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Original Article

AKAP95 promotes cell cycle progression via interactions with cyclin E and low molecular weight cyclin E

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Abstract: AKAP95 in lung cancer tissues showed higher expression than in paracancerous tissues. AKAP95 can bind with cyclin D and cyclin E during G1/S cell cycle transition, but its molecular mechanisms remain unclear. To identify the mechanism of AKAP95 in cell cycle progression, we performed AKAP95 transfection and silencing in A549 cells, examined AKAP95, cyclin E1 and cyclin E2 expression, and the interactions of AKAP95 with cyclins E1 and E2. Results showed that over-expression of AKAP95 promoted cell growth and AKAP95 bound cyclin E1 and E2, low molecular weight cyclin E1 (LWM-E1) and LWM-E2. Additionally AKAP95 bound cyclin E1 and LMW-E2 in the nucleus during G1/S transition, bound LMW-E1 during G1, S and G2/M, and bound cyclin E2 mainly on the nuclear membrane during interphase. Cyclin E2 and LMW-E2 were also detected. AKAP95 over-expression increased cyclin E1 and LMW-E2 expression but decreased cyclin E2 levels. Unlike cyclin E1 and LMW-E2 that were nuclear located during the G1, S and G1/S phases, cyclin E2 and LMW-E1 were expressed in all cell cycle phases, with cyclin E2 present in the cytoplasm and nuclear membrane, with traces in the nucleus. LMW-E1 was present in both the cytoplasm and nucleus. The 20 kDa form of LMW-E1 showed only cytoplasmic expression, while the 40 kDa form was nuclear expressed. The expression of AKAP95, cyclin E1, LMW-E1 and -E2, might be regulated by cAMP. We conclude that AKAP95 might promote cell cycle progression by interacting with cyclin E1 and LMW-E2. LMW-E2, but not cyclin E2, might be involved in G1/S transition. The binding of AKAP95 and LMW-E1 was found throughout cell cycle.

Keywords: AKAP95, cyclin E, low molecular weight cyclin E, cell cycle, proliferation

Introduction

AKAP95, which is an AKAP (A-kinase anchoring protein) family member localizing in the nucleus [1], is a PKA anchoring protein that participates in chromatin recruitment and condensation during the cell cycle [2], and its binding to chromatin and function in chromosome condensation does not require the anchoring nor the activating functions of PKA [2]. AKAP95 can form various complexes with different proteins to regulate cell signaling. Our previous studies showed that the expression of AKA95 in lung cancer tissues was significantly higher than that found in paracancerous tissues. Moreover, AKA95 expression was associated with differential degree and histological types of lung cancer [3, 4].

The E-type cyclins, including subtype E1 and E2, play a key role in G1/S transition [5, 6]. Cyclin E can be digested by trypsin-like proteases into various LMW-E [7], and over-expression of cyclin E in many serious tumor cells indicated a pro-tumor functional property of cyclin E [8-10]. In addition, cyclin E2 was previously shown to display high expression levels in lung cancer cells [11]. Cyclin E1 and E2 employ different mechanisms to induce chromosomal instability [12], and their degradation patterns were also different [13].

Further, in CHO cells, AKAP95 interacted with cyclin D and E during the G1/S phase of the cell cycle, and CDK2 inhibited the binding of AKAP95 to cyclin E [14]. It was also found that CDK4 competes with AKAP95 in binding to cyclin D [15].

In this study, we have explored the potential mechanism of AKAP95 in tumor promotion, and analyzed the interaction between AKAP95 and cyclin E1, cyclin E2, LMW-E1 and LMW-E2 in different phases of the cell cycle. These studies have provided evidence for the existence of a novel subtype of cyclin E2.

Materials and methods

Antibodies and agents

Mouse anti cyclin E1 (HE12), cyclin E2 (A-9), AKAP95 (22-Z), CDK2 (D-12) and CDK4 (DCS-35) antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA); rabbit anti-cyclin E1 (ab33911), cyclin E2 (ab40890), AKAP8 (ab140628) and β-tubulin (ab52901) antibodies were purchased from Abcam Company (Cambridge, UK); Lamin B1 (L75) was purchased from Bioworld Technology (Nanjing, Jiangsu, China); RB1 (Phospho-Ser795, AB55-056) were purchased from Sangon Biotech (Shanghai, China); FITC and TRITC was purchased from MultiSciences Biotech (Hangzhou, Zhejiang, China); Protein A/G Plus-Agarose (sc-2003) was from Santa Cruz (Dallas, Texas, USA), L-Mimosine (0253), Aphidicolin (A0781), Nocodazole (M1404), Colchicine (C9754), H89 (B1427) and Forskolin (F6886) were from Sigma (Santa Clara, CA, USA); Cell lysis buffer for Western blot and IP (P0013) were purchased from Beyotime Institute of Biotechnology (Haimen, China); Nuclear-Cytosol Extraction Kit (P1200) was from Applygen (Beijing, China); proximity ligation assay technology kit (DU-092101) was from Sigma (Santa Clara, CA, USA). Restore™ Western Blot Stripping Buffer (21059) was purchased from Thermo Scientific (Rockford, IL, USA).

Plasmid construction

The pcDNA3.1 plasmid was used to construct the pcDNA3.1-AKAP95 over-expression plasmid (NCBI, NM_005858.3); pcDNA™ 6.2-GW/EmGFP-miR-RNAi-AKAP95 was used to silence the 1587-1608 fragment of the AKAP8 gene (NCBI, NM_005858.3); the pTT5 plasmid was used to construct the pTT5-cyclin E2 over expression plasmid (NCBI, NM_057749.2); and pSuper-shRNA-cyclin E2 was used to silence the 753-776 fragment of the cyclin E2 gene (NCBI, NM_057749.2).

Inoculation of cells immobilized on glass slides

The lung cancer cell-line A549 and the gastric adenocarcinoma cell-line BGC823 were routinely cultured until they reached 60% confluence. Then, the medium was discarded, cells were washed with PBS three times, fixed in 100% ethanol for 15 min, washed three times in PBS and further used for immunofluorescence and proximity ligation assays.

Separation of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins were extracted according to the instructions provided with the Nuclear-Cytosol Extraction Kit. Briefly, cells were collected, then treated with CEB-A solution, mixed and vortexed, and then treated with the CEB-B solution, mixed and vortexed again, followed by centrifugation. The supernatant contained the cytoplasmic proteins (including several nuclear membrane proteins, if CEB-B were not added, there would be no nuclear membrane protein) and the precipitant was further added with the NEB solution, incubated, vortexed and centrifuged. The resultant supernatant contained nuclear proteins.

Working concentration of agents

The working concentrations of L-mimosine was 100 μg/ml [33, 34], colchicine 0.5 μg/ml [35, 36]; aphidicolin 1 μg/ml [37], nocodazole 1 μg/ml, and H89 and forskolin, which were both used at a concentration of 20 μM.

Western blot

When cultured to 80% confluence, cells were collected and lysed with the WB/IP lysate buffer. Protein quantification was performed using the BCA kit (Thermo, NCI1059CH). After denaturation, proteins were SDS-PAGE separated, electro-transferred to a membrane, and then incubated with primary antibody at 4°C overnight, incubated with secondary antibody at room temperature for 1 h, ECL-developed, exposed, and imaged using the BioRadChemi DOC XRS + Imaging System (BioRad, Hercules, CA, USA). Gray values of the blots were scanned using the Image Lab version 5.0 software program (BioRad, Hercules, CA, USA).

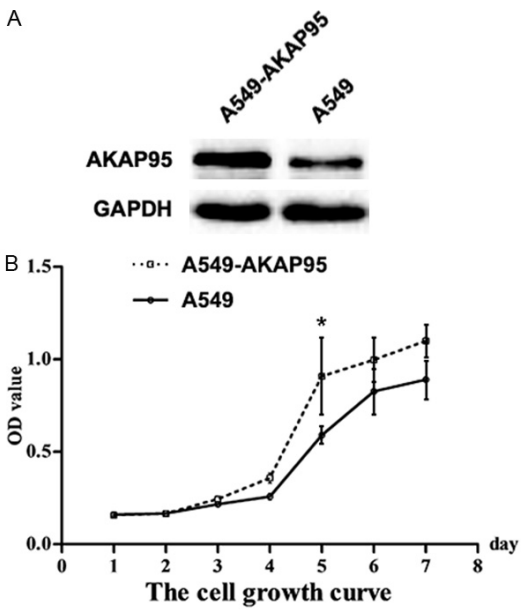


Figure 1. Growth curve of A549 cells before and after AKAP95 transfection. Cells were synchronized by 24 h of serum starvation. After changing to complete medium, cells were collected each day and the optical density (OD) value was measured using the MTT assay for seven days. The OD value of A549 and A549-AKAP95 cells began to increase at day four. The A549-AKAP95 cells displayed a more significant increase as compared to A549 cells. The experiment was repeated three independent times.

Co-IP

Proteins that were extracted from the cells (500 μg) were incubated with the antibody for 1 h (on a shaker), and then incubated with Protein A/G Plus-Agarose overnight. After centrifuging at 4°C, the precipitant was washed in PBS alone, centrifuged again, and then resuspended by using a sample buffer and identified by Western blot assay.

Immunofluorescence and confocal microscope observation

Cells grown on slides were treated with 0.5% Triton-X 100 at room temperature for 20 min, blocked in 1% BSA, incubated with primary antibody at 4°C overnight, and labeled by FITC and TRITC fluorescent antibody (at a dilution of 1:100) at 37°C in a dark room. Nuclei were counter-stained by DAPI and smears were observed under a confocal microscope (LSM5, Zeiss).

Proximity ligation assay

Cells grown on slides were treated with 0.5% Triton-X 100 at room temperature for 20 min, blocked by 1% BSA, incubated with primary antibody at 4°C overnight, and then incubated with PLA probes at 37°C, following which the linking-agent-diluted ligase was added to allow ligation at 37°C for 30 min. Next, the amplifying-agent-diluted polymerase was added to allow amplification at 37°C for 100 min, which was then blocked in Duolink *in situ* blocking solution (containing DAPI), and observed by confocal microscopy (LSM5, Zeiss).

Statistical analysis

Statistical analysis was performed using the SPSS version 13.0 software program (SPSS Inc., Chicago, IL, USA), comparison of gray values of the western blot bands was made by one-way analysis of variance (ANOVA). An alpha value of $P<0.05$ was considered statistically significant between paired or groups of data.

Results

AKAP95 transfection increased the growth rate of A549 cells

The growth rate of A549 cells transfected with AKAP95 is shown in **Figure 1**. At 5 d, the OD value of A549-AKAP95 cells (0.908) significantly exceeded that of the A549 cells (0.590), which indicated that AKAP95 promoted cell growth.

AKAP95 transfection enhanced expression of cyclin E1 and LMW-E2

The expression of cyclin E1, cyclin E2, CDK2 and CDK4 was measured in AKAP95-transfected and AKAP95-silenced A549 cells (**Figure 2A**). After AKAP95 transfection, the expression of cyclin E1 was significantly higher than that found in control or in AKAP95-silenced cells. Additionally, AKAP95-silenced cells displayed a lower level of cyclin E1 expression when compared with the control. The expression of cyclin E2 was examined using two different antibodies, A-9 from Santa Cruz and ab40890 from Epitomic, and the results were very different. It was found that detection with ab40890 suggested cyclin E2 (about 42 kDa, referred to as lower-molecular-weight

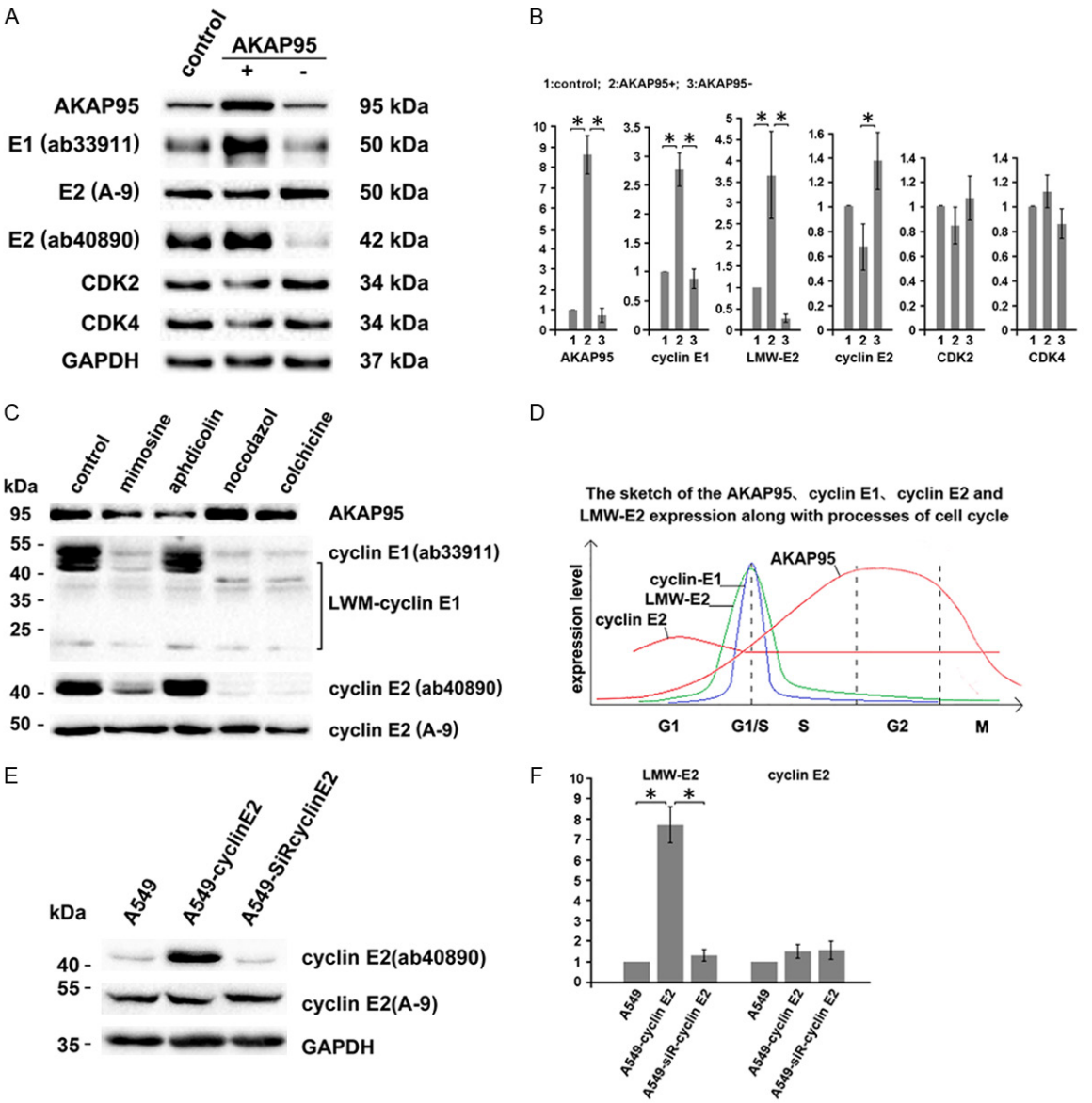
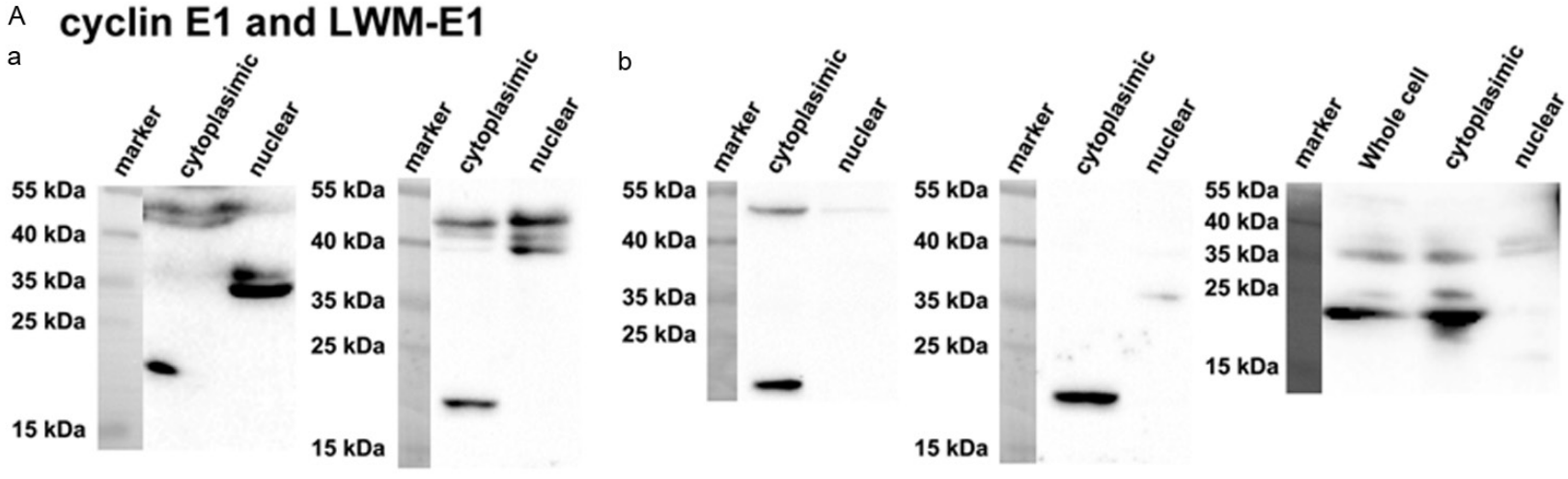


Figure 2. The influence of AKAP95 on cyclin E1, cyclin E2, CDK2 and CDK4 in A549 cells. A. After AKAP95 transfection and silencing, the following antibodies were used to determine the expression of their specific proteins: anti-AKAP95 (22-Z, 1:1000), cyclin E1 (ab33911, 1:1000), cyclin E2 (A-9, 1:500 and ab40890, 1:2000), CDK2 (D-12, 1:500) and CDK4 (DCS-35, 1:500). Proteins were loaded at 30 μg per sample. Blots in rows 2-6 show proteins on the same membrane that were incubated with the corresponding antibodies following treatment with Restore™ Western Blot Stripping Buffer. B. Shows statistical results of protein expression. After AKAP95 transfection, cells exhibited significantly increased expression of cyclin E1, LMW-E2 and CDK4 while the expression of cyclin E2 and CDK2 were decreased. However, AKAP95 silencing showed converse results. Experiments were repeated three times. C. Cells were synchronized for 24 h by treatment with mimosine (100 μg/ml), aphdicolin (1 μg/ml), nocodazol (1 μg/ml) and colchicine (0.5 μg/ml), following which, the proteins were collected and loaded at 30 μg (with the exception of 50 μg for cyclin E1). D. Shows the variation in expression of AKAP95, cyclin E1, cyclin E2 and LMW-E2 with the progression of cell cycle. E. Shows the detection of cyclin E2 and LMW-E2 after over-expression and silencing of cyclin E2 gene that was synthesized by referring to the NCBI database (NCBI, NM_057749.2) in A549 cells. F. Shows the statistical results of cyclin E2 and LMW-E2 expression. Cells transfected with AKAP95 showed a 7.7-fold higher expression of LMW-E2 than was found in the control. In addition, LMW-E2 expression was not significantly different from that of the control after AKAP95 silencing. The difference in cyclin E2 expression, and in AKAP95 over-expression, gene silencing and control groups were not significantly different from each other. Experiments were repeated eight times.



c The frequency of bands with different molecular weight in 20 results

kDa	cytoplasimic	nuclear	cytoplasimic + nuclear
50	16	10	8
45	5	2	0
40	0	5	0
35	4	9	2
20	15	0	0

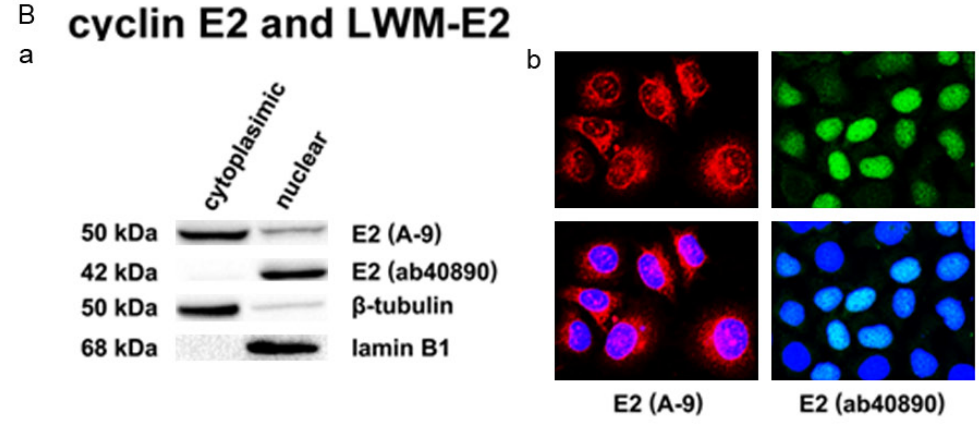


Figure 3. The expression of cyclin E1/E2 and LMW-E1/E2 in A549 and BGC823 cells. (Aa and Ab) Show the expression of cyclin E1 and LMW-E1 in A549 cells and BGC823 cells respectively. After routine culture, cells were lysed by the WB/IP lysate procedure, and proteins were collected, separated (cytoplasmic and nuclear fractions) and loaded in each well of the gel at 30 μg each. The expression of cyclin E1 was detected by using ab33911 (1:1000) and HE12 (1:1000). As shown in (Aa and Ab), cyclin E1 and LMW-E1 were expressed in both the cytoplasm and nucleus. (Ac) Shows the frequency of cyclin E1 and LMW-E1 expression out of 20 detections (i.e., three repeats for HE12 and 17 repeats for ab33911). (B) Shows the expression of cyclin E2 and LMW-E2 in A549 cells. Proteins were separated, loaded at 30 μg and incubated with the A-9 antibody (1:500). Then, the film was treated with the Restore™ Western Blot Stripping Buffer and again incubated with antibody ab40890 (1:2000). As shown at the top of (Ba), use of A-9 detected the cytoplasmic expression and trace nuclear expression of cyclin E2, while ab40890 detected expression of LMW-E2 only in the nucleus. The bottom part of (Ba) shows cytoplasmic and nuclear protein separation. The intracellular localization of cyclin E2 and LMW-E2 are shown in (Bb). TRITC-labeled A-9 (1:100) detected cytoplasmic localized cyclin E2, which exhibited high expression near to, or on the nuclear membrane, while low expression was found in the nucleus. FITC-labeled ab40890 (1:100) suggested that LMW-E2 was localized to the nucleus. The nucleus was counter-labeled by DAPI staining.

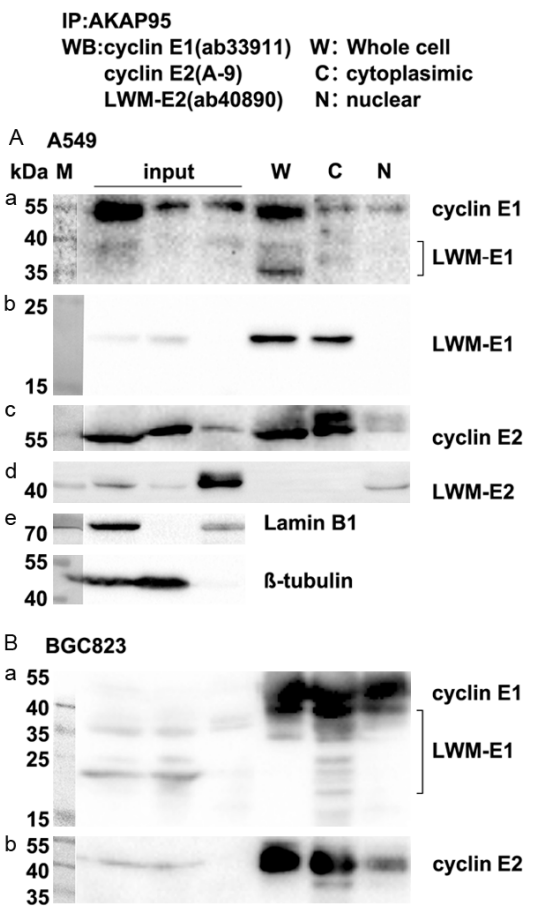


Figure 4. IP Determination of the interaction between AKAP95 and cyclin E1/E2 in the cytoplasm and nucleus in A549 and BGC823 cells (n = 4). Nuclear membrane proteins were included in cytoplasmic proteins during protein separation. In these studies AKAP95 was co-immunoprecipitated with antibody 22-Z, and then the expression of cyclin E1 (1:1000), cyclin E2 (1:500) and LMW-E2 were examined by Western blot. As shown in (Aa), cyclin E1 is shown in the cytoplasmic and nuclear inputs, and in the corresponding IP products. LMW-E1 of 40-35 kDa, were detected in the whole cell and cytoplasmic inputs. (Ab) Shows that the 20 kDa LMW-E1 was detected in the cytoplasmic input as well as the corresponding IP products. (Ac) Indicates that in the input, cyclin E2 was mainly expressed in the cytoplasm and slightly expressed in the nuclear fraction. Thus, cyclin E2 was detected in whole cells and cytoplasmic IP products, while trace cyclin E2 was detected in the nucleus. (Ad) Suggests that LMW-E2 was only expressed in the nucleus, and thus LMW-E2 was detectable only in the nuclear IP product. (Ba) Shows the results of BGC823 cell examination. As shown in (Ba), cyclin E1 and LMW-E1 of 45-20 kDa were detected in whole cells, cytoplasm and the nucleus, and LMW-E1 of 25-15 kDa was particularly detected in the cytoplasm, showing several bands on the membrane. In (Bb), cyclin E2 was detected in the whole cell, cytoplasm and nucleus, although the expression was most significant in the cytoplasm and less in the nucleus.

cyclin E2 or LMW-E2) expression in AKAP95-transfected cells had significantly exceeded that found in the control or in AKAP95-silenced cells. Also, LMW-E2 expression in AKAP95-silenced cells was lower than that found in controls; however, A-9 detection suggested that cyclin E2 expression (50 kDa, we named it after cyclin E2) in AKAP95-transfected cells was lower than that found in control or in AKAP95-silenced cells, and cyclin E2 expression in AKAP95-silenced cells exceeded that of the control. The level of CDK2 decreased after AKAP95 transfection, while it increased after silencing. The results of the effect on the phosphorylation of s795 of Rb (Figure S1) by AKAP95 showed that when AKAP95 genes were transfected into A549 cells, the expression of pRb-s795 was increased; however, the observation the converse was found when silencing AKAP95. It can be inferred that functional expression of the AKAP95 protein promotes s795 phosphorylation of Rb. Statistical results are shown in **Figure 2B**. Since cyclin E1 promotes the G1/S transition, our results suggested that by up-regulating the expression of cyclin E1, then AKAP95 induced the transition from G1 to the S phase of the cell cycle, thereby promoting cell cycle progression. LMW-E2 might be involved in cyclin E1-mediated G1/S transition. Specific blockers of cycle phase were used to synchronize A549 cells, and the results showed that the expression of LMW-E2 and cyclin E1 were very similar (**Figure 2C**), as both showed high expression in the S phase and yet low expression in G1 and G2/M phases. However, the 50 kDa cyclin E2 showed an expression pattern different from those of cyclin E1 and LMW-E2, as its expression level did not vary significantly in the G1, S, G2 and M phases, and only showed a slight increase in the G1 phase. However, LMW-E1 of varying molecular weights were detected in all four phases. Only LMW-E1 with a molecular weight of about 45 kDa showed relatively high expression in the S phase of the cell cycle, and LMW-E1 of other molecular weights were expressed at very low levels in the G1, G2 and M phases.

Figure 2D shows the expression of AKAP95, cyclin E1, cyclin E2 and LMW-E2 in different phases. **Figure 2E** suggests that cyclin E2 (50 kDa) expression did not vary significantly before and after cyclin E2 gene expression, which was synthesized according to reference to the NCBI database (NCBI, NM_057749.2). However,

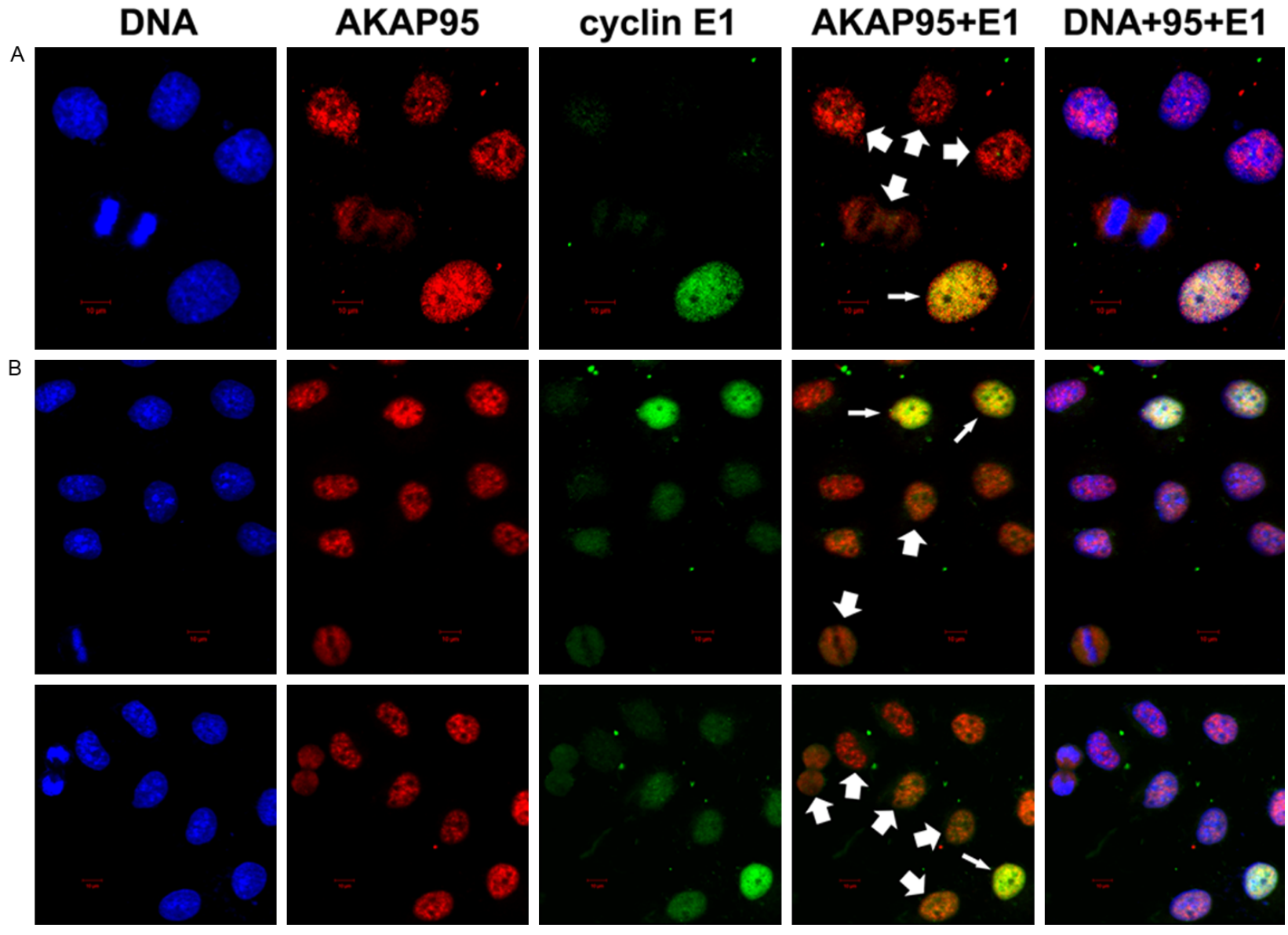


Figure 5. Co-localization of AKAP95 and cyclin E1 in A549 cells. The antibody used to detect AKAP95 was 22-Z (1:75), and for cyclin E1, HE12 (A, 1:100) and ab33911 (B, 1:100) were used. TRITC-labeled AKAP95 and FITC-labeled cyclin E1 co-localize to the nucleus in the G1/S phase (thin arrow). Co-localization was detected in other phases of the cell cycle/cell division, including early G1 phase, metaphase, anaphase and telophase (bold arrow). Magnification is indicated as (A: 200 × 2; B: 200 ×).

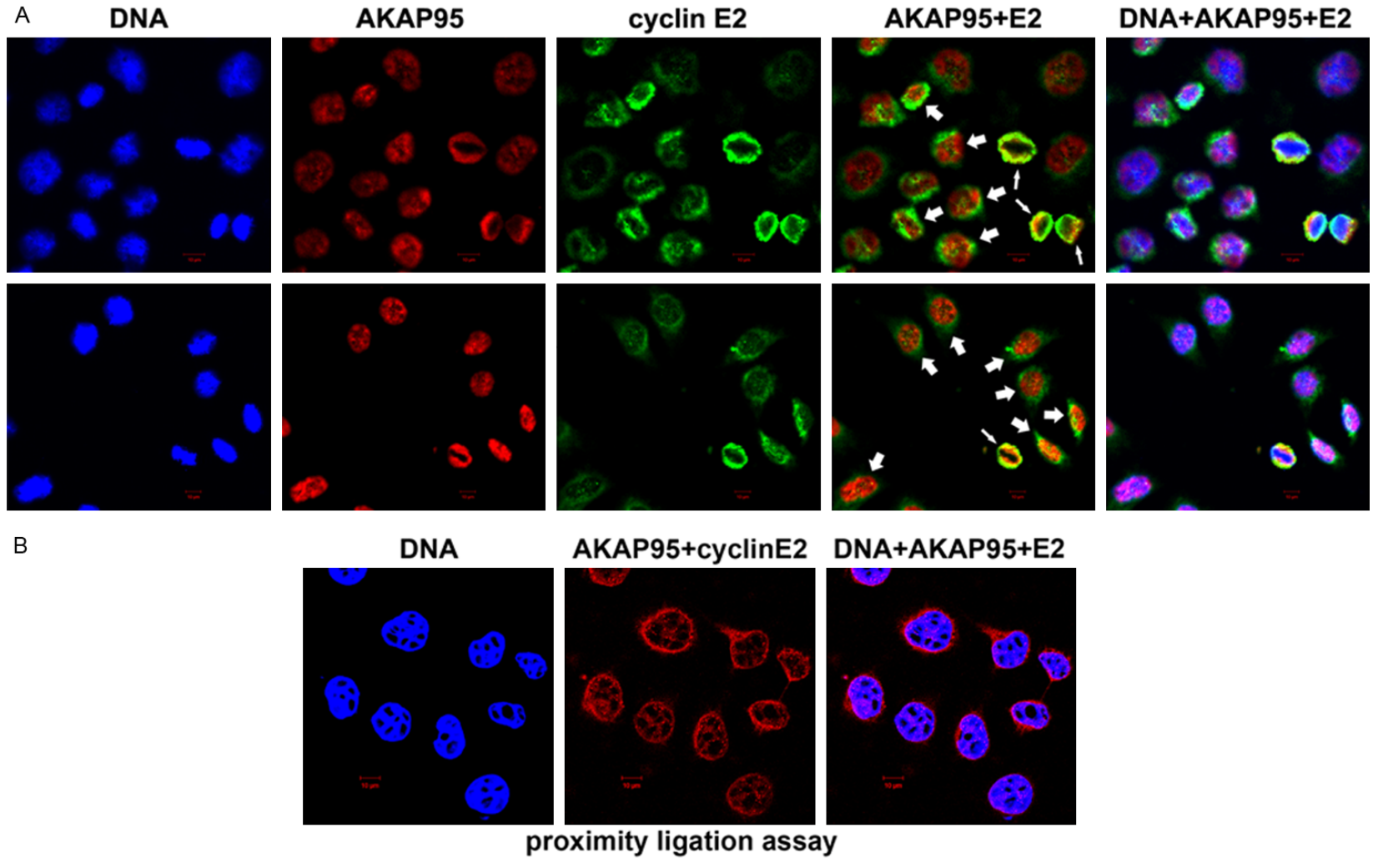


Figure 6. Co-localization of AKAP95 and cyclin E2 in A549 cells. (A) Shows that TRITC-labeled AKAP95 (ab140628, 1:100) was expressed inside the nucleus in G1 and S phases, and was detected in the cytoplasm during the metaphase and telophase due to the disappearance of the nuclear membrane. FITC-labeled cyclin E2 (A-9, 1:100) showed cytoplasmic expression and was thus not co-localized with AKAP95 during the G1 and S phases (bold arrow). In metaphase and telophase, both proteins were co-localized, as indicated by the thin arrow. (B) Shows the results of proximity ligation of AKAP95 (ab140628, 1:100) with cyclin E2 (A-9, 1:100). TXRD fluorescence suggested the binding of AKAP95 and cyclin E2 on the nuclear membrane, and traces of binding were detected inside the nucleus. Magnification is indicated as (A: 200 ×; B: 200 × 2).

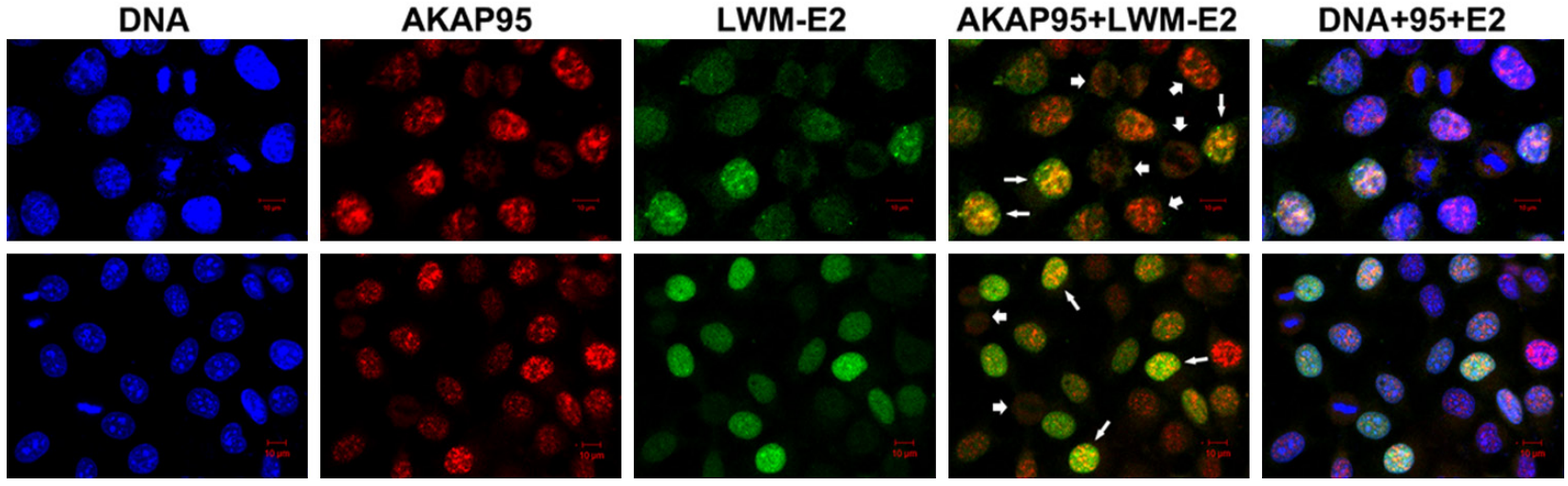


Figure 7. Co-localization of AKAP95 and LMW-E2 in A549 cells, 200 ×. Based on nuclear morphology and AKAP95 expression levels, FITC-labeled LMW-E2 (ab40890, 1:100) displayed high intra-nuclear expression only in the G1/S phase of the cell cycle, and co-localized with TRITC-labeled AKAP95 (22-Z, 1:75; as shown by the thin arrow). In other phases, LMW-E2 was expressed at a very low level, and co-localization with AKAP95 was not detected (bold arrow). Magnification is indicated as 200 ×.

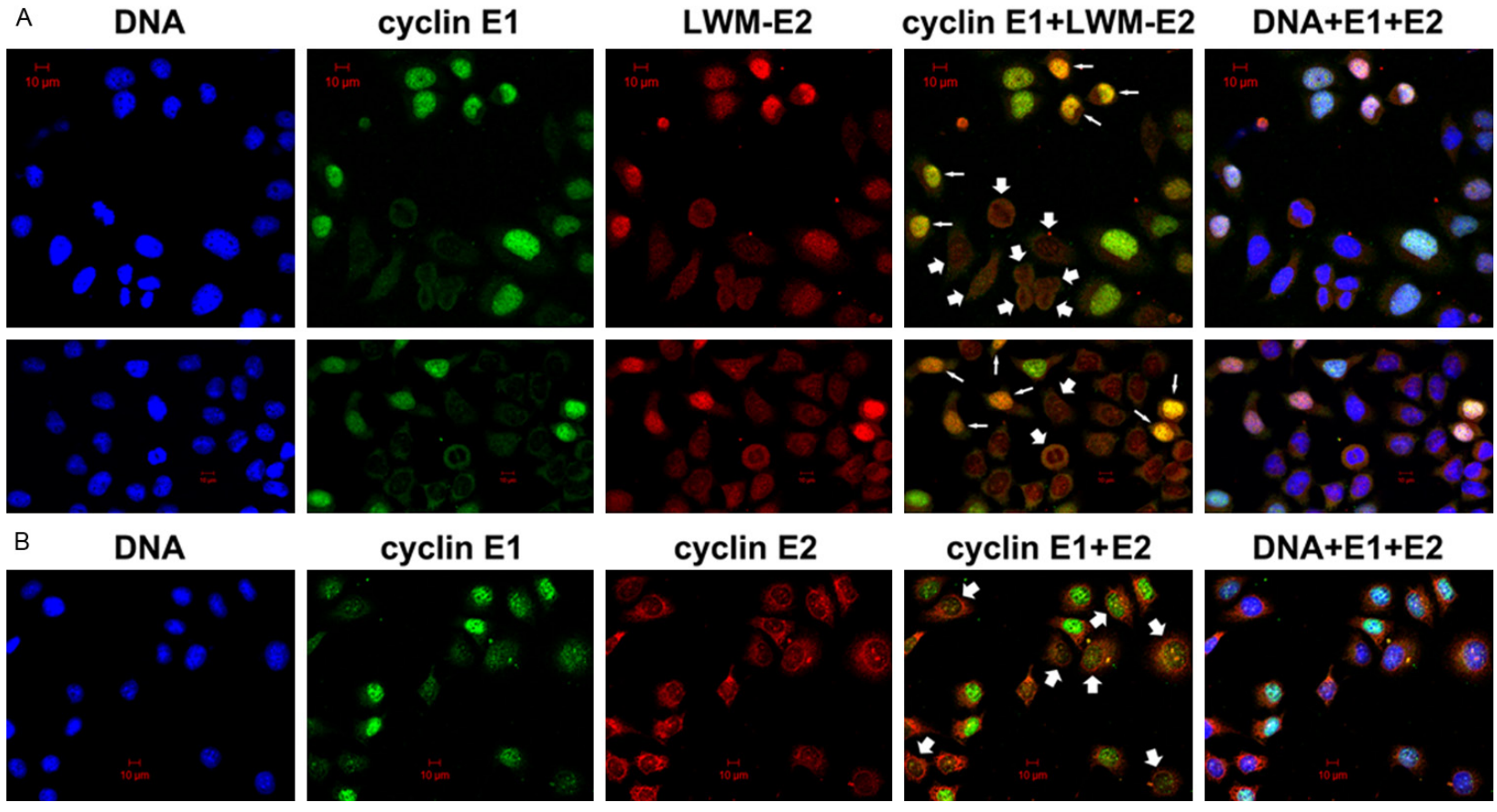


Figure 8. Co-localization of cyclin E1, LMW-E2 and cyclin E2 in A549 cells. Based on nuclear morphology, (A) showed that FITC-labeled cyclin E1 (HE12, 1:100) and TRITC-labeled LMW-E2 (ab40890, 1:100) displayed high intra-nuclear expression in the G1/S phase, and were co-localized (thin arrow). However, in other phases such as early G1 phase, late S phase and the G2/M phase, both proteins were expressed at a very low level and no co-localization was detected (bold arrow). (B) Shows that FITC-labeled cyclin E1 had high intra-nuclear expression in the G1/S phase, while TRITC-labeled cyclin E2 (A-9, 1:100) was mainly expressed in the cytoplasm, most on the nuclear membrane, and in each phase, the expression levels did not vary significantly. No co-localization of cyclin E1 or E2 was detected during the G1/S phase (bold arrow). Magnification is indicated as 200 ×.

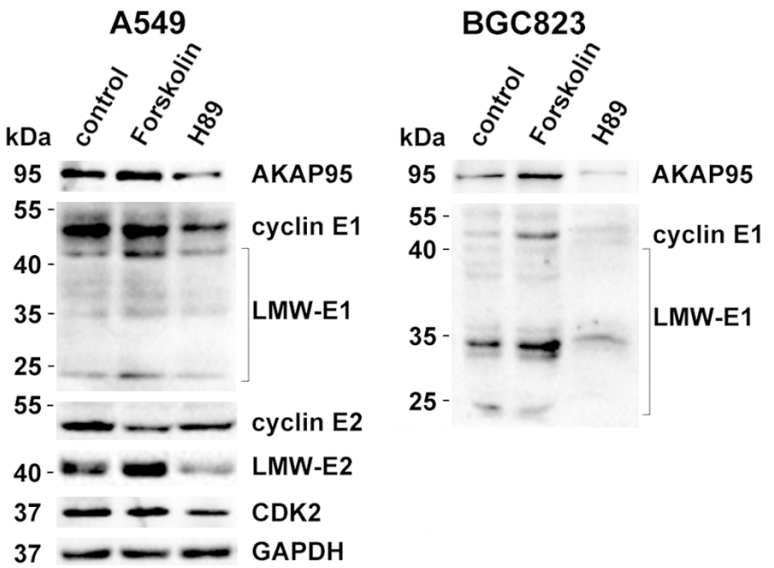


Figure 9. Expression of cyclin E1/E2 and LMW-E1/E2 after forskolin and H89 treatment. Routinely cultured A549 and BGC823 cells were treated with forskolin (20 μM) and H89 (20 μM) for 24 h, and proteins were collected from the WB/IP lysate and equally loaded at 30 μg. After incubation with AKAP95 antibody 22-Z (1:1000), the membrane was treated with the Restore™ Western Blot Stripping Buffer and again incubated with antibodies for cyclin E1 (ab33911, 1:1000), cyclin E2 (A-9, 1:500; ab40890, 1:1000) and CDK2 (D-12, 1:500).

LMW-E2 expression was significantly enhanced after cyclin E2 over-expression, and was insignificantly different from the control after cyclin E2 gene silencing. These results indicated the following key points: 1. AKAP95 promoted cell cycle progression by up-regulating cyclin E1 and LMW-E2 expression; 2. LMW-E1 was expressed throughout the entire cell cycle, but only LMW-E1 at 45-48 kDa showed a relatively high expression in the S phase, while it was expressed at very low levels in the G1 and G2/M phases; 3. The expression of LMW-E2 and cyclin E1 were very similar, although the expression pattern of cyclin E2 was significantly different from that of cyclin E1 and LMW-E2, which indicated that LMW-E2 and cyclin E1 may participate in G1/S transition while cyclin E2 might not; 4. LMW-E2 and cyclin E2 might be derived from different genes. In addition, LMW-E2 might not be transformed from cyclin E2, and AKAP95 over-expression could increase LMW-E2 expression.

Expression of cyclin E1/E2 and LMW-E1/E2 in A549 and BGC823 cells

Antibodies HE-12 (3 repeats) and ab33911 (17 repeats) detected the expression of cyclin E1 in A549 and BGC823 cells. The expression of cyclin E1 (50 kDa) and LMW-E1 (48-20 kDa) in the cytoplasm or nucleus are shown in **Figure 3Aa** and **3Ab**. The expression frequencies of cyclin E1 (50-20 kDa) are shown in **Figure 3Ac**. Repeated examination of the same protein sample showed good reproducibility regarding the expression of LMW-E1 of the same molecular weight; however, examination of different protein samples showed poor reproducibility that indicated prompt expression of LMW-E1.

LMW-E1, with a molecular weight of 40 kDa was only expressed in the nucleus, and LMW-E1 of about 20 kDa was only expressed in the cytoplasm. In addition, LMW-E1 of 35-50 kDa was expressed in both the nucleus and the cytoplasm, indicating that the degradation of cyclin E1 to 40 kDa LMW-E1 only occurred in the nucleus, while degradation to that of the 20 kDa form of LMW-E1 only occurred in the cytoplasm. With the exception of the 20 kDa form of LMW-E1 that showed a relatively darker band than other LMW-E1 species, LMW-E1 of other molecular weights all showed a gray value that was lower than that of cyclin E1, which indicated low expression levels of LMW-E1.

Moreover, the 20 kDa LMW-E1 was detected only by antibody ab33911 (15 out of 17 repeats) other than the HE-12 antibody (0 out of 3 repeats). This indicated that the 20 kDa LMW-E1 was more stable as compared other forms of LMW-E1. The expression of cyclin E2 that was detected by antibody A-9 and ab40890 are shown in **Figure 3Ba**. Here, it was found that A-9 only detected cytoplasmic 50 kDa cyclin E2 (cyclin E2), while ab40890 only detected the 42 kDa form of cyclin E2 (LMW-E2) in the nucleus. **Figure 3Bb** shows the confocal microscopic observations of cyclin E2 and LMW-E2, which suggested that cyclin E2 was mainly localized to the cytoplasm and accumulated around the nuclear membrane, while it

was also found that LMW-E2 mainly localized to the nucleus and was not expressed in the cytoplasm. These results indicated that following key points: 1. LMW-E1 was produced in the S phase, and were unstable since LMW-E1 of larger molecular weights might be degraded to LMW-E1 of smaller sizes. LMW-E1 was also expressed in both the cytoplasm and nucleus, and the 20 kDa form of LMW-E1 might also exist in the cytoplasm; 2. Cyclin E2 was mainly localized to the cytoplasm, accumulated around the nuclear membrane, and was only slightly expressed in the nucleus. In addition, LMW-E2 was mainly localized to the nucleus.

Interaction of AKAP95 with cyclin E1/E2 and LMW-E1/E2

AKAP95 is generally expressed in the nucleus, the IP product of AKAP95 was used to detect cyclin E1 and E2 expression (**Figure 4**). In A549 cells, cyclin E1, LMW-E2, cyclin E2 and LMW-E2 all bound to the AKAP95 protein. In addition, since nuclear membrane proteins were included in the cytoplasmic proteins and to the bands derived from the cytoplasm, we considered that the proteins that interacted with AKAP95 were formed on the nuclear membrane, such as cyclin E1 (C of **Figure 4Aa** and **4Ba**), LMW-E1 (C of **Figure 4Aa**, **4Ab**, **4Ba**) and cyclin E2 (C of **Figure 4Ac** and **4Bb**). By contrast, cyclin E1 (N of **Figure 4Aa**) and LMW-E2 (N of **Figure 4Ad**) bound to nuclear expressed AKAP95. The results also suggested that cyclin E2 (**Figure 4Ac** input) was mainly expressed in the cytoplasm and slightly expressed in the nucleus, while LMW-E2 (**Figure 4Ad** input) was only expressed in the nucleus, which were consistent with the results described in **Figure 3Bb**.

These results indicated the following issues: 1. Cyclin E1 and LMW-E1 both bound the AKAP95 protein. Since LMW-E1 was expressed throughout the whole cell cycle and had high bioactivity [16], it was unclear the binding of LMW-E1 and AKAP95 promoted cell cycle progression in other phases outside of the G1/S transition; 2. LMW-E2 bound to AKAP95 inside the nucleus, indicating that AKAP95 might bind with cyclin E1 and LMW-E2 to form a ternary complex. 3. Cyclin E2 bound to the AKAP95 protein on the nuclear membrane.

Immunofluorescent detection

Co-localization of AKAP95 and cyclin E1 in A549 cells is shown in **Figure 5**. The results suggested that cyclin E1 showed high expression only in the G1/S phase, co-localized with the AKAP95 protein inside the nucleus, and that no cyclin E1 was detected in other phases of the cell cycle.

Figure 6A suggested that in the G1, S and G2 phases, AKAP95 was expressed in the nucleus while cyclin E2 was expressed in the cytoplasm. Thus, the two proteins were not co-localized. During the M phase, the disappearance of the nuclear membrane led to the co-localization of AKAP95 and cyclin E2. Results of the proximity ligation assay (**Figure 6B**) indicated that AKAP95 and cyclin E2 co-localized to the nuclear membrane in G1 and S phases of the cell cycle, and showed slight co-localization to the nucleus. LMW-E2 exhibited high intra-nuclear expression (**Figure 7**), but only in the G1/S phase and was co-localized with AKAP95, and a trace to no expression at all of LMW-E2 was detected in other phases.

Figure 8A shows the co-localization of cyclin E1 and LMW-E2. The intra-nuclear expression of cyclin E1 and LMW-E2 increased significantly in the G1/S phase, and the two proteins bound to each other. After entering the G2/M phase, the level of cyclin E1 and LMW-E2 declined significantly, and co-localization was not detected. These results showed that the expression and intracellular localization of LMW-E2 and cyclin E1 were highly similar, indicating that both proteins interacted with each other to regulate the G1/S transition. **Figure 8B** shows the co-localization of cyclin E1 and E2. Although cyclin E1 exhibited high intra-nuclear expression in the G1/S phase, cyclin E2 was mainly expressed in the cytoplasm. Therefore both proteins were not co-localized. These results suggested the following: 1. AKAP95 expression increased significantly in the middle and late phases of G1, and as the cell entered the G1/S phase, cyclin E1 and LMW-E2 demonstrated peak expression to form a ternary complex with AKAP95. Since the expression of cyclin E1 and LMW-E2 declined rapidly to trace levels after G1/S transition, we speculated that at the end of the G1 phase transition, the ternary complex that was formed by AKAP95, cyclin E1 and LMW-E2 promoted the G1/S transition. The role of AKAP95 and LMW-E2 in G1/S conversion is illustrated in Figure S2. In addition, this complex might not involve the whole-length cyclin E2; 2. AKAP95 and whole-length cyclin E2 co-localized to the nuclear membrane in both the G1 and S phases of the cell cycle.

The flow cytometry results (Figure S2) showed that compared with the control group (A), when AKAP95 and LMW-E2 were simultaneously over-expressed (B), the ratio of cells in G1 phase significantly decreased; and the corresponding ratio of S phase increased.

When AKAP95 was over-expressed and LMW-E2 was silenced (C), there was a slight decrease in the ratio of cells in G1 phase. Even though, the ratio of cells in S phase increased correspondingly, the change was smaller than under conditions where AKAP95 and LMW-E2 were simultaneously over-expressed. The same result was seen in the AKAP95-silenced/LMW-E2 over-expressed group (D). These results showed that AKAP95 and LMW-E2 could promote G1/S phase transition independently; and the effect was stronger when the two proteins were simultaneously over-expressed. However, when AKAP95 and LMW-E2 were simultaneously silenced (E), the ratio of cells in G1 phase was continued to decrease, the ratio of S phase was increasing correspondingly. However, the change was lower when compared with other groups. Thus, we speculate that other cyclin proteins or pathways were activated in this process.

Influence of PKA activity on cyclin E1/E2 and LMW-E1/E2

Since AKAP95 is the carrier protein for PKA, we used forskolin [17, 18] to up-regulate the activity of PKA, while H89 was used [17, 19-21] to down-regulate the activity of PKA, thereby determining whether the influence of AKAP95 on cyclin E1, LMW-E1 and LMW-E2 was associated with PKA. The expression of AKAP95, cyclin E1, LMW-E1 and LMW-E2 are shown in **Figure 9**.

When compared with the control, forskolin increased the expression of AKAP95 (A549 and BGC823), cyclin E1 (BGC823), LMW-E1 (A549 and BGC823) and LMW-E2 (A549), but the level of cyclin E2 (A549) was reduced. By contrast, CDK2 expression showed no significant difference as compared with the control. H89 treatment showed a contrasting result with that of forskolin since the expression of AKAP95, cyclin E1 and LMW-E1 (A549 and BGC823), and the expression of LMW-E2 and CDK2 (A549) were significantly lower than that found for control cells or cells that were treated with forskolin. However, the expression of cyclin E2 (A549) was much higher as compared that of the forskolin group. These results indicated that PKA activity influenced the expression of AKAP95, cyclin E1, LMW-E1, LMW-E2, cyclin E2 and CDK2.

Discussion

AKAP95 is a chaperon protein for cyclin D [15] that co-localizes with cyclin D1 on chromatin in the G1 phase [22, 23]. During cell cycle progression, AKAP95 also interacts with cyclin E [14], which forms a complex with CDK2 and regulates the transition of the G1 phase to the S phase of cell cycle [24, 25]. Our previous studies found that AKAP95 expression was associated with cyclin E1 and E2 in lung cancer tissues [3, 4], and in this study, we demonstrated that AKAP95 accelerated cell cycle progression and promoted cell growth rate. We also found that over-expression of AKAP95 enhanced the expression of cyclin E1, LMW-E1 and LMW-E2 (42 kDa), and inhibited the expression of cyclin E2 (50 kDa). In addition, AKAP95 silencing showed an opposite effect to that of AKAP95 over-expression, and finally, AKAP95 formed a ternary complex with cyclin E1 and LMW-E2 during the G1/S transition. These results suggested that AKAP95 might promote G1/S transition by up-regulating cyclin E1 and LMW-E2 expression and binding with them; observations that was consistent with our previous studies [3, 4].

In human breast cancer tissues, cyclin E was degraded into LMW-E with varying molecular weights that ranged from 30 kDa to 50 kDa [26-28]. It has been reported that when compared with full-length cyclin E, cyclin E-T1 and cyclin E-T2 respectively increased the activity of CDK2 by 7- and 10-fold, and elevated the efficiency of histone 1 (H1) phosphorylation by 2- and 12-fold [16]. Protease-digested cyclin E lacked the nuclear localization sequence [26], but LMW-E localized to the nucleus, and exerted its biological function there [7].

In this study, we detected LMW-E1 expression not only in the G1/S phase, but also in G1, S, G2 and M phases of the cell cycle, although LMW-E1 only showed high expression in the G1/S phase and was expressed at a very low level in the other phases. Our study also showed that LMW-E1 of different molecular weights

could all bind AKAP95. However, it remained unknown whether this binding helped them to exert their biological functions, and this clearly requires further study.

Moreover, 40 kDa LMW-E1 only showed intra-nuclear expression, indicating that it might only function inside the nucleus. Interestingly, apart from the LMW-E1 that displayed molecular weights of between 35-48 kDa, antibody ab33911 also detected stable cytoplasmic expression of a 20 kDa form of LMW-E1 that was not detected by antibody HE12. A plausible reason for this might be due to the ab33911 antibody recognizing the 100-150 amino acid sequence of cyclin E1, while HE12 does not. Since the 20 kDa form of LMW-E1 included the 100-150 amino acid sequence of cyclin E1, the 45-44 kDa form of LMW-E1 was digested at the 40-45 Asp of cyclin E1, and the 40 kDa form of LMW-E1 was digested at the 46 Met of cyclin E1. By contrast the 33-35 kDa form of LMW-E1 was digested at the 70 Asp of cyclin E1 [7]. Furthermore, the LMW-E1 that was detected by ab33911 included the 100-150 amino acid sequence, indicating that the 20 kDa form of LMW-E1 should be derived from whole-length cyclin E1 or LMW-E1 with a larger molecular size. Also, the 20 kDa form of LMW-E1 was stably expressed, and it bound AKAP95 at the nuclear membrane.

The mechanism employed by cyclin E2 at inducing chromosomal instability differed from that employed by cyclin E1 [12]. Over-expression of cyclin E2 accelerated G1 progression in mammalian cells [11], but unlike cyclin E1, cyclin E2 was slightly, and in some cases, not even expressed in non-transformed cells [11]. The mRNA of cyclin E2 displayed a periodic expression pattern throughout the cell cycle, with its expression level peaking at the G1/S phase [29, 30]. The activity of cyclin E2-associated kinase increased at the late G1 phase, and peaked at a level very close to that of cyclin E [31], which suggested that the function and mechanism of cyclins E1 and E2 are probably different.

In this study, antibodies A-9 (Santa Cruz) and ab40890 (Epitomics) respectively detected cyclin E2 with a respective molecular weight of 50 kDa and approximately 42 kDa. In order to distinguish two different cyclin E2 isoforms, we named the 50 kDa form after cyclin E2, and another, which was the 42 kDa form, was named after the low molecular weight cyclin E2 (LMW-E2). Both forms of cyclin E2 exhibited significantly different expression and localization in different phases, as the cyclin E2 (50 kDa) was present throughout the cell cycle, showing high expression in the G1/S phase and stable expression in G1, S, G2 and M phases, and which were mainly localized to the cytoplasm and nuclear membrane while at the same time exhibiting slight intra-nuclear expression (**Figure 3B**), and binding with the AKAP95 protein on the nuclear membrane (**Figures 5**, **6B**). However, the LMW-E2 (42 kDa) only exhibited intra-nuclear expression in the late G1 phase and early S phase (**Figures 7**, **8A**), which showed the highest expression during G1/S transition, and which was undetectable in other phases nor in the cytoplasm.

Moreover, the expression phases (i.e., late G1 and early S phases), the occurrence of peak expression (G1/S transition), and localization of LMW-E2 were highly similar to those of cyclin E1. Additionally, the complex formed by LMW-E2, cyclin E1 and AKAP95 during G1/S transition did not include the 50 kDa form of cyclin E2. This indicated that LWM-E2, but not cyclin E2, interacted with cyclin E1 and AKAP95 to regulate G1/S progression.

Could cyclin E2 (50 kDa) be digested into active LMW-E2 (42 kDa), which could then permit its entry into the nucleus to exert its biological function? Over-expression of the cyclin E2 gene which was synthesized refer to NCBI database (NCBI, NM_057749.2) significantly increased the protein level of LMW-E2 (42 kDa) but not that of cyclin E2 (50 kDa). This indicated that LMW-E2 (42 kDa) might not be derived from cyclin E2 (50 kDa). The results of LMW-E2 (42 kDa) detection that were determined in this study were very similar to the cyclin E2 that was reported previously [29, 30]; however, the expression of cyclin E2 (50 kDa) that we named was inconsistent with those previous studies [29, 30]. Thus, whether other family members of cyclin E2 exist requires further study.

Forskolin activates PKA by increasing cAMP concentration [17, 18], while H89 inhibits PKA activity by specific binding with PKA [17, 19-21]. It was previously shown that cAMP induces the early activation of ERK, and reduces the degradation of cyclin E via the MEK/ERK pathway,

thereby elevating the level of cyclin E [31]. Increased intracellular levels of cAMP could induce P21Cip1 to bind the cyclin/CDK2 complex, which would serve to inhibit the activity of CDK2 and the dephosphorylation of the Rb protein [32]. After forskolin treatment, cells showed enhanced expression of AKAP95, cyclin E1, LMW-E1 and LMW-E2, which might all be associated with increased cAMP levels. However, after H89 treatment, the expression of the above proteins declined, which might be due to dampened inhibition of PKA and cAMP levels by a feedback mechanism. This indicates that AKAP95 and LMW-E2 might be expressed in a cAMP-dependent manner just like cyclin E1. The increase in LMW-E1 after forskolin treatment could be associated with heightened levels of cyclin E1 in the G1/S phase.

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Disclosure of conflict of interest

None.

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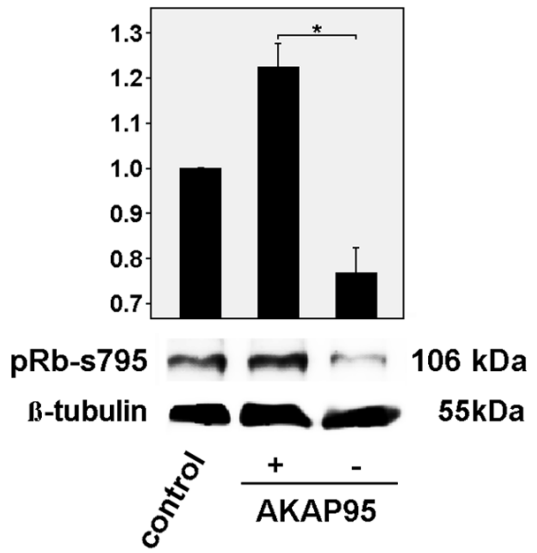

Figure S1. The influence of AKAP95 on pRb-s795. After transfecting and silencing AKAP95 in A549 cells, the cells were cultured for 24 h, then pRb-s795 (AB55056) was expressed. The experiment was repeated three times.

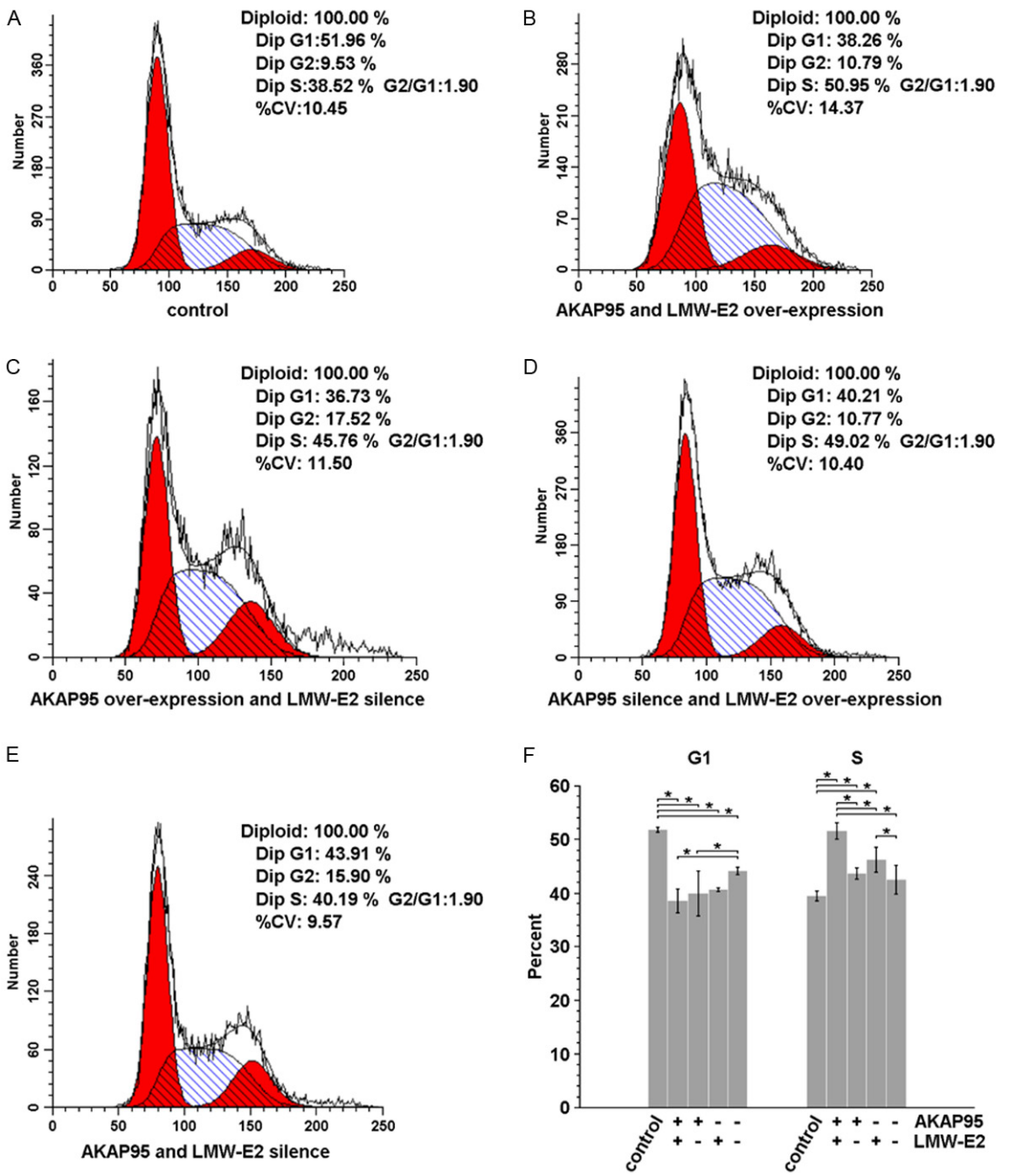

Figure S2. The influence of AKAP95 and LMW-E2 on cell cycle. After transfecting and silencing AKAP95 in A549 cells, cells were cultured for 15 h, and analyzed by Flow Cytometry. A. Control. B. Over-expressed AKAP95 and LMW-E2. C. Simultaneously over-expressed AKAP95 and silenced LMW-E2. D. Simultaneously silenced AKAP95 and over-expressed LMW-E2. E. Silenced AKAP95 and LMW-E2. F. Percentage of G1 and S phases of the cell cycle. The experiment was repeated three times.