

SEC61G participates in endoplasmic reticulum stress by interacting with CREB3 to promote the malignant progression of lung adenocarcinoma

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Received November 2, 2021; Accepted December 23, 2021

DOI: 10.3892/ol.2022.13316

Abstract. As the most common type of lung cancer, lung adenocarcinoma (LUAD) poses a great threat to human health worldwide and severely compromises the quality of life of the patients. The present study aimed to explore the potential pathogenesis of LUAD. Reverse transcription-quantitative PCR and western blotting were applied to measure the expression levels of SEC61 translocon subunit γ (SEC61G) and cyclic AMP-responsive element-binding protein 3 (CREB3). Western blotting was also used to determine the expression of endoplasmic reticulum (ER) stress-, apoptosis- and migration-related proteins. Cell Counting Kit-8, colony formation, TUNEL, wound healing and Transwell assays were used, respectively, to determine the viability, proliferation, apoptosis, migration and invasion of LUAD A549 cells. The association between SEC61G and CREB3 was verified by co-immunoprecipitation assay. The results revealed that SEC61G was upregulated in A549 cells and its downregulation could activate ER stress. It was also found that silencing SEC61G inhibited the malignant development of LUAD through ER stress. In addition, SEC61G was verified to participate in ER stress in LUAD via CREB3 and silencing SEC61G exerted inhibitory effects on the malignant progression of LUAD by regulating CREB3. In summary, SEC61G participated in ER stress and its knock-down exerted inhibitory effects on A549 cells via regulating CREB3, which suggests that SEC61G may be a potential therapy for patients with LUAD.

Introduction

As the most frequently diagnosed type of cancer, lung cancer has the highest cancer-related incidence and mortality worldwide (1). It is reported that there were 220,000 newly diagnosed lung cancer cases and >140,000 mortalities due to lung cancer in USA in 2019 (2). Lung adenocarcinoma (LUAD), which accounts for >40% of lung cancer cases, is the most common histological subtype of lung cancer (3). Although significant progress has been made in the diagnostic and treatment methods in recent years, the average 5-year relative survival rate among patients with lung cancer is only ~18% (4). Thus, it is important to explore the mechanism of LUAD and to identify an optimal therapy to improve the treatment of LUAD.

As a member of SEC61 translocon, SEC61 translocon subunit γ (SEC61G) consists of three subunits in mammals, namely Sec61 α , Sec61 β and Sec61 γ (5). The SEC61 complex, which serves as the core component of the protein translocation apparatus of the endoplasmic reticulum (ER) membrane (6), is involved in protein folding, modification and translocation and in the unfolded protein response, particularly under conditions of hypoxia and nutrient deprivation in the tumor microenvironment (7,8). SEC61 serves a critical role in numerous types of cancer. For example, SEC61 is overexpressed in hepatocellular carcinoma and its silencing can suppress cell proliferation and induce apoptosis (9). Meng *et al* (10) report that SEC61 is highly expressed in human kidney tumor tissues, whereas its knockdown exerts inhibitory effects on cell proliferation, migration and invasion in kidney cancer. According to the UALCAN database, SEC61 is upregulated in LUAD and SEC61 upregulation is associated with poor prognosis of LUAD patients, which suggests that SEC61G may be involved in the progression of LUAD. However, the biological roles and special mechanism of SEC61 in LUAD remains to be elucidated.

Cyclic AMP-responsive element-binding protein 3 (CREB3), also named LZIP or LUMAN, is an ER stress-related protein that is considered to be a transcriptional coregulator (10). According to the Biological General Repository for Interaction Datasets (BioGRID) database (<https://thebiogrid.org/>), SEC61G, a membrane transporter on the ER, may interact with multiple proteins. Silencing the expression

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Key words: SEC61 translocon subunit γ , cyclic AMP-responsive element-binding protein 3, lung adenocarcinoma, endoplasmic reticulum stress

of CREB3, a central protein in ER stress, is able to induce ER stress and cell apoptosis (11). Thus, it was hypothesized that SEC61G may interact with CREB3 to induce ER stress. In addition, since CREB3 had been found to be increased in LUAD, it was inferred that SEC61G could participate in ER stress by interacting with CREB3 to promote the malignant progression of LUAD.

Materials and methods

Bioinformatic analysis. The Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn>) version 1.0 was used to analyze the mRNA expression of SEC61G and CREB3 in LUAD samples from The Cancer Genome Atlas (TCGA) database (<https://www.tcg.org/>). The BioGRID database (<https://thebiogrid.org/>) version 4.4 was used to predict potential proteins that interact with SEC61G. In addition, the expression of CREB3 in LUAD was analyzed by the UALCAN database (<http://ualcan.path.uab.edu>).

Cell culture, treatment and transfection. Human LUAD cell line A549 was from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO₂. Subsequently, 4-phenylbutyric acid (4-PBA; 5 mmol/l), an inhibitor of ER stress, was used to treat the A549 cells.

For transfection, small interfering RNA (siRNA)-negative control (si-NC), overexpression plasmid (Oe)-NC, si-SEC61G-1/2 and Oe-CREB3 at a concentration of 20 μ M were obtained from Shanghai GenePharma Co., Ltd. Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection. Cells were incubated with 5% CO₂ at 37°C for 8 h and were used in subsequent experiments 48 h post-transfection. The sequences of siRNAs were: si-SEC61G-1: 5'-GCAAUAGGAUUUGCUAUAUG-3'; si-SEC61G-2: 5'-CUUAGAGAUUGGUGACAAGU-3'; si-NC: 5'-UUCUCCGACGUGUCACGU-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from A549 cells (5x10⁶ cells) using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Synthesis of complementary DNA was conducted with PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocols. Next, SYBR Premix Ex Taq reagents (Takara Bio, Inc.) were employed to perform qPCR on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols and all reaction was repeated three times. The following thermocycling conditions were used for qPCR: 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 60 sec. The primer sequences for PCR were: SEC61G: 5'-AAAGGACTCCATTCGGCTGGTT-3' (forward) and 5'-CAAAGAAGCCAATGAATCCC-3' (reverse); CREB3: 5'-ACCCTTTCCGTAGTTGTCCC' (forward) and 5'-GAA TGTTCACGACGCTGGG-3' (reverse); GAPDH: 5'-GGG

AAACTGTGGCGTGAT-3' (forward) and 5'-GAGTGGGTGTCGCTGTTGA-3' (reverse). GAPDH served as an internal control for normalization and relative gene expression was evaluated with the 2^{- $\Delta\Delta$ C_q} method (12).

Western blot assay. Total proteins were extracted from A549 cells with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) and were quantified with a BCA protein assay kit (Thermo Fisher Scientific, Inc.). After being subjected to 10% SDS-PAGE, the proteins (30 μ g per lane) were transferred onto PVDF membranes, which were then blocked with 5% non-fat milk for 2 h at room temperature and then incubated at 4°C overnight with primary antibodies: anti-SEC61G (1:500; cat. no. 11147-2-AP; ProteinTech Group, Inc.), anti-phosphorylated (p)-PERK (1:1,000; cat. no. 3179; Cell Signaling Technology, Inc.), anti-PERK (1:1,000; cat. no. ab229912; Abcam), anti-p-eukaryotic initiation factor 2 α (EIF2 α ; 1:1,000; cat. no. 3398; Cell Signaling Technology, Inc.), anti-EIF2 α (1:1,000; cat. no. ab169528; Abcam), anti-Activating transcription factor 4 (ATF4; 1:1,000; cat. no. ab184909; Abcam), anti-B cell lymphoma-2 (Bcl-2; 1:1,000; cat. no. ab32124; Abcam), anti-BCL-2-associated X (Bax; 1:1,000; cat. no. ab32503; Abcam), anti-C-Caspase 3 (1:500; cat. no. ab32042; Abcam), anti-Caspase 3 (1:5,000; cat. no. ab32351; Abcam), anti-matrix metalloproteinase 2 (MMP2; 1:1,000; cat. no. ab92536; Abcam), anti-matrix metalloproteinase 2 (MMP9; 1:1,000; cat. no. ab76003; Abcam), anti-CREB3 (1:2,000; cat. no. ab180119; Abcam) and anti-GAPDH (1:2,500; cat. no. ab9485; Abcam). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature (1:2,000; cat. no. ab6721; Abcam). Finally, the protein bands were visualized by using enhanced chemiluminescence (MilliporeSigma) and quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.).

Cell counting kit-8 (CCK-8) assay. A549 cells were inoculated into 96-well plates and cultured overnight at 37°C before being treated with 5 mmol/l 4-PBA. After 24, 48 and 72 h, 10 μ l CCK-8 reagent (Beyotime Institute of Biotechnology) was added into each well and the cells were further incubated for additional 2 h. Finally, the absorbance at 450 nm was determined with a microplate reader (Thermo Fisher Scientific, Inc.).

Colony formation assay. A549 cells were inoculated in 6-well plates and incubated for 10 days. Subsequently, the cell colonies were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.5% crystal violet solution, for 30 min at room temperature. Finally, the number of colonies (defined as >50 cells) was counted under an inverted microscope (magnification, x10).

TUNEL assay. Following the corresponding treatment and transfection, A549 cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.25% Triton X-100 for 20 min at room temperature. Cells were incubated with 5% bovine serum albumin (Yeast Biotech Co., Ltd.) and then stained with TUNEL reagent. Subsequently, DAPI staining solution (Beyotime Institute of Biotechnology)

was used to counterstain the sections in dark. Images of apoptotic cells were captured under a fluorescence microscope (Nikon Corporation; magnification, x200).

Wound healing assay. A549 cells were seeded into 6-well plates and incubated until reaching a confluence of 90-100%. A linear scratch in the cell monolayer was created with a 10 μ l pipette tip. Subsequently, PBS was applied to wash the cells three times to remove cell debris. The cells were then incubated at 37°C in the presence of 5% CO₂ and recorded at 0 and 24 h. ImageJ software (version 1.46; National Institutes of Health) was used to evaluate the area occupied by the migrated cells.

Transwell assay. The invasiveness of A549 cells was detected with Transwell assay. The upper chamber was used to inoculate and incubate A549 cells, while 10% FBS was added to the lower chamber. After 24 h, fixation and staining of A549 cells were conducted with 4% paraformaldehyde for 10 min and 0.1% crystal violet for 10 min at room temperature, respectively. The invaded cells across the filter were observed in three random fields under a light microscope (magnification, x100).

Co-immunoprecipitation (Co-IP) assay. According to the BioGRID and UALCAN databases, CREB3 was found to interact with SEC61G. Thus, Co-IP assay was used to verify this hypothesis. Total proteins that had been isolated with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), centrifuged at 1,000 x g at 4°C for 10 min and quantified with a BCA kit (Beyotime Institute of Biotechnology) were incubated overnight at 4°C with 2 μ g appropriate antibodies: Anti-SEC61G (cat. no. PA5-106309; Thermo Fisher Scientific, Inc.) and anti-CREB3 (cat. no. LS-C664784; LifeSpan BioSciences, Inc.). Normal rabbit IgG (cat. no. sc-2027; Santa Cruz Biotechnology, Inc.) were used as control. Next, the cell lysates were incubated with 40 μ l Protein G/A Agarose Beads (Invitrogen; Thermo Fisher Scientific, Inc.). Following centrifugation at 600 x g at 4°C for 10 min, PBS was then used to wash the beads three times and the precipitated proteins were then re-suspended in 2X SDS-PAGE loading buffer, boiled in Laemmli buffer for 5 min and eluted from the beads. Finally, the immunoprecipitated products were determined using western blotting.

Statistical analysis. Data were expressed as the mean \pm standard deviation. SPSS 22.0 (IBM Corp.) was employed for data analysis. One-way ANOVA followed by Tukey's post hoc test was used for the comparisons of multiple groups, while Student's t-test was employed for comparisons between two groups. Mantel-Cox test was to determine the overall survival rate of LUAD patients. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SEC61G is upregulated in LUAD and its downregulation can trigger ER stress. According to the GEPIA database, SEC61G expression was greatly increased in LUAD (Fig. 1A and B). The RT-qPCR and western blot

results revealed that the mRNA and protein expression levels of SEC61G were increased in LUAD A549 cells (Fig. 1C and D). Compared with those of si-NC, the mRNA and protein expression levels of SEC61G were greatly decreased after transfection with SEC61G interfering plasmids (Fig. 1E and F). In addition, the expression levels of ER stress-related proteins, including phosphorylated (p)-protein kinase RNA-like ER kinase (PERK), p-eukaryotic initiation factor-2 α (eIF2 α) and activating transcription factor 4 (ATF4), were significantly increased upon SEC61G knockdown (Fig. 1G).

SEC61G silencing inhibits the malignant progression of LUAD via ER stress. As depicted in Fig. 2A, cell viability was greatly decreased by SEC61G silencing, while 4-PBA treatment partially recovered the viability of A549 cells. The decreased colony number in SEC61G-silenced A549 cells was significantly increased after treatment with 4-PBA (Fig. 2B). The results from Fig. 2C and D showed that the increased apoptosis rate caused by SEC61G knockdown was decreased by 4-PBA treatment, which indicated that 4-PBA abolished the promoting effects of SEC61G knockdown on the apoptosis rate of A549 cells. Additionally, SEC61G silencing increased Bcl-2 expression but decreased Bax and cleaved caspase 3 expression in comparison with the results of si-NC and these findings were subsequently reversed by 4-PBA treatment (Fig. 2E). Compared with those of si-NC, the migration and invasion rate of A549 cells were reduced after transfection with SEC61G interfering plasmids, while 4-PBA treatment reversed the inhibitory effects of SEC61G silencing on cell migration and invasion (Fig. 2F and G). Furthermore, the decreased expression levels of MMP9 and MMP2 in SEC61G-silenced A549 cells were increased after 4-PBA treatment (Fig. 2H).

SEC61G participates in ER stress via CREB3 in LUAD. According to the BioGRID database, SEC61G is predicted to interact with CREB3 (Fig. 3A). The UALCAN database revealed that CREB3 expression was significantly increased in LUAD (Fig. 3B). As shown in Fig. 3C and D, the mRNA and protein expression levels of CREB3 were markedly high in A549 cells. A Co-IP assay was adopted to further verify the binding of SEC61G and CREB3. As shown in Fig. 3E, CREB3 existed in anti-SEC61G and SEC61G existed in anti-CREB3, revealing that SEC61G could bind to CREB3 in LUAD. Compared with the si-NC group, CREB3 expression was greatly decreased by SEC61G silencing (Fig. 3F). In addition, Fig. 3G and H revealed that the expression of CREB3 markedly increased after transfection with CREB3 overexpression plasmids. Furthermore, the increased expression levels of p-PERK, p-eIF2 α and ATF4 in SEC61G-silenced A549 cells were decreased upon CREB3 overexpression (Fig. 3I).

SEC61G knockdown inhibits the malignant progression of LUAD via CREB3. As demonstrated in Fig. 4A, the decreased cell viability of SEC61G-silenced A549 cells was increased by CREB3 overexpression. SEC61G silencing decreased the number of colonies, while CREB3 overexpression partially abolished the inhibitory effects of SEC61G silencing (Fig. 4B).

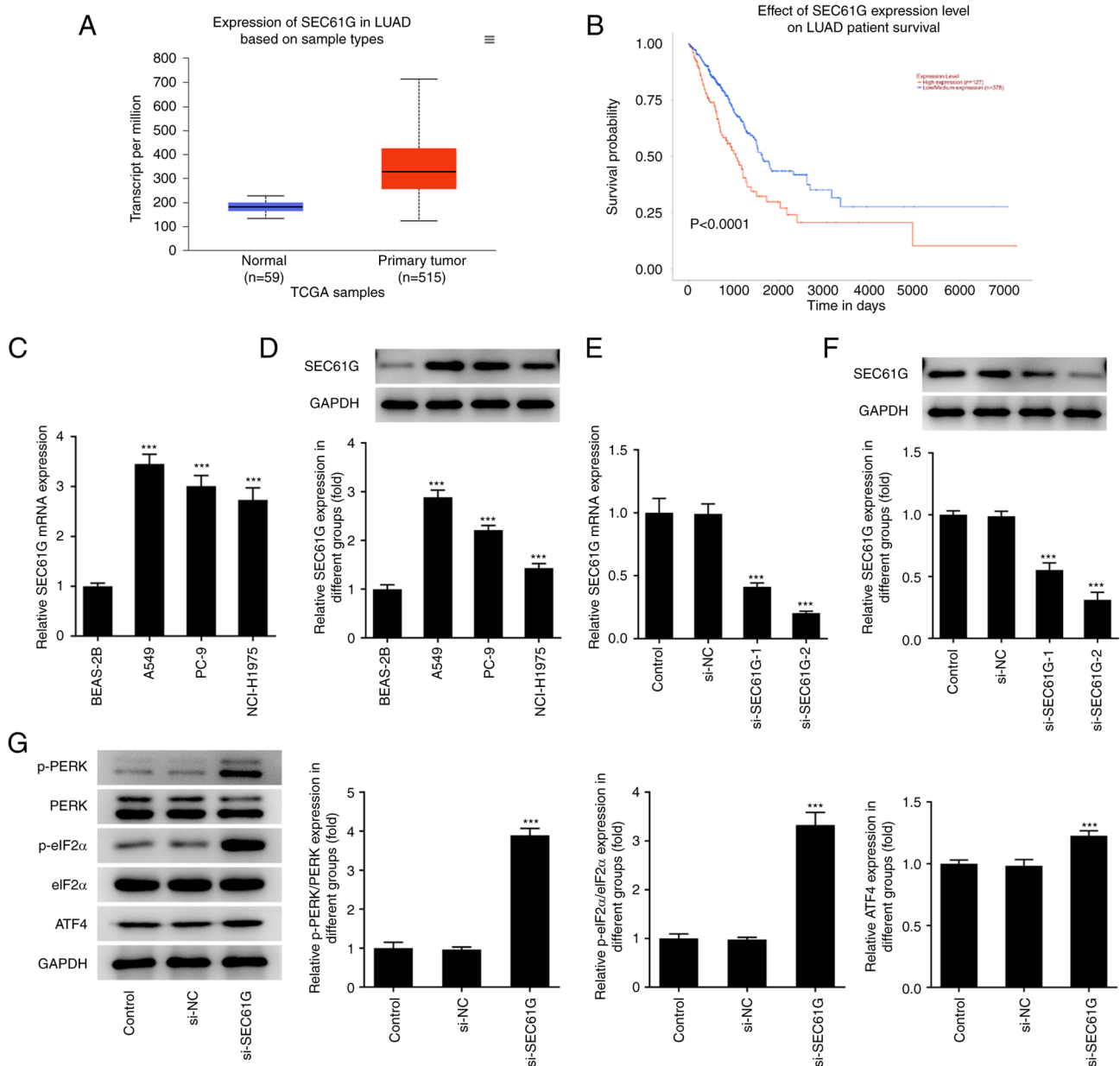


Figure 1. SEC61G is upregulated in lung adenocarcinoma and its downregulation can activate endoplasmic reticulum stress. (A and B) According to GEPIA database, SEC61G was found to be increased in LUAD. mRNA and protein expressions of SEC61G in lung adenocarcinoma cells were measured by (C) RT-qPCR and (D) western blotting, respectively. (E) mRNA and (F) protein expressions of SEC61G were measured by RT-qPCR and western blotting. (G) The expressions of ER stress-related proteins were measured by western blotting. Data are expressed as mean \pm standard deviation. ***P < 0.001 vs. BEAS-2B or si-NC. SEC61G, SEC61 translocon subunit γ ; GEPIA, Gene Expression Profiling Interactive Analysis; LUAD, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; ER, endoplasmic reticulum; si, small interfering; NC, negative control; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; eIF2 α , eukaryotic initiation factor-2 α ; TCGA, The Cancer Genome Atlas.

SEC61G knockdown greatly increased the apoptosis rate of A549 cells compared with that of si-NC. However, CREB3 overexpression reversed the promoting effects of SEC61G silencing on cell apoptosis, as evidenced by the decreased apoptosis rate of si-SEC61G + Oe-CREB3 (Fig. 4C and D). Fig. 4E revealed that the increased expression of Bcl-2 and the decreased expression of Bax and cleaved caspase 3 were reversed by CREB3 overexpression. In addition, the decreased cell migration and invasion rate caused by SEC61G silencing were increased after transfection with Oe-CREB3 plasmids (Fig. 4F and G). Furthermore, the expression levels of MMP9 and MMP2 were markedly increased by CREB3

overexpression in comparison with those of si-SEC61G + Oe-NC (Fig. 4H).

Discussion

As the type of cancer with the highest incidence worldwide, LUAD remains the main cause of cancer-associated mortality globally (13). Effective methods to improve the efficacy of LUAD treatment remain to be explored (14). The present study evaluated the expression of SEC61G and CREB3 in LUAD, as well as their association. It was found that SEC61G was upregulated in A549 cells and its downregulation could

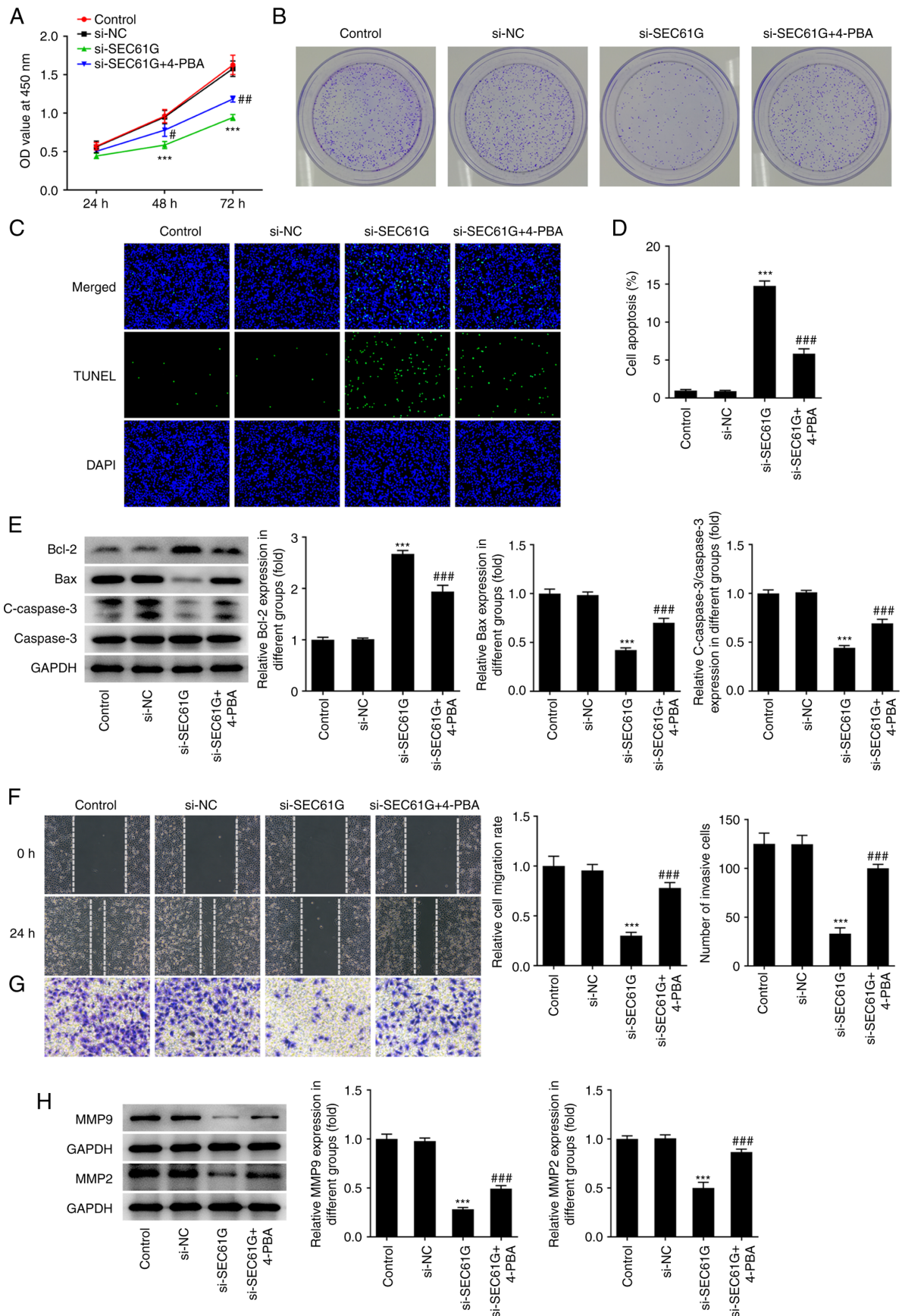


Figure 2. SEC61G silence inhibits malignant development of lung adenocarcinoma via endoplasmic reticulum stress. (A) The cell viability was detected using CCK-8. (B) The colony formation was detected using colony formation assay (magnification, x10). (C and D) The apoptosis was detected using TUNEL (magnification, x200). (E) The expressions of apoptosis-related proteins were measured by western blotting. The (F) migration and (G) invasiveness were evaluated by wound healing and Transwell assays (magnification, x100). (H) The expressions of MMP9 and MMP2 were measured using western blotting. Data are expressed as mean \pm standard deviation. *** P <0.001 vs. si-NC. # P <0.05, ## P <0.01, ### P <0.001 vs. si-SEC61G. SEC61G, SEC61 translocon subunit γ ; si, small interfering; NC, negative control; 4-PBA, 4-phenylbutyric acid; C-, cleaved.

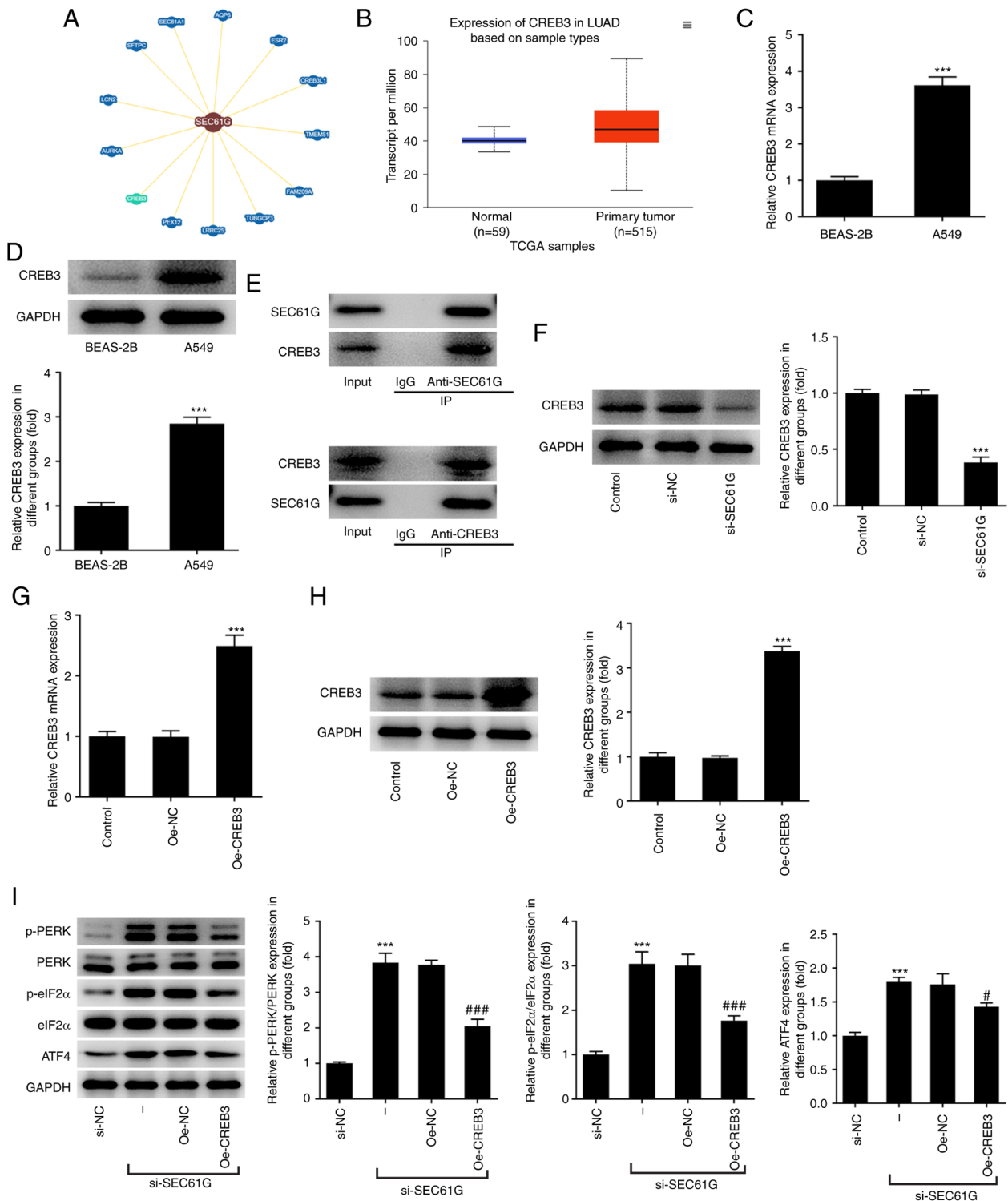


Figure 3. SEC61G participates in endoplasmic reticulum stress via CREB3 in lung adenocarcinoma. (A) According to BioGRID database, SEC61G could interact with CREB3. (B) According to UALCAN database, CREB3 was upregulated in LUAD. (C and D) The mRNA and protein expressions of CREB3 were measured by RT-qPCR and western blotting, respectively. ***P<0.001 vs. BEAS-2B. (E) The binding of SEC61G and CREB3 was verified by Co-immunoprecipitation. (F) The expression of CREB3 was measured by western blotting. ***P<0.001 vs. si-NC. (G and H) The mRNA and protein expressions of CREB3 were measured by RT-qPCR and western blotting, respectively. ***P<0.001 vs. Oe-NC. (I) The expressions of ER stress-related proteins were measured by western blotting. ***P<0.001 vs. si-NC. #P<0.05, ###P<0.001 vs. Oe-NC. Data are expressed as mean \pm standard deviation. SEC61G, SEC61 translocon subunit γ ; CREB3, cyclic AMP-responsive element-binding protein 3; LUAD, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; Oe, overexpression plasmid; TCGA, The Cancer Genome Atlas; p-, phosphorylated; PERK, protein kinase RNA-like ER kinase; eIF2 α , eukaryotic initiation factor-2 α ; ATF4, activating transcription factor 4.

trigger ER stress. SEC61G silencing could suppress LUAD cell viability. Knockdown of SEC61G inhibited colony formation, migration, invasiveness and the expression of

migration-related proteins, while it promoted the apoptosis of A549 cells, which was reversed after treatment with 4-PBA. According to the BioGRID and UALCAN databases,

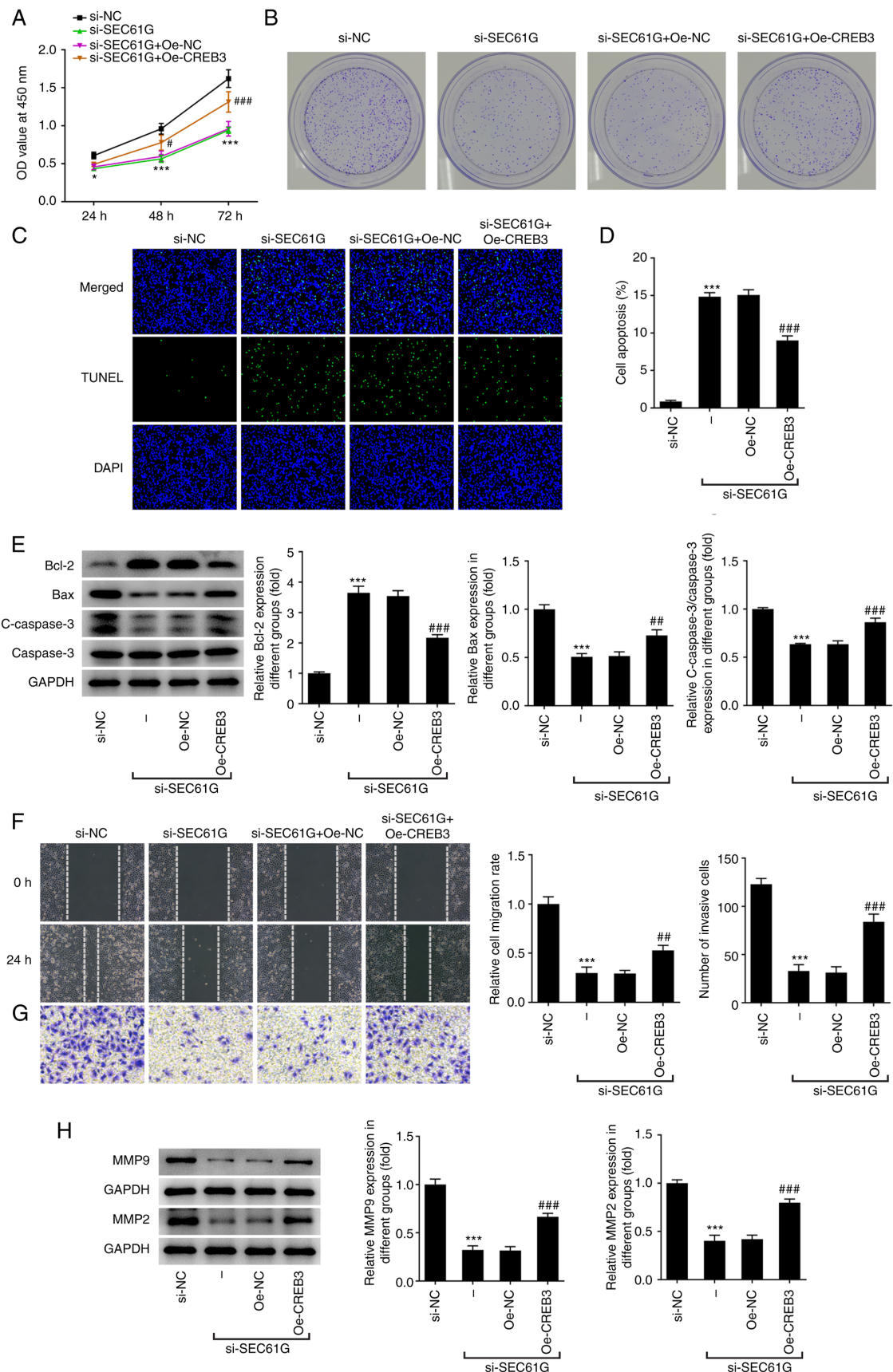


Figure 4. SEC61G knockdown inhibits malignant development of lung adenocarcinoma via CREB3. (A) The cell viability was detected using CCK-8. * $P < 0.05$, *** $P < 0.001$ vs. si-NC. # $P < 0.05$, ### $P < 0.001$ vs. si-SEC61G + Oe-NC. (B) The colony formation was detected using colony formation assay (magnification, $\times 10$). (C and D) The apoptosis was detected using TUNEL (magnification, $\times 200$). *** $P < 0.001$ vs. si-NC. ### $P < 0.001$ vs. Oe-NC. (E) The expressions of apoptosis-related proteins were measured by western blotting. The (F) migration and (G) invasiveness were evaluated by wound healing and Transwell assays (magnification, $\times 100$). (H) The expressions of MMP9 and MMP2 were measured using western blotting. *** $P < 0.001$ vs. si-NC. # $P < 0.01$, ### $P < 0.001$ vs. Oe-NC. Data are expressed as mean \pm standard deviation. SEC61G, SEC61 translocon subunit γ ; CREB3, cyclic AMP-responsive element-binding protein 3; si, small interfering; Oe, overexpression plasmid; NC, negative control; C-, cleaved.

CREB3 was upregulated in LUAD and could interact with SEC61G. CREB3 overexpression partially reversed the effects of SEC61G silencing on the viability, colony formation, apoptosis, migration and invasiveness of A549 cells. The present study revealed that SEC61G acted as an oncogene in the development of LUAD and that silencing SEC61G could suppress LUAD progression via the regulation of CREB3.

Various studies have demonstrated that SEC61G has an abnormal expression in numerous cancer types. Shi *et al* (15) reported that SEC61G expression was increased in head and neck squamous cell carcinoma. It has also been found that SEC61G serves an important role in the progression and prognosis of head and neck squamous cell carcinoma, thus serving as an effective biomarker to predict patient survival (15). In addition, Sheu *et al* (16) report that SEC61G is important in oral squamous cell carcinoma. In the present study, SEC61G expression was detected in A549 cells and it was found that SEC61G levels were increased in LUAD cells. To explore the specific role of SEC61G in LUAD, the expression of SEC61G in A549 cells was silenced. The results indicated that SEC61G knockdown inhibited cell viability, colony formation, migration and invasiveness and promoted apoptosis in A549 cells via ER stress.

As an ER membrane-bound transcription factor (17), CREB3 induces apoptosis by activating ER stress (18). Penney *et al* (19,20) suggest that CREB3 can alter stress sensitivity, thus serving as a potential factor in the development of stress-related pathologies. The present study found that CREB3 level was increased in LUAD cells. According to the BioGRID database, SEC61G could bind to CREB3; therefore, further experiments were conducted to verify this hypothesis. The results revealed that the decreased cell viability, colony formation, migration and invasiveness caused by SEC61G silencing were reversed by CREB3 overexpression, which indicated that SEC61G knockdown exerted inhibitory effects on the malignant progression of LUAD via targeting CREB3. However, the present study only produced the results for SEC61G knockdown and CREB3 overexpression. These experiments of SEC61G overexpression and CREB3 silencing will provide more evidence to support the hypothesis of the present study and will supplement related experiments in further studies.

In conclusion, SEC61G and CREB3 were upregulated in LUAD cells and SEC61G could interact with CREB3. Therefore, SEC61G silencing could inhibit LUAD progression via regulating CREB3.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Natural Science Foundation of China, China (grant no. 82070076).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QZ and ZG designed the research, performed the experiments, drafted and revised the manuscript. QZ searched the literature and analyzed the data. ZG guided the experiments. QZ and ZG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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