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Original Article

Geranylgeranylation signaling promotes breast cancer cell mitosis via the YAP-activated transcription of kinetochore/centromere genes

Jing Wei, Song Xia, Aiqin Sun, Yaping Qu, Jinyi Gao, Genbao Shao, Wannian Yang, Qiong Lin

School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu, China

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Abstract: Geranylgeranylation signaling plays an important role in cancer cell proliferation. Our previous studies have shown that the YAP is one of the geranylgeranylation signal transducers in breast cancer cells (Mi W, et al., Oncogene. 2015; 34(24): 3095-3106). However, the downstream effectors that mediate the promoting effect of the geranylgeranylation/YAP signal axis on breast cancer cell proliferation remain elusive. In this report, we investigated the pathway that mediates the effect of the geranylgeranylation on breast cancer cell proliferation. The results have shown that inhibition of geranylgeranyl biosynthesis inactivates transcription of a set of kinetochore/centromere genes. Further biochemical and cell biological studies demonstrated that inhibition of geranylgeranyl biosynthesis significantly reduced the level of key kinetochore/centromere proteins, thus caused a defect in mitosis. Knockdown of YAP caused similar inhibitory effects on the kinetochore/centromere gene expression and mitosis to that of inhibition of geranylgeranyl biosynthesis. Furthermore, we found that *E2F1*, the gene coding for E2F1 that is known to activate expression of cell cycle genes, is a target gene of YAP. Knockdown of E2F1 also reduced expression of the kinetochore/centromere genes, suggesting that the activation effect of YAP on expression of the kinetochore/centromere genes may be mediated by E2F1. Our studies have proposed a novel geranylgeranylation-dependent cancer cell proliferation signaling pathway in which geranylgeranylation signaling promotes cancer cell mitosis via the YAP-activated transcription of kinetochore/centromere genes.

Keywords: Geranylgeranylation, YAP, cell proliferation, mitosis, kinetochore/centromere genes

Introduction

Geranylgeranylation is a lipid modification process for many important cellular signaling proteins, including Rho and Rab family small GTPases and gamma subunits of heterotrimeric GTPases [1, 2]. Geranylgeranyl pyrophosphate, the donor lipid molecule for geranylgeranylation, is synthesized by the mevalonate pathway [3]. Statins, a class of the inhibitors for the key enzyme hydroxylmethylglutaryl coenzyme A (HMG-CoA) reductase in the mevalonate pathway, have been broadly used for inhibition of biosynthesis of geranylgeranyl pyrophosphate and determine the effect of geranylgeranylation in cancer cells [4, 5]. Early studies observed that statins inhibited cell proliferation and caused cellular apoptosis in multiple types of cancer, including leukemia, mesothelioma, breast cancer, colon cancer, pancreatic cancer, prostate cancer, melanoma, and glioma cells [6-13]. Inhibiting biosynthesis of geranylgeranyl, not farnesyl, was identified as the cause for statin-induced apoptosis and inhibition of cancer cell proliferation [4, 14, 15]. These studies indicate that protein geranylgeranylation, not farnesylation, is pivotal for cancer cell survival and proliferation.

It has been extensive interest in targeting the geranylgeranylation signaling for cancer therapy [16-19]. The cellular signaling pathway mediated by geranylgeranylation that controls cancer cell survival and proliferation was not identified until recent studies discovered that the YAP/TAZ signaling [20-22]. The YAP/TAZ signaling is known to promote cancer progression in many types of cancer [23-25]. Our previous studies found that geranylgeranylation is required for nuclear translocation and transcrip-

tional activity of YAP [22]. Further studies observed that YAP is mainly involved in promoting breast cancer cell proliferation while TAZ for breast cancer cell migration and invasion [22]. The YAP/TAZ target gene product CYR61 was found to overexpress in gastric cardia adenocarcinoma (GCA), and the overexpression of CYR61 is inversely associated with the overall survival and positively correlated with metastasis of GCA [26]. Knockdown of CYR61 severely impaired migration and invasion of gastric cancer cells [26]. These studies demonstrate that the YAP/TAZ signaling mediates the geranylgeranylation-dependent cancer progression.

However, it remains elusive how YAP/TAZ mediates the effect of geranylgeranylation on cancer cell proliferation. In this report, we show that YAP, as the down-stream protein of geranylgeranylation signaling, promotes breast cancer cell mitosis by activation of the kinetochore/centromere gene expression. Our studies have revealed a novel mechanism underlying the geranylgeranylation-facilitated breast cancer growth.

Materials and methods

Materials

Geranylgeraniol (G3278) was purchased from SigmaAldrich. Atorvastatin calcium (AT) was from WuXi Sigma. Polybrene, doxycycline (Dox), dimethyl sulfoxide (DMSO), nocodazole, and Hochest33342 were purchased from Sigma-Aldrich. DAPI and ECL reagents were purchased from Beyotime Biotechnology. Anti-TAZ (4883S) and anti-P53 (2527S) were purchased from Cell Signaling; anti-CYR61 (SC-13100), anti-BUB1 (SC-365685), anti-CENPM (SC-398754) and anti-E2F1 (sc-251) from Santa Cruz; anti-actin (RLM3028) from Ruiying Biological; anti-PLK1 from Millipore; anti-YAP (ab52771) and anti-CENPA (ab45694) from Abcam. The shRNA expressing lentiviral vector pLKO-Tet-On and pLKO.1 were purchased from Addgene. The breast cancer cell lines MDA-MB-231 and MDA-MB-453 were purchased from ATCC.

Cell culture and treatment

Breast cancer cell lines MDA-MB-231 and MDA-MB-453 were cultured in DMEM medium (HyClone) supplemented with 10% fetal bovine serum (FBS) (ExCellBio) and 100 units/ml penicillin and streptomycin in 5% CO_2 at 37°C.

For treatment, cells were seeded and incubated for 24 h, and then treated with atorvastatin (AT) (10 μM) or AT plus GGOH (10 μM) for 48 h. In controls, the same amount of solvent (DMSO) was added in parallel with the treatments. For cell cycle synchronization, cells were treated with 0.4 μg/ml of nocodazole for 16 h at 37°C. The cell cycle progression was released by removing nocodazole from the medium at indicated time. All the treated cells were harvested at the same time.

Cell lysate preparation and immunoblotting

After culture medium was removed, cells were washed with cold PBS and lysed in precooled mammalian cell lysis buffer (40 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 25 mM beta-glycerolphosphate, 1 mM Na-orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Cells were lysed in SDS sample buffer and denatured at 100°C for 10 min. The lysates were cleared by centrifugation at 12,000 rpm for 15 min. The lysate proteins were separated by an SDS-PAGE, transferred to PVDF (Millipore) membrane, and probed with kinetochore/centromere protein or other indicated antibodies.

Lentiviral shRNA cloning, production, and infection

Recombinant lentiviral particles containing non-target (luciferase), human YAP, TAZ, E2F1, and P53 targeted shRNA were made using transient transfection of HEK 293T cells (ATCC). Cells were plated at 1×10^6 in a six-well plate and incubated overnight. Then cells were transfected with 1.0 g psPAX2, 0.5 μg PMD2.G (Addgene), and 1.5 μg of shRNA plasmid DNA. All the shRNA oligos were synthesized by ShengGong Company. The shRNA targeting sequences were shown below: YAP#1: GACCAATAGCTCAGATCCTTT; YAP#2: CCCAGTTAAATGTTCACCAAT; TAZ: GCCCTTTCTAACCTGGCTGTA; E2F1#1: ACCTCTTCGACTGTGACTTTG; E2F1#5: CAGGATGGATATGAGATGGGA.

The YAP, TAZ, and E2F1 shRNAs were cloned into the pLKO.1 vector. After 8 h the medium was changed and the medium containing the recombinant lentivirus was harvested at 24,

48, and 72 hrs after transfection. The medium was centrifuged at 1250 rpm for 5 min to remove cell debris. For infection, MDA-MB-231 or MDA-MB-453 cells ($1×10^5$) were plated in a 35 mm tissue culture dish overnight. One ml lentiviral particle solution was used for infecting cells in presence of 6 μg/ml polybrene. The infected cells were selected with 2 μg/ml puromycin. Expression of inducible shRNA vector was induced by addition of Doxycycline (100 ng/mL) for 48 hrs. The effect of knockdown was detected by immunoblotting the cell lysates or detection of the mRNA level using RT-PCR.

GeneChip microarray assay

The Affymetrix microarray assay with GeneChip human Gene 1.0 ST (Affymetrix) was used for determination of the mRNA expression profiles. Treatment of cells with DMSO, atorvastatin or atorvastatin plus geranylgeraniol was carried out for 40 hrs. The mRNA was isolated using Qiagen RNeasy mRNA Purification Kit and the cDNA was synthesized using Qiagen QuantiTect Reverse Transcription Kit.

Cell proliferation assay

The control or the knockdown cells were cultured in DMEM with 10% FBS at 37°C plus 5% CO_2 and seeded in a 12 well culture plate. After cultured at indicated time points, the cells were trypsinized and counted under a phase microscope with a hemocytometer. Only live cells were counted. The counted cell numbers were used for evaluation of the cell proliferation rate. The proliferation assay was repeated at least three times. The histogram was drawn by Prism5.

Live-cell staining and immunofluorescence staining

The cells were cultured and treated as indicated in specific experiments. For live-cell DNA staining, cells were resuspended in the Hoechst staining solution (10 μg/ml) and incubated for 15 minutes in dark at 22°C. Rinse cells twice with PBS and then analyze under fluorescence microscopy.

In immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 20 min. After washing with PBS, the cells were incubated with a primary antibody at 4°C overnight. Cells were washed with PBS 3 times and each time for 5 min, and incubated with a fluorescent dye-conjugated secondary antibody for 2 h at 22°C. The nucleus was stained with DAPI. The fluorescent staining was visualized under a confocal microscope (GE Healthcare).

Preparation of RNA and RT-PCR

Cells were lysed and total RNA was extracted using the total RNA extraction Kit (ShengGong Company). cDNA was generated by cDNA Synthesis Kit (ABM), quantitative real-time PCR was performed using the EvaGreen qPCR MasterMix (ABM) and CFX96 real-time PCR system (Bio-Rad). PCR reaction system was performed using 2× PCR Taq plus MasterMix (ABM). The amplification products were analyzed by agarose gel electrophoresis. The primers for PCR are as follows: YAP1 forward primer: 5'-ATGGATCCCGGGCAGCAGC-3'; YAP1 reverse primer: 5'-AGCTCCCAACTGCAGAGAAG-3'; TAZ forward primer: 5'-ATCACCACATGGCAAGACCC-3'; TAZ reverse primer: 5'-TCTGGATTCTCTGAAGCCGC-3'; BUB1 forward primer: 5'-GCTGCACAACTTGCGTCTAC-3'; BUB1 reverse primer: 5'-TGGAGCCCAGCAATAGCATC-3'; PLK1 forward primer: 5'-GGTTTTCGATTGCTCCCAGC-3'; PKL1 reverse primer: 5'-TCATTGAAGAGCACCCCCAC-3'; ZWINT forward primer: 5'-AGGAGGACACTGCTAAGGGT-3'; ZWINT reverse primer: 5'-TTCTGGACTGCTCTGCGTTT-3'; AURK1 forward primer: 5'-AATACAGTCCCACCTTCGGC-3'; AURK1 reverse primer: 5'-GGAGCATGTACTGACCACCC-3'; CENPA forward primer: 5'-TGCGATGCTGTCTGGACTTT-3'; CENPA reverse primer: 5'-AGCCTTTTCTCCCATACCACA-3'; CENPF forward primer: 5'-ACCCAGGAGTTACAGCAAGC-3'; CENPF reverse primer: 5'-GGCAGACTTCTCTGGCCTTT-3'; CENPM forward primer: 5'-GTGGAAGGCTTTAGGGCCAC-3'; CENPM reverse primer: 5'-AAGCCCTGACTGGACATCCT-3'; E2F1 forward primer: 5'-AAGAGCAAACAAGGCCCGAT-3'; E2F1 reverse primer: 5'-TGGGGAAAGGCTGATGAACTC-3'; GAPDH forward primer: 5'-AACGGATTTGGTCGTATTG-3'; GAPDH reverse primer: 5'-GGAAGATGGTGATGGGGAT-3'.

Statistical analysis of experimental data

The Student *t*-test was used in statistical analysis of experimental data for pair comparison.

Table 1. The top 32 kinetochore/centromere genes down-regulated upon inhibition of geranylgeranyl biosynthesis in MDA-MB-231 cells

Gene	Gene description	Down-regulated by AT (fold of control)	Rescued by GGOH (fold of control)
SPC25	SPC25, NDC80 kinetochore complex component	0.05	1.19
KIFC1	kinesin family member C1	0.06	0.92
CENPM	centromere protein M	0.06	0.99
SGOL1	shugoshin-like 1	0.06	0.97
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	0.07	1.26
PLK1	polo-like kinase 1	0.07	1.02
KIF2C	kinesin family member 2C	0.07	1.19
BIRC5	baculoviral IAP repeat-containing 5	0.07	0.99
NDC80	NDC80 homolog, kinetochore complex component	0.07	1.56
AURKB	aurora kinase B	0.08	1.04
BUB1	budding uninhibited by benzimidazoles 1 homolog	0.08	1.19
NUF2	NUF2, NDC80 kinetochore complex component	0.08	1.34
KIF4A	kinesin family member 4A	0.08	1.49
CENPA	centromere protein A	0.09	1.11
KIF18B	kinesin family member 18B	0.09	1.15
CENPF	centromere protein F, 350/400 ka (mitosin)	0.10	1.33
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta	0.11	1.42
KIF15	kinesin family member 15	0.11	1.21
ZWINT	ZW10 interactor	0.11	1.06
KIF23	kinesin family member 23	0.12	1.20
AURKA	aurora kinase A	0.12	0.78
CENPI	Centromere protein I	0.13	1.66
KIF11	Kinesin family member 11	0.14	1.43
MAD2L1	MAD2 mitotic arrest deficient-like 1	0.14	0.69
PLK4	polo-like kinase 4	0.14	0.75
KIF20B	Kinesin family member 20B	0.14	1.77
SPC24	SPC24, NDC80 kinetochore complex component	0.15	0.80
CENPE	centromere protein E, 312 kDa	0.16	0.71
KIF18A	Kinesin family member 18A	0.18	1.62
SGOL2	shugoshin-like 2	0.18	0.83
SKA3	spindle and kinetochore associated complex subunit 3	0.18	1.28
SKA1	spindle and kinetochore associated complex subunit 1	0.19	1.60

The $P<0.05$ is considered as statistically significant.

Results

Inhibition of geranylgeranyl biosynthesis down-regulates transcription of kinetochore/centromere genes

Our previous studies have shown that inhibition of biosynthesis of geranylgeranyl by the HMG-CoA reductase inhibitor atorvastatin or geranylgeranylation by the geranylgeranyltransferase I (GGTase I) inhibitor GGTI-293 impaired proliferation and migration of breast cancer cells [22]. To search for the signaling candidates that may mediate the geranylgeranylation effect on the cell proliferation, we performed the GeneChip microarray assay in MDA-MB-231 cells upon treatment with atorvastatin or atorvastatin with GGOH to determine the geranylgeranyl biosynthesis-dependent and the proliferation-associated gene expression profiles. As shown in **Table 1**, expression of a cluster of the kinetochore/centromere genes, including both the assembly and the regulation genes that are pivotal in mitosis and cytokinesis during cell cycle progression,

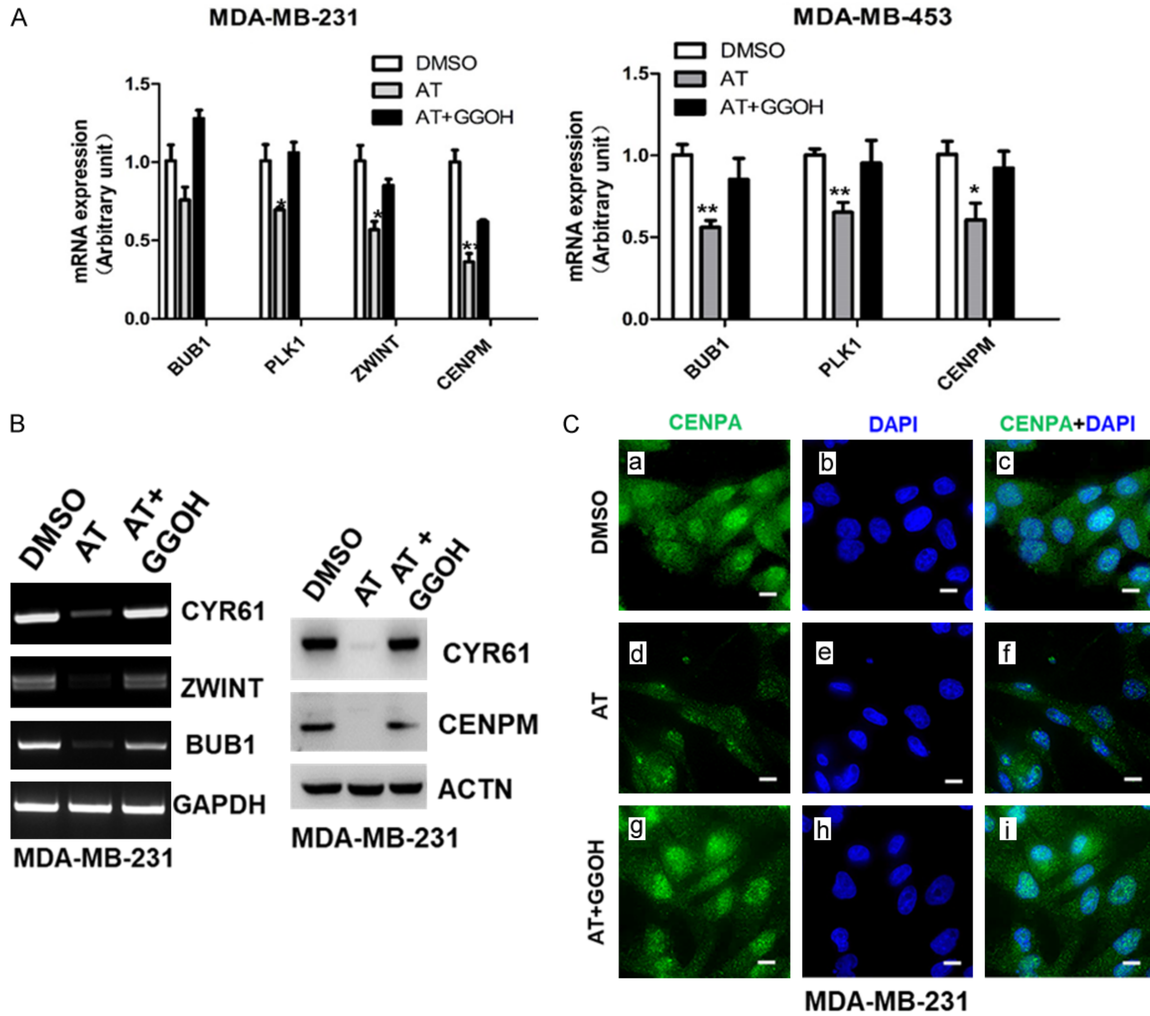

Figure 1. Inhibition of geranylgeranyl biosynthesis down-regulates expression of the kinetochore/centromere-related proteins in breast cancer cells. A. The quantitative RT-PCR (qRT-PCR) assay of expression of the mRNA of kinetochore/centromere-related protein in the breast cancer MDA-MB-231 and MDA-MB-453 cells upon treatment with the solvent (DMSO), 10 μM atorvastatin (AT), and 10 μM atorvastatin plus 10 μM geranylgeraniol (AT+GGOH) for 48 hours. Data are from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$. B. The conventional RT-PCR assay of expression of the YAP/TAZ target gene *CYR61* and the kinetochore/centromere regulatory genes *ZWINT* and *BUB1* (the left panel). The Western blot assay of CYR61 and CENPM upon the treatments (the right panel). C. The immunofluorescent staining assay of the level and nuclear localization of the kinetochore/centromere protein CENPA upon the treatments. Bar, 10 μm.

was dramatically reduced upon treatment with atorvastatin, and the reduction was rescued by addition of geranylgeraniol. The results suggest that geranylgeranylation signaling may promote breast cancer cell proliferation by activation of transcription of the kinetochore/centromere genes.

To confirm that the geranylgeranylation signaling regulates transcription of the kinetochore/centromere genes, we examined the mRNA level of the known important kinetochore/centromere genes, such as *BUB1*, *PLK1*, *CENPM*, and *ZWINT*, in two breast cancer cell lines MDA-MB-231 and MDA-MB-453 by the quantitative RT-PCR (qRT-PCR) or the conventional PCR assay upon treatment with atorvastatin or atorvastatin plus geranylgeraniol. As shown in **Figure 1A**, the mRNA level of the kinetochore/centromere genes was significantly reduced by atorvastatin treatment, and addition of geranylgeraniol either partially or fully rescued the inhibitory effect of atorvastatin in both breast cancer cell lines as determined by the qRT-PCR assay. The conventional PCR assay in MDA-MB-231 cells also confirmed that the

atorvastatin treatment reduced more than 90% of the mRNA level of *ZWINT* and *BUB1*, the two genes whose products are crucial for kinetochore/centromere assembly [27, 28], and addition of geranylgeraniol fully rescued the inhibitory effect of atorvastatin (the left panel, **Figure 1B**). These data indicate that geranylgeranylation signaling is activating transcription of the kinetochore/centromere genes.

We also examined the level of the kinetochore/centromere structural proteins CENPM and CENPA with immunoblotting and immunofluorescent staining respectively. As shown in the **Figure 1B** (the right panel) and **1C**, treatment with atorvastatin dramatically reduced the protein amount of both CENPM and CENPA, while addition of geranylgeraniol recovered the protein level. These results indicate that geranylgeranylation signaling is important for the kinetochore/centromere assembly.

Inhibition of geranylgeranyl biosynthesis severely impairs mitotic progression

Because inhibition of the geranylgeranyl biosynthesis by atorvastatin inactivates transcription of the kinetochore/centromere genes and reduces the protein level of kinetochore/centromere proteins, we hypothesize that geranylgeranylation signaling promotes breast cancer cell proliferation by facilitating the mitotic progression. Thus, we treated breast cancer MDA-MB-231 cells with atorvastatin or atorvastatin plus geranylgeraniol, synchronized the cells at the mitotic phase by treatment with nocodazole, stained the nuclei with DAPI, and counted the M-phase cells that contain chromosomes (**Figure 2A**). As expected, treatment with atorvastatin significantly reduced the mitotic phase cells, while addition of geranylgeraniol rescued the reduction effect (**Figure 2A**). To further confirm the effect of geranylgeranylation signaling on mitotic progression, we collected the nocodazole-synchronized round-shaped suspension cells as shown in **Figure 2A**, and re-plated the cells without nocodazole (**Figure 2B**). If mitosis is not impaired, the cells will move forward to the G1 phase in the nocodazole-free medium and spread on the plate. As expected, most of the cells treated with atorvastatin were unable to pass the M-phase, and went into apoptosis, while addition of geranylgeraniol along with atorvastatin recovered the ability of the cells to progress in mitosis (**Figure 2B**). These results indicate that geranylgeranylation signaling plays a key role in breast cancer cell mitosis, which is consistent with its role in activation of expression of kinetochore/centromere genes as shown in **Figure 1**.

The effect of geranylgeranylation signaling on promoting transcription of the kinetochore/centromere genes is mediated by YAP

Our previous studies have found that inhibition of geranylgeranylation blocked the YAP nuclear translocation and diminishes its transcriptional activity [22], Knockdown of YAP and TAZ impairs the geranylgeranylation-promoted breast cancer cell proliferation and migration [22]. We here observed that atorvastatin treatment significantly reduced transcription of the known YAP/TAZ target gene *CYR61* and its protein product along with the kinetochore/centromere genes, and addition of geranylgeraniol rescued the reduction effect (**Figure 1B**). Therefore, we examined if the YAP/TAZ axis is the downstream effector mediating geranylgeranylation signaling on transcription of the kinetochore/centromere genes by the YAP/TAZ shRNA knockdown assay in the breast cancer MDA-MB-231 cells. As shown in **Figure 3A**, knockdown of YAP with two YAP shRNAs shYAP#1 and shYAP#2 significantly reduced transcription of the kinetochore/centromere genes. Consistently, the knockdown significantly reduced the kinetochore/centromere protein level detected either by Western blot or immunofluorescent staining (**Figure 3B** and **3C**). To our surprise, knockdown of TAZ, a similar transcriptional co-activator to YAP, did not cause any significant change in both transcription and the protein product level of the kinetochore/centromere genes (**Figure 3D**). The data suggest that YAP is the specific downstream effector of the geranylgeranylation signaling for activation of the kinetochore/centromere gene expression.

Knockdown of YAP impaired the mitosis of breast cancer cells

Next, we examined if depletion of YAP by shRNA knockdown in the breast cancer cells produces a similar effect of atorvastatin on cell proliferation and mitosis. As shown in **Figure 4A**, knockdown of YAP in MDA-MB-231 cells significantly inhibited cell proliferation. To assess the effect on mitosis, we used nocodazole

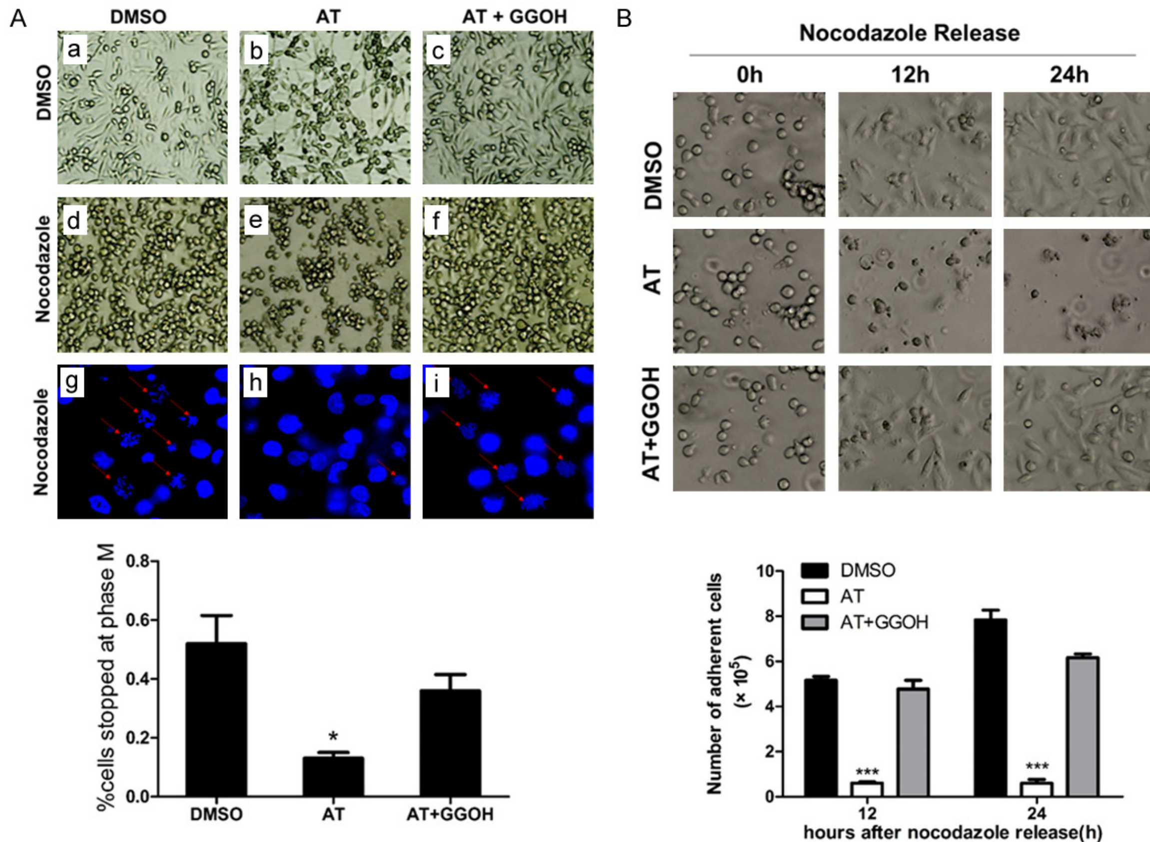

Figure 2. Inhibition of geranylgeranyl biosynthesis impairs mitosis of breast cancer MDA-MB-231 cells. A. MDA-MB-231 cells treated with the solvent (DMSO), 10 μM atorvastatin (AT), 10 μM atorvastatin plus 10 μM geranylgeraniol (AT+GGOH) for 48 hours, followed by nocodazole (1 μg/ml) or DMSO for 12 h. The cells were fixed, stained with DAPI and visualized under a fluorescent microscope. The red arrows indicate the chromosome-containing mitotic cells. Quantification of the mitotic cells upon the treatments for 48 hours was from three independent experiments. $^{*}P<0.05$. B. Cells arrested at the mitotic phase by nocodazole were released by removing nocodazole from the medium. Same numbers of the arrested cells from each of the treatments were collected and cultured in the nocodazole-free medium for 12 hours and 24 hours. Quantification of the adherent cells upon the nocodazole release for 12 hours and 24 hours was from three independent experiments. $^{***}P<0.001$.

to synchronize the cells at the mitotic phase, then observed and counted the round suspension cells (mitotic cells). As shown in **Figure 4B**, knockdown of YAP remarkably reduced the number of round suspension cells, indicating that knockdown of YAP severely impaired mitosis. To confirm the effect of YAP knockdown on mitosis, we collected an equal amount of the nocodazole treatment-induced round suspension cells shown in **Figure 4B** and re-plated the cells in the nocodazole-free medium to allow the cells proceed in mitosis for evaluation of ability of the cells in mitotic progression. As shown in **Figure 4C**, knockdown of YAP severely impaired ability of the cells in mitotic progression. These results suggest that YAP is the mediator for geranylgeranylation signaling in activation of the kinetochore/centromere gene transcription and promotion of mitosis.

The YAP-activated transcription of the kinetochore/centromere genes is mediated by E2F1

From our gene expression profile studies, we noticed that treatment with atorvastatin dramatically reduced the mRNA level of *E2F1*, a transcriptional factor gene, and addition of geranylgeraniol rescued the reduction. We speculate that *E2F1* may be a target gene of YAP mediating the effect of YAP on the kinetochore/centromere gene transcription. To test this hypothesis, we examined the effect of atorvastatin or YAP knockdown on the mRNA level of *E2F1* by the qRT-PCR assay. As shown in **Figure 5A**, treatment with atorvastatin in breast cancer MDA-MB-231 cells significantly reduced the mRNA level of *E2F1*. Consistent with the atorvastatin treatment, the YAP knockdown dramatically reduced the mRNA level

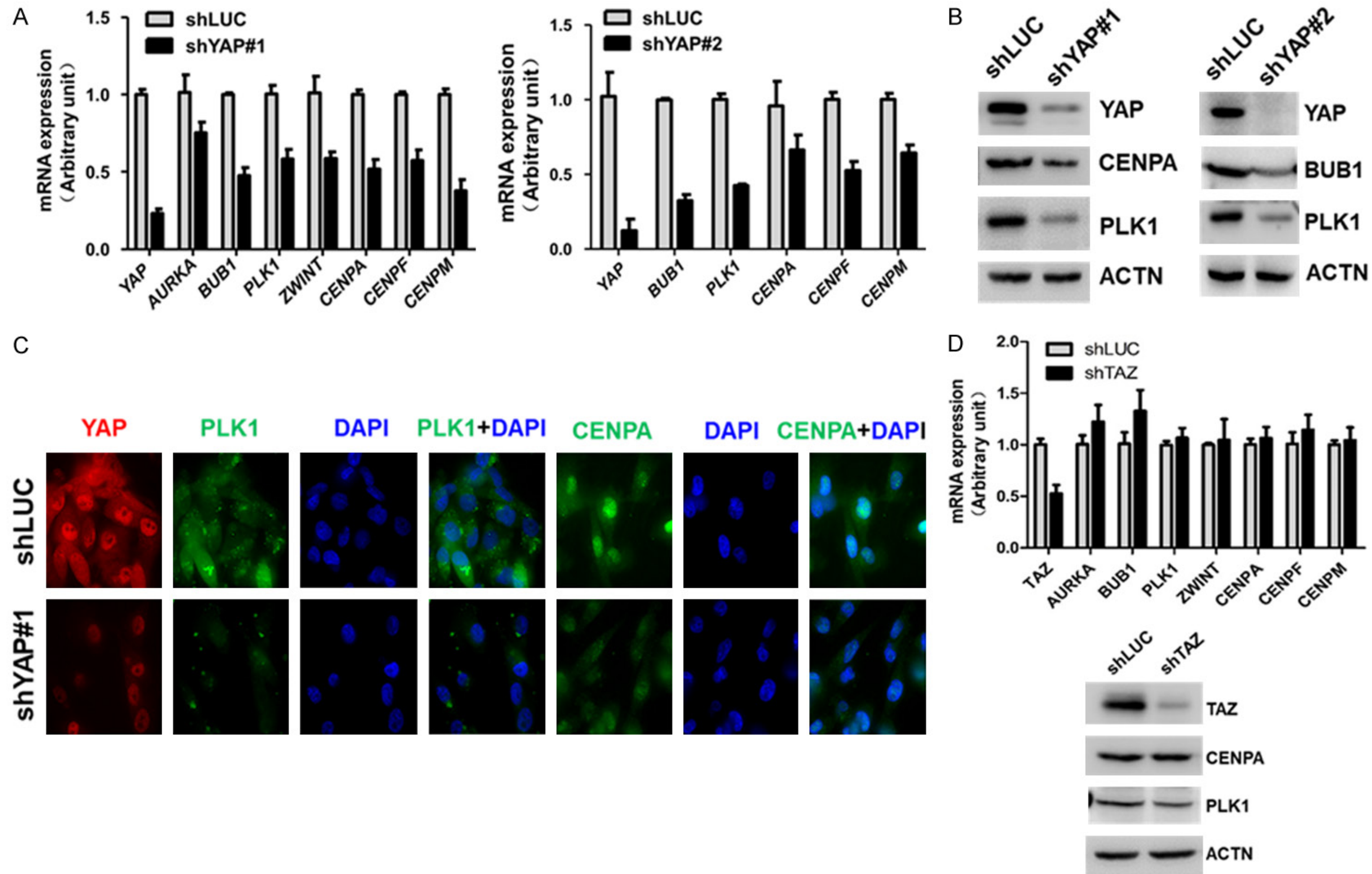

Figure 3. Knockdown of YAP in breast cancer MDA-MB-231 cells down-regulates expression of the kinetochore/centromere-related genes. A. The quantitative RT-PCR (qRT-PCR) assay of expression of the mRNA of kinetochore/centromere-related genes upon knockdown of *YAP* by two shYAPs. B. The Western blot assay of the level of kinetochore/centromere-related proteins upon knockdown of *YAP*. C. The immunofluorescent staining assay of the kinetochore/centromere-proteins in MDA-MB-231 cells. Bar, 10 μm. D. Knockdown of TAZ by shTAZ had no effect on the expression of the kinetochore/centromere-related genes in breast cancer MDA-MB-231 cells detected by the qRT-PCR and Western blot assays.

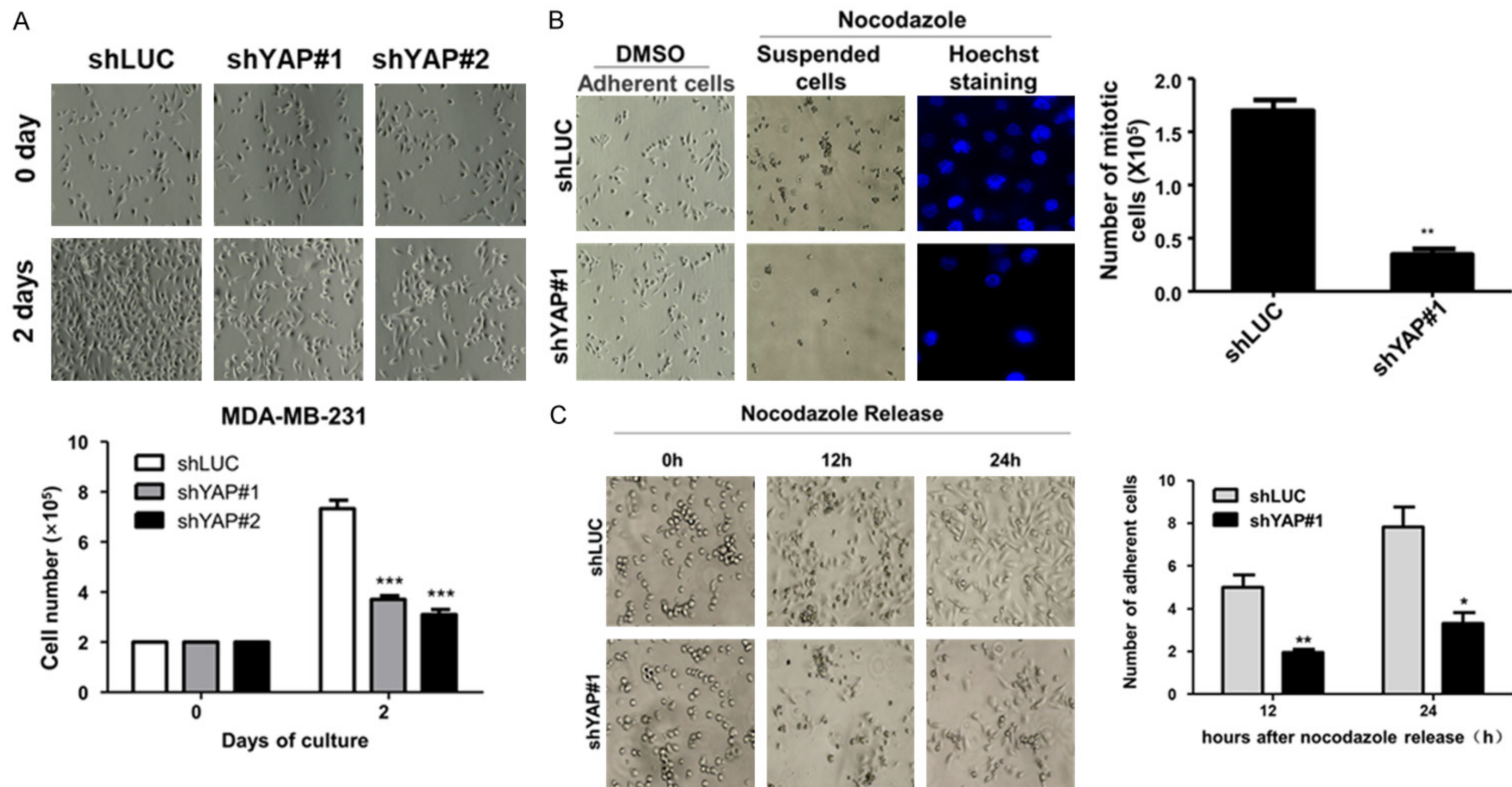

Figure 4. Knockdown of YAP inhibits proliferation and mitosis of breast cancer MDA-MB-231 cells. A. The effect of YAP knockdown on cell proliferation. The top panel, the microscopic images of cells upon YAP knockdown. The bottom panel, quantification of cell proliferation upon knockdown of YAP by from three independent experiments. $^{***}P<0.001$. B. The effect of YAP knockdown on mitosis. Cells were treated with nocodazole (1 μg/ml) for 12 hours. The phase microscopic images of cells and the Hoechst-stained fluorescence images of DNA were taken from both the control and the YAP knockdown samples (the left panel). The suspension cells upon the treatments with nocodazole for 12 hours in both the control and the YAP knockdown samples were quantified from three independent experiments. $^{**}P<0.01$. C. The nocodazole-arrested cells were released for 12 and 24 hours by culturing in the nocodazole-free medium. Equal numbers of the nocodazole-arrested suspension cells from both the control and the YAP1 knockdown samples were collected and cultured. The left panel, the phase microscopic images of the cells; the right panel, the adherent cells upon culture in the nocodazole-free medium for 12 and 24 hours were quantified from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$.

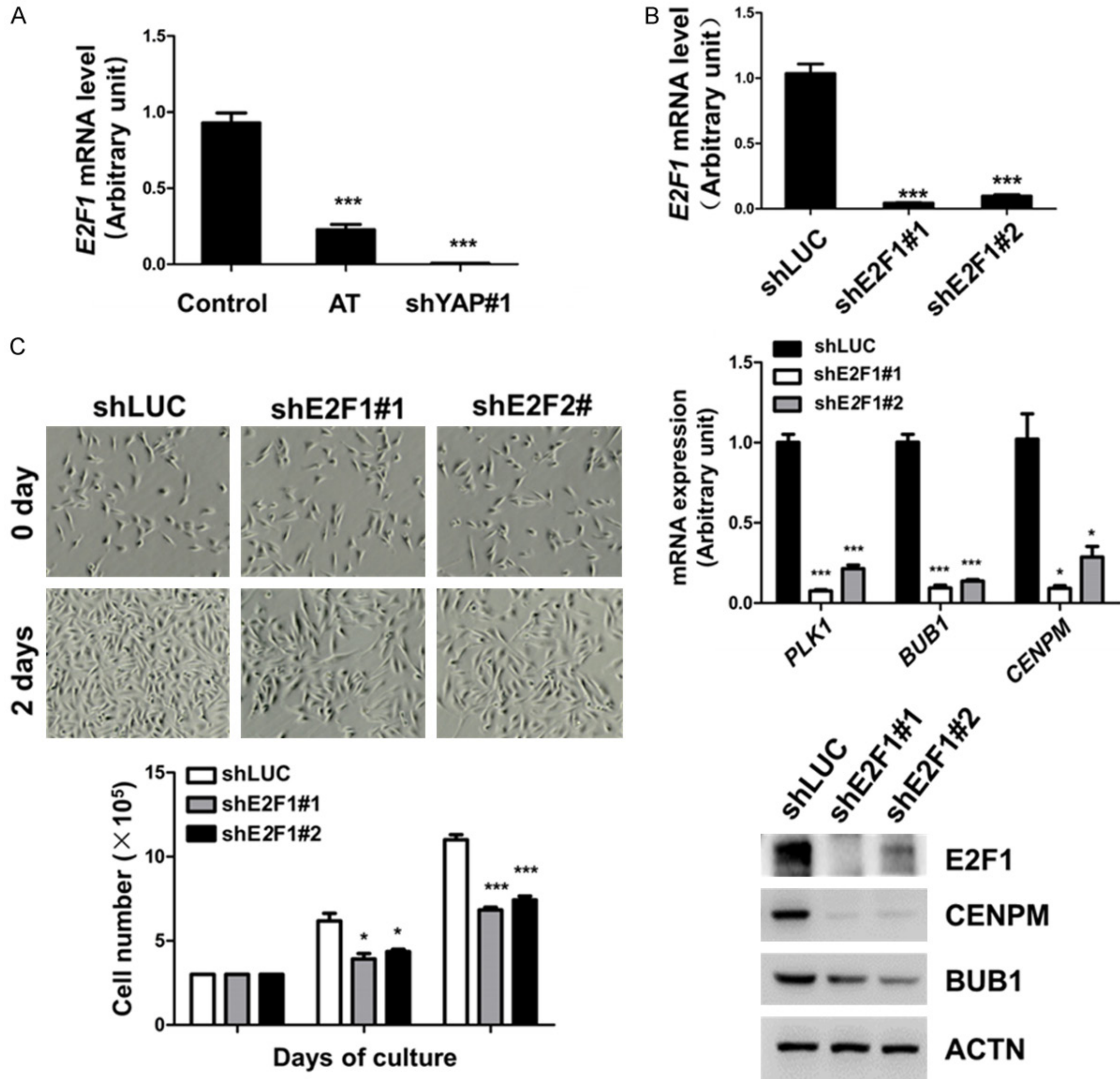

Figure 5. E2F1 mediates the YAP-activated transcription of kinetochore/centromere genes. A. Inhibition of the mevalonate pathway by atorvastatin or knockdown of YAP dramatically down-regulated expression of *E2F1* in MDA-MB-231 cells. $^{***}P<0.001$. B. Knockdown of E2F1 by shE2F1s remarkably reduced the mRNA level of the kinetochore/centromere genes PLK1, BUB1, and CENPM, and the protein amount of CENPM and BUB1 in MDA-MB-231 cells. $^{***}P<0.001$. C. Knockdown of E2F1 inhibits proliferation of MDA-MB-231 cells. The top panel, the phase microscopic images of the cells upon knockdown of E2F1; the bottom panel, quantification of the effect on proliferation of the cells upon knockdown of E2F1. $^{*}P<0.05$; $^{***}P<0.001$. All the data in the quantification analysis were from three independent experiments.

of *E2F1*. We further detected the effect of knockdown of *E2F1* by the shRNAs on the kinetochore/centromere gene expression and the protein product level. As expected, knockdown of *E2F1* by either of the two E2F1 shRNAs remarkably reduced the mRNA level of kinetochore/centromere genes *PLK1*, *BUB1*, and *CENPM* and the amount of the kinetochore/centromere proteins BUB1 and CENPM (**Figure 5B**). Furthermore, knockdown of E2F1, similar to knockdown of YAP, dramatically inhibited proliferation of MDA-MB-231 cells (**Figure 5C**). All these data suggest that *E2F1* is the target gene of YAP and mediates the effect of the geranylgeranylation/YAP signaling on the kinetochore/centromere gene transcription and cell proliferation.

Discussion

Geranylgeranylation has been found to play a key role in cancer cell survival and prolifera-

tion for more than two decades [29]. Small GTPases including Rho and Rab GTPase families and some of the gamma subunits of the trimeric GTPases are known as the direct downstream signaling effectors of geranylgeranylation [1, 2]. Recent studies have found that the YAP/TAZ pathway mediates the geranylgeranylation signaling-dependent breast cancer cell proliferation through the Rho GTPase and beta/gamma subunits of the heterotrimeric GTPases [22]. However, how the YAP/TAZ pathway promotes the geranylgeranylation-dependent cancer cell proliferation has been puzzled for years. In this report, we discovered the YAP/E2F1 signaling axis that mediates the geranylgeranylation-dependent breast cancer cell proliferation. Our data have demonstrated for the first time that geranylgeranylation signaling promotes breast cancer cell proliferation through facilitating mitosis via the YAP-activated kinetochore/centromere gene transcription. The findings in our studies have provided a new vision of the role of geranylgeranylation in promoting tumorigenesis.

One of the interesting findings in our studies is that YAP, but not TAZ, mediates the effect of geranylgeranylation on the kinetochore/centromere gene transcription (**Figure 3**). YAP and TAZ seem to have no difference as the downstream effectors of the Hippo kinase signaling shown by many studies. However, our previous studies found that YAP and TAZ have a very differential role in the effect of geranylgeranylation on cell proliferation and migration in breast or gastric cancer cells [22, 30]. For example, YAP seems to favorably mediate the effect of geranylgeranylation on cell proliferation, while TAZ tends to mediate the effect of geranylgeranylation on cell migration. The regulatory mechanism and the upstream signals for this differential effect of YAP and TAZ currently are unknown. We speculate that YAP and TAZ may interact with additional specific interactive partners besides TEAD in the TEAD-cored transcriptional complex, thus activate differential sets of gene transcription and cause differential cellular effects. Further investigation is needed to clarify the mechanism by which how geranylgeranylation signaling confers the activation specificity between YAP and TAZ.

Our studies suggest that the kinetochore/centromere genes may not be the direct target genes of YAP. It seems that YAP activates the transcription of the *E2F1* gene, and E2F1 transcribes the kinetochore/centromere genes (**Figure 5**). Thus, proliferation signals of geranylgeranylation in breast cancer cells are transduced by the YAP/E2F1 transcriptional axis. E2F1 is a known transcriptional factor involved in regulation of cell cycle progression, including the G1/S transition and mitosis [31, 32]. E2F1 has been identified as an important driver for aneuploidy in breast tumors, particularly in the TP53 mutant-containing breast tumors [33]. However, how E2F1 facilitates aneuploidy in breast tumors remains elusive. The YAP/E2F1 transcriptional cascade shown in our studies for activation of kinetochore/centromere gene expression not only provides a novel geranylgeranylation signaling-activated proliferation pathway but also suggests a possible mechanism underlying the E2F1-facilitated aneuploidy in breast tumors.

Although we found that YAP mediates the geranylgeranylation-promoted breast cancer cell proliferation through facilitating mitosis, the mechanism by which geranylgeranylation transduces signals to YAP remains elusive. Our previous studies have shown that geranylgeranylation of both Rho A and beta/gamma subunits might account for geranylgeranylation signaling that activates the YAP/TAZ pathway [22]. We also demonstrated that the Hippo pathway is activated when geranylgeranylation is inhibited [22]. There are two possible signaling pathways connecting the geranylgeranylation to the Hippo kinase cascade: one is the Rho A/ROCK/PP1A axis that dephosphorylates YAP/TAZ and facilitates the nuclear localization of YAP/TAZ [34, 35], the other one is the Gβγ/RAP1A/RASSF1A axis that disrupts the dimerization of MST1/2 [36, 37]. However, more research needs to be done to define these two possible pathways connecting geranylgeranylation signaling to the Hippo kinase cascade.

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Disclosure of conflict of interest

None.

Abbreviations

AT, atorvastatin; BUB1, budding uninhibited by benzimidazoles 1 homolog; CENP, centromere protein; GGOH, geranylgeraniol; HMG-CoA, hydroxylmethylglutaryl-coenzyme A; PLK, polo-like kinase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TAZ, transcriptional coactivator with PDZ-binding motif; TEAD, transcriptional enhanced associate domain protein; YAP, YES-associated protein; ZWINT, ZW10-interactor.

Address correspondence to: Qiong Lin, School of Medicine, Jiangsu University, No. 301, Xuefu Road, Zhenjiang 212013, Jiangsu, China. Tel: +86-182-61972370; E-mail: humandock11@gmail.com

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