

## ORIGINAL RESEARCH

# Chikusetsusaponin Iva induces apoptosis and inhibits proliferation in endometrial cancer *via* promoting reactive oxygen species (ROS) production

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**Abstract**

Endometrial carcinoma (EC) is an epithelial malignant tumor that occurs in the endometrium. It is of great significance to explore new targets for improving the prognosis of endometrial cancer. Chikusetsusaponin Iva (CHI) is a plant compound extracted from *Panax japonica* which has multiple biological activities, including anti-inflammation and anti-tumor effects. However, its possible effects on EC are unclear. Herein, we found that CHI inhibited EC cell proliferation and cell cycle, confirmed by colony formation and flow cytometry (FCM) assays. The results further confirmed that CHI stimulated the apoptosis of EC cells. Furthermore, we noticed that CHI treatment induced reactive oxygen species (ROS) in EC cells. In addition, it could suppress the Microtubule-Associated Protein Kinase (MAPK) pathway in EC cells, thereby affecting cell proliferation and apoptosis. Therefore, CHI could serve as a potential drug for EC treatment.

**Keywords**

Endometrial carcinoma (EC); Chikusetsusaponin Iva (CHI); Proliferation; Reactive oxygen species (ROS) production; MAPK pathway

## 1. Introduction

Endometrial carcinoma (EC) is an epithelial tumor in the endometrium, accounting for about 8% of the malignant tumors in women [1]. With the continuous improvement of living standards, the female obesity rate is increasing, which makes the incidence of EC continue to increase [2, 3]. Patients with early-stage endometrial cancer generally have a good prognosis, with a survival rate of 95%, but patients with late-stage cancer have a poor prognosis, with a lower survival rate [4]. Although the current medical science has been very advanced, the molecular mechanism of the occurrence of EC is not very clear [5]. Further exploration of the pathogenesis of EC and finding new targets for treatment are of vital significance for improving the prognosis of EC [6].

Chikusetsusaponin Iva (CHI), a plant compound extracted from *Panax japonica*, is the main active component of triterpenoid Chikusetsusaponin [7]. CHI is the main active component of triterpenoid saponins, which acts as an activator of protein kinase and has resistance to thrombus and some metabolic diseases. Previous study showed that Chikusetsusaponin Iva of *Panax japonicus* had neuroprotective effects [8]. For example, CHI of *S. panax* reduces isoflurane-induced neurotoxicity and cognitive deficits in developing rats by modulating the Sirtuin 1 (SIRT1)/Extracellular regulated protein kinase (ERK) 1/2 pathway [9]. CHI may reduce the expression of inflammatory genes and the secretion of inflammatory factors by inhibiting

the activation of MAPK and signal transducer and activator of transcription 1 (STAT1), thereby achieving anti-inflammatory effect [9]. CHI alleviates sevofluran-induced neuroinflammation and cognitive impairment [9].

CHI suppressed the progression of multiple types of cancers, including prostate cancer and ovarian cancer [10, 11]. therapy-in addition, CHI triggers apoptosis in human prostate cancer by producing ROS and inhibits the proliferation of tumor cells [12]. CHI induces G1 cell cycle arrest, triggers cell apoptosis and inhibits the motility of ovarian cancer cells [11]. However, the role of Chikusetsusaponin Iva in EC is rarely reported, and the mechanism is unclear.

In this study, we explored the role of CHI in EC progression and found that it triggered ROS accumulation in EC cells by inhibiting p38 MAPK pathway, thereby inhibiting EC cell proliferation and promoting apoptosis. The findings suggested that CHI could serve as a drug for EC treatment.

## 2. Materials and methods

### 2.1 Cell culture and drug treatment

Human EC cell line HEC1B was purchased from Chinese Academy of Sciences. HEC1B cells were cultured with the Roswell Park Memorial Institute (RPMI)-1640 complete medium. After 12 hours of culture, cells were treated with CHI (A51415-02-2, Bought from sigma, St. Louis, MO, USA,

storage concentration: 50 mM) for 24 h at 0, 12.5, 25 and 50  $\mu$ M concentration. Then, the effect of CHI was verified for subsequent experiments.

## 2.2 Western blotting

Radio Immunoprecipitation Assay (RIPA) buffer was used to fully lysate cells to extract protein. The equal amount of proteins from each sample was separated by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), and further transferred onto the polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with TBS+Tween (TBST) containing 5% milk for 1 h, and then the antibodies were added. Primary antibodies including p38 (Abcam, Cambridge, ab32142; 1:1000), p-p38 (Abcam, Cambridge, ab178867; 1:500), ERK (Abcam, Cambridge, ab184699; 1:1000), p-ERK (Abcam, Cambridge, ab201015; 1:1000), JNK (Abcam, Cambridge, ab179461; 1:1000), p-JNK (Abcam, Cambridge, ab307802; 1:1000), Bax (Abcam, Cambridge, ab32503; 1:1000), Bcl-2 (Abcam, Cambridge, ab32124; 1:500), Cleaved caspase 3 (Abcam, Cambridge, ab32042; 1:1000), and  $\beta$ -actin (Abcam, Cambridge, ab8226; 1:3000) were incubated with membranes for overnight at 4 °C, and then secondary antibodies were incubated for 1 h and photographed after chemiluminescence. The reagents used in this were purchased from Wuhan Google Co., LTD.

## 2.3 Cell counting kit-8 (CCK-8) assay

1000 cells/well HEC1B cells were plated in 96-well plated and maintained for 48 h. Cells were subsequently incubated with CCK-8 (C0040, Beyotime, Beijing, China) for 3 h. Then the optical density (OD) 450 value was measured by a microplate reader (BD).

## 2.4 Colony formation assay

HEC1B cells were plated into the 6-well plates (500 cells per well) and maintained in media (10% Fetal bovine serum, FBS) for 14 days at 37 °C. Then cells were fixed for 15 min and stained with 0.1% crystal violet for 20 min.

## 2.5 Cell apoptosis and cell cycle assay

The cells were washed with phosphate buffer saline (PBS). Subsequently cells were fixed with 70% ethanol at -20 °C for 2 h and stained with the apoptosis kit at 4 °C and the apoptosis levels were measured using FACSCalibur flow cytometer and CellQuest Pro 5.1. For cell cycle assays, cells were stained with PI at 4 °C for 20 min and the cell cycle was assessed using FACSCalibur flow cytometer (8.0, BD Biosciences, Inc., Franklin Lake, NJ, USA) and CellQuest Pro 5.1 (8.0, BD Biosciences, Inc., Franklin Lake, NJ, USA).

## 2.6 ROS detection

EC cells were incubated with ROS kit (MAK143, Sigma, St. Louis, MO, USA). The proportion of ROS positive nuclei in 8 random fields was analyzed. The GSH/GSSG ratio was detected in EC cells by the kit (ab138881, Abcam, Cambridge, UK).

## 2.7 Immunostaining

Cells were fixed with 4% paraformaldehyde (PFA) and 0.5% Triton X100, followed by incubation with 2% bovine serum albumin (BSA) for 0.5 h. Cells were then stained with Cleaved caspase-3 antibody (Abcam, UK) at room temperature for 2 h, followed by the incubation of secondary antibody for 2 h. 4',6-diamidino-2-phenylindole (DAPI) staining was performed, and the staining was observed under the microscope.

## 2.8 Statistics

GraphPad 5.0 software (Graphpad, La Jolla, CA, USA) was used and performed. Data were represented as mean  $\pm$  standard deviation (SD).  $p < 0.05$  was considered as statistically significant.

## 3. Results

### 3.1 CHI inhibits EC cell proliferation as well as cell cycle

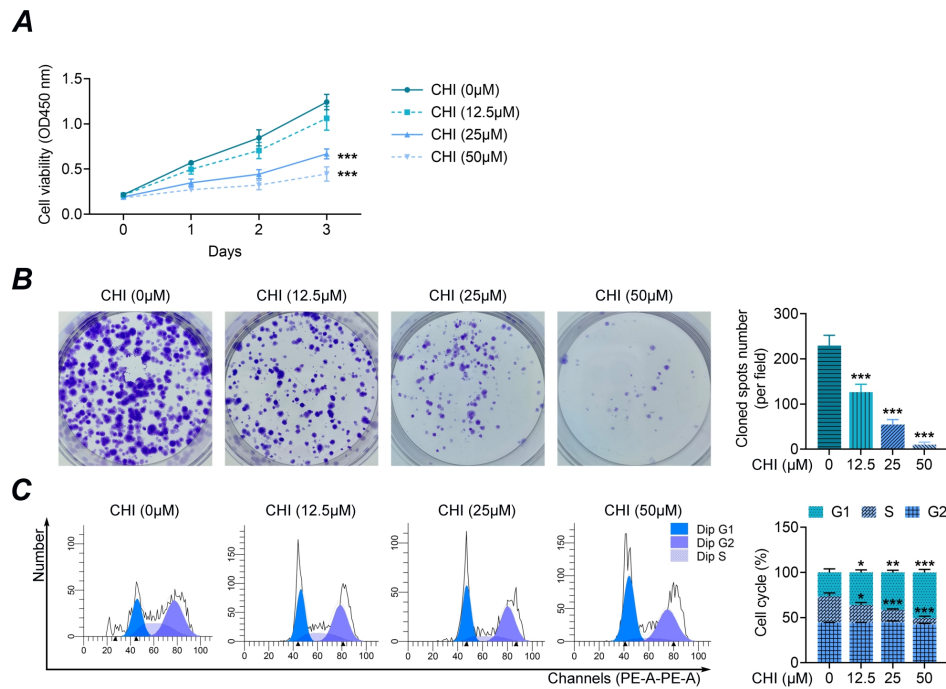
To evaluate the effects of CHI on the EC cell proliferation, we first detected its effects on the viability of HEC1B cells at the concentration of 0, 12.5, 25 and 50  $\mu$ M *via* CCK-8 assays. Interestingly, we noticed that CHI treatment decreased the OD value of the HEC1B cells in a dose-dependent manner (Fig. 1A). We further performed the colony formation assays, and the results showed that CHI treatment decreased the colony numbers at the high concentration of CHI (Fig. 1B). Through FCM assays, we found that CHI treatment induced EC cell cycle arrest at G1 phase (Fig. 1C). Therefore, CHI treatment restrained EC cell proliferation and affected cell cycle.

### 3.2 The treatment of CHI stimulated apoptosis of EC cells

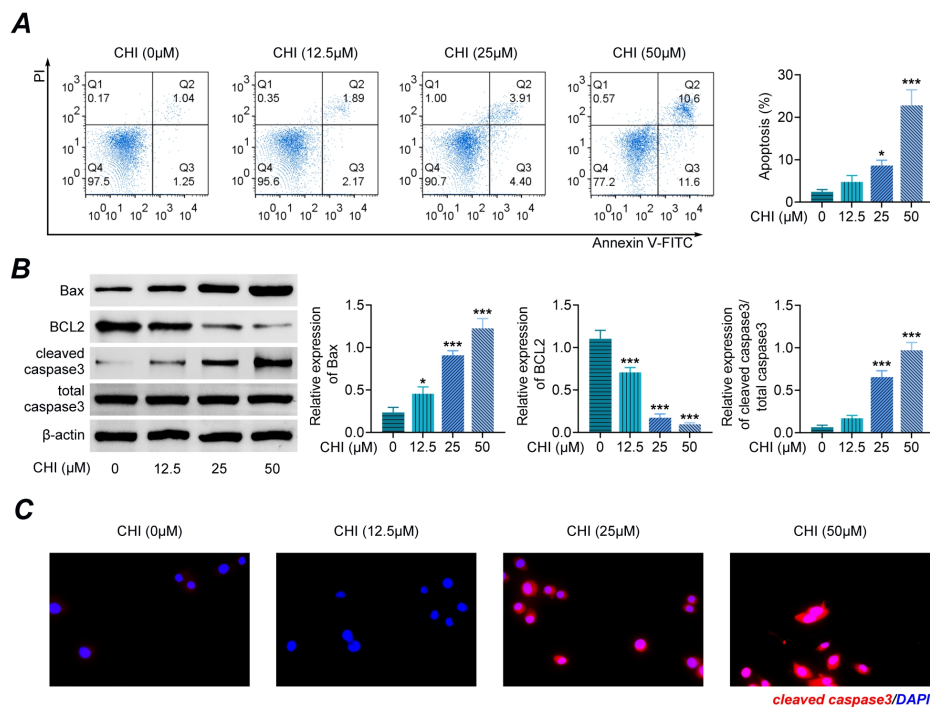
Interestingly, we further performed FCM assays to detect the effects of CHI on the apoptosis of HEC1B cells. CHI treatment stimulated the apoptosis of HEC1B cells in a concentration-dependent manner (Fig. 2A). Immunoblot assays also confirmed the decreased expression of Bcl-2 and increased expression of Bax and cleaved caspase-3 expression upon CHI treatment, suggesting the promoting effects on apoptosis (Fig. 2B). Through Immunostaining assays, we noticed the increased expression of cleaved caspase-3 expression upon CHI treatment (Fig. 2C). Therefore, CHI stimulated apoptosis of EC cells.

### 3.3 CHI treatment promoted the production of ROS in EC cells

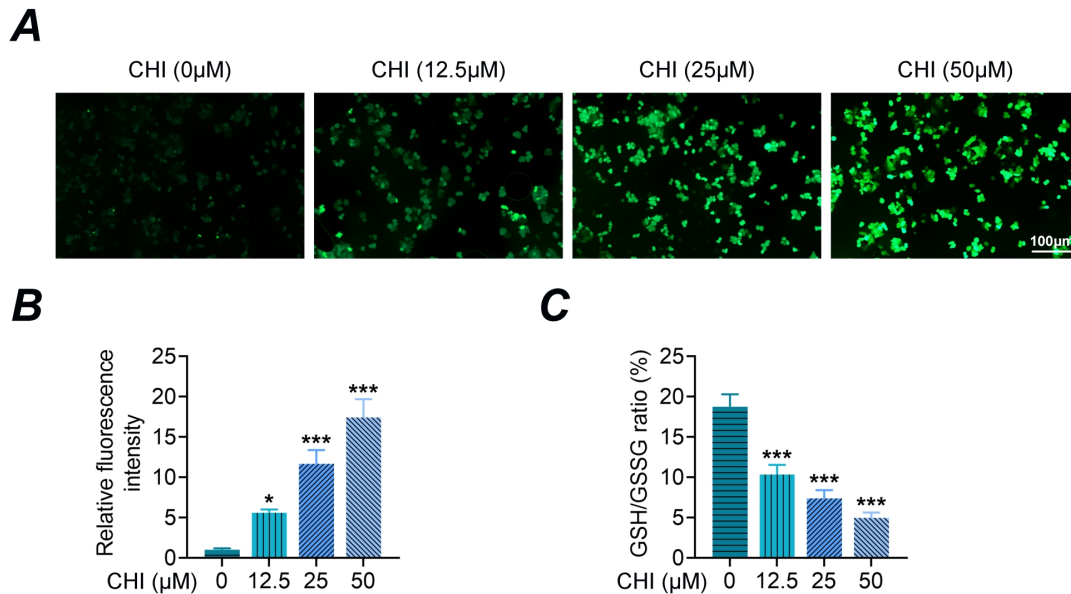
We then performed Immunostaining assays to detect the effect of CHI on the ROS and GSH/GSSG ratio in EC cells, which could affect tumor progression. We found that the addition of CHI in HEC1B cells increased the intracellular ROS generation in a dose-dependent manner (Fig. 3A,B). Through the detection of GSH/GSSG ratio by the kit, we noticed that CHI decreased the ratio in EC cells, suggesting the promoting of ROS (Fig. 3C). Therefore, CHI induced intracellular ROS generation in EC cells.



**FIGURE 1. CHI inhibits EC cell proliferation and cell cycle.** (A) CCK-8 assays showed the effects of CHI on the OD value at 450 nm wavelength at the concentration of 0, 12.5, 25 and 50  $\mu\text{M}$  for 24 h in HEC1B cells. (B) Colony formation assays showed the effects of CHI on the viability of HEC1B cells at the indicated concentration in HEC1B cells. Data were represented as mean  $\pm$  SD.  $***p < 0.001$ . (C) FCM assays showed the effects of CHI on the cell cycle of HEC1B cells in HEC1B cells. Data were represented as mean  $\pm$  SD.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , CHI vs. control. CHI: Chikusetsusaponin Iva; OD: optical density.



**FIGURE 2. The treatment of CHI stimulated the apoptosis in EC cells.** (A) Flow cytometry (FCM) assays showed the effects of CHI on the apoptosis of HEC1B cells in HEC1B cells. The percentage of apoptosis cells are quantified. (B) Immunoblot assays showed the effects of CHI on the expression of indicated proteins in HEC1B cells. (C) Immunostaining showed the expression of cleaved caspase 3 in HEC1B cells upon the indicated treatment. Data were represented as mean  $\pm$  SD.  $**p < 0.01$ ,  $***p < 0.001$ , CHI vs. control. CHI: Chikusetsusaponin Iva. PI: Propidium iodide; V-FITC: V-Fluorescein isothiocyanate isomer; Bcl-2: B-cell lymphoma-2; DAPI: 4',6-diamidino-2-phenylindole.



**FIGURE 3. CHI treatment promoted the production of ROS in EC cells.** (A,B). Immunostaining assays showed the effects of CHI on the ROS production of HEC1B cells in HEC1B cells. The positive stained cells were ROS production cells (A). The staining intensity is quantified (B). (C). The GSH/GSSG ratio in HEC1B cells upon the treatment of indicated drugs for 24 h was measured. Data were represented as mean  $\pm$  SD. \* $p < 0.05$ , \*\*\* $p < 0.001$ , CHI vs. control. CHI: Chikusetsusaponin Iva; GSH: L-Glutathione; GSSG: oxidized glutathione.

### 3.4 CHI treatment suppressed MAPK pathway in EC cells

We lastly detected the effects of CHI on MAPK pathway in HEC1B cells. Through Immunoblot assays, we found that CHI treatment decreased the phosphorylation levels of p38, ERK and JNK in HEC1B cells in a dose-dependent way (Fig. 4). Therefore, CHI suppressed the MAPK pathway in EC cells.

## 4. Discussion

In developed countries, the incidence of endometrial cancer ranks first among gynecological malignancies [13]. In recent years, with the change of people's living habits and diet structure, informal hormone replacement therapy and other factors, the incidence of endometrial cancer has significantly increased, and tends to be younger, becoming a serious reproductive tract malignant diseases that threatens women's health [14]. The pathogenesis of endometrial cancer is still unclear. Currently, the main therapeutic methods include surgical therapy, chemoradiotherapy and drug therapy, but the effects of improving the survival of patients are relatively limited [15]. Therefore, there is an urgent need to explore effective drugs for EC treatment. Here, we noticed that Chikusetsusaponin Iva (CHI), a plant compound extracted from *Panacis japonica*, could affect EC progression.

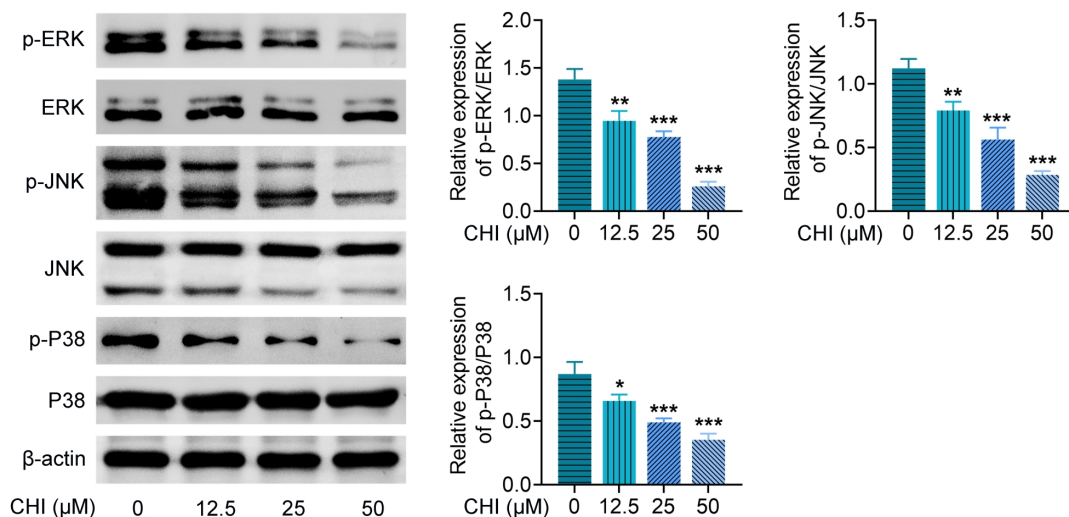
CHI is a glycoside compound whose parent nucleus is triterpenoid, and it is the main active component in *S. japonica* [8]. It has pharmacological activities such as lowering blood glucose, lowering blood lipid and antiviral [8]. Meanwhile, CHI also has a certain anti-inflammatory effect, but its anti-inflammatory molecular mechanism needs to be further explored [9]. CHI can reduce the expression of inflammatory genes and the secretion of inflammatory factors by inhibiting

the activation of MAPK and STAT1, thereby achieving anti-inflammatory effect [7]. In addition, CHI is an interleukin (IL)-6R antagonist, which has good inhibitory activity on IL-6/STAT3 pathway and can effectively inhibit the expression of downstream genes of this pathway [16]. In this study, we found that CHI induced apoptosis and inhibited proliferation of EC, and the mechanism needs further study.

In addition, the anti-tumor activities of CHI have been widely revealed. CHI triggers apoptosis of prostate cancer cells by producing ROS and inhibits the proliferation of tumor cells [12]. CHI also induces G1 cell cycle arrest, and triggers cell apoptosis of ovarian cancer cells [11]. Through FCM and CCK-8 assays, we noticed that CHI affected the apoptosis and proliferation of EC cells through the MAPK pathway, which was consistent with the previous study. Next, we will clarify its possible effects on tumor growth of EC in mice.

Moreover, our study also revealed that CHI treatment decreased the phosphorylation levels of p38, ERK and JNK in HEC1B cells. The p38, ERK and JNK are three key regulators in the MAPK pathway, which could be phosphorylated to affect this pathway [17, 18]. Therefore, our results suggested that CHI mediated MAPK pathway, thereby affecting EC progression. The Pten/PI3K pathway is key in EC progression, and we next need to detect whether CHI could affect this pathway.

The balance between ROS production and ROS clearance is critical [19]. It is important to note that ROS may play a dual role in cancer biology [19]. At high concentrations, they induce programmed cell apoptosis and necrosis [20]. Previous studies have indicated that excessive accumulation of ROS in EC cells can induce autophagy, leading to apoptosis and death of EC cells [21]. Notably, the levels of ROS are closely linked to tissue homeostasis, so the possibility of off-target



**FIGURE 4. CHI treatment suppressed MAPK pathway in EC cells.** Immunoblot assays showed the effects of CHI on the expression of indicated proteins in HEC1B cells. Data were represented as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , CHI vs. control. CHI: Chikusetsusaponin Iva; ERK: extracellular regulating kinase; JNK: c-Jun N-terminal kinase.

effects should be noticed in the next study. The regulation of ROS level plays important role in tumor therapy. Through the detection of ROS in EC cells, we noticed that CHI triggered apoptosis in EC cells by producing ROS.

## 5. Conclusions

In summary, our results demonstrated that CHI triggers ROS accumulation in EC cells, thus inhibiting the proliferation of EC cells and promoting apoptosis through inhibiting the p38 MAPK pathway. Our results confirmed that CHI has the potential to be explored as a promising drug for the treatment of EC.

## AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

## AUTHOR CONTRIBUTIONS

LLX—designed the study and carried them out; LLX, FH, CHL and XPL—supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors. Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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