RETRACTED ARTICLE: DGUOK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY

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ABSTRACT

Background: Cervical cancer is identified as one of the most familiar female malignancies. And a number of studies have elucidated that long non-coding RNAs (lncRNAs) participated in the initiation and progress of incurable tumors, so lncRNAs have attracted more and more attention from researchers. The purpose of the study is to figure out the role and molecular regulation mechanism of DGUOK-AS1 in cervical cancer.

Methods: The role of DGUOK-AS1 in cervical cancer was validated by CCK-8, colony formation, EdU, flow cytometry and western blot assays. The interactions among DGUOK-AS1, miR-653-5p and EMSY were confirmed by qRT-PCR, western blot, RNA pull down, RIP and luciferase reporter assays.

Results: The expression of DGUOK-AS1 is significantly high in cervical cancer cells. Further, shortage of DGUOK-AS1 repressed cell proliferation and stimulated cell apoptosis in cervical cancer. Then miR-653-5p, which low expressed in cervical cancer cells, was found to be sponged by DGUOK-AS1. Similarly, miR-653-5p mimics inhibited cervical cancer proliferation. Moreover, EMSY served as the target gene of miR-653-5p, and DGUOK-AS1 could regulate EMSY expression via sponging miR-653-5p. Besides, EMSY possessed high obviously high expression in cervical cancer cells. Final rescuing assays illustrated that both pcDNA3.1/EMSY and miR-653-5p inhibitor could remedy the suppressive role of DGUOK-AS1 down-regulation in cervical cancer cell proliferation.

Conclusions: DGUOK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY, which may be helpful to make a deep research about the development of cervical cancer.

Introduction

Cervical cancer belongs to a malignancy, and its prevalence ranks only second to that of breast cancer. About 469,000 newly diagnostic cases and 232,000 patients died from cervical cancer annually all over the world. Moreover, around 90.1% of which occurred in underdeveloped countries and patients become younger in average age trend gradually. In China, about 149,000 newly diagnosed cases every year, which constitutes a severe threat to women’s health. With the popularity of cervical cancer prevention and treatment knowledge, the incidence of cervical cancer in developed areas has dropped significantly. Whereas its incidence rate keeps high in developing and under-developed countries owing to the shortage of or irregular cancer screenings. For the past few years, in spite of much advancement in therapy ways and techniques, the patients with medium or advanced cervical cancer still receive a poor prognosis. Hence, it is extremely imperative for us to find out effective molecular markers to screen out cervical cancer in early stage.

Long non-coding RNAs (lncRNAs) possessing over 200 nucleotides are considered as non-coding RNAs. The features of lncRNAs include structural complexity and expression-regulation diversity. Studies have demonstrated that lncRNAs exert vital roles, like regulating protein function, genome imprinting, transcription as well as post-transcriptional regulation [11]. Previous studies have illustrated that lncRNAs can act as a competitive endogenous RNAs (ceRNAs) to bind with microRNAs (miRNAs) and reverse the inhibited or degraded function caused by miRNAs on target genes, thus modulating the messenger RNAs (mRNAs) and protein level of target genes. For illustration, IncRNA 00152 palys the role of a ceRNA and regulates the expression of PTEN via cooperating with miR-197-5p to release E2 F1. LncRNA HULC expedites liver cancer via modulating the expression of PTEN via cooperating with miR-361. The considerable biological functions of lncRNAs have been explored stage by stage, however, the regulation mechanisms of them in cervical cancer keep vague.

Further, DGUOK-AS1 belongs to a fresh and underexplored IncRNA in cancer studies, besides, we obtained that the expression of DGUOK-AS1 is obviously upregulated in CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma) tissues than corresponding non-tumor tissues according to TCGA public database. Therefore, we aimed to research the molecular mechanism of DGUOK-AS1 in cervical cancer, and our findings might assist in open new chapter about researching DGUOK-AS1 in cervical cancer.

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Materials and methods

Cell culture

Normal human keratinocyte (HaCaT) was brought from Cell lines service (CLS) and cervical cancer cells (CaSki, SiHa, HeLa, C33A and MS751) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Under humidity containing 5% CO₂, cells were cultivated at 37°C in RPMI 1640 medium (Gibco, Grand Island, NY, USA) with additional 1% penicillin/streptomycin (Gibco) and 10% FBS (Gibco).

Cell transfection

Specific shRNAs against DGUOK-AS1 (sh-DGUOK-AS1#1/2) and their corresponding NC (sh-NC), along with pcDNA3.1/DGUOK-AS1, pcDNA3.1/EMSY and the empty vector, were obtained from Genechem (Shanghai, China). Simultaneously, miR-653-5p mimics, miR-499a-5p mimics and their corresponding NC mimics, miR-653-5p inhibitors and NC inhibitors were obtained from GenePharma (Shanghai, China). Plasmids were transfected into HeLa or C33A cells by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extracted applying Trizol reagent (Invitrogen) was reverse-transcribed through TransScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). Later, qRT-PCR was implemented by use of TransScript Top Green qPCR SuperMix (TransGen) with 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Results were analyzed using 2⁻ΔΔCt. GAPDH and U6 were separately used to normalize the genes’ expression.

Cell-counting kit 8 (CCK-8) assay

Transfected HeLa or C33A cells were inoculated into 96-well plates (2 × 10⁴ cells/well), and each well was incubated for 1 h with 10 μL CCK-8 solution (Dojindo, Kumamoto, Japan) before detection. A450 values were read using Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Colony formation assay

Transfected HeLa or C33A cells were planted into 6-well plates (600 cells/well), followed by being cultured for 14 days. Subsequently, cells were fixed applying methanol (Sigma-Aldrich, St. Louis, MO, USA) and dyed using 0.1% crystal violet (Sigma-Aldrich). Colony numbers were counted.

EdU assay

Transfected HeLa or C33A cells were incubated for 2 h by adopting 50 μM EdU (RiboBio, Nanjing, China), and sequentially dyed in Apollo (RiboBio) and DAPI (Invitrogen). The number of EdU-positive cells was explored with a fluorescence microscopy (Nikon, Tokyo, Japan).

Flow cytometry

Apoptotic cell rate was measured and analyzed using the Annexin-V and propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich). Transfected HeLa or C33A cells were harvested and re-suspended in binding buffer (Invitrogen) after being washed with ice-cold PBS (Sigma-Aldrich). In the dark, cells were subjected to Annexin V–FITC (5 μl) and propidium iodide (PI; 10 μl) for 15 min. Prepared cellular samples were explored using the CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA).

Western blot

Transfected HeLa or C33A cells washed using cold PBS were lysed via RIPA buffer (Sigma-Aldrich) for protein extraction. Following assessment of protein concentration applying the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), protein was divided through SDS-PAGE gel (Bio-Rad, Hercules, CA, USA), after which they were blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Upon this, membranes were blocked by 5% nonfat milk powder, followed by being incubated with primary antibodies against Bcl-2 (ab32124, Abcam, Cambridge, MA, USA), Bax (ab32503, Abcam), Cleaved caspase-3 (ab2302, Abcam), Cleaved caspase-6 (ab2326, Abcam), Total-caspase-6 (ab232539, Abcam), Cleaved caspase-9 (ab2323, Abcam), Total-caspase-9 (ab232539, Abcam), Cleaved PARP (AB3620, Sigma-Aldrich), Total-PARP (ab74290, Abcam), EMSY (ab123, Abcam), PPP1 CC (SAB1406283, Sigma-Aldrich) and GAPDH (ab9485, Abcam). After being rinsed by TBS-Tween 20 buffer (Sigma-Aldrich), membranes were further incubated with secondary antibody. At last, protein blots were examined by use of ECL (Beyotime, Shanghai, China) and quantified via Image Lab software (Bio-Rad).

Subcellular fractionation

Isolation of RNAs from cytoplasm or nucleus of HeLa or C33A cells was conducted by using the Cytoplasmic and Nuclear RNA Purification Kit (Norgenbiotek, Thorold, ON, Canada). Cytoplasmic and nuclear fractions were assayed via qRT-PCR.

Fluorescence in situ hybridization (FISH)

Cy3-labeled DGUOK-AS1 probe was synthesized (RiboBio) and signal was investigated using the Fluorescence in Situ Hybridization Kit (Exonbio Lab, Guangzhou, China). Hoechst (Invitrogen) was applied for staining the nucleus. Photos were captured by a confocal microscope (Olympus, Tokyo, Japan).

RNA pull-down

Cell lysates of HeLa or C33A cells were incubated with Biotin-DGUOK-AS1 sense/antisense. For further incubation, magnetic beads (Invitrogen) were added. MiR-653-5p or miR-499a-5p level was attested through conducting qRT-PCR.
Luciferase reporter assay

The wild-type (WT) or mutant (Mut) interacting sequences of miR-653-5p or miR-499a-5p within DGUOK-AS1 sequence were sub-cloned into pmirGLO luciferase vector (Promega, Madison, WI, USA) to establish pmirGLO-DGUOK-AS1-Wt/Mut vectors, which were then co-transfected into HeLa or C33A cells along with miR-653-5p mimics, miR-499a-5p mimics or NC mimics. Besides, the vectors pmirGLO-EMSY 3’UTR-Wt/Mut were formed and transfected with specific transfection plasmids. Dual luciferase reporter assay system (Promega) was applied so that the luciferase activities were examined.

RNA immunoprecipitation (RIP)

RIP was conducted as the guide of Imprint RNA Immunoprecipitation kit (Sigma-Aldrich) requested. At 4°C, protein Magnetic beads were mingled for 4 h with anti-Ago2 (Millipore) or anti-IgG (Millipore). The complex was added to cell lysates for immunoprecipitation. Enrichments of DGUOK-AS1, miR-653-5p and EMSY were determined using qRT-PCR.

Statistical analysis

Student’s t-test (two groups) or one-way ANOVA (more than two groups) was utilized for comparing difference in groups. Data were analyzed statistically by Prism 5 (GraphPad, La Jolla, CA, USA), expressed as mean ± SD. P < .05 was considered statistically significant, and all assays were run in triplicate.

Results

DGUOK-AS1 knockdown restrains cell proliferation in cervical cancer

To affirm the role of DGUOK-AS1 in cervical cancer, according to TCGA database (http://gepia.cancer-pku.cn/index.html), the expression of DGUOK-AS1 was markedly upregulated in CESC tissues compared with normal controls (Figure 1a). Then qRT-PCR measured that DGUOK-AS1 was more expressed in cervical cancer cells (CaSkI, SiHa, HeLa, C-33A and MS751) than that in epidermal cell (HaCaT), besides, DGUOK-AS1 expressed the most obviously in HeLa and C-33A cells (Figure 1b). Therefore, sh-NC or sh-DGUOK-AS1#1/#2 was transfected into HeLa and C-33A cells so that the expression of DGUOK-AS1 was silenced (Figure 1c). Then followed the functional assays, CCK-8 tested that DGUOK-AS1 depletion decreased the optical density (OD) value of HeLa and C-33A cells (Figure 1d), colony formation assay detected that DGUOK-AS1 absence reduced the colony number in HeLa and C-33A cells (Figure 1e), and EdU assay measured that DGUOK-AS1 deficiency lessened positive stained cell percent of HeLa and C-33A cells (Figure 1f). The three assays verified that DGUOK-AS1 knockdown impeded cervical cancer cell proliferation. With respect to cell apoptosis, flow cytometry illustrated that DGUOK-AS1 insufficiency promoted HeLa and C-33A cell apoptosis (Figure 1g). Meanwhile, western blotting testified the level of apoptosis-related protein (Bcl-2, Bax, caspase-3, caspase-6, caspase-9 and PARP), and that of Bcl-2 was decreased, but that of other proteins were raised in sh-DGUOK-AS1 transfected cells (Figure 1h). Above findings attested that DGUOK-AS1 knockdown restrains cell proliferation in cervical cancer.

DGUOK-AS1 could bind with miR-653-5p in cervical cancer

To further comprehend the molecular mechanism of DGUOK-AS1 in cervical cancer, first of all, subcellular fraction and FISH assays confirmed that DGUOK-AS1 mainly distributed in cytoplasm (Figure 2A and 2B), which was further validated through bioinformatic analysis (http://starbase.sysu.edu.cn/bioinf/Inclocator/) (Figure 2c). Afterwards, the two microRNAs (miRNAs), miR-653-5p and miR-499a-5p, that possibly bind with DGUOK-AS1 were obtained by starBase (http://starbase.sysu.edu.cn/index.php). Subsequently, qRT-PCR detection showed that sh-DGUOK-AS1 stimulated the expression of miR-653-5p, but there were no changes in that of miR-499a-5p (Figure 2d). And RNA pull down assay detected the overtly enrichment RNA of miR-653-5p in Biotin-DGUOK-AS1 groups, in a similar way, there was little existence of miR-653-5p RNA enrichment (Figure 2e). As shown in figure 2f, starBase predicted the binding sequences between DGUOK-AS1 and miR-653-5p or miR-499a-5p, in addition, the overexpression efficiency of miR-653-5p/miR-499a-5p were measured and identified (Figure 2g). And luciferase reporter assay found that the luciferase activity of DGUOK-AS1-WT was visibly inhibited by miR-653-5p mimics, and miR-499a-5p mimics didn’t affect that of DGUOK-AS1-WT at all. Additionally, there were no changes in the luciferase activity of DGUOK-AS1-WT under any condition (Figure 2h). What’s more, qRT-PCR determined that miR-653-5p exhibited lower expression in cervical cancer cells than that of HaCaT cell (Figure 2i). Then cell proliferation assays proved the repressive effect of miR-653-5p mimics on cell proliferation (Figure 2j–l). In the meantime, flow cytometry and western blotting ensured that cervical cancer cell apoptosis was accelerated by overexpressing miR-653-5p (Figure 2m, Figure 2n). In a summary, DGUOK-AS1 could exert as a sponge of miR-653-5p which acted as an anti-tumor regulator in cervical cancer.

DGUOK-AS1 could regulate EMSY via sponging miR-653-5p

Based on above findings about the role of miR-653-5p in cervical cancer, the messenger RNA (mRNA) that was able to bind with miR-653-5p needed to be explored. By employing PIPA, miRanda, TargetScan and miRmap databases, two qualified mRNAs, EMSY and PPP1CC, were screened out (Figure 2c). Afterward, the two microRNAs (miRNAs), miR-653-5p and miR-499a-5p, that possibly bind with DGUOK-AS1 were obtained by starBase (http://starbase.sysu.edu.cn/index.php). Subsequently, qRT-PCR detection showed that sh-DGUOK-AS1 stimulated the expression of miR-653-5p, but there were no changes in that of miR-499a-5p (Figure 2d). And RNA pull down assay detected the overtly enrichment RNA of miR-653-5p in Biotin-DGUOK-AS1 groups, in a similar way, there was little existence of miR-653-5p RNA enrichment (Figure 2e). As shown in figure 2f, starBase predicted the binding sequences between DGUOK-AS1 and miR-653-5p or miR-499a-5p, in addition, the overexpression efficiency of miR-653-5p/miR-499a-5p were measured and identified (Figure 2g). And luciferase reporter assay found that the luciferase activity of DGUOK-AS1-WT was visibly inhibited by miR-653-5p mimics, and miR-499a-5p mimics didn’t affect that of DGUOK-AS1-WT at all. Additionally, there were no changes in the luciferase activity of DGUOK-AS1-WT under any condition (Figure 2h). What’s more, qRT-PCR determined that miR-653-5p exhibited lower expression in cervical cancer cells than that of HaCaT cell (Figure 2i). Then cell proliferation assays proved the repressive effect of miR-653-5p mimics on cell proliferation (Figure 2j–l). In the meantime, flow cytometry and western blotting ensured that cervical cancer cell apoptosis was accelerated by overexpressing miR-653-5p (Figure 2m, Figure 2n). In a summary, DGUOK-AS1 could exert as a sponge of miR-653-5p which acted as an anti-tumor regulator in cervical cancer.

DGUOK-AS1 could regulate EMSY via sponging miR-653-5p
efficiency of GUOK-AS1 was measured in pcDNA3.1/DGUOK-AS1 transfected cells (Figure 3f). When performing luciferase reporter assay, DGUOK-AS1 upregulation counteracted the obstructive effects of miR-653-5p mimics on the luciferase activity of EMSY 3’UTR-WT. In the same way, no changes occurred in luciferase activity of EMSY 3’UTR-Mut (Figure 3g). Finally, the RNA expression and protein level of EMSY was found to upregulated in cervical cancer cells.
Figure 2. DGUOK-AS1 could bind with miR-653-5p in cervical cancer. (a) Subcellular fraction assay measure the distribution of DGUOK-AS1 in nucleus and cytoplasm. (b, c) FISH assay and bioinformatic analysis (http://www.csbio.sjtu.edu.cn/bioinf/LncLocator/) confirmed that DGUOK-AS1 mainly located in cytoplasm. GAPDH or U6 was an internal control. (d) qRT-PCR detected the expression of miR-653-5p and miR-499a-5p. (e) RNA pull down detected the enrichment of miR-653-5p and miR-499a-5p in biotin-DGUOK-AS1 group. (f) The binding sites of miR-653-5p/miR-499a-5p and DGUOK-AS1 were predicted by utilizing starBase. (g) qRT-PCR detected the expression of miR-653-5p and miR-499a-5p. (h) Luciferase reporter assay measured that the luciferase activity of DGUOK-AS1-WT and DGUOK-AS1-Mut. (i) qRT-PCR detected the expression of miR-653-5p. (j–l) CCK-8, colony formation and EdU assays measured the proliferation in HeLa and C-33A cells. (m) Flow cytometry assay tested the apoptosis in HeLa and C-33A cells. (n) Western blot assay measured the level of apoptosis-related proteins in HeLa and C-33A cells. Error bars represent the mean ± SD of at least three independent experiments. **P < .01.
DGUOK-AS1 could regulate EMSY via sponging miR-653-5p. (a) EMSY and PPP1CC that may bind with miR-653-5p were screened out through PIPA, miRanda, TargetScan and miRmap databases. (b,c) qRT-PCR and western blot assays measured the expression and protein level of EMSY and PPP1CC. (d) RIP assay detected the relative enrichment of DGUOK-AS1, miR-653-5p and EMSY. (e) The binding sites of miR-653-5p and EMSY were predicted by utilizing starBase. (f) qRT-PCR detected the expression of DGUOK-AS1. (g) Luciferase reporter assay measured that the luciferase activity of EMSY 3′UTR-WT and EMSY 3′UTR-Mut. (h) qRT-PCR and western blot assays measured the expression and protein level of EMSY. GAPDH was an internal control. Error bars represent the mean ± SD of at least three independent experiments. **P < .01.
Figure 4. DGUOK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY. (a,b) qRT-PCR detected the expression of miR-653-5p and EMSY. (c–e) CCK-8, colony formation and EdU assays measured the proliferation in HeLa and C-33A cells. (f) Flow cytometry assay tested the apoptosis in HeLa and C-33A cells. (g) Western blot assay measured the level of apoptosis-related proteins in HeLa and C-33A cells. GAPDH was an internal control. Error bars represent the mean ± SD of at least three independent experiments. **P < .01.
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DGUK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY

To clearly elucidate the regulation mechanism of competing endogenous RNA (ceRNA) in cervical cancer, rescuing assays were carried out. As displayed in Figure 4a,b, miR-653-5p expression was downregulated in miR-653-5p inhibitor transfected groups, and EMSY expression was escalated in pcDNA3.1/EMSY transfected groups. After that, on one hand, CCK-8, colony formation and EdU assays demonstrated that both miR-653-5p inhibitor and pcDNA3.1/EMSY could reversed the impetuous function caused by DGUK-AS1#1 depletion to cervical cancer cell proliferation (Figure 4c–Figure 4e). On the other hand, flow cytometry and western blotting gave the evidence that miR-653-5p inhibitor and pcDNA3.1/EMSY could remedy the stimulating effects of DGUK-AS1#1 silencing on cell apoptosis (Figure 4f,g). As a whole, DGUK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY.

Discussion

The true pathogenesis of cervical cancer remains elusive by researchers. Although numerous molecular medical studies have explained its pathogenesis, the effective targets for cervical cancer are indistinguishable, leading to inaccurate treatment strategies for clinicians. Epigenetic regulation of cervical cancer may uncover this puzzle and make it public.

In multiple cancers, the aberrant expression of long-non coding RNAs (lncRNAs) possesses referential significance for finding effective treatment targets for cancers. For illustration, lncRNA RHPN1-AS1 functions as a negative regulator in head and neck squamous cell carcinoma by hampering cell migration and proliferation. LncRNA LINC01089 serves as the mark of colorectal cancer cell proliferation, and migartion via Wnt/β-catenin signaling pathway.10 And lncRNA Malat1 stimulated autophagy to pro

MiRNA is getting more and more attention by people in cancer studies. And its regulation role in cancer, including cervical cancer, development has been reported in numerous researches. For example, miR-3154, miR-7-3, and miR-600 act as potential biomarkers for cervical cancer prognosis.15 MiRNA-433 interferes with cervical cancer development via targeting metadherin and regulating the AKT/β-catenin pathways.16 And miRNA-874 is low expressed in cervical cancer and hampers cancer through targeting ETS1.17 Furthermore, the inhibiting role of miR-653-5p has been validated in non-small cell lung cancer. And neuroblastoma.19 However, its role in cervical cancer still was a mystery. In our research, firstly, we confirmed that DGUK-AS1 mainly distributed in cytoplasm, and then validated that DGUK-AS1 could bind with miR-653-5p. Moreover, the expression of miR-653-5p was downregulated in cervical cancer cells when comparing that in HaCaT cell. Besides, overexpression of miR-653-5p suppressed cell proliferation but motivated cell apoptosis in cervical cancer, which demonstrated that miR-653-5p served as a tumor suppressor gene in cervical cancer.

In general, the target gene, messenger RNA (mRNA) does the actual work in cells. For example, lncRNA SNHG1 accelerates non-small cell lung cancer via upregulating the expression MTDH and inhibiting the expression of miR-145-5p. LncRNA PCAT6 serves as an oncogene in non-small-cell lung cancer via binding with EZH2 and downregulating LATS2. And lncRNA AWPPH activates hepatocellular carcinoma progression through YBX1 and is considered as a prognosis biomarker. In this research, by utilizing PIPA, miRanda, TargetScan and miRmap databases, EMSY and PPP1CC were screened out, nonetheless, on one hand, the expression of PPP1CC wasn’t affected by miR-653-5p mimics or sh-DGUK-AS1 at all, on the other hand, that of EMSY was obviously inhibited. What’s more, it confirmed that EMSY exerted cancer-promoting function in breast cancer23 and ovarian cancer. Therefore, EMSY was selected to carry out following assays. And EMSY was high expressed in cervical cancer cells and validated as the target gene of miR-653-5p, furthermore, DGUK-AS1 could upregulate EMSY through sequestration of miR-653-5p. Finally, rescuing experiments verified that miR-653-5p insufficiency or EMSY upregulation could neutralize the interruptive effects of DGUK-AS1 silencing on cell proliferation and stimulating effects of that on cell apoptosis.

To be concluded, DGUK-AS1 promotes the proliferation cervical cancer through regulating miR-653-5p/EMSY, which may help to bring new ideas for cervical cancer etiology research.

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Disclosure of potential conflicts of interest

Authors declare no conflicts of interest in this study.
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