Analysis of the epidermal growth factor receptor/phosphoinositide-dependent protein kinase-1 axis in tumor of the external auditory canal in response to epidermal growth factor stimulation

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Abstract
Objectives: The epidermal growth factor receptor (EGFR) is related to the invasion and metastasis of external auditory canal (EAC) squamous cell carcinoma (SCC). The phosphoinositide-dependent protein kinase-1 (PDPK1) accelerates tumor cell growth through anti-apoptotic signaling under the influence of downstream EGFR-mediated signaling pathways. In this study, we investigated the EGFR/PDPK1 axis in the EAC under EGF stimulation.

Methods: We confirmed EGFR and PDPK1 expression in human EACSCC specimens immunohistochemically. We next transfected the EGF expression vector in the mouse EAC and then conducted a PDPK1 inhibitory experiment. Immunohistochemical analysis was performed in the mouse EAC, using anti-EGF, anti-EGFR, anti-PDPK1, and anti-Ki67 antibodies. Immunohistochemical analysis of cleaved caspase-3 and terminal deoxy(d)-UTP nick end labeling (TUNEL) detection assays were also performed for the assessment of apoptosis in the inhibitory experiment.

Results: Immunohistochemical analysis revealed overexpression and colocalization of EGFR and PDPK1 in human EACSCC specimens. The growth of a protuberant tumor was observed in the mouse EAC in which EGF expression vector was transfected, and EGFR, EGFR, PDPK1, and Ki67 labeling indexes (LIs) were significantly increased. PDPK1 inhibition then induced normal epithelial appearance in the EAC. Moreover, EGF, EGFR, PDPK1, and Ki67 LIs were decreased, and cleaved caspase-3 and TUNEL LIs were increased in the EAC.

Conclusion: We demonstrated the possibility that PDPK1 plays an important role in EACSCC.
Level of Evidence: NA.

Keywords
EGFR, external auditory canal squamous cell carcinoma, PDPK1
1 | INTRODUCTION

Carcinoma of the external auditory canal (EAC) is a rare disease, representing 1–5 cases per million people per year. Squamous cell carcinoma (SCC) is the most common histologic type of this disease. The pathogenesis of EACSCC is not fully understood because of the rarity of this disease. EACSCC is locally aggressive due to the anatomical features of the temporal bone which facilitates tumor spread via microscopic diffusion through the bony canals as well as nerve and intraosseous vessels. Surgical resection with or without adjuvant radiotherapy is the main strategy for EACSCC. Although the prognosis for patients has been improved by advances in surgery and multidrug chemoradiotherapy, it is still poor in the advanced stages.

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases and is activated following its binding with peptide growth factors of the EGF family. Activated EGFR is involved in the pathogenesis and progression of EACSCC, and is the same as in head and neck SCC (HNSCC). Several anti-EGFR therapeutic strategies have been developed in HNSCC, targeting the extracellular or intracellular domain of the EGFR. However, the efficacy of these drugs is still limited because of intrinsic and acquired drug resistance, which is mainly due to tumor heterogeneity and genetic instability. In addition to the direct activation of EGFR by ligands, EGFR can be transactivated by G protein-coupled receptors mediated by phosphoinositide 3-kinase (PI3K), and activating alterations to the PI3K-signaling pathway are observed in HNSCC. Recently, Basura et al. screened gene mutation profiles of EACSCC and reported recurrent alterations of EGFR and PI3K. Phosphoinositide-dependent protein kinase (PDPK1) is a downstream target of PI3K and plays a critical role in the tumor cell migration and invasion of many cancer cells, including HNSCC cells, and is expected to be a new therapeutic target for HNSCC.

In this study, we demonstrated the immunohistochemistry of EGFR and PDPK1 in human EACSCC specimens. We then transfected the EGF expression vector in the mouse EAC and analyzed the EGFR/PDPK1 axis immunohistochemically. We also conducted inhibitory experiments targeting to PDPK1 by systemic administration of the PDPK1 inhibitor in this model.

2 | MATERIALS AND METHODS

2.1 | Patients, specimen harvest, and tissue preparation

Between February 2009 and May 2021, 10 patients (4 males and 6 females; age range 35–83 years [y]; mean age 53 y) with EACSCC were treated surgically at the Department of Otorhinolaryngology, Jikei University Hospital. All of the EACSCC tissue samples harvested from the patients during surgery were diagnosed by histopathologic examination. This study protocol was approved by the Human Ethics Review Committee of the Jikei University School of Medicine and signed informed consent was obtained from all of the patients or their guardians for this study (approval number 31-139 9638). Samples were fixed, embedded in paraffin, and 5-μm thick serial sections were prepared in the standard manner. For the histological examination, hematoxylin and eosin (H&E) staining was performed using a standard procedure.

2.2 | Animals

The experiments were conducted in 14 male BALB/c mice (6 weeks, 30–35 g) with a normal tympanic membrane (TM). The animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Jikei University with approval guidelines (No. 2015-139C5).

2.3 | Electroporatical transfection of hEGF-expression vector to the mice ears

The 3X FLAG human (h) full-length EGF vector was constructed by inserting the cDNA to the p3XFLAG-CMV14 vector (Sigma Chemical Co., Saint Louis, MO). Flag-h EGF DNA plasmid driven by a CMV14 promoter (0.5 μg/ml) (EGF-expression vector) was transfected once (n = 2, 4 ears) or five times every fourth day into the epithelial region of the mouse ear canals (n = 12, 12 ears) using a Nepa21 Electroporator (Nepa Gene Co., Chiba, Japan) under anesthesia using a combination anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol), according to the protocol of a previous study. As a control, a null plasmid driven by a CMV14 promoter (0.5 μg/ml) (empty vector) was transfected into the other ears (n = 12, 12 ears) of the mice.

2.4 | Otoendoscopic examination

At every time-point after the EGF-expression vector or empty vector transfection and after PDPK1 inhibitor GSK2334470 (Cayman Chemical, Ann Arbor, MI) administration, an otoendoscopic examination was performed with a rigid rod 0° otoendoscope (AVS Co., Tokyo, Japan) under anesthesia.

2.5 | Administration of PDPK1 inhibitor GSK2334470 in vivo

EGF-expression vector was transfected five times every fourth day into the epithelial region of the right EAC (n = 8, 8 ears) and empty vector was transfected five times in the left EAC as control in mice (n = 8, 8 ears). After confirming tumor formation in the right EAC by otoendoscopy, PDPK1 inhibitor GSK2334470 (40 mg/kg/day) or phosphate-buffered saline (PBS) (n = 4, each) was administered intra-peritoneally for five consecutive days.
2.6 | Tissue preparation of the animal model

The mice were eututututed using an intraperitoneal injection of 200 mg/kg pentobarbital and their ears and temporal bones were removed and fixed with 4% paraformaldehyde in PBS at 4°C overnight. The ears were embedded in paraffin and 5 μm serial sections were prepared in a standard manner. The temporal bones were decalcified by 10% ethylenediaminetetraacetic acid (EDTA) at 4°C for 7 days and paraffin sections were prepared as described previously.

2.7 | Immunohistochemistry

Immunohistochemistry was performed with the indirect enzyme-labeled antibody method, as described previously. In brief, after deparaffinization and rehydration, the sections were autoclaved in a Tris-EDTA buffer (pH 9.0) at 120°C for 10 min to detect cleaved caspase3, a 0.01 M citrate buffer (pH 6.0) at 90°C for 20 min to detect EGFR (A-10) and PDPK1 (EP569Y), or in the HistoVT one (Nacalai Tesque, Kyoto, Japan) at 90°C for 20 min to detect FLAG, Ki67, EGFR, EGFR (EP38Y), and PDPK1 (E3). They were preincubated with 500 μg/ml normal goat IgG in 1% bovine serum albumin (Sigma) for 60 min to block a nonspecific reaction and treated with the first antibodies listed in Table 1 overnight. After washing the slides with 0.05% Tween 20 in PBS, the slides were reacted with the secondary antibodies listed in Table 1, and washed with 0.05% Tween 20 in PBS. For visualization, Alexa Fluor 488-goat anti-mouse IgG or Alexa Fluor 555-goat anti-rabbit IgG was used instead of the first antibodies, respectively, in every experiment.

2.8 | Terminal deoxy(d)-UTP nick end labeling

For terminal deoxy(d)-UTP nick end labeling (TUNEL) detection, an In Situ Cell Death Detection Kit, fluorescein (Roche Applied Science, Penzberg, Germany) was used. The sections were immersed in 0.2 mol/ml proteinase K (Dako, Santa Clara, CA) in PBS at 37°C in a water bath after deparaffinization. The TUNEL reaction mixture was then added to the samples and the slides were incubated for 60 min at 37°C in accordance with the manufacturer’s instructions. After being washed with PBS, the slides were counterstained with DAPI.

2.9 | Microscopy, image analysis, and statistical analysis

Bright-field images were obtained using a Zeiss microscope and fluorescent images were obtained using a Zeiss LSM 880 confocal laser scanning microscope connected to a CCD camera (AxioCam, Carl Zeiss, Jena, Germany). Image analysis was performed using Zeiss acquisition analysis software (Zen 2.1 black edition). For each section, more than 1000 cell nuclei were counted in three equal epithelial or stromal regions at 400× magnification in bright-field images or three 10,000 μm² areas (100 × 100 μm²) in fluorescent images. The number of positive cells was expressed as a percentage of positive cells per total number of counted cells, respectively (labeling index [LI]; mean ± standard deviation). The thickness of the epithelium was measured at four locations (central and at each extreme) using NIH Image/ ImageJ software as described previously (ver. 1.46r). Differences between the groups were examined for statistical significance using the one-way analysis of variance test followed by the unpaired t-test. A p-value of less than 0.05 denoted a statistically significant difference. All analyses were performed using a statistical software package (JMP version 13; SAS Institute Japan, Tokyo, Japan).

| TABLE 1 | Antibodies used for immunostaining |
|---------------------------------|-----------------------------------|
| **Antigen, clone name, or immunogen** | **Manufacturer, species, catalog no.** | **Dilution used** | **Type** |
| FLAG, M2 | Sigma-Aldrich, St. Louis, MO, mouse, F1804 | 1:500 | Primary |
| Ki67, mouse Ki67 aa.1850-1950 | Novus Biologicals, Centennial, CO, rabbit, NB110-89717 | 1:400 | Primary |
| EGFR, 90-140/208 | Bios Antibodies, Woburn, MA, rabbit, bs-3576R | 1:200 | Primary |
| EGFR, A-10 | Santa Cruz Biotechnology, Dallas, TX, mouse, sc-373746 | 1:50 | Primary |
| EGFR, EP38Y | Abcam, Cambridge, UK, rabbit, ab52894 | 1:100 | Primary |
| PDPK1, E3 | Santa Cruz Biotechnology, Dallas, TX, mouse, sc-17765 | 1:100 | Primary |
| PDPK1, EP569Y | Abcam, Cambridge, UK, rabbit, ab52893 | 1:150 | Primary |
| Cleaved caspase-3, Asp175 | Cell signaling Technology, Danvers, MA, rabbit, #9661 | 1:200 | Primary |
| HRP-goat anti-mouse IgG | Abcam, Cambridge, UK, goat, ab6789 | 1:100 | Secondary |
| HRP-goat anti-rabbit IgG | Abcam, Cambridge, UK, goat, ab6721 | 1:100 | Secondary |
| Alexa Fluor 488-goat anti-mouse IgG | Thermo Fisher Scientific, Hudson, NH, goat, A-11008 | 1:500 | Secondary |
| Alexa Fluor 555-goat anti-rabbit IgG | Thermo Fisher Scientific, Hudson, NH, goat, A-21422 | 1:500 | Secondary |
FIGURE 1   Analysis of human squamous cell carcinoma (SCC) of the external auditory canal (EAC). Representative images of hematoxylin and eosin (H&E) stainings of human EACSCC. Squamous cells arising from the epidermis and extending into the dermis exhibiting architectural abnormalities, cytological atypia, and chromatin condensation. The yellow-boxed area is shown in the immunofluorescence stainings. EGFR-positive (+) cells were mainly observed in the lower layers of the epidermis. In the double immunofluorescence staining of EGFR (red) and PDPK1 (green), EGFR+/PDPK1+ cells were mainly observed in the lower layers of the epidermis (asterisks). Negative control was obtained by normal mouse (Ms) IgG and normal rabbit (Rb) IgG instead of first antibodies. The nuclei were stained with DAPI (blue). Scale bars: 100 μm
3 | RESULTS

3.1 | Localization of EGFR- and PDPK1-positive cells in human EACSCC tissues

Representative H&E stainings of the human EACSCC specimen are shown in Figure 1. Squamous cells were noted arising from the epidermis and extending into the dermis, exhibiting architectural abnormalities, cytological atypia, and chromatin condensation (Figure 1, yellow-boxed area). EGFR and PDPK1-positive (+) cells were detected in all tissues (10/10, 100%). In the double immunofluorescent staining of EGFR and PDPK1, EGFR+/PDPK1+ cells were almost found to colocalize in the epidermis (Figure 1). No staining was detected with normal mouse IgG and normal rabbit IgG instead of with the first antibody (Figure 1).

3.2 | Evaluation of the EGF-expression vector-transfected ears

Flag protein and EGF-expression were detected immunohistochemically in almost all epithelial cells and some stromal cells in the sections of the
EGF-expression vector-transfected ears (4/4, 100%, data not shown), and it was indicated that EGF-expression vector was successfully transfected in the epithelial and subepithelial regions of the EAC. In the EGF-expression vector-transfected ears (n = 4, EGF group), the growth of a protuberant tumor was observed at the fifth transfection (4/4: 100%), whereas a normal appearance of the EAC and TM was observed in all of the ears in which empty vector was transfected (4/4: 100%) (n = 4, empty group) by otoendoscopic analysis (Figure 2A). Representative H&E staining of the EAC in the EGF group showed thickened epithelium as compared with the EAC in the PBS group (Figure 2B). An enzyme immunohistochemistry of EGF, EGFR, PDPK1, and Ki67 was performed in the empty group and EGF group (Figure 2B). According to the results, EGF+ cells and EGFR+ cells were observed in the lower layers of the EAC epithelium in the EGF group, whereas EGF+ and EGFR+ cells were scarcely found in the empty group (Figure 2B). PDPK1+ cells were also observed in the lower layers of the EAC epithelium in the EGF group, **Figure 3** Effects of PDPK1 inhibitor (GSK2334470) against EGF-expression in the vector-transfected ears. (A) Otoendoscopic views of EGF-expression vector-transfected ears after PBS or GSK2334470 administration. The area within the dotted lines indicates the remains of protuberant tumor formation within the EAC in the PBS group, while tumor formation was not detected in the GSK2334470 group. (B) Morphological and immunohistochemical analysis. Hematoxylin and eosin (H&E) staining revealed a reduction of the thickened epithelium of the EAC in the GSK2334470 group, but thickened epithelium remained in the PBS group. Immunohistochemical analysis revealed the number of EGF-positive (+), EGFR+, PDPK1+, and Ki67+ cells was decreased in the GSK2334470 group as compared with the PBS group. Negative control was obtained by normal mouse (Ms) IgG and normal rabbit (Rb) IgG instead of first antibodies. (C) The bar graph shows the average of the epithelial thickness in the sections of the PBS group and the GSK2334470 group (n = 4 for each). (D) Labeling indexes (LIs) of EGF, EGFR, PDPK1, and Ki67 in the epithelium of the EAC in the PBS group versus in the GSK2334470 group (n = 4, each). Asterisks: tympanic membrane. Dashed lines: basement membrane. Arrows: positive cells. Scale bar: 20 μm. Error bars: 95% confidence interval. **p < .01, ***p < .001, ****p < .0001
but PDPK1+ cells were scarcely found in the empty group (Figure 2B). Ki67+ cells were increased in the EGF+/EGFR+/PDPK1+ layers of the EAC epithelium in the EGF group (Figure 2B). EGF LI of the EAC epithelial cells in the EGF group was significantly higher than in the empty group (39.2 ± 10.7 vs. 6.0 ± 1.1, p < .001, t-test, Figure 2C). EGFR LI was also higher in the EGF group than in the empty group (42.1 ± 4.7 vs. 8.8 ± 1.4, p < .0001, t-test, Figure 2C). Moreover, PDPK1 LI was significantly increased in the EGF group as compared with the empty group (43.3 ± 5.0 vs. 3.5 ± 1.4, p < .0001, t-test, Figure 2C). As expected, Ki67 LI of the EAC epithelium was also higher in the EGF group than in the empty group (45.0 ± 13.8 vs. 5.3 ± 1.8, p < .001, t-test, Figure 2C). These results indicated that the EGF-expression vector induced EGF- and EGFR-overexpression and led to PDPK1 upregulation. Moreover, PDPK1 upregulation led to an increase in cell proliferative activities in the EAC.

3.3 Effects of GSK2334470 against EGF transfected ears

To assess the effects of PDPK1 inhibitor against EGF-expression in the vector-transfected ears, we administered the PDPK1 inhibitor GSK2334470 intraperitoneally (n = 4, GSK2334470 group), while PBS was used as the control (n = 4, PBS group). According to the results, a normal appearance of the EAC was observed in the GSK2334470 group as compared with the PBS group by otoendoscopic analysis (Figure 3A). H&E staining showed a notably reduced thickness of the epithelial region in the GSK2334470 group (Figure 3B), although a thickened epithelium remained in the PBS group (22.7 ± 5.3 μm vs. 87.3 ± 17.0 μm, p < .001, t-test, Figure 3C). The number of PDPK1+ cells was decreased in the GSK2334470 group as compared with the PBS group (44.4 ± 3.7 vs. 3.9 ± 2.2, p < .0001, t-test, Figure 3D). Ki67 LI was also reduced significantly in the GSK2334470 group as compared with the PBS group (39.0 ± 8.7 vs. 8.6 ± 4.7, p < .001, t-test, Figure 3D). Interestingly, EGF and EGFR expression levels were also reduced in the GSK2334470 group as compared with the PBS group (Figure 3B), and EGF LI and EGFR LI were significantly reduced in the GSK2334470 group (34.0 ± 10.2 vs. 16.3 ± 3.2, p < .01, 46.4 ± 9.2 vs. 23.0 ± 6.6, p < .01, t-test, Figure 3D).

3.4 GSK2334470 induced apoptosis in the EGF-expression vector-transfected ears

According to the results, we hypothesized that tumor regression by PDPK1 inhibition might be due to apoptosis. To assess this hypothesis, we analyzed apoptotic efficacy. As a result, TUNEL+ cells and cleaved caspase-3+ cells were increased both in the epithelial cells (Figure 3A). H&E staining showed a notably reduced thickness of the epithelial region in the GSK2334470 group (Figure 3B), although a thickened epithelium remained in the PBS group (22.7 ± 5.3 μm vs. 87.3 ± 17.0 μm, p < .001, t-test, Figure 3C). The thickness of the epithelial region was significantly reduced in the GSK2334470 group as compared with the PBS group (22.7 ± 5.3 μm vs. 87.3 ± 17.0 μm, p < .001, t-test, Figure 3C). The number of PDPK1+ cells was decreased in the GSK2334470 group as compared with the PBS group (44.4 ± 3.7 vs. 3.9 ± 2.2, p < .0001, t-test, Figure 3D). Ki67 LI was also reduced significantly in the GSK2334470 group as compared with the PBS group (39.0 ± 8.7 vs. 8.6 ± 4.7, p < .001, t-test, Figure 3D). Interestingly, EGF and EGFR expression levels were also reduced in the GSK2334470 group as compared with the PBS group (Figure 3B), and EGF LI and EGFR LI were significantly reduced in the GSK2334470 group (34.0 ± 10.2 vs. 16.3 ± 3.2, p < .01, 46.4 ± 9.2 vs. 23.0 ± 6.6, p < .01, t-test, Figure 3D).

**FIGURE 4** Apoptosis assay of the GSK2334470 effects against EGF-expression in the vector-transfected ears. (A) Terminal deoxy(d)-UTP nick end labeling (TUNEL) and immunofluorescent staining of cleaved caspase-3 of the EAC. GSK2334470 (GSK2334470 group, n = 4) or PBS (PBS group, n = 4) was administered intraperitoneally after EGF-expression vector transfection. TUNEL-positive (+) cells increased both in the epithelial cells and stromal cells. The number of TUNEL+ cells and cleaved caspase-3+ cells was increased in the epithelial and stroma in the GSK2334470 group as compared with the PBS group. Negative control was obtained by normal mouse (Ms) IgG and normal rabbit (Rb) IgG instead of the first antibodies. The nuclei were stained with DAPI (blue). (B) TUNEL labeling index (LI) and cleaved caspase-3 LI of the GSK2334470 group and the PBS group. The red- and yellow-boxed areas: higher magnification view. Arrows: positive cells. Scale bars: 50 μm. Error bars: 95% confidence interval. *p < .05, **p < .01, ***p < .001
and in the stromal cells in the GSK2334470 group as compared with the PBS group (Figure 4A). TUNEL LIs both in the epithelial cells and stromal cells increased significantly in the GSK2334470 group as compared with the PBS group (28.7 ± 15.1 vs. 8.0 ± 6.8, p < .05, 14.7 ± 1.9 vs. 5.2 ± 1.2, p < .001, t-test, Figure 4B). Cleaved caspase-3 LIs in both the epithelial cells and stromal cells also increased significantly in the GSK2334470 group (13.8 ± 3.4 vs. 1.9 ± 0.6, p < .001, 11.4 ± 2.3 vs. 4.8 ± 1.1, p < .01, t-test, Figure 4B). These results indicated that GSK2334470 induced an increase of apoptotic cells both in the epidermis and dermis of the EAC into which the EGF-expression vector was transfected.

4 | DISCUSSION

Activated EGFR is involved in the pathogenesis and progression of EACSCC the same as HNSCC, but the pathogenesis of EACSCC is not fully understood. EACSCC is often accompanied by a marked inflammatory response and is suggested to be correlated with mechanical stimulations to the EAC. In carcinoma tissues, the close correlation between chronic inflammation and a high risk of carcinogenesis has now been widely confirmed. EGFR acts in an autocrine and paracrine manner on their specific cell membrane receptor and mounts an effective reparative response to inflammatory change. In this study, protuberant tumor formation and thickened epithelium were observed after the fifth transfection of the EGF-expression vector (4/4: 100%). We demonstrated EGFR LI was significantly increased in the EGF-expression vector-transfected ears compared with the empty vector-transfected ears immunohistochemically. These results indicated that EGFR overexpression was induced by transfection of the EGF-expression vector in the EAC.

PDPK1 plays an important role in tumor-cell growth downstream of the EGFR-mediated signaling pathways. Expression of PDPK1 is higher in renal cell carcinoma, bladder cancer, prostate cancer, lung cancer, breast cancer, hepatocellular carcinoma, and HNSCC compared with related normal tissues. In this study, it was confirmed that EGFR and PDPK1 were expressed in the human EACSCC specimens and colocalization was observed in the tumor region of the EAC immunohistochemically. Moreover, PDPK1+ cells was apparently decreased and PDPK1 LI was significantly decreased in the GSK2334470 group compared with the PBS group. Moreover, Ki67 LI was significantly lower in the GSK2334470 group compared with the PBS group. These results indicated that systemic administration of GSK2334470 inhibited PDPK1 expression and induced tumor reduction effectively. In previous studies, it has been reported that PDPK1 inhibition induced apoptosis to suppress tumor formation in breast cancer and colorectal cancer. To confirm apoptosis in the effects of GSK2334470 against EACSCC in this animal model, we performed a TUNEL assay and immunofluorescence staining using an anti-cleaved caspase-3 antibody. According to the results, TUNEL LIs both in the squamous cells and stromal cells were significantly increased in the GSK2334470 group. Interestingly, TUNEL+ cells were mainly observed in the outer layers of the squamous cells of the GSK2334470 group, whereas TUNEL+ cells were scarcely found in the PBS group. Cleaved caspase-3 LIs both in the epithelial cells and stromal cells were also significantly increased in the GSK2334470 group.

In conclusion, it is suggested that the EGFR/PDPK1 axis plays an important role in EACSCC, and the PDPK1 inhibitor, GSK2334470 could reduce the EACSCC inducing apoptosis. These results indicated that PDPK1 could be a therapeutic target of EACSCC and provide clinical benefits for patients in the future, although further investigation is required.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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