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RARg Downregulation Contributes to Colorectal Tumorigenesis and Metastasis by Derepressing the Hippo–Yap Pathway

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Abstract

The Hippo–Yap pathway conveys oncogenic signals, but its regulation during cancer development is not well understood. Here, we identify the nuclear receptor RARg as a regulator of the Hippo–Yap pathway in colorectal tumorigenesis and metastasis. RARg is downregulated in human colorectal cancer tissues, where its expression correlates inversely with tumor size, TNM stage, and distant metastasis. Functional studies established that silencing of RARg drove colorectal cancer cell growth, invasion, and metastatic properties both *in vitro* and *in vivo*. Mechanistically, RARg controlled Hippo–Yap signaling to inhibit colorectal cancer development, acting to promote phosphorylation and binding of Lats1 to its transcriptional coactivator Yap and thereby inactivating Yap target gene expression. In clinical specimens, RARg expression correlated with overall survival outcomes and expression of critical Hippo–Yap pathway effector molecules in colorectal cancer patients. Collectively, our results defined RARg as tumor suppressor in colorectal cancer that acts by restricting oncogenic signaling by the Hippo–Yap pathway, with potential implications for new approaches to colorectal cancer therapy. *Cancer Res; 76(13); 3813–25. ©2016 AACR.*

Introduction

Retinoic acid receptor g (RARg), a member of the nuclear receptor subfamily, plays a critical role in mediating cell proliferation, differentiation, and apoptosis (1–4). Like other nuclear receptors, RARg can act in the nucleus as a transcription factor to regulate its target gene expression by binding to its DNA response elements as a heterodimer with retinoid X receptor (RXR; refs. 5, 6). However, the pleiotropic effects mediated by RARg in human diseases cannot be fully explained by genomic regulation due to a limited number of target genes. Interestingly, our previous findings showed that RARg has a unique cytoplasmic localization depending on the cell microenvironment (7), suggesting that RARg can function in the cytoplasm to mediate the pleiotropic effects of retinoids. Recent findings indicated that RARg can nongenomically regulate some rapid biologic responses through its cytoplasmic action. For example, cytoplasmic RARg interacts with b-catenin and leads to Wnt/b-catenin activation, which is required for cholangiocarcinoma cell growth and metastasis (8). RARg interacts with and activates c-SRC kinase in the cytoplasm during neuritogenic differentiation (9). We recently also reported that RARg nongenomically activates the PI3K/Akt and NF-kB signaling pathway through its interaction with the p85a regulatory subunit of PI3K in hepatocellular carcinoma (HCC; ref. 10).

There is conflicting evidence regarding RARg's role in cancer. On one hand, overexpression of RARg could significantly induce neuroblastoma cell death in response to retinoic acid (RA; ref. 11). RARg inhibits melanoma invasion by inducing carbohydrate sulfotransferase 10 (CHST10) expression (12). RARg is also suggested to suppress Ras-induced squamous cell carcinoma (13), suggesting RARg may act as a tumor suppressor in tumorigenesis. Conversely, RARg is often overexpressed in many human cancers, such as HCC and cholangiocarcinoma, and functions as a tumor promotor to drive cancer cell growth and metastasis (8, 10). Combined, these data indicate that RARg is involved in the regulation of tumorigenesis and its potential functions depend on different cancer types and a cell-specific context. However, whether and how RARg regulates colorectal cancer development and progression remains unclear.

The Hippo pathway is initially considered as an important player in organ growth and size maintenance (14, 15). Recently, accumulating evidence has strongly suggested that dysregulation of this pathway contributes to cancer development. Attenuated expression of the Hippo pathway components by epigenetic silencing or DNA mutations have been reported in human cancer. For example, hypermethylation of Mst1/2 in human soft tissue sarcomas (16) and hypermethylation of Lats1/2 in breast cancer and astrocytoma led to a decrease in their expression (17, 18). Mst1/2 deficiency in mice caused HCC (19). Moreover, Yap, the major downstream effector of the Hippo pathway, is known to be overexpressed in several human cancers, including liver cancer, prostate cancer, lung cancer, and ovarian cancer (20–23), and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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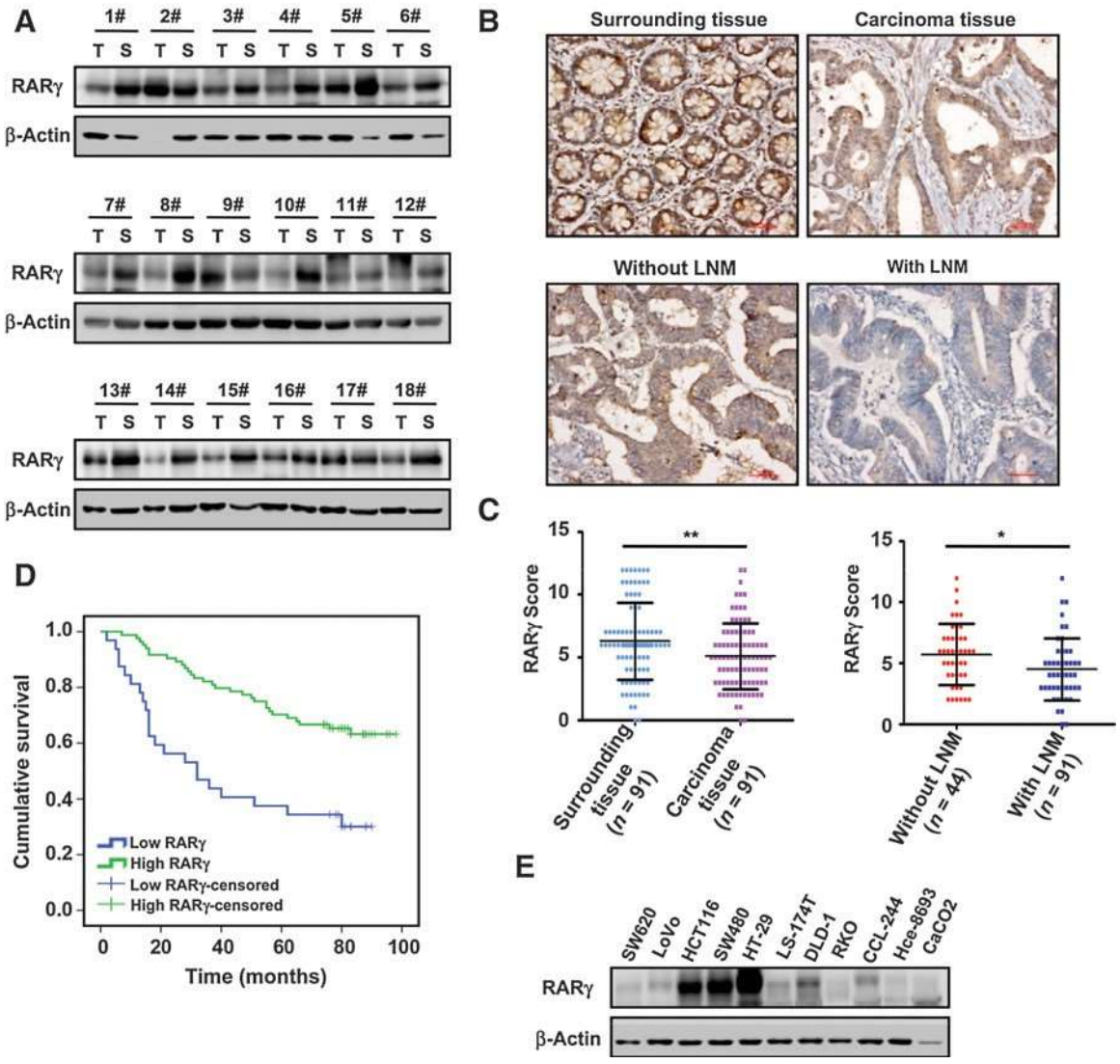


Figure 1.
RARg is downregulated in colorectal cancer and predicts a poor clinical outcome. A, Western blotting for RARg protein in colorectal cancer tumors. Eighteen randomly selected pairs of colorectal cancer tumors (T) and matched surrounding tissues (S) are presented. B, immunohistochemical staining of RARg in representative carcinoma and the surrounding tissues of colorectal cancer (magnification, x100). C, scatter plot analysis of RARg levels in 91 colorectal cancer tissue samples and their surrounding tissues. Statistical significance was determined by a two-tailed, paired or unpaired Student *t* test. *, $P < 0.05$; **, $P < 0.01$. D, Kaplan–Meier survival curve of colorectal cancer patients with low ($n = 32$) and high ($n = 84$) RARg expression. E, Western blotting for RARg protein in 11 colorectal cancer cell lines.

signi*fi*cantly correlates with poor survival for HCC patients (24). Experimentally, ectopic Yap expression in cancer cell lines drives tumor growth and metastasis (25–27). Tissue-speci*fi*c expression of Yap in transgenic mice led to tumor formation (28, 29). These *fi*ndings suggest that the Hippo pathway plays an important role in tumorigenesis.

Several types of signaling and molecules are involved in regulation of the Hippo–Yap pathway. Extracellular diffusible signals, such as serum-borne lysophosphatidic acid and sphingosine 1-phosphate (S1P), act through G-protein–coupled receptors (GPCR) to inhibit Lats1/2 kinase activity, and subsequently activate YAP/TAZ transcription coactivators (30). EGFR signaling inhibits the Hippo pathway through activation of the PI3K–PDK1 pathway (31). Recent studies also showed that the receptor tyrosine kinase ERBB4 induces breast cancer cell migration through interaction with Yap (32). Leukemia inhibitory factor receptor (LIFR) inhibits breast cancer metastasis by triggering a Hippo kinase cascade (33). Despite all of these efforts, however, how the Hippo–Yap pathway is regulated in cancer remains to be further explored.

In this study, we investigated the role of nuclear receptor RARg in colorectal cancer development. Our *in vitro* and *in vivo* studies identi*fi*ed RARg functions as a novel regulator for the Hippo–Yap pathway to mediate colorectal cancer cell growth and metastasis. In human colorectal cancer, loss of RARg is associated with poor survival.

Materials and Methods

Antibodies and reagents

Antibodies for Myc-tag, HA-tag, E-cadherin, RARg, vimentin, connective tissue growth factor (CTGF), p-Yap, Yap, Lats1, b-actin, and All-*trans*-RA (ATRA), Lipofectamine 2000, TRIzol LS, and WesternBright ECL reagents were used in this study. Detailed information on the antibodies and reagents is provided in Supplementary Data.

Cell culture

The SW620, LoVo, HCT116, SW480, HT-29, LS174T, DLD-1, RKO, CCL-244, Hce-8693, and Caco-2 human colon cancer cell lines and HEK293T human embryonic kidney cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), which performs routine cell line authentication testing with SNP and short tandem repeat analyses. These cells were cultured as described previously (34, 35). All cell lines were passaged in the laboratory for fewer than 4 months after resuscitation and were used at the *fi*fth through tenth passage in culture for this study.

Tissue samples and evaluation

Expression levels of RARg, E-cadherin, vimentin, and CTGF were measured using IHC in 91 human colorectal cancer tissues that were collected immediately after surgical resection between 2003 and 2007 at the First Aff*i*liated Hospital of Soochow University (Suzhou, Jiangsu, China). The clinical characteristics of all patients are listed in Supplementary Table S1. A colorectal cancer tissue microarray was used to illustrate the correlation of RARg expression and patients' survival outcomes. The colorectal cancer tissue microarray containing 116 colorectal cancer samples with survival times were purchased from Shanghai Outdo Biotech Co., Ltd.. These samples were collected from 2006 to 2007. The study was approved by Soochow University for Biomedical Research Ethics Committee, and all of the patients provided informed consent. Tissues were probed with the indicated antibodies, and the staining score was evaluated as described previously (35, 36). Representative images were taken by a Nikon ECLIPSE Ni scope with color camera, and were processed with NIS-Elements D 4.10.00 software.

Generation of stable cell lines

SW480 and HT-29 cell lines stably expressing RARg-speci*fi*c shRNA (shRNA/RARg) or scrambled shRNA control (shRNA/Control) were constructed using a lentiviral shRNA technique (GeneChem), as described previously by our laboratory (34). The human RARg shRNA target sequences are listed in Supplementary Table S2.

Cell extraction and Western blotting

The experiments were performed as described previously (34). Representative blots are shown from several experiments, and the images were taken and analyzed by an image-forming system with Chemiscope analysis V1.01 software. Detailed protocol is provided in Supplementary Data.

RNA extraction and qPCR analysis

Total RNAs were isolated and reverse transcribed as described previously (34). qPCR was carried out using gene-speci*fi*c primers for E-cadherin, vimentin, amphiregulin (AREG), and CTGF with SYBR Green RT-PCR kits (TaKaRa) and ran on a StepOne Plus Real-Time PCR System (Life Technologies). Relative transcript levels of those genes were normalized to b-actin mRNA levels. The primers for those genes are listed in Supplementary Table S3.

Confocal microscopy

Colorectal cancer cells seeded on coverslips in 24-well plates overnight were transfected for 24 hours, and then the cells were *fi*xed, permeabilized, and stained with the indicated antibodies. The details have been described previously (7). The images were taken with a Nikon ECLIPSE Ni scope with color camera and were processed by NIS-Elements D 4.10.00 software.

Immunoprecipitation

Cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. Whole-cell lysates were incubated with the indicated antibodies overnight, followed by incubation with protein A/G beads (Santa Cruz Biotechnology) for 2 hours. The beads were washed using RIPA lysis buffer for at least three times, and then boiled in SDS loading buffer. Immunoprecipitated protein complexes were detected using Western blotting. Images of the blots were taken with an image-forming system (Bioshine).

Colony formation assay

Colorectal cancer cells (1×10^3) were placed in 6-well plates for 14 days and then *fi*xed and stained with Wright-Giemsa. The number of foci containing >50 cells was determined at 40× magni*fi*cation using an optical microscope (Nikon), and the images were taken by a digital camera (Nikon).

Transwell migration and Matrigel invasion assays

Transwell migration and Matrigel invasion assays were used in this study. Detailed protocol is provided in Supplementary Data.

Xenografts

Nude mice (BALB/c, SPF grade, 16–18 g, 4–5 weeks old, and male) were purchased from Shanghai SLRC Laboratory Animal Co., Ltd. and housed in a pathogen-free room with a 12-hour light/dark cycle. For tumor growth, nude mice were injected subcutaneously with 2×10^6 colorectal cancer cells (n ¼ 6 mice per group). After 7 days of transplantation, body weight and tumor sizes were measured every 3 days. Mice were killed after 4 weeks, and tumors were removed for assessments. For tumor metastasis, nude mice were injected with 2×10^6 colorectal cancer cells into the lateral vein in the nude mouse tail (n ¼ 6 mice per group). The mice were sacri*fi*ced after 7 weeks. Lung tissues were collected for metastatic foci evaluation and standard histopathologic study. The details have been described recently (36–38). All animal experiments were approved by the Animal Care and Use Committee of Soochow University (Suzhou, Jiangsu, China).

Statistical analysis

Each assay was performed in three independent experiments. Data are presented as mean ± SD. Statistical signi*fi*cance was analyzed using a Student t test (unpaired, two-tailed) or one-way ANOVA. Pearson c^2 test was used to analyze the relationships between RARg expression and clinicopathologic factors, and Spearman rank correlation analysis was used to calculate the correlations between the expression levels of RARg and E-cadherin, vimentin, or CTGF. A Kaplan–Meier survival analysis was

used to illustrate the prognostic relevance of RARg in univariate analysis. $P < 0.05$ was considered statistically significant.

Results

RARg is downregulated in human colorectal cancer and correlates with tumor size, metastasis, and poor survival

To investigate the role of RARg in the development of colorectal cancer, we first used Western blotting to examine RARg expression in human colorectal cancer tissues. Our results showed that abundant RARg expression was observed in the matched surrounding tissues of colorectal cancer. In comparison, RARg expression was obviously lower in the primary colorectal cancer tumors (Fig. 1A). Immunohistochemical staining confirmed that the expression of RARg is downregulated in tumor tissues, and is further reduced in those with lymph node metastasis (LNM; Figs. 1B and C). We next investigated the relationship between RARg expression levels and the clinicopathologic status of patients with colorectal cancer. Pearson c^2 test analysis showed that reduced RARg in colorectal cancer cells was significantly correlated with tumor size, TNM stage, and distant metastasis (Supplementary Table S1). Kaplan–Meier analysis revealed that the low RARg expression level in colorectal cancer tissues markedly correlated with a reduction in patient survival (Fig. 1D). in addition, cytologic experiments showed that there was low RARg expression in most of the colorectal cancer cell lines (Fig. 1E). These data suggest that reduced RARg expression is associated with the development of colorectal cancer.

RARg deficiency promotes colorectal cancer cells growth and metastasis *in vitro* and *in vivo*

The above findings indicated reduced RARg expression in colorectal cancer. We thus asked whether RARg has a causal role in regulating colorectal cancer cell phenotypes. We first stably knocked down RARg expression using a lentivirus vector–based shRNA technique in SW480 and HT-29 cell lines that express a high level of RARg protein. The knockdown efficiency was confirmed by Western blotting (Supplementary Fig. S1). RARg 's ability to affect cell proliferation, migration, and invasion was first tested *in vitro*. MTT assays showed that silencing RARg in SW480 cells significantly enhanced the ability of cellular proliferation (Supplementary Fig. S2). In agreement with this result, colony formation assays revealed that the ability of SW480 cells to form foci was greatly enhanced when cells lacked RARg (Fig. 2A). In a Transwell migration assay, RARg-depleted SW480 cells migrated approximately twice as much as the control cells (Fig. 2B), which is consistent with the observations that knocking down RARg potently enhanced colorectal cancer cells' invasive property (Fig. 2C). These results suggested that RARg deficiency promotes colorectal cancer cell proliferation, migration, and invasion *in vitro*.

We also assessed the role of RARg in colorectal cancer cell growth and metastasis in an *in vivo* mice model. We first implanted the same number of SW480 cells that stably expressed RARg shRNA or control scrambled shRNA onto the subcutaneous sites of nude mice. Growth of the implanted tumors was monitored and tumor sizes were measured every 4 days. The results showed that depletion of RARg greatly promoted tumor growth as revealed by the fact that the sizes (Fig. 2D) and weight (Fig. 2E) of tumors derived from SW480/shRNA/RARg cells were markedly increased than control cells. In a xenografted metastasis model, knocking down RARg expression significantly promoted cancer metastasis to the lungs. As shown in Fig. 2F, SW480/shRNA/RARg cells formed more and larger pulmonary micrometastases than the control cells. These findings are also summarized in Fig. 2G. These results indicated that RARg deficiency enhanced colorectal cancer cell growth and metastasis *in vivo*.

Overexpression of RARg impaired colorectal cancer cells growth and metastasis *in vitro* and *in vivo*

We further performed a gain-of-function study and investigated the effects of RARg overexpression on growth and metastasis of colorectal cancer. In colony formation assays, ectopic expression of RARg in DLD-1 and RKO cells significantly inhibited the ability of those cells to form foci (Supplementary Figs. S3A and S3B). The Transwell migration and invasion assays showed that forced expression of RARg markedly suppressed the colorectal cancer migration and invasion (Supplementary Figs. S3C–S3F). Moreover, in agreement with the functional link between RARg and colorectal cancer cell growth, migration, and invasion *in vitro*, our *in vivo* observations showed that ectopic expression of RARg greatly suppressed tumor growth and metastasis as evidenced by the fact that the sizes (Supplementary Fig. S3G) and weight (Supplementary Fig. S3H) of tumors derived from DLD-1/RARg cells were greatly decreased compared with the control cells, and the ability of DLD-1/RARg cells to metastasize to the lungs was significantly impaired compared with the control cells (Supplementary Figs. S3I and S3J). Together, these experiments demonstrated that overexpression of RARg inhibits colorectal cancer cell growth and metastasis *in vitro* and *in vivo*, strongly suggesting that RARg functions as a tumor suppressor in colorectal cancer development.

RARg promotes Yap phosphorylation and cytoplasmic retention

Aberrant Yap activity and defects in the Hippo pathway underlies the development of cancer (14, 39). We thus asked whether RARg modulates Yap activity on colorectal cancer development and progression. Surprisingly, the basal Yap phosphorylation was significantly influenced by altering RARg expression in colorectal cancer cells. As shown in Fig. 3A and B, silencing RARg in SW480 cells markedly impaired Yap phosphorylation (Fig. 3A), while ectopic RARg expression in DLD-1 and RKO cells greatly increased Yap phosphorylation (Fig. 3B). These results were further confirmed by *in vivo* studies showing that RARg expression is positively correlated with Yap phosphorylation in SW480 xenografts (Fig. 3C). We next studied the effect of ATRA, an agonist for RARs, on Yap phosphorylation. Our results showed that treatment of RKO cells with ATRA enhanced RARg-induced Yap phosphorylation (Fig. 3D). However, silencing RARg expression in SW480 cells reduced ATRA-induced Yap phosphorylation (Fig. 3E), suggesting that RARg-mediated Yap phosphorylation can be regulated by its ligands.

This phosphorylation could cause cytoplasmic retention of YAP, thereby preventing its function as a transcriptional coactivator in the nucleus (21). Compared with the control cells, silencing RARg expression in SW480 cells significantly increased the levels of nuclear Yap and reduced the levels of cytoplasmic Yap, as gauged by immunofluorescent staining (Fig. 3F) and fractionation assays (Fig. 3G), which were consistent with *in vivo* observations that increased levels of nuclear Yap in the tumors derived from SW480/shRNA/RARg cells (Fig. 3H).

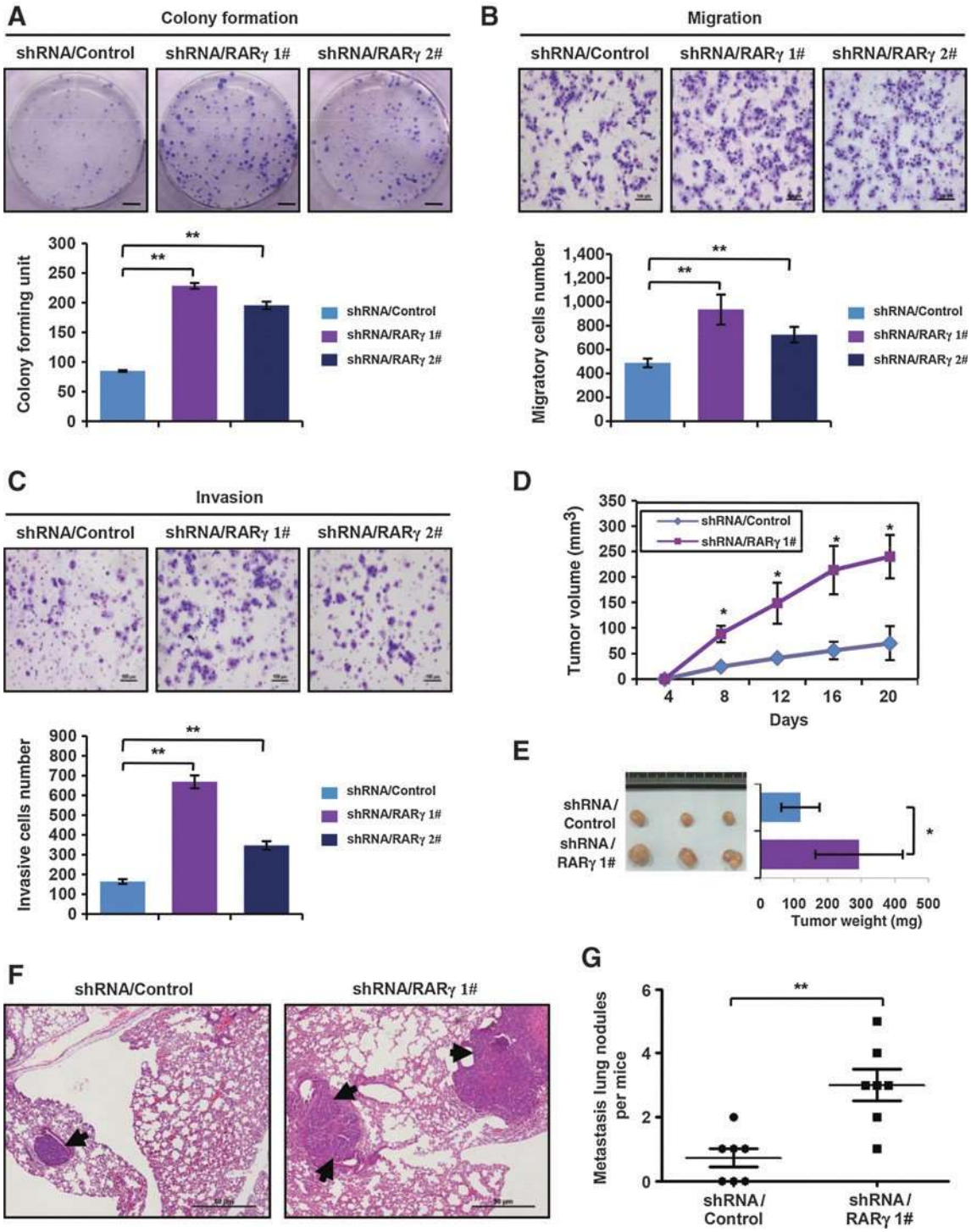
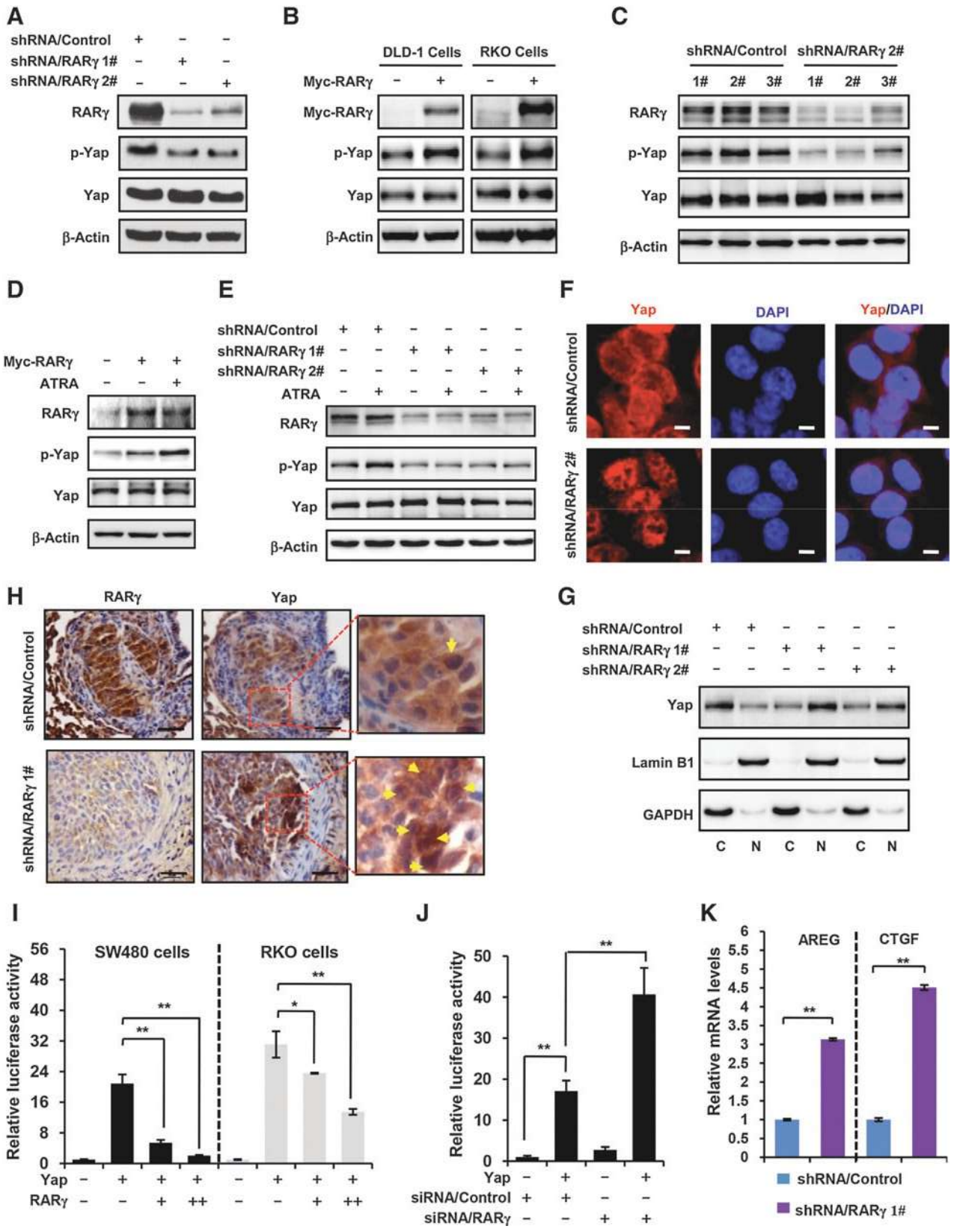


Figure 2.

Knockdown of RARg promotes colorectal cancer growth and metastasis *in vitro* and *in vivo*. A, SW480 cells stably expressing the shRNA vector were maintained in culture media for 14 days and then fixed and stained with Wright-Giemsa. The representative photographs are presented (top; magnification, x1), and the relative number of colonies was counted (bottom). B and C, migration (B) and invasion (C) assays were performed in wild-type cells (shRNA/Control) and in cells with stable knockdown of RARg (shRNA/RARg). Representative photographs are presented (B and C, top; magnification, x100) and the relative number of migratory cells (B, bottom) and invasive cells (C, bottom) were counted. D and E, silencing RARg promotes colorectal tumorigenesis. SW480 cells stably expressing RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control) were transplanted into nude mice. The volumes of the tumors were measured every 4 days during the indicated period (D), and the representative images of tumors and the average tumor mass of each group are shown (E). F and G, silencing RARg promotes colorectal cancer metastasis. F, representative micrographs of lung tissues with metastatic nodules are shown by hematoxylin and eosin staining (magnification, x40). G, the number of pulmonary micrometastases in individual mice was counted under a microscope. Statistical significance was determined by a two-tailed, unpaired Student *t* test. *, $P < 0.05$; **, $P < 0.01$.


A
shRNA/Control + − −
shRNA/RARγ 1# − + −
shRNA/RARγ 2# − − +
RARγ
p-Yap
Yap
β-Actin
B
DLD-1 Cells
RKO Cells
Myc-RARγ − + − +
Myc-RARγ
p-Yap
Yap
β-Actin
C
shRNA/Control
shRNA/RARγ 2#
1# 2# 3# 1# 2# 3#
RARγ
p-Yap
Yap
β-Actin
D
Myc-RARγ − + +
ATRA − − +
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shRNA/Control + + − − − −
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Yap
Lamin B1
GAPDH
C N C N C N
I
SW480 cells
RKO cells
Relative luciferase activity
56 48 40 32 24 16 8 0
**
**
*
**
Yap − + + + − + + +
RARγ − − + ++ − − + ++
J
Relative luciferase activity
50 40 30 20 10 0
**
**
Yap − + − +
siRNA/Control + + − −
siRNA/RARγ − − + +
K
Relative mRNA levels
6 5.5 5 4.5 4 3.5 3 2.5 2 1.5 1 0.5 0
AREG
CTGF
**
**
shRNA/Control
shRNA/RARγ 1#

RARg inhibits Yap-TEAD transcriptional activity and Yap target gene expression

Yap phosphorylation led to its cytoplasmic sequestration and inactivation of its functions in the nucleus (21). Yap facilitates regulation of downstream target gene expression by directly binding to the transcription factor TEADs (40, 41). Through a TEAD4-responsive luciferase report, we found that overexpression of Yap significantly enhanced the activity of luciferase reporter (Fig. 3I), as described elsewhere (41). However, ectopic expression of RARg led to a dramatic decrease of Yap-induced luciferase activity (Fig. 3I). Consistent with these results, silencing RARg remarkably increased the luciferase activity induced by Yap (Fig. 3J). We further determined whether RARg could modulate the expression of Yap target genes, such as AREG and CTGF (40, 42). qPCR assays revealed that silencing RARg expression induced a 3-fold or 4.5-fold increase in AREG mRNA and CTGF mRNA, respectively (Fig. 3K). Thus, the above data indicated that RARg can diminish the transcriptional activity of TEADs and the expression of Yap target genes through its modulation of Yap activity.

RARg regulates EMT through a Yap-dependent pathway

Because RARg is involved in colorectal cancer metastasis, it is possible that RARg may regulate the epithelial-to-mesenchymal transition (EMT), which is an early event in the metastasis of cancer (34, 43, 44). To test this, the expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin were analyzed using qPCR and Western blotting assays. The results revealed that silencing RARg reduced E-cadherin expression, but increased vimentin expression (Figs. 4A and B). Consistently, ectopic expression of RARg did the opposite (Figs. 4C and D). These results indicated an important role of RARg in modulating the EMT.

Given the evidence linking aberrant Yap activity to tumor metastasis (27, 45), we sought to test whether Yap is implicated in the RARg-mediated EMT. Western blotting assays revealed that knocking down Yap expression in DLD-1 cells significantly enhanced RARg-induced E-cadherin increases and vimentin reduction (Fig. 4E). Consistent with these results, silencing RARg expression induced E-cadherin losses and vimentin increases, which were markedly reversed by the depletion of Yap in HT-29 cells (Fig. 4F). To further support this notion, we tested whether Yap is involved in RARg-derived migratory and invasive behaviors of colorectal cancer cells. Results from migration assays showed that silencing RARg greatly enhanced the migratory ability of colorectal cancer cells. However, silencing RARg showed only a limited effect on colorectal cancer cell migration when Yap was depleted (Fig. 4G, top). Similar results were also observed in invasion assays (Fig. 4G, bottom). These findings are summarized in Fig. 4H. Together, these results indicated that the effects of RARg on the EMT are dependent on Yap.

RARg interacts with Yap

To study the mechanisms by which RARg inactivates Yap activity in colorectal cancer, we examined the possibility whether RARg interacts with Yap. Surprisingly, coimmunoprecipitation (co-IP) assays showed a constitutive interaction between endogenous RARg and Yap in SW480 cells (Fig. 5A). Consistent with these results, exogenous interaction between RARg and Yap was also observed in HEK293T cells that were cotransfected with Myc-RARg and HA-Yap (Fig. 5B). We previously reported that RARg often resides in the cytoplasm of cancer cells (7). The cytoplasmic localization of RARg has also been observed in colorectal cancer cells (Supplementary Fig. S4A), implicating that cytoplasmic RARg may function by interacting with Yap. Indeed, confocal microscopy showed approximately 75% colocalization of RARg and Yap in the cytoplasm of colorectal cancer cells (Supplementary Fig. S4B). These results suggested that RARg can interact with Yap in the cytoplasm. Interestingly, our results further showed that the interaction between RARg and Yap could be greatly enhanced after ATRA treatment in SW480 and HCT116 cells (Fig. 5C), indicating that the interaction between RARg and Yap could be regulated by ligands for RARg.

To further explore the molecular basis of the interaction of RARg with Yap, we defined the regions of RARg and Yap that are required for their interaction. First, a series of RARg deletion mutants (Fig. 5D) were cotransfected with Yap into HEK293T cells. Co-IP assays revealed that the mutant lacking the LBD domain completely lost its capacity to bind Yap, whereas other mutants were able to bind Yap (Fig. 5E), suggesting that the LBD domain of RARg is responsible for the association with Yap. To identify the regions of Yap responsible for its interaction with RARg, we constructed a series of Yap domain-deletion mutants (Fig. 5F). Our results showed that the Yap mutant (1–292 aa), Yap mutant (48–488 aa), and Yap mutant (102–488 aa) could interact with RARg, whereas the Yap mutant (263–488 aa) abolished this particular interaction (Fig. 5G), indicating that the region (102–263 aa) covering the WW domains in Yap is indispensable for its interaction with RARg.

RARg potentiates Lats1 binding to and phosphorylating Yap

It is unlikely that RARg serves as a kinase to directly regulate Yap phosphorylation. A possible mechanism underlying

Figure 3.

RARg regulates Yap phosphorylation and functionally inactivates Yap in colorectal cancer cells. A, Western blotting of phosphorylated Yap (p-Yap), Yap, RARg, and b-actin in SW480 cells that stably express RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control). B, Western blotting of phosphorylated Yap (p-Yap), Yap, RARg, and b-actin in RARg-overexpressed DLD-1 and RKO cells. C, Western blotting of phosphorylated Yap (p-Yap), Yap, RARg, and b-actin in tumors. Six shRNA/Control and six shRNA/RARg xenografts were randomly selected for determination of the indicated protein. D and E, association of RARg and Yap phosphorylation in response to ATRA. Western blotting of phosphorylated Yap (p-Yap), Yap, RARg, and b-actin in RARg-transfected RKO cells (D) or in RARg-silenced SW480 cells (E) treated with vehicle or 1 mmol/L ATRA. F, immunofluorescent staining of Yap (red) in SW480 cells stably expressing RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control). Nuclei were stained with DAPI (blue). Representative images are shown (magnification, x400). G, subcellular fractionation analysis of Yap expression in SW480 cells stably expressing RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control). Immunoblottings of GAPDH and Lamin B1 served as controls for the purity of cytoplasmic (C) and nuclear (N) fractions, respectively. H, immunohistochemical staining of RARg and Yap in the metastatic tumors derived from SW480/shRNA/RARg and SW480/shRNA/Control cells. Representative photographs are presented (magnification, x100). I and J, TEAD4-dependent luciferase reporter activity was measured in RKO cells transfected with the increasing amount of RARg (I) or in RARg siRNA-transduced SW480 cells (J). K, qPCR analysis of the relative AREG and CTGF mRNA expression in SW480 cells stably expressing RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control). Statistical significance was determined by a two-tailed, unpaired Student *t* test. **, $P < 0.01$.

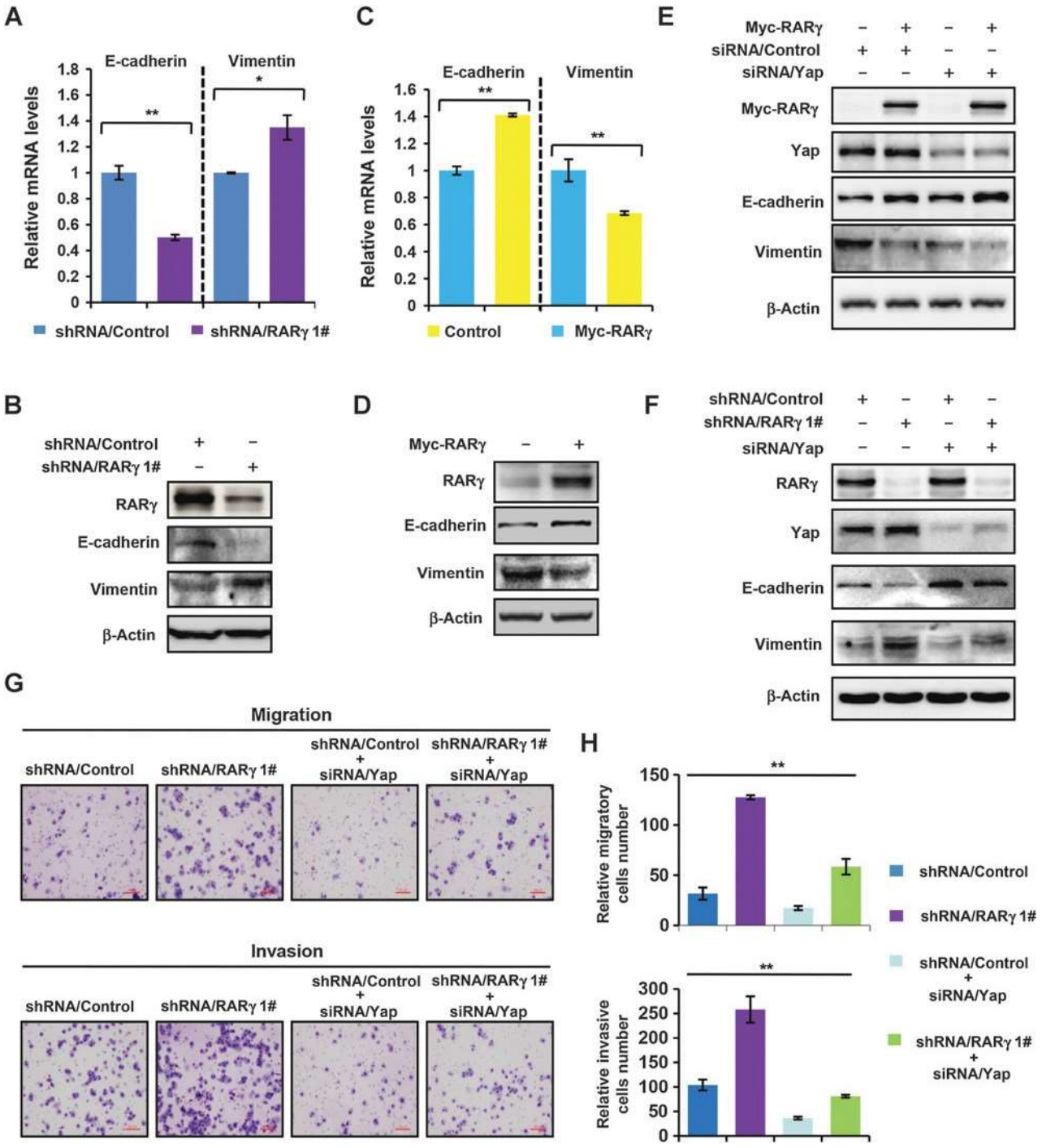

Figure 4.
RARg regulates Yap-dependent EMT. A and B, qPCR (A) and Western blotting (B) analysis of the E-cadherin and vimentin expression in HT-29 cells stably expressing RARg shRNA (shRNA/RARg) or shRNA (shRNA/Control). C and D, qPCR (C) and Western blotting (D) analysis of E-cadherin and vimentin expression in RARg-transfected DLD-1 cells. E and F, Western blotting of the indicated protein in RARg-overexpressed DLD-1 cells with or without Yap siRNA (E), or in RARg shRNA-transduced HT-29 cells in the presence and absence of Yap siRNA (F). G and H, migration and invasion assays were performed in RARg shRNA-transduced SW480 cells with or without Yap siRNA. The representative photographs are presented (G; magnification, x100) and statistical analysis (H) are shown. Statistical significance was determined by a two-tailed, unpaired Student *t* test or one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$.

RARg-mediated Yap phosphorylation may be due to its modulation of some kinases to phosphorylate Yap. The kinase Lats1/2, a core component of the Hippo pathway, interacts with and phosphorylates Yap, thereby resulting in Yap inactivation (46). We thus asked whether RARg is involved in the process. Interestingly, our results showed that ectopic expression of RARg in RKO cells

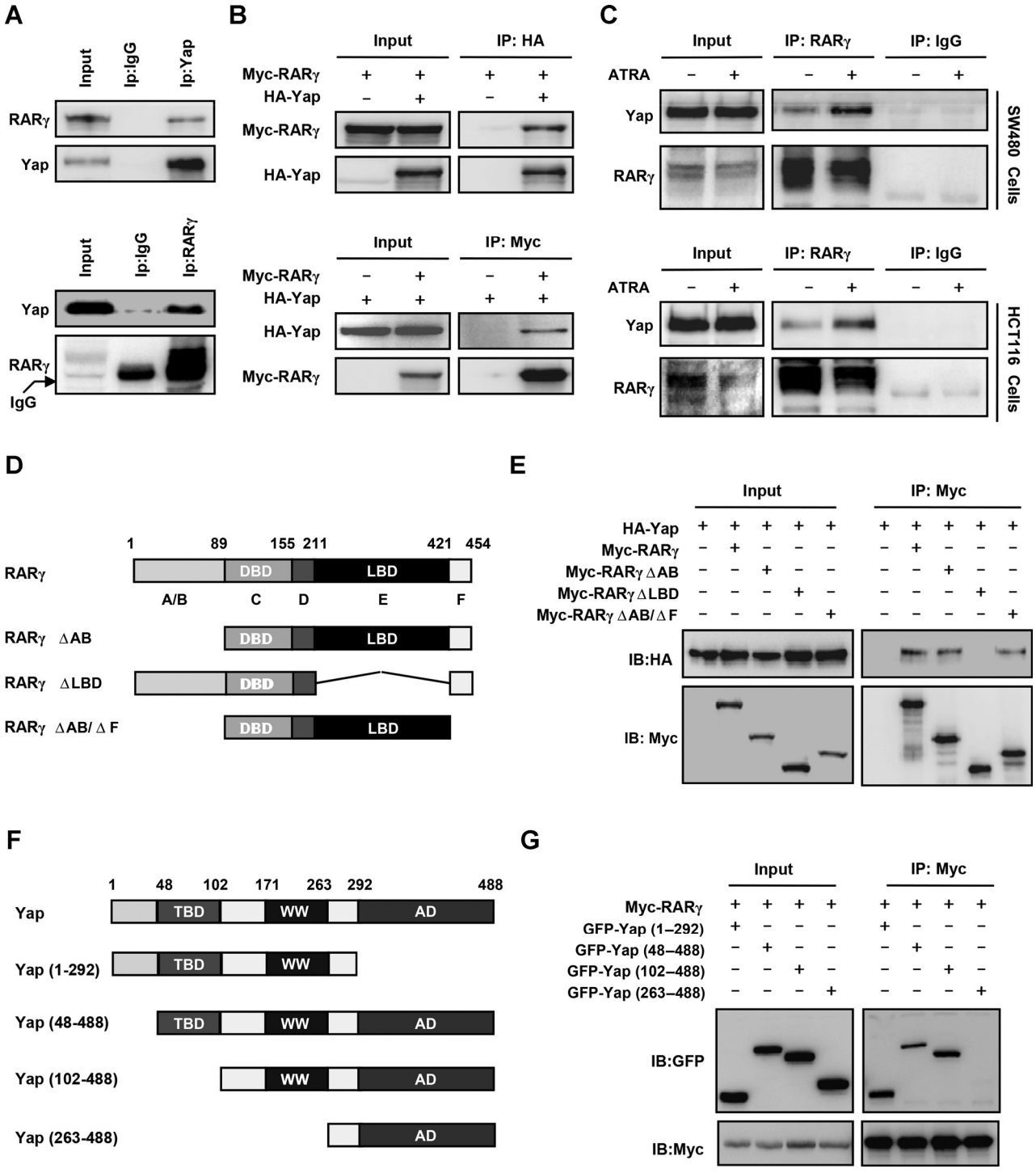

Figure 5.
RARg interacts with Yap. A and B, RARg interacts with Yap at both endogenous levels and exogenous levels. A, cell lysates from SW480 cells were immunoprecipitated with anti-Yap or anti-RARg antibodies, followed by immunoblotting with anti-RARg or anti-Yap antibody. IgG was used as a control. B, immunoblotting analysis of lysates after immunoprecipitation from HEK293T cells transfected with Myc-RARg and HA-Yap. C, interaction of endogenous RARg and Yap is enhanced by ATRA. Immunoprecipitation was conducted by using anti-RARg antibody in colorectal cancer cells treated with vehicle or 1 mmol/L ATRA. IgG was used as a control. D and E, identification of the Yap-binding sites on RARg. Different RARg mutants as indicated (D) were cotransfected with Yap into HEK293T cells, and then immunoprecipitation analyses were carried out (E). F and G, mapping of regions of Yap binding with RARg: structures of deletion mutants of Yap (F), immunoprecipitation, and immunoblot of cell lysates from HEK293T cells expressing Myc-tagged RARg and deletion mutants of Yap with GFP-tag (G). The experiments were performed at least three times and the representative images of blots are presented.

could significantly potentiate Lats1 interactions with Yap in a dose-dependent manner (Fig. 6A). However, silencing RARg expression in SW480 cells impaired the interaction between Lats1 and Yap (Fig. 6B), indicating the role of RARg as a modulator of the interaction of Lats1 and Yap. We next analyzed the phosphorylation status of Yap. Overexpression of Lats1 significantly induced Yap phosphorylation, which was further enhanced by ectopic expression of RARg in RKO cells (Fig. 6C). Inversely, inhibition of RARg expression in SW480 cells remarkably impaired the phosphorylation of Yap by Lats1 (Fig. 6D). Collectively, these results demonstrated that the inhibitory effect of RARg on Yap activity was likely due to its modulation that RARg promoted Lats1 binding to Yap and phosphorylating Yap.

RARg expression is positively correlated with E-cadherin and negatively correlated with vimentin and CTGF

To further confirm the correlation of RARg expression and the critical molecules downstream of Hippo–Yap signaling, including E-cadherin, vimentin, and CTGF, we examined the expression of these proteins in 91 cases of colorectal cancer tissues using immunohistochemical staining. Our results showed that low expression levels of RARg correlated with low expression levels of E-cadherin but correlated with high expression levels of vimentin and CTGF. Inversely, high expression levels of RARg correlated with both high expression levels of E-cadherin and low expression levels of vimentin and CTGF (Fig. 7A). Spearman rank correlation analysis confirmed the positive correlation between RARg and E-cadherin expression and the negative correlation between RARg and vimentin or CTGF were significant (Fig. 7B). Thus, these observations agreed with the finding that RARg inhibits colorectal cancer tumorigenesis and metastasis through regulation of Hippo–Yap signaling.

Discussion

RARg has well-characterized roles in the regulation of various biologic functions such as osteoclastogenesis, skeletal development, and limb bud formation (47, 48). Recent evidence indicated the critical roles of aberrant expression of RARg in tumorigenesis (8, 10, 13). However, the expression profile and role of RARg in colorectal cancer remain unknown. In the current study, we found that reduced RARg expression in colorectal cancer is associated with clinical tumor size, TNM stage, and distant metastasis. Significantly, colorectal cancer patients with low RARg expression levels have shorter survival time than those with high

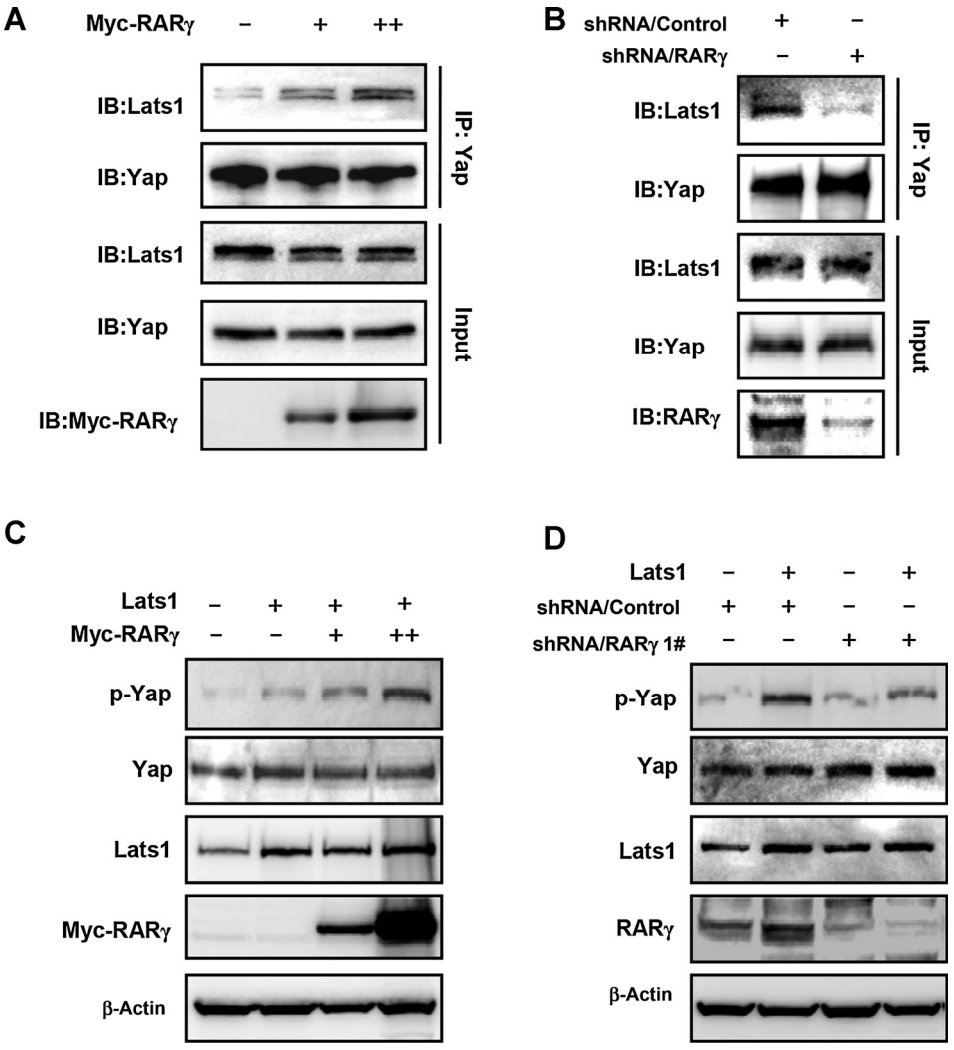

Figure 6.
RARg enhances Lats1 interacting with and phosphorylating Yap. A and B, immunoprecipitation analysis of the interaction of endogenous Lats1 and Yap in RKO cells with increasing amounts of RARg (A) or in RARg shRNA-transduced SW480 cells (B). C and D, Western blotting of phosphorylated Yap (p-Yap) in RKO cells cotransfected with Lats1 and the increasing amounts of RARg (C) or in RARg shRNA-transduced SW480 cells with or without overexpressed Lats1 (D).

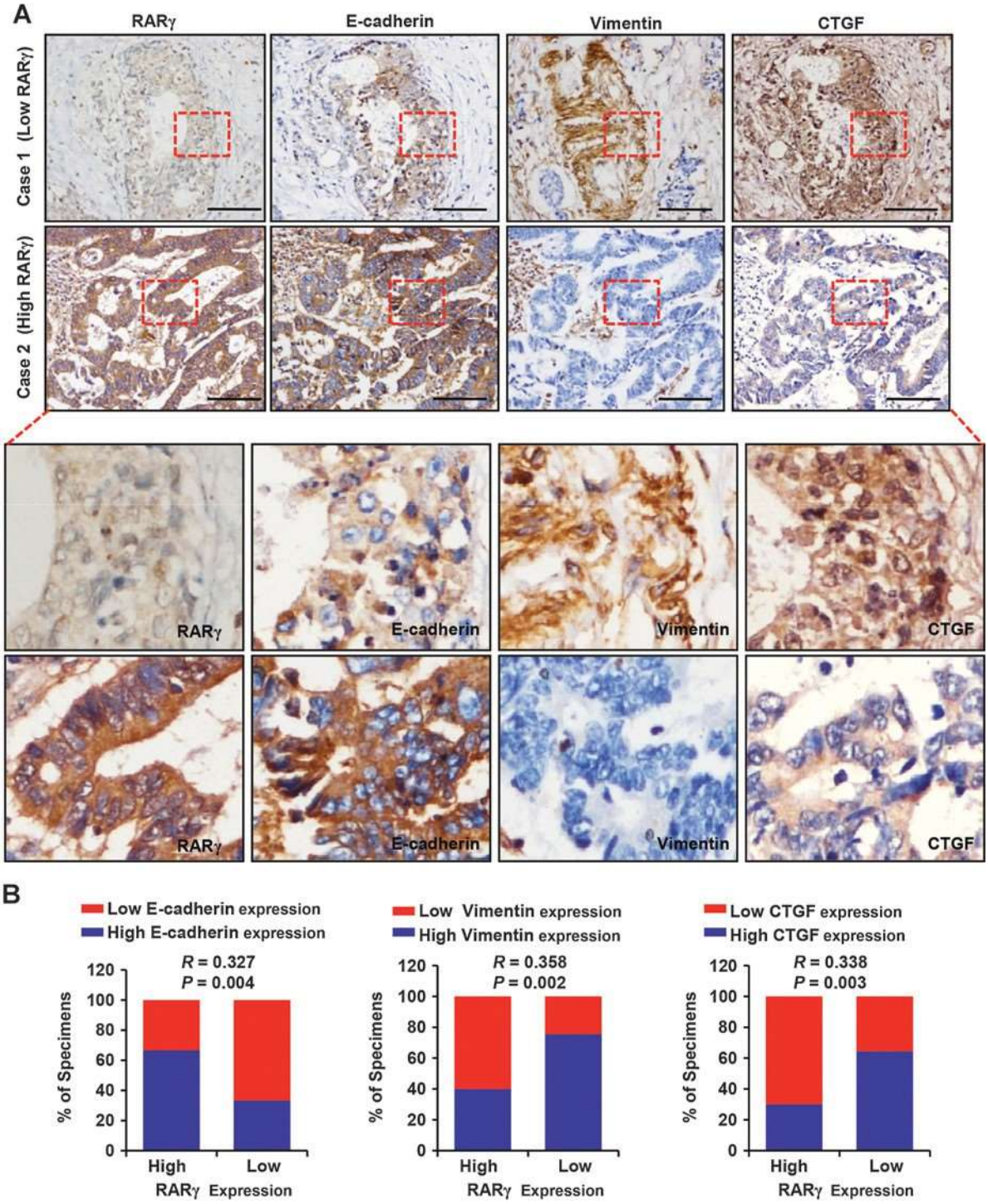

Figure 7.
The expression levels of RARg, E-cadherin, vimentin, and CTGF in clinical colorectal cancer tissues. A, immunohistochemical staining of RARg, E-cadherin, vimentin, and CTGF in human colorectal cancer tissues. Representative images of immunohistochemical staining from the same tumor samples are shown (magnification, x100). B, Spearman correlation analysis between RARg and E-cadherin, RARg and vimentin, RARg and CTGF in 91 cases of colorectal cancer tissues.

RARg expression levels. Thus, these results strongly indicated that RARg has an important role in colorectal cancer development. The results from *in vitro* and *in vivo* studies showed that silencing RARg expression significantly enhanced colorectal cancer cell growth, migration, invasion, and metastasis, whereas ectopic expression of RARg did the opposite, suggesting that RARg functions as a novel tumor suppressor in colorectal cancer. Consistently, in neuroblastoma, RARg is believed to mediate the effect of RA on

antiproliferative activity (11). Ablation of RARg in Ras-transformed keratinocytes induced tumorigenesis through resisting RA-mediated growth arrest and apoptosis (13).

Accumulating evidence has indicated the critical role of dysregulation of the Hippo–Yap pathway in tumorigenesis (14). Components of the Hippo–Yap pathway, such as Mst1/2, Lats1/2 and Yap, are deregulated in various human cancers, and their expression levels and activities correlate with tumor development and progression (14). However, how the Hippo–Yap pathway is regulated in cancer is largely undercharacterized. One of our key findings is that we identified RARg as a novel regulator of the Hippo–Yap pathway in colorectal tumorigenesis and metastasis. RARg functions through its interaction with Yap to suppress colorectal cancer cell growth and metastasis. The interaction between RARg and Yap leads to Yap phosphorylation and cytoplasmic sequestration, which prevents Yap's nuclear translocation and function as a transcriptional coactivator to stimulate the expression of proproliferation and antiapoptosis genes, such as *AREG* and *CTGF*. Disruption of this interaction in RARg-silencing cells resulted in acceleration of colorectal cancer cell growth and metastasis. Our results further revealed that the constitutive interaction between RARg and Yap could be regulated by RARg agonists, emphasizing a significant clinical application regarding the RARg-mediated Hippo–Yap pathway to treat colorectal cancer. Previous studies showed that AMOT p130 interacted with the WW domains of Yap to promote Yap phosphorylation and inhibit Yap transcriptional activity (49). Similar to AMOT p130, our findings suggested that the region (102–263 aa) covering the two WW domains in Yap are indispensable for its interaction with RARg.

The role of RARg in colorectal cancer depends on Yap. Silencing RARg in colorectal cancer cells induced EMT, and enhanced colorectal cancer cell migratory and invasive behaviors, which was reversed by knocking down of Yap. These observations are compatible with the idea that Yap acts as an oncogenic regulator for cancer development. Increased expression and activity of Yap is associated with the growth, metastatic potential, and poor prognosis of several cancer types, including liver cancer and colorectal cancer (19, 24, 50).

Extensive studies have identified several types of signaling and molecules that regulate the Hippo–Yap pathway (30–33). However, whether nuclear receptors are involved in the regulation of the Hippo–Yap pathway remains unclear. Our results established the coupling of nuclear receptors with the Hippo–Yap pathway for the first time. Although the nuclear receptor RARg does not have kinase activity to directly modulate Yap phosphorylation, our results showed that RARg promotes Yap cytoplasmic retention through their physical interactions, which may confer Lats1 more opportunity to bind to and phosphorylate Yap.

The biologic activities of nuclear receptors, including RARg, are tightly controlled not only at the transcriptional levels but also at the posttranslational levels (51–53). Of note, we previously reported that RARg, rather than RARa and RARb, has a unique cytoplasmic localization in many cancer cell types (7). Cytoplasmic accumulation of RARg in HCC cells plays an oncogenic role via nongenomic activation of Akt/NF-kB signaling (10). We here further revealed a surprising nongenomic role of RARg in colorectal cancer, wherein RARg nongenomically regulates the Hippo–Yap pathway via interacting with and sequestering Yap in the cytoplasm. Although RARg is downregulated in human colorectal cancer, elevating its expression in the cytoplasm or alteration of its subcellular localization to a nongenomically targeted Hippo–Yap pathway may be useful for blocking colorectal cancer tumorigenesis and metastasis.

In summary, our *in vitro* and *in vivo* studies indicated that RARg functions as a novel tumor suppressor in colorectal cancer development and progression. We revealed a critical mechanism for RARg in the regulation of colorectal cancer tumorigenesis and metastasis through its nongenomic action to regulate the Hippo–Yap pathway. This may highlight a new entry point for treating colorectal cancer by targeting the RARg/Hippo–Yap signaling axis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.-M. Li, H. Wu
Development of methodology: P.-D. Guo, H. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.-D. Guo, X.-X. Lu, W.-J. Gan, X.-M. Li, X.-S. He, S. Zhang, Q.-H. Ji, F. Zhou, Y. Cao, J.-R. Wang, J.-M. Li, H. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.-D. Guo, W.-J. Gan, X.-M. Li, H. Wu
Writing, review, and/or revision of the manuscript: J.-M. Li, H. Wu
Study supervision: J.-M. Li, H. Wu

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