G-Protein-Coupled Receptor 5 (LGR5) Overexpression Activates β-Catenin Signaling in Breast Cancer Cells via Protein Kinase A

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Background: Targeting cancer stem cells (CSCs) in breast cancer (BrCa) may improve treatment outcome and patient prognosis. Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a well-recognized adult stem cell and CRC marker, and previous reports have suggested the cancer-promoting role of LGR5 in breast cancer, but the mechanism remains unclear.

Material/Methods: Potential LGR5-associating genes were explored using STRING database, and LGR5 overexpression and knockdown was constructed in MCF-7 and MDA-MB-453 human BrCa cells, respectively. PKA catalytic subunit activation and PKA kinase activity in human BrCa cells was examined by Western blot and PKA kinase activity assay, respectively. Protein expression level or activation of β-catenin and GSK-3β in human BrCa cells was investigated by Western blot. Cell proliferation, colony formation, Transwell migration, cisplatin sensitivity, and in vivo tumor formation of human BrCa cells were examined.

Results: LGR5 overexpression increased PKA activation and its kinase activity in human BrCa cells, which was decreased by LGR5 knockdown. LGR5 expression level or PKA kinase activity were correlated with β-catenin Ser 552 phosphorylation but inversely correlated with GSK-3β Ser9 phosphorylation in human BrCa cells in vitro. LGR5/PKA increased cell proliferation, colony formation, Transwell migration, and cisplatin sensitivity in vitro, as well as tumor formation in vivo, of human BrCa cells.

Conclusions: LGR5 activates the Wnt/β-catenin signaling pathway in human BrCa cells in vitro via PKA.

MeSH Keywords: beta Catenin • Inflammatory Breast Neoplasms • Nod Signaling Adaptor Proteins • Ribosomal Protein S6 Kinases, 90-kDa • Wnt Signaling Pathway

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Background

Post-operative recurrence and spread remains a major challenge for breast cancer management [1–3]. The revolutionary cancer stem cell (CSC) theory suggests that CSCs account for the chemo- or targeted-therapy resistance, metastasis, and relapse of most cancer types, and targeting the stemness of breast cancer has proved promising in improving the clinical outcome and patient survival [3–5]. Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), a G-protein-coupled receptor located on the cell membrane with the R-spondin ligand is a well-recognized stem cell marker, and LGR5+ CSCs have been suggested to drive tumor growth in cancers origin-ated from the digestive tract, liver, lung, and ovary, primarily by increasing activation of the Wnt/β-catenin signaling pathway [6–8]. LGR5 is not only considered as the stem cell marker for breast cancer [9,10], but its cancer-promoting role in breast cancer has also been suggested by several recent studies: by analyzing the KEGG signaling pathway data, Chen et al. raised the possibility that LGR5 might enhance the signal transduction downstream of HER-2 and VEGF receptor by increasing the expression of β-catenin [11], and Yang et al. revealed that LGR5 is not only over-expressed in breast cancer tissue compared to non-cancerous counterparts, its expression is also essential for the stemness of breast cancer cells, and its expression level inversely correlates with breast cancer patient survival, which was further confirmed by Hou et al. [12], who found that β-catenin expression level was correlated with that of LGR5, and LGR5 expression was further increased in triple-negative breast cancer tissue compared to other breast cancer types. Yang et al. also suggested that LGR5 could potentially activate the Wnt/β-catenin signaling pathway in breast cancer, but this effect needs further verification in breast cancer and the molecular mechanism underlying this effect remains to be clarified.

In the present research, we investigated how LGR5 activates Wnt/β-catenin in breast cancer. By exploring the gene association network potentially associated with LGR5 using STRING database [13], we found that PKA catalytic subunits α, β, and γ are located in the intersection of the estrogen signaling pathway, Hedgehog signaling pathway, and Wnt signaling pathway enriched by potential LGR5-associating genes in the KEGG enrichment analysis. We further investigated whether PKA catalytic subunits were involved in LGR5-mediated stimulation of the Wnt/β-catenin signaling pathway in breast cancer, and we found that PKA catalytic subunits were activated upon LGR5 overexpression, which was required for the LGR5-promoted β-catenin activation and cancer cell growth. Our results suggest that LGR5 can activate β-catenin, at least in part through PKA.

Material and Methods

Cell culture and preparation.

The MCF-7 human breast adenocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). The MDA-MB-453 human breast cancer cell line was purchased from Thermo Fisher Scientific (Shanghai, China). MCF-7 and MDA-MB-453 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 g/ml streptomycin, in a cell incubator with humidified atmosphere (37%, 5% CO2). DMEM medium, FBS, and penicillin-streptomycin for cell culture were purchased from Sigma (Merck Life Science, Shanghai, China). LGR5 overexpression in MCF-7 cells as well as LGR5 knockdown in MDA-MB-453 cells were performed by Genecopoeia (Guangzhou, China). Briefly, for LGR5 overexpression, LGR5 cDNA-carrying pcDNA3.1 vector was transfected into MCF-7 cells, and cells transfected with empty vector were used as wild-type control (WT). For LGR5 knockdown, short hairpin RNA (shRNA) targeting LGR5 mRNA was transfected into MDA-MB-453 cells, and cells transfected with non-targeting shRNA was used as wild-type control (WT). Equal LGR5 mRNA and protein expression in non-transfected cells and transfected control cells were confirmed by RT-qPCR and Western blot (data not shown).

Western blot and PCR

Efficiency of LGR5 gene overexpression or knockdown was evaluated by Western blot and RT-qPCR detecting the protein and mRNA expression of LGR5 in each cell line, respectively. Primary antibody against human LGR5 protein (ab75732) was purchased from Abcam (Cambridge, UK). Primer pairs for detecting human LGR5 and β-actin mRNA by RT-qPCR were purchased from Genecopoeia (Guangzhou, China), and the primer sequences were as follows: LGR5: 5'-TGGTGCTGCTGTGATGATGCC-3' (forward), 5'-GCCAGCGGAGCAAGACAAA-3' (reverse). β-actin: 5'-CTGGGACGACATGAGAAA-3' (forward), 5'-AAGGAAGCTGGAAGACTGC-3' (reverse). Antibodies for detecting the protein expression level (ab71764) and Thr197 phosphorylation level (ab5815) of PKA α and β catalytic subunits were purchased from Abcam. Antibodies for detecting the protein expression level (E247, 3D10) and Thr197 phosphorylation level (D8E11, D85E12) of β-catenin and GSK-3β were purchased from Cell Signaling (Danvers, USA). Secondary antibodies against rabbit, mouse, or rat primary antibody were purchased from Abcam and preserved at -20°C until use.

PKA kinase activity assay

PKA kinase activity assay was performed using a PKA Kinase Activity Assay Kit (ab139453) purchased from Abcam, following the manufacturer’s instructions. Briefly, a cell lysis buffer (20 mM
MOPS, 50 mM, β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 μg/mL leupeptin and aproatin, dissolved in MilliQ water) was prepared for cell lysis performed on ice to minimize protein degradation. Cell lysates were pre-cleared by centrifugation at 10,000×g for 1 min to remove cell debris before being stored at -20°C. Supernatants were co-incubated with ATP solution on the PKA substrate plate for 90 min in a cell incubator, and the purified active PKA was used as positive control and for constructing the standard curve. The substrate phosphorylation level was detected by phospho-specific primary antibody and HRP-conjugated secondary antibody, followed by incubation with TMB solution in the dark for 30 min at room temperature. After stop solution was added, the substrate phosphorylation level was evaluated by optical density (OD) at 450 nm using a microplate reader.

**In vitro and in vivo assays**

Cell proliferation and cytotoxicity assay were performed by the CCK-8 method. CCK-8 solution was purchased from Dojindo (Shanghai, China) and applied following the manufacturer’s instructions. For cell proliferation assay, approximately 5000 cells were added in each well on a 96-well plate and pre-incubated for 6, 12, 24, 48, or 72 h under cell culture conditions. Cells in each well were then incubated with 10 μl of CCK-8 solution for 2 h under culture conditions. For cytotoxicity assay, about 5000 cells were added in a 96-well plate and pre-incubated for 16 h (overnight). Cells were then incubated with 0, 25, 50, or 100 μM of cisplatin for 36 h. The abundance of viable cells in each well was evaluated by measuring the OD at 450 nm using a microplate reader. For clonogenicity assay, about 1500 cells suspended in culture medium were added in each well on a 6-well plate and cultured for 12 days under culture conditions. Cell colonies were then counted under a phase-contrast microscope after crystal violet staining. Transwell assay was performed using Corning Transwell polycarbonate membrane inserts (Sigma-Aldrich) following the manufacturer’s instructions. Briefly, a total of 1.03×10⁴ cells suspended in DMEM medium supplemented with 0.5% FBS were added onto the membrane of the insert, the bottom of which was submerged in DMEM medium supplemented with 0.5% FBS and 40 μg/mL collagen I in a well on a 24-well plate. Cells were incubated for 3 h under culture conditions, and cells that did not migrate were gently removed with a cotton swab, and cells that migrated through the membrane were fixed with glutaraldehyde and stained with crystal violet for cell counting under a microscope. Xenotransplantation assay was performed following the procedure described by Hsu et al. and Yang et al., with minor modifications [12,14]. Briefly, about 5.02×10⁶ cells were injected into the fat pads of 4-week-old nude mice, and tumor formation at 1, 2, 3, and 4 weeks post-injection was evaluated by measuring the tumor mass. Animal experiments were approved by the Ethics Review Committee of the First Affiliated hospital of Zhengzhou University.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (Ver 7.04). Data in each panel represent at least 5 independent replicates, and all data are presented as mean ±SD, unless otherwise indicated. The t test was used for comparisons between 2 groups, and one-way ANOVA with Dunnett correction was used for multiple comparisons. A p<0.05 was the threshold for statistical significance.

**Results**

**LGR5 activates PKA in MCF-7 and MDA-MB-453 breast cancer cells in vitro**

To investigate the molecular mechanisms of LGR5 promoting breast cancer, we first explored the gene interaction network associated with potential LGR5-interacting genes using the STRING database. By sorting out the top 50 genes most likely interacting with LGR5 and performing the KEGG enrichment analysis of these genes, we found that these genes can be significantly enriched to several KEGG terms that are related to breast cancer development, such as “estrogen signaling pathway”, “Wnt signaling pathway”, “ hedgehog signaling pathway”, and “Pathways in cancer”. By cross-comparing the proteins within these pathways that potentially interact with LGR5, we found the 3 catalytic subunits of PKA lying in the cross-section of these pathways, and these PKA catalytic subunits were all suggested to be potentially interacting with β-catenin (data not shown). To verify this possible link between LGR5 and PKA catalytic subunits, we constructed an LGR5-overexpressing MCF-7 cell line and an LGR5-knockdown MDM-453 cell line, considering that Yang et al. have reported MCF-7 cells as LGR5 low and MDA-MB-453 cells as LGR5 high [12]. Successful LGR5 overexpression (LGR5 O/E) and knockdown (sh-LGR5) were confirmed by RT-qPCR (Figure 1A, 1B) and Western blot (Figure 1C–1E). Using these 2 LGR5 gene-manipulated cell lines, as well as their wild-type counterparts, we first tested our hypothesis that LGR5 activates or sensitizes the Wnt/β-catenin signaling pathway by activating PKA or influencing its protein expression level. We employed Western blot analysis to examine the protein expression level of the α and β catalytic subunit of PKA (PKA Cat-α and Cat-β) and PKA activation status by evaluating Thr197 phosphorylation of PKA Cat-α and Cat-β [15]. Our Western blot results showed that LGR5 overexpression in MCF-7 cells or LGR5 knockdown in MDA-MB-453 cells did not affect PKA Cat-α and Cat-β protein expression level in the 2 cell lines, but the phosphorylation level of PKA Cat-α and Cat-β at Thr197 was significantly correlated with...
LGR5 expression level (p<0.05, Figure 2A–2C). We further performed a PKA activity assay to confirm that the catalytic activity of PKA can be influenced by change in LGR5 expression level, and the results showed that LGR5 overexpression significantly increased PKA catalytic activity in MCF-7 cells, while it was decreased in MDA-MB-453 cells by LGR5 knockdown (p<0.05, Figure 2D). These results preliminarily support our hypothesis that LGR5 can activate PKA in breast cancer cells by increasing its phosphorylation level.

**LGR5 increases β-catenin activation in breast cancer cells by activating PKA**

We next examined whether this LGR5/PKA axis could activate or increase the sensitivity of β-catenin to the downstream signaling mediated by Wnt, as Brudvik et al. have reported that PKA can catalyze the phosphorylation of β-catenin at Ser552 and Ser675, which activates β-catenin in human colon cancer cells in vitro [16]. Our Western blot results demonstrated that...
LGR5 overexpression or knockdown affected both the protein expression level of β-catenin and its phosphorylation level at Ser552; moreover, application of a specific PKA kinase inhibitor, myr-PKA, significantly blocked the increase in β-catenin protein level and phosphorylation in MCF-7 cells raised by LGR5 overexpression, while an AC/PKA activator rescued the decrease in β-catenin protein level and phosphorylation level in MDA-MB-453 cells caused by LGR5 knockdown (Figure 3A–3D). As the RT-qPCR results indicated that mRNA expression level of β-catenin in none of these experimental groups was significantly changed compared to wild-type and un-treated control groups, we hypothesized that this increase or decrease in β-catenin protein level along with its phosphorylation level was due to changes in its degradation. We therefore examined the influence of changes in LGR5 expression level and PKA activity on the activation of GSK-3β, a dominant β-catenin deactivator, whose activation by phosphorylation at Ser9 triggers the ubiquitin-mediated β-catenin degradation [16,17].

Our results showed that the phosphorylation level of GSK-3β at Ser9 is inversely correlated with either LGR5 expression level or PKA kinase activity in MCF-7 and MDA-MB-453 cells, suggesting that GSK-3β activity can be inhibited by the LGR5/PKA axis (Figure 3E–3H). Thus, our results suggest that PKA increases the activation of β-catenin and decreases its degradation mediated by GSK-3β, and PKA catalytic activity can be regulated by LGR5 expression level.

LGR5 increases the aggressiveness and cisplatin resistance of breast cancer cells through PKA

We next investigated the impact of this LGR5/PKA axis on the aggressiveness of breast cancer cells by evaluating cell proliferation, colony formation, and transwell migration activity of MCF-7 and MDA-MB-453 cells with different LGR5 expression levels and PKA kinase activity. LGR5 overexpression significantly increased cell proliferation, colony formation,
Figure 3. LGR5/PKA axis increased β-catenin activation while decreasing GSK-3β activation in human breast cancer cells in vitro. 

(A, B) Western blot results showing that LGR5 overexpression significantly increased Ser552 phosphorylation of β-catenin in MCF-7 cells, which could be attenuated by application of the specific PKA inhibitor myr-PKI. LGR5 knockdown significantly decreased Ser552 phosphorylation of β-catenin in MDA-MB-453 cells, which could be rescued by application of cAMP/PKA activator forskolin. Alterations in LGR5 expression level or PKA activity showed no significant influence on protein expression level of β-catenin in the 2 cells. (C, D) Representative result of Western blot evaluating protein expression level and Ser552 phosphorylation level of β-catenin in each group of cells. (E, F) Western blot results showing that LGR5 overexpression significantly decreased Ser9 phosphorylation of GSK-3β in MCF-7 cells, which could be reversed by application of the specific PKA inhibitor myr-PKI. LGR5 knockdown significantly increased Ser9 phosphorylation of β-catenin in MDA-MB-453 cells, which could be inhibited by application of cAMP/PKA activator forskolin. Alterations in LGR5 expression level or PKA activity showed no significant influence on protein expression level of GSK-3β in the 2 cells. (G, H) Representative result of Western blot evaluating protein expression level and Ser9 phosphorylation level of GSK-3β in each group of cells. Western blot data analysis was performed using the same method as in Figure 1. For significance test, the t test (*) was used for comparison between 2 groups, and one-way ANOVA with Dunnett correction (#) was used for multiple comparisons. *, # p<0.05; ### p<0.0001.
Figure 4. LGR5/PKA increased the aggressiveness of human breast cancer cells in vitro. (A, B) Cell proliferation assay results showing that increase in cell proliferation in MCF-7 cells caused by LGR5 overexpression could be impaired by myr-PKI application, while the decrease in that in MDA-MB-453 cells induced by LGR5 knockdown could be rescued by forskolin application. (C–F) Colony formation assay and Transwell assay results showing that LGR5/PKA axis regulated colony formation and Transwell migration ability of MCF-7 and MDA-MB-453 cells in vitro. C and E are representative results of colony formation assay and Transwell assay. The t test was used for significance test. * p<0.05; ** p<0.01.
and Transwell migration of MCF-7 cells, which was attenuated by the application of PKA inhibitor myr-PKI. LGR5 knockdown significantly decreased these cancer cell aggressiveness parameters in MDA-MB-453 cells, which were partially rescued by the adenyl cyclase/PKA activator forskolin (Figure 4). These results suggest that PKA activity is involved in the cancer-promoting effect of LGR5.

LGR5 is broadly considered as a cancer stem cell marker in different cancer types, and chemotherapy resistance is believed to be an important aspect of cancer cell stemness, so we next tested whether increase or decrease in LGR5 expression level or PKA kinase activity could influence the cisplatin sensitivity of MCF-7 and MDA-MB-453 cells by cell viability and apoptosis assays. Our result showed that LGR5 overexpression can significantly increase cell viability while decreasing apoptosis in MCF-7 cells treated by relatively low concentrations of cisplatin, and LGR5 knockdown showed the opposite effects in MDA-MB-453 cells with the same treatment; the necessity of PKA kinase activity for the LGR5 overexpression-mediated cisplatin resistance effect was revealed by the application of PKA inhibitor, and application of forskolin significantly increase cell viability while decreasing apoptosis in MDA-MB-453 cells with LGR5 knockdown (Figure 5). These results suggest that PKA kinase activity is involved in the LGR5 overexpression-mediated cisplatin resistance.

We performed the xenotransplantation assay to further verify the cancer-promoting role of LGR5 in human breast cancer cells. MCF-7 and MDA-MB-453 cells with or without artificial change in LGR5 gene expression were injected in nude mice, and tumor growth was evaluated by measuring the tumor mass at 1, 2, 3, and 4 weeks post-injection. Our result showed that LGR5 overexpression significantly accelerated tumor cell growth of MCF-7 cells, whereas that of MDA-MB-453 cells was repressed by LGR5 knockdown (Figure 6). Collectively, our data indicate that LGR5 promotes human breast cancer cell growth by activating β-catenin via PKA.

**Discussion**

LGR5 is a well-recognized marker for adult stem cells as well as cancer stem cells, and has been confirmed to facilitate cancer cell growth, metastasis, and relapse in different cancer types, but these cancer-promoting effects of LGR5 were only very recently observed in breast cancer, and the molecular mechanisms behind these effects remain to be explored [11,12,18]. As an estrogen-driven cancer type in most cases, breast cancer may acquire a different strategy to thrive compared to cancers originating from the digestive track, in which LGR5 was initially recognized as a CSC marker and cancer-promoting gene [19–21]. In the present research, we attempted to investigate the
molecular mechanism underlying the cancer-promoting role of LGR5 in breast cancer. Previous reports have suggested that overexpression of LGR5 may activate β-catenin in breast cancer cells in vitro [11,12,18]; therefore, we first attempted to find links between LGR5 and the β-catenin signaling pathway by exploring the LGR5 interacting genes using the STRING database, which provides both known and predicted interactions between genes and proteins. We found that the PKA catalytic subunit genes were located in the cross-section of several signaling pathways enriched by possible LGR5 interacting genes, and these pathways – Estrogen signaling pathway, Hedgehog signaling pathway, and Wnt signaling pathway – have already been confirmed to facilitate the stemness gain and development of breast cancer [5,22,23]. Notably, the STRING prediction results suggested the interaction between PKA catalytic subunits and β-catenin, which has also been suggested by multiple reports in different cancer types [16,24,25]. Based on these findings, we hypothesized that LGR5 activates or potentiates β-catenin in breast cancer cells through PKA, either by activating it or increasing its protein expression. There currently

Figure 6. LGR5 regulated tumor growth of human breast cancer cells after xenotransplantation in nude mice. (A) Growth of xenograft by MCF-7 cells was increased by LGR5 overexpression. (B) Growth of xenograft by MDA-MB-453 cells was decreased by LGR5 knockdown. (C) Xenograft of each group at week 4 post-transplantation.
is no other evidence suggesting or implying these interactions, but, considering that LGR5 has been identified as a G-protein-coupled receptor, the possibility does exist that LGR5 activates PKA through adenyly cyclase or NF-κB.

Results of the present research support our hypothesis that LGR5 overexpression increases PKA activation, which can increase the activation of β-catenin in response to Wnt signaling. In this research, we first evaluated the activation of PKA in MCF-7 and MDA-MB-453 breast cancer cells with different LGR5 expression levels by measuring phosphorylation at Thr197 on the α and β subunit by Western blot, considering that phosphorylation on this site has been confirmed to activate PKA catalytic subunits [26], as well as by PKA kinase activity assay. The results showed that phosphorylation at Thr197 on PKA catalytic subunits and PKA kinase activity were increased by LGR5 overexpression in MCF-7 cells and decreased by LGR5 knockdown in MDA-MB-453 cells, compared to their wild-type counterparts. We believe this is the first evidence of LGR5 as a G-protein-coupled receptor activating PKA in human breast cancer cells. However, the mechanism of LGR5 activating PKA remains unresolved. Considering that application of forskolin, the adenyly cyclase activator, can partially resume the decrease in β-catenin phosphorylation level and tumor cell activity of MDA-MB-453 cells due to LGR5 knockdown, it seems reasonable that LGR5 activates PKA via adenyly cyclase, but this hypothesis needs further verification.

Our data also suggest that LGR5-mediated PKA activation downregulated GSK-3β activity in MCF-7 and MDA-MB-453 breast cancer cells. The protein complex formed by activated GSK-3β, Axin, and APC, is responsible for triggering the ubiquitin-mediated proteolysis of β-catenin, which is the dominant mechanism of inhibition of the Wnt/β-catenin signaling. Our results showed that application of myr-PKI or forskolin did not affect β-catenin or GSK-3β protein expression in MCF-7 or MDA-MB-453 cells, but the activation of β-catenin as well as inhibition of GSK-3β was significantly correlated with LGR5 expression level and PKA activity, suggesting that the LGR5/PKA axis can activate Wnt/β-catenin signaling in human breast cancer cells. Cell functional assay and xenotransplantation assay also showed that the LGR5/PKA axis could increase the aggressiveness of breast cancer cells both in vitro and in vivo. It is currently unknown how LGR5/PKA activates β-catenin. Brudvik et al. have demonstrated that PKA can activate beta-catenin by phosphorylation at Ser552 in colorectal cancer; however, considering that GSK-3β can be inhibited either by LGR5 overexpression or PKA activation, as shown by our results, and that several previous reports have raised the possibility that active PKA could inhibit GSK-3β by decreasing its activation [27–30], we inferred from these results that LGR5/PKA may also activate β-catenin by inhibiting the Wnt/β-catenin inhibitor GSK-3β. Notably, LGR5/PKA decreases GSK-3β activation, which is reported to cause an increase in β-catenin protein expression level. However, we did not observe this effect in our investigation on β-catenin protein expression level in LGR5 overexpressing MCF-7 cells in comparison to that in WT counterparts, which is also different from Yang et al’s report [12]. We believe that this is probably due to the limited number of replicates in our experiments. Further investigation should be made to verify these results.

Conclusions

Overall, our data suggest that LGR5 overexpression can activate β-catenin in MCF-7 human breast cancer cells, leading to the increase in β-catenin activation and cancer cell aggressiveness, whereas LGR5 knockdown showed the opposite effect; these effects of LGR5 are at least partially mediated by activating the kinase activity of PKA. These results suggest that LGR5 is more of a molecular marker for cancer stem cells but may also promote cancer cell growth and tumor development in breast cancer. Targeting LGR5 may thus be a potential therapeutic strategy for breast cancer treatment.

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