tumor suppressor, RARRES3 is induced by diverse stimuli, such as retinoid and p53.[30,31] Although p53 and canonical Wnt/β-catenin signaling have been extensively studied, the two pathways were considered to be independent until recently.[55] In this study, 5-FU upregulated p53, which in turn induced RARRES3 expression and resulted in the suppression of Wnt/β-catenin signaling. Our results provide evidence for the functional role of the p53–RARRES3 axis and show a connection between p53, protein acylation and Wnt/β-catenin signaling (Figure 4g). In summary, the present study validates that RARRES3 has a crucial role in the suppression of cellular growth and Wnt/β-catenin signaling, apparently through its ability to modulate the protein acylation status.

Materials and Methods

Reagents. 5-FU and most chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used in this study: RARRES3, pan-cadherin, calnexin, cyclin D (Abcam, Cambridge, UK); β-actin (Millipore, Billerica, MA, USA); JNK1/2/3 and p-JNK1/2/3 (JNK1/Tyr185, JNK2/Tyr185, JNK3/Tyr223), p38 MAPK and p-p38 MAPK (Thr180/Tyr182), ERK1/2, p-ERK1/2 (ERK1/Thr202, ERK2/Tyr204), AKT, p-AKT (Ser473), Slug (Cell Signaling Technology, Danvers, MA, USA); LRP6, H-Ras, c-myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Wnt1, Wnt3a, Wnt7b, LRP6, β-catenin, p-β-catenin (Ser33/37), vimentin, E-cadherin, N-cadherin, Axin2, Lamin B (GeneTex, Irvine, CA, USA); and FITC mouse anti-human CD44 and PE mouse anti-human CD24 (BD Pharmingen, San Diego, CA, USA). As secondary antibodies, anti- mouse and anti-rabbit coupled to horseradish peroxidase (HRP) (GeneTex), Alexa Fluor 555 goat anti-mouse IgG (Invitrogen, Irvine, CA, USA) and Alexa Fluor 647 goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used. The cDNA encoding human RARRES3 in the pCR3.1 and pEGFP vectors were kindly provided by Dr. Shun-Yuan Jiang (Department of Medical Education and Research, The Buddhist Tzu Chi General Hospital, Taipei). The pCR3.1 vector containing the RARRES3 cDNA with specific mutations, ΔHC-RARRES3 (H23L and C113S), was kindly provided by Richard L. Eckert (Department of Biochemistry and Molecular Biology, School of Medicine, University of Maryland, Baltimore, MD, USA). The ΔHC-RARRES3 cDNA from pCR3.1-ΔHC-RARRES3 was subcloned into the Hind III and Bam HI sites of the pEGFP vector (Clontech Laboratories, Mountain View, CA, USA), and the new vector was named pΔHC-RARRES3-EGFP.

Cell culture. In this study, human breast cancer MDA-MB 231 and MCF-7 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories). To study the effect of RARRES3 on cell proliferation, MDA-MB 231 and MCF-7 cells were stably transfected with either RARRES3 or empty vector (pCR3.1) to obtain the RARRES3-overexpressing cells (R-MDA, R-MCF) or the corresponding vector control cells (MDA, MCF). After transfection, the cells were maintained for 2 weeks in medium with a lethal dosage of G-418 (3 mg/ml for MDA-MB 231 cells and 4 mg/ml for MCF-7 cells), and the selected clones were maintained in DMEM with half G-418 lethal dose for each cell line. The expression levels of RARRES3 in these selected clones were further verified by immunoblotting. For transient expression, cells were plated in 35-mm dishes and transfected with the appropriate amounts of pRARRES3 or pΔHC-RARRES3. Forty-eight hours after transfection, the cells were harvested, washed in phosphate-buffered saline (PBS) and analyzed. For cell proliferation analysis, 2000 cells were plated in triplicate in 24-well plates, 1 nM of the EGFR kinase inhibitor erlotinib (OSI-744, Selleck Chemicals, Houston, TX, USA) or 10 nM of the Wnt antagonist C-59 (BioVision, Milpitas, CA, USA) was added. The growth curves of the cells were determined by counting the viable cells using trypan blue exclusion and a hemocytometer. Each well was counted thrice, and the experiments were repeated on 3 separate days.

Lentivirus particle production and infection. Human kidney HEK 293 cells were transfected with the pLKO.1-puro–based vector, pCMV-dR-8.91 packaging vector and pMD2G-VSVG envelope vector. The pLKO.1-puro-based vector encoded an shRNA specific for human RARRES3 (TRCN0000003492, TRCN0000009063; Taiwan National RNAi Core Facility) or no shRNA. The virus particle-containing medium was harvested, cleared of debris by low-speed centrifugation and filtered through 0.45-μm filters. For cell infection, MDA-MB 231 and MCF-7 cells (1×10^6) were incubated in the presence of virus particle-containing medium for 48 h and then washed. Non-infected cells were eliminated from the culture by keeping the cells in medium containing a lethal dose of puromycin for 4 weeks (5 μg/ml for MDA-MB 231 cells and 15 μg/ml for MCF-7 cells), and the surviving clones were selected to obtain the control (shC-MDA and shC-MCF) or RARRES3 knockdown cell lines (shR-MDA and shR-MCF).

Flow cytometric analysis. The cell cycle distribution and the percentage of apoptotic cells were analyzed using propidium iodide (PI) staining as described previously.[56] Briefly, the cells (1×10^6) were washed and resuspended in 1 ml hypotonic fluorochrome solution (50 μg/ml of PI in 0.1% sodium citrate plus 0.1% Triton-X-100). The samples were placed at 4 °C in the dark, and the PI fluorescence of individual nuclei was analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). FACS analysis for the TIC-enriched $CD44^{hi}$/$CD24^{lo}$ subpopulation was performed as described previously.[57] The cells were harvested by trypsinization and washed twice with PBS. The cells then were fixed and stained with FITC- or PE-conjugated monoclonal antibodies against CD44 and CD24 and subjected to flow cytometry analysis.

Immunoprecipitation and Immunoblotting. Cells were lysed in lysis buffer (1% Nonidet P-40, 50 mM Tris-Cl, pH 7.4, 180 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 10 mM Na_3VO_4) for 30 min on ice, and the lysates were collected. For the immunoprecipitation, the samples were precleared with 50 μl Sepharose beads for 2 h and then incubated with primary RARRES3 antibody over night at 4 °C. Seventy microliters of Sepharose beads were then added to each sample, and the lysate-bead mixtures were incubated at 4 °C under rotary agitation for 2 h. After incubation, the supernatants were removed, the beads were washed three times with lysis buffer and the precipitated proteins were then eluted by lysis buffer containing 1% SDS. For immunoblotting, 10 μl of 6× loading buffer was added to denature the samples, the samples were incubated for 5 min at 95 °C and the proteins were separated on 12% polyacrylamide gels and then blotted onto polyvinylidene fluoride membranes (Millipore). The blots were preblocked and incubated with primary antibodies at 4 °C overnight followed by 30 min of incubation with one of the following secondary antibodies: anti-rabbit-HRP or anti-mouse-HRP. Detection of luminescence was carried out using enhanced chemiluminescence (Millipore).

Reporter assay. The cells were plated in 24-well plates for 24 h and then transfected with the appropriate amounts of control, pCR3.1-RARRES3 or pCR3.1-ΔHC-RARRES3 expression vector. One microgram each of TOP Flash (Millipore, 21170) and FOP Flash (Millipore, 21169) were cotransfected into the cells. After 24 h of transfection, a Wnt agonist (100 μM; Millipore 681665) or 5-FU (10 μg/ml) was added, and the luciferase readings were recorded 48 h

posttransfection. The luciferase activity is expressed as the relative luminescence unit/μg normalized to the constant transfection efficiency of FOP Flash. The protein concentrations were determined using the BCA assay reagent kit (Pierce, Rockford, IL, USA).

Fractionation. The preparation of PM and post-PM fractions were performed as described previously.[58] In brief, after washing three times with PBS, the cells were lysed with Buffer A (Tris, pH 8.0, 50 mM; dithiothreitol, 0.5 mM; NP-40, 0.1%). The lysates were then centrifuged at $1000 \times g$ for 10 min at 4 °C. The pellets were re-suspended in NP-40-free Buffer A on ice for another 10 min with occasional vortexing and then re-centrifuged at $1000 \times g$ for 10 min at 4 °C. The pellets were re-suspended in Buffer A and left on ice for 3 h with occasional vortexing and then centrifuged at $16\,000 \times g$ for 20 min at 4 °C. The supernatant was collected as the PM fraction and stored at −80 °C until use. The supernatants from the first and second spins at $1000 \times g$ were combined and spun at $16\,000 \times g$ for 20 min at 4 °C. The resulting supernatant was collected and used as the post-PM fraction.

Immunofluorescence. MDA-MB 231 and MCF-7 cells growing on 35-mm glass dishes were transfected with the pRARRES3-EGFP or pΔHC-RARRES3-EGFP vectors. After 24 h, the cells were washed, fixed with 3% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. The glass dishes were then incubated for 1 h with the appropriate primary and secondary antibodies. After washing, the cells were incubated in PBS containing 90% glycerol, and fluorescence was visualized using a Zeiss Axiovert 100M Confocal Laser Scanning Microscope (Carl Zeiss GmbH, Jena, Germany).

ABE analysis. ABE analysis was performed as previously described.[59] Briefly, cells were lysed with 100 μl lysis buffer containing 1% SDS and 10 mM *N*-ethylmaleimide (NEM) and then diluted with 900 μl lysis buffer containing 2% Triton X-100 and 10 mM NEM. The lysates were incubated for 1 h at 4 °C and then precipitated (chloroform–methanol method). The precipitates were solubilized with 200 μl solving buffer (50 mM Tris–HCl, 4% SDS, 5 mM EDTA, pH 7.5) containing 10 mM NEM for 10 min at 37 °C and then diluted with 800 μl of lysis buffer containing 0.2% Triton X-100 and 1 mM NEM. The mixtures were incubated overnight at 4 °C and then precipitated. The pelleted proteins were dissolved with 200 μl of solving buffer. One of the duplicated samples was mixed with 800 μl of 1 M hydroxylamine and 1 mM Biotin–HPDP. The other sample was mixed with 1 M Tris–HCl and 1 mM Biotin–HPDP (used as a negative control). The mixtures were incubated for 1 h at room temperature with gentle shaking and then precipitated. The precipitated proteins were solubilized with 50 μl Tris buffer and diluted with 450 μl lysis buffer containing 0.2% Triton X-100. The samples were incubated at 4 °C for 30 min and then centrifuged at $20\,000 \times g$ for 5 min. Fifty microliters of the supernatant was mixed with 25 μl of 3× Laemmli buffer supplemented with 6% 2-mercaptoethanol and incubated at 95 °C for 5 min (input samples). The remnants were mixed with 30 μl streptavidin–agarose slurry equilibrated with lysis buffer containing 0.1% SDS and 0.2% Triton X-100. The precipitates were eluted and analyzed by immunoblotting. Cytoskeletal lamin, which is acylated in the cell nucleus, served as a negative control.[60]

Cell migration. For transwell assays, 5×10^4 cells were added to the upper polycarbonate membrane insert (0.8-μm pore size; Costar, Boston, MA, USA) of the cell migration assay kit in a 24-well/plate. In the lower well, 700 μl DMEM with 10% FBS was used as a chemoattractant. After 48 h of incubation, the numbers of migrated cells were observed and counted. Three fields were randomly chosen, and the numbers of penetrated cells were counted. The experiments were performed in triplicate on at least 3 separate days.

Wound-healing assay. Cells were seeded in six-well plates and grown to confluency, and the monolayers were scratched. Pictures of nine randomly chosen wound edges per condition were taken at time 0 h and at the indicated time points, and the recovered area was quantified using Image J (NIH, Bethesda, MD, USA).

Mammosphere. Single-cell suspensions were suspended at a density of 1000 cells/ml in MammoCult medium (Stem Cell Technologies, Vancouver, BC, Canada) with supplemented heparin (2 μg/ml) and hydrocortisone (100 μM) and then seeded into 10-cm plates coated with 1.2% poly-Hema. Visible spheres (>0.45 μm) were counted in 10 different views under a microscope on day 7. The experiments were repeated three times, and each experiment was triplicated.

Statistical analysis. Statistical analysis was performed utilizing the Student's *t*-test with the SPSS 11.0 software system for Windows (IBM, Endicott, NY, USA). Some data were analyzed by one-way ANOVA. Significant differences compared with the controls were calculated and are marked by asterisks (**P*-value ≤ 0.05; ***P*-value ≤ 0.01; ****P*-value ≤ 0.005).

Conflict of Interest

The authors declare no conflict of interest.

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