microRNA-224 Promotes Cell Proliferation and Tumor Growth in Human Colorectal Cancer by Repressing PHLPP1 and PHLPP2

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Abstract

Purpose: To investigate the clinicopathologic significance, role, and mechanism of action of microRNA-224 (miR-224) in colorectal cancer.

Experimental Design: Real-time PCR was used to quantify miR-224 expression. The association of miR-224 with the clinicopathologic features and survival was evaluated in 110 colorectal cancer patients. The role of miR-224 in colorectal cancer was investigated using in vitro and in vivo assays. Luciferase reporter assays were conducted to confirm target gene associations.

Results: miR-224 was overexpressed in colorectal cancer. High-level expression of miR-224 was significantly associated with an aggressive phenotype and poor prognosis. Overexpression of miR-224 promoted colorectal cancer cell proliferation in vitro and tumor growth in vivo. Specifically, miR-224 accelerated the G1–S phase transition through activation of AKT/FOXO3a signaling, downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1. Moreover, both PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, antagonists of PI3K/AKT signaling, were confirmed as bona fide targets of miR-224. miR-224 directly targeted the 3′-untranslated regions of the PHLPP1 and PHLPP2 mRNAs and repressed their expression.

Conclusion: This study reveals functional and mechanistic links between miRNA-224 and the tumor suppressors PHLPP1 and PHLPP2 in the pathogenesis of colorectal cancer. miR-224 not only plays important roles in the regulation of cell proliferation and tumor growth in colorectal cancer, but also has potential as a prognostic marker or therapeutic target for colorectal cancer.

Introduction

Colorectal cancer is one of the most common causes of cancer-related deaths worldwide (1). Although progress has been made in diagnostic and therapeutic strategies, the clinical outcome and prognosis of colorectal cancer patients with advanced stage disease still remains poor (2). Thus, understanding the molecular mechanisms which regulate the initiation and progression of colorectal cancer represents a pivotal step in the discovery and exploration of novel molecular targets, which may help to generate more effective therapies. Similarly to many other solid tumors, the initiation of colorectal cancer is a multigene and multistep process. Activation of oncogenes or inactivation of tumor suppressor genes affects multiple aspects of colorectal cancer tumorigenesis, such as cell proliferation, apoptosis, invasion, and metastasis (3). However, although numerous mutations are well recognized to be associated with the development of colorectal cancer, the other genetic and epigenetic alterations responsible for this disease remain largely unknown.

microRNAs (miRNA) are a class of small-regulatory RNA molecules that repress protein translation through binding to the 3′-untranslated region (UTR) of their target mRNAs in a sequence-specific manner. miRNAs are highly conserved across species and participate in the regulation of a variety of basic biological processes such as development, cellular differentiation, proliferation, apoptosis, and metabolism (4, 5). In recent years, miRNAs have been proven to play...
Translational Relevance

Dysregulation of microRNA-224 (miR-224) has been implicated in the pathogenesis of hepatocellular carcinoma and breast cancer. However, the biological functions of miR-224 in the development and progression of colorectal cancer remains unknown. This study revealed that miR-224 is overexpressed in colorectal cancer. High level of miR-224 expression is associated with aggressive phenotype of colorectal cancer and is an independent prognostic factor for poor survival of patients with colorectal cancer. Overexpression of miR-224 enhances the proliferation and tumorigenicity of colorectal cancer cells both in vitro and in vivo. In addition, overexpression of miR-224 leads to downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1 via activation of AKT/FOXO3a signaling. Moreover, both PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, antagonists of PI3K/AKT signaling, were confirmed as bona fide targets of miR-224. Our data highlight an important role for miR-224 in the pathogenesis of colorectal cancer, and miR-224 could be considered as a potential prognostic marker or therapeutic target for colorectal cancer.

Materials and Methods

Tissue specimens and cell cultures

For the use of clinical materials for research purposes, prior approval was obtained from the Southern Medical University Institutional Board (Guangzhou, China). All samples were collected and analyzed with the prior written informed consent of the patients.

A total of 230 patients with histologically diagnosed with colorectal columnar adenocarcinoma and undergoing surgical treatment were recruited for this study from the Department of General Surgery, Nanfang Hospital (China). Exclusion criteria included smoking, psychiatric disease, cerebrovascular disease, abnormal electrolytes, anemia, or hypertension. A total of 110 cases of archived colorectal cancer tissue samples were collected between March 2005 and October 2007. 24.5% (27 of 110) of patients with rectal cancer received neoadjuvant therapy and 78.2% (86 of 110) of patients received postoperative adjuvant therapy. A total of 43 pairs of colorectal cancer biopsies and their matched adjacent normal tissues, and 77 cases of fresh colorectal cancer biopsies were collected between January 2011 and May 2013. All the tissue biopsies were freshly frozen in liquid nitrogen and stored at −80°C until further use. The medical records of the patients were reviewed to collect the following clinicopathologic information: age, gender, pathologic stage, Dukes’ stage, T stage, lymph node metastases, and distant metastasis. Survival data were available for the cohort of 110 patients. The median follow-up time was 56.7 months (range, 2–87 months).

Two colorectal cancer cell lines SW620 and HCT116 were purchased from American Type Culture Collection Cell Biology Collection and were maintained in Department of Pathology, Southern Medical University. Cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (HyClone) and 1% penicillinestreptomycin (Invitrogen) at 37°C with 5% CO2.

RNA extraction and real-time quantitative PCR

For miRNA quantification, total miRNA was extracted from the cells and tissues using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions, and then cDNA was synthesized from 5 ng of total RNA using the Taqman microRNA reverse transcription kit (Applied Biosystems). The expression levels of miR-224 were quantified using the miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems). The relative miR-224 expression levels after normalization to U6 small nuclear RNA were calculated using 2−[(Ct of miR-224) − (Ct of U6)]. Real-time quantitative PCR for target genes was conducted as previously described (13). Primer sequences were summarized in Supplementary Table S1.

Plasmids and transfection

To generate a miR-224 expression vector, a 281 bp genomic fragment covering the region coding for pri-miR-224 and its upstream and downstream regions was PCR amplified and cloned into the pLtvhM vector (Addgene). The full-length PHLPP1 3′-UTR is 1,012 bp long and the PHLPP2 3′-UTR is 3,883 bp long. The miR-224 binding site in the PHLPP1 3′-UTR is located at 5,917 to 5,924 bp, and 7,540 to 7,546 bp in the PHLPP2 3′-UTR. The region of the human PHLPP1 3′ UTR from 5,857 to 6,318 bp and PHLPP2 3′-UTR
from 7,525 to 7,835 bp were generated by PCR amplification and subcloned into the \textit{Sac}I/\textit{Xma}I sites of the pGL3-basic luciferase reporter plasmid (Promega). The primers used to generate these constructs are listed in Supplementary Table S2. The miR-224 mimics, negative control, and anti-miR-224 inhibitors were purchased from Genecopoeia (Genecopoeia Co. Ltd.) and transfected into colorectal cancer cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**Western blotting**

Protein lysates were prepared, subjected to SDS-PAGE, transferred onto PVDF membranes, and blotted according to standard methods using anti-PHLPP1 (ab71972), anti-PHLPP2 (ab71973; Abcam), anti-p21 (#2947), anti-p27 (#3688), anti-cyclinD1 (#2978), anti-p-AKT (#4056), anti-AKT (#9272) antibodies (Cell Signaling Technology), anti-p-FOXO3a (BS5019), or anti-FOXO3a (BS5520) antibodies (Bioworld Technology). Anti-\(
\alpha\)-tubulin monoclonal antibody (T6199; Sigma) was used as a loading control.

**MTT assay, colony formation assay, soft-agar colony formation assay, flow cytometry, and immunohistochemistry**

The MTT assay, colony formation assay, soft-agar colony formation assay, flow cytometry, and immunohistochemistry (IHC) were conducted according to previously described methods (13). Further details are provided in the Supplementary Materials and Methods section.

**Luciferase assays**

Cells were seeded in triplicate in 24-well plates (1 \( \times \) 10\(^5\)/well) and cultured for 24 hours. The pGL3-luciferase reporter gene plasmids pGL3.0-PHLPP1-3’-UTR, pGL3-PHLPP2-3’-UTR, or the control-luciferase plasmid were cotransfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000 reagent (Invitrogen). Luciferase and Renilla activities were assayed 48 hours after transfection using the Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer’s instructions. All experiments were conducted at least 3 times and the data are presented as mean \( \pm \) SD.

**Colorectal cancer xenograft model in nude mice**

Animal experiments were conducted as previously described (13). Details are provided in the Supplementary Materials and Methods.

**Statistical analysis**

All statistical analyses were conducted using SPSS13.0 for Windows. The two-tailed paired Student \( t \) test was used for analyzing 2 groups. The Mann–Whitney \( U \) test and
Spearman’s correlation analyses were used to analyze the relationship between miR-224 expression and the clinicopathologic features of colorectal cancer. Survival curves were plotted by the Kaplan–Meier method and compared with the log-rank test. The significance of various survival-related variables was assessed using a multivariate Cox regression model. \( P < 0.05 \) was considered statistically significant.

**Results**

**miR-224 is overexpressed in colorectal cancer**

The expression of miR-224 was detected in 43 pairs of colorectal cancer biopsies and their matched adjacent normal tissues, and 77 cases of fresh colorectal cancer biopsies by real-time PCR revealed that miR-224 was significantly overexpressed in 76.7\% (33/43) of the colorectal cancer tissue samples examined (\( T/N > 2\)-fold) compared to the matched adjacent normal tissues from the same patient, with up to 68-fold increases observed in the colorectal cancer tissue samples (Fig. 1A). Student \( t \) test revealed that the miR-224 expression levels were significantly lower in the adjacent normal tissues than the colorectal cancer tissue samples (\( P = 0.004 \), Fig. 1B). In addition, miR-224 was expressed at relatively low levels in tumors with an early T classification (T1 and T2), and markedly increased in T3 tumors and further elevated in T4 tumors (Fig. 1C).

**High levels of miR-224 expression are associated with progression and poor prognosis in colorectal cancer**

To further investigate the clinicopathologic and prognostic significance of miR-224 expression in colorectal cancer patients, the levels of miR-224 were quantified in a cohort of 107 colorectal cancer tissue samples using real-time PCR. The median relative expression level of miR-224 in all 110 colorectal cancer samples was chosen as the cut-off point for separating tumors with low expression of miR-224 and high expression of miR-224. Mann–Whitney \( U \) tests and Spearman correlation analysis showed that low expression of miR-224 was significantly associated with pathologic stage, Dukes’ stage, \( T \) classification, and distant metastasis in colorectal cancer (\( P < 0.05 \); Table 1 and Supplementary Table S3). However, there were no significant correlations between the miR-224 expression level and age, gender, histology, or \( N \) classification in colorectal cancer. Kaplan–Meier survival analysis revealed that patients with low miR-224 expression levels had a better clinical outcome (Fig. 1D). Multivariate survival analysis indicated that the miR-224 expression level, \( T \) stage, and pathologic stage were independent prognostic factors for outcome in patients with colorectal cancer (Supplementary Table S4).

**Overexpression of miR-224 enhances the proliferation and tumorigenicity of colorectal cancer cells in vitro and in vivo**

We transfected the colorectal cancer cell lines SW620 and HCT116 with hsa-miR-224 mimic oligonucleotides and examined the effects on cellular proliferation. MIT and colony formation assays revealed that overexpression of miR-224 significantly increased the growth rate of both colorectal cancer cell lines, compared to negative control–transfected cells (Supplementary Fig. S1A and S1B). Moreover, the expression of Ki-67, a well-known marker of proliferation, was dramatically increased in miR-224–overexpressing SW620 and HCT116 cells compared with negative control–transfected cells (Supplementary Fig. S1C). Furthermore, the BrdUrd incorporation assay showed that a higher percentage of miR-224–overexpressing SW620 and HCT116 cells contained newly synthesized DNA, compared with negative control cells (Supplementary Fig. S1D).

We next examined the effect of miR-224 on the tumorigenicity of colorectal cancer cells using an anchorage-independent growth assay. Overexpression of miR-224 significantly enhanced the anchorage-independent growth ability of SW620 and HCT116 cells, as indicated by increased colony number and size (Fig. 2A). To test whether miR-224 could promote the growth of colorectal cancer tumors in vivo, we engineered SW620 and HCT116 cells to stably overexpress miR-224. These stably miR-224–overexpressing and control cells were subcutaneously implanted in nude mice.}

**Table 1. Correlation between clinicopathologic features and miR-224 expression in 110 colorectal cancer tissues**

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inoculated into nude mice. As shown in Fig. 2B, the tumors in the SW620/miR-224 group grew more rapidly than the tumors in the SW620/vector group ($P = 0.004$). Similar results were obtained using HCT116 cells ($P < 0.001$; Fig. 2D). IHC staining confirmed that the tumors of the control group displayed much lower Ki-67 indexes than the tumors from the miR-224–overexpressing group (Fig. 2C and E).

**Upregulation of miR-224 accelerates colorectal cancer cell-cycle progression and regulated cell-cycle factors: AKT/FOXO3a activation in colorectal cancer cells was involved**

We determined the cell-cycle distribution of miR-224–overexpressing SW620 and HCT116 cells by flow cytometry, to explore the possible mechanism by which miR-224 regulates colorectal cancer cell proliferation. A significant decrease in the percentage of cells in the G$_1$–G$_0$ phase and increase in the percentage of cells in the S-phase were observed in miR-224–overexpressing cells (Fig. 3A). The expression levels of a number of critical cell-cycle regulators were also detected. As expected, p21Cip1 and p27Kip1 were strikingly downregulated, whereas cyclin D1 was significantly upregulated at both the protein and mRNA levels in miR-224–overexpressing cells (Fig. 3B and C). It has been well documented that the expression of p27 Kip1 and cyclin D1 can be transcriptionally regulated by FOXO3a and, in turn, the transcriptional activity of FOXO3a is modulated by AKT phosphorylation (14). Thus, we hypothesized that upregulation of miR-224 may activate AKT/FOXO3a signaling. As shown in Fig. 3B and C, the phosphorylation levels of both FOXO3a and AKT increased in miR-224–overexpressing colorectal cancer cells. In addition, FOXO3a activity was strongly repressed by overexpression of miR-224, as shown by a FOXO-luciferase reporter gene (Fig. 3C).
Inhibition of miR-224 reduces the growth of colorectal cancer cells

To confirm the effects of miR-224 on the proliferation of colorectal cancer cells, endogenous miR-224 was inhibited by ectopically expressing a miR-224 inhibitor in SW620 and HCT116 cells. As shown in Supplementary Fig. S2, inhibition of miR-224 evidently increased the mRNA expression levels of both p21Cip1 and p27Kip1, and decreased the level of cyclin D1 mRNA in both cell lines. In addition, inhibition of miR-224 dramatically increased the percentage of cells in the G0–G1 phase and decreased the percentage of cells in the S-phase, indicating that silencing of miR-224 induced G1–S arrest in colorectal cancer cells (Fig. 4A). Moreover, MTT assays (Fig. 4B) and the colony formation assay (Fig. 4C) confirmed that inhibition of miR-224 reduced the growth rate of SW620 and HCT116 colorectal cancer cells, compared to negative control–transfected cells.

miR-224 directly targets the tumor suppressors PHLPP1 and PHLPP2 in colorectal cancer cells

Recently, the PHLPP phosphatase family members PHLPP1 and PHLPP2 have been identified to directly dephosphorylate and serve as negative regulators of AKT.
This prompted us to investigate whether the modulation of AKT/FOXO3a signaling activity by miR-224 were due to regulation of PHLPP1 and PHLPP2. The 3 bioinformatic algorithms (miRBase, Pictar, and TargetScan) were used to predict miR-224 target sites. Gene ontology enrichment analysis showed that PHLPP1 and PHLPP2 were theoretical target genes of miR-224 (Fig. 5A). Western blotting analyses showed that the protein levels of both PHLPP1 and PHLPP2 were dramatically downregulated in miR-224–overexpressing cells, whereas PHLPP1 and PHLPP2 were upregulated after inhibition of miR-224 in both cell lines (Fig. 5B). RNA-immunoprecipitation (RIP) analysis revealed that overexpression of miR-224 increased the recruitment of PHLPP1 mRNA and PHLPP2 mRNA to miRNP complexes (Fig. 5C). To further analyze the relationship between miR-224 and PHLPP1 and PHLPP2, the PHLPP1 or PHLPP2 3'-UTR fragments containing the wild-type or mutant miR-224 binding sites were subcloned into the pGL3.0-Basic luciferase reporter vector. As shown in Fig. 5D, a dose-dependent reduction in both wild-type PHLPP1 and PHLPP2 reporter gene luciferase activity was observed upon overexpression of miR-224 in both colorectal cancer cell lines, whereas inhibition of miR-224 increased wild-type PHLPP1 and PHLPP2 luciferase activity, compared to negative control–transfected cells. However, when miR-224-binding sites in the 3'-UTRs were mutated, we observed a dramatic relief of silencing of PHLPP1 and PHLPP2 genes, suggesting that these are probably true miR-224 target sites (Fig. 5D).

Repression of PHLPP1 and PHLPP2 play essential roles in miR-224–induced proliferation of colorectal cancer cells

Further analyses revealed that overexpression of PHLPP1 or PHLPP2 dramatically abrogated the miR-224–mediated regulation of AKT and FOXO3a phosphorylation (Fig. 6A and B) and modulation of the cell-cycle regulators p21, p27, and cyclin D1 (Supplementary Fig. S3). In addition, flow cytometry analysis showed that overexpression of PHLPP1 or PHLPP2 in miR-224–overexpressing colorectal cancer cells decreased the percentage of cells in S-phase (Fig. 6C). MTT assays indicated that overexpression of PHLPP1 or PHLPP2 significantly reduced the growth rate of miR-224–overexpressing SW620 and HCT116 cells (Fig. 6D).

We further investigated the expression levels of miR-224, PHLPP1, and PHLPP2 in clinical colorectal cancer samples. Analyses of the 43 paired colorectal cancer samples and matched adjacent normal tissues in which miR-224 was upregulated revealed that miR-224 expression correlated negatively with PHLPP1 and PHLPP2 mRNA expression (Supplementary Fig. S4). We also measured the expression of PHLPP1 and PHLPP2 in the xenograft tumors formed by SW620/vector cells, SW620/miR-244 cells, HCT116/vector cells, and HCT116/miR-244 cells. Real-time PCR and IHC...
showed that PHLPP1 and PHLPP2 mRNA and protein were expressed at lower levels in the tumors generated from miR-224–overexpressing cells, compared to the vector control cells (Supplementary Fig. S5).

Discussion

miRNAs are a large family of gene regulators that negatively regulate their target mRNAs in a sequence-specific manner (16). miRNAs may also function as tumor suppressors or oncogenes (17). Recent evidence has shown that miRNAs play essential roles in multiple biological processes related to cancer, including cell differentiation, proliferation, tumorigenesis, angiogenesis, invasion, and metastasis (18–20). Upregulation of miR-224 has only been observed in a few tumor types, such as hepatocellular carcinoma, pancreatic ductal carcinoma, breast and renal cancer (21–24). Importantly, in hepatocellular carcinoma, p65/NF-κB has been shown to act as a direct transcriptional regulator of miR-224 expression, which links upregulation of miR-224 with cell migration and invasion (21). In addition, miR-224 can promote cell invasion and the expression of metastasis-related genes by targeting the Raf kinase inhibitor protein (RKIP) in human breast cancer cells (22). However, it was uncertain whether dysregulation of miR-224 was associated with the progression of colorectal cancer. In this study, we found that miR-224 was upregulated in colorectal cancer tissues, and high miR-224 expression levels were found to be significantly associated with aggressive characteristics and poor patient prognosis in colorectal cancer. In addition, ectopic overexpression of miR-224 promoted cell proliferation, proliferation, and tumorigenesis in colorectal cancer cells. Thus, our data are consistent with previously published studies which associated changes in miR-224 expression with tumor formation and progression (21, 22), implicating miR-224 as an oncomir.

More specifically, we showed that the molecular mechanism by which miR-224 promotes colorectal cancer cell proliferation was due, at least in part, to acceleration of the G1–S phase transition, downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1 in cells overexpressing miR-224. Previous studies revealed that the PI3K/AKT signal transduction cascade was required for cell-cycle progression through the G1-phase (25). In addition, activation of PI3K/AKT decreases the cellular levels of p21Cip1 and p27Kip1, and induces cyclin D1 expression, thereby promoting cell proliferation (14, 26). The modulation of these cell-cycle regulators by PI3K/AKT is accomplished either directly or indirectly through inhibiting the phosphorylation and activation of FOXO3a (14, 27, 28). Our data showed that miR-224 activates PI3K/Akt signaling and promotes cell survival through modulation of FOXO3a, p21Cip1, p27Kip1, and cyclin D1 expression. This pathway represents a new mechanism which may possibly underlie the development of colorectal cancer.

We further explored the mechanism by which miR-224 could activate the PI3K/Akt signaling cascade. Bioinformatic algorithms predicted that both isoforms of PHLPP, PHLPP1 and PHLPP2, were bona fide target genes of miR-224. PHLPP1 and PHLPP2 belong to a novel family of Ser/Thr protein phosphatases and play central roles in...
maintaining cell survival suppression through negatively regulating the signaling pathways activated by AKT, PKC, MAPK, and Mst1 (29–32). Evidence has recently emerged to suggest that PHLPP1 and PHLPP2 act as tumor suppressor genes, and their expression is frequently depleted in a variety of human cancers, including breast cancer, prostate cancer, and colorectal cancer (26, 33). As Ser/Thr protein phosphatases, PHLPP1 and PHLPP2 can directly dephosphorylate AKT to inhibit AKT signaling activity, which promotes apoptosis (15, 30, 33). Overexpression of PHLPP1 and PHLPP2 in cancer cell lines decreases cell proliferation and tumorigenesis both in vitro and in xenograft models (26, 30, 33–37). In colorectal cancer cells, stable overexpression of PHLPP1 or PHLPP2 blocked the G2-M transition or induced G1 cell-cycle arrest, thus decreasing the rate of cell proliferation (26). Collectively, these studies suggest that restoration of the expression or function of the PHLPPs may represent a potential novel therapeutic intervention strategy for colorectal cancer. However, precise details of the mechanisms which regulate PHLPP1 and PHLPP2 remain elusive.

Our results also confirmed that both PHLPP1 and PHLPP2 were direct targets of miR-224. In addition, ectopic overexpression of PHLPP1 or PHLPP2 (without the 3'-UTR) significantly abrogated the miR-224–induced proliferation of colorectal cancer cells in vitro. Finally, expression analyses of miR-224, PHLPP1, and PHLPP2 in clinical colorectal cancer tissues revealed significant negative correlations between miR-224 and the expression of PHLPP1 and PHLPP2. Taken together, these results suggest that the
effects of miR-224 on the proliferation of colorectal cancer cells may be mediated via downregulation of PHLPP1 and PHLPP2 via miR-224 directly targeting the 3’-UTRs of these genes. Thus, our current study uncovers what we believe to be a novel mechanism leading to downregulation of PHLPP1 and PHLPP2 in cancer cells.

In summary, this study provides, for the first time, an essential link between miR-224-mediated tumor growth and downregulation of PHLPP1 and PHLPP2 in colorectal cancer. Our findings suggest an important role for miR-224 in the proliferation of colorectal cancer cells. Understanding the precise roles played by miR-224 in the initiation and progression of colorectal cancer will not only increase our understanding of the biology of this tumor type, but may also allow the development of a novel therapeutic strategy based on inhibition of miR-224.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W.-T. Liao, Z.-G. Wang, C. Zhang, Y. Ding

References
**Clinical Cancer Research**

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