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SHORT COMMUNICATION

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microRNA-377-3p downregulates the oncosuppressor T-cadherin in colorectal adenocarcinoma cells

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Abstract

Colorectal cancer (CRC) is the second leading cause of death of malignant tumors worldwide. Recent studies point to a role for the adiponectin-receptor axis in colorectal carcinogenesis, and in particular to the oncosuppressive properties of the T-cadherin receptor. In addition, the loss of T-cadherin expression in tumor tissues has been linked to cancer progression and attributed to aberrant methylation of its promoter. Recognizing the pivotal role of microRNAs in CRC, this study explores their possible contribution to the downregulation of T-cadherin. A systematic bioinformatics analysis, restricted by microRNA expression data in the colon or in cultured colorectal cell lines, predicted twelve top-ranking target miRNA sites within the 3′ UTR of T-cadherin. Experimental validation analyses based on luciferase reporter constructs and miRNA mimic or miRNA inhibitor transfections toward colorectal adenocarcinoma cell lines indicated that miR-377-3p was able to directly bind to the T-cadherin sequence, and thus downregulating its expression. Given the oncogenic activity of miR-377 and the oncosuppressive activity of T-cadherin in CRC, the regulatory circuit highlighted in this study may add new insights into molecular mechanisms driving colorectal carcinogenesis, and perspectively it could be exploited to identify novel biomarkers and therapeutic targets.

KEYWORDS
cancer, RNA

1 | INTRODUCTION

Colorectal cancer (CRC) is the most frequent digestive malignancy and the second leading cause of death of malignant tumors worldwide (Bray et al., 2018). Most patients develop CRC as a consequence of tumor progression from adenoma into colorectal adenocarcinoma (COAD). Much effort has been dedicated to uncovering genetic and epigenetic alterations driving the development of CRC from normal epithelial cells of intestinal mucosa into colorectal cancer cells (Markowitz & Bertagnolli, 2009). Environmental factors, including lifestyle, have also been implicated in CRC pathogenesis (Świerczyński et al., 2020); in particular, obesity and metabolic-related disorders are strictly related to an increased risk of CRC, highlighting the functional cross-talk existing between the

The first two authors contributed equally to this study.

adipose tissue and peripheral organs, mainly related to the hormonal function of the adipose tissue and the dysregulated production of adipokines (Karczewski et al., 2019). Indeed, recent studies revealed that the overexpression or loss of several adipokines and the altered expression of their receptors may lead to CRC, pointing to a role for adipokine-receptor axis in colorectal carcinogenesis (Kawashima et al., 2017).

Adiponectin (APN), the major adipocytes-secreted adipokine, is the most abundant circulating peptide hormone; it is found in the serum as a trimeric, a hexameric, and a high-molecular-weight (HMW, composed of 12–18 adiponectin molecules) oligomers, with the last endowed with the highest biological activity (Kawashima et al., 2017; Nigro et al., 2014). Adiponectin has pleiotropic, beneficial functions in human health, ranging from antidiabetic, antiatherogenic, anti-inflammatory, and anticancer properties (Parida et al., 2019). Different studies suggest that higher circulating APN levels or activating APN-induced signaling pathways can balance metabolic dysfunctions and delay cancer progression in experimental models (Hebbard & Ranscht, 2014); moreover, clinical studies have established the correlation between low APN levels and obesity-related cancers, with cancers of the digestive system primarily affected (Dalamaga et al., 2012). In particular, recent studies indicate that adiponectin may work as a tumor suppressor in colorectal carcinogenesis (Otani et al., 2017; Polito et al., 2020). In fact, it has been shown that adiponectin inhibited the growth of colorectal cancer cells in vitro by activating adenosine monophosphate-activated protein kinase (AMPK) and suppressing the mammalian target of the rapamycin (mTOR) pathway (Kim et al., 2010; Sugiyama et al., 2009); we also demonstrated that adiponectin reduced the growth of colorectal cancer cells by inducing apoptosis and inhibited cell migration (Nigro et al., 2018). Indeed, a low level of circulating APN was associated with the risk of colorectal cancer in men, and a stronger risk factor than a high triglyceride level or body mass index in patients with adenoma and early colorectal cancer (Otake et al., 2010).

Adiponectin works by AdipoR1 and AdipoR2, the canonical and ubiquitary expressed receptors; Hug et al. identified a third effective receptor, T-cadherin (also known as cadherin 13, CDH13, and H-cadherin), an atypical member of the cadherin superfamily, lacking transmembrane and cytosolic domain, and able to bind the hexameric and HMW isoforms of adiponectin, but not trimeric or globular adiponectin forms (Hug et al., 2004; Kadowaki & Yamauchi, 2005). In particular, T-cadherin's main role in vivo is docking adiponectin and present it directly or indirectly to AdipoR1/R2 to inhibit signaling pathways activated in cancer (Hebbard & Ranscht, 2014). In colorectal cancer, AdipoR1 and AdipoR2 are expressed in both normal colon epithelium and tumor tissues, whereas loss of T-cadherin expression has been consistently reported by many studies (Andreeva & Kutuzov, 2010; Yoneda et al., 2008). T-cadherin downregulation in tumor tissues has been linked to hypermethylation of the promoter region, strongly associated with CRC risk, progression, and poor prognosis (Duan et al., 2017; Hibi et al., 2004; Ren & Huo, 2012; Toyooka et al., 2002; Wang et al., 2012; Xu et al., 2004; Ye et al., 2017). Promoter aberrant methylation has been frequently found also in other different tumors, where the silencing of T-cadherin is linked to increased tumorigenicity and cancer progression in in vitro and in vivo models (Andreeva & Kutuzov, 2010; Hebbard & Ranscht, 2014). Consistent with a tumor suppressor role, re-expression of T-cadherin in tumor cells can repress cancer-promoting pathways (Andreeva & Kutuzov, 2010). Deletion of 16q24 locus containing T-cadherin gene and histone modification additionally contribute to T-cadherin downregulation in cancer (Philippova et al., 2009). So far, possible mechanisms regulating T-cadherin aberrant expression at the transcriptional level have been extensively investigated; by contrast, potential posttranscriptional regulation, particularly via miRNA, has been very poorly studied (Liu et al., 2018; Shi et al., 2014) and never in CRC context, although the well-recognized role of microRNAs in carcinogenesis, included CRC onset, progression, and outcome (Wai Hon et al., 2020). The aim of this study is to unveil possible miRNAs contributing to downregulation of T-cadherin in COAD and thus potentially involved in colorectal carcinogenesis.

2 | MATERIALS AND METHODS

2.1 | Reporter constructs

The T-cadherin segments potentially targeted by human miRNAs were chemically synthesized and cloned into psiCheck-2 vector (Promega). In detail, couples of oligonucleotides, representing the target sites for the selected miRNAs, and carrying additional upstream *Xho*I and *Eco*RV restriction sites and a downstream *Not*I site, were annealed and ligated into *Xho*I and *Not*I sites of the psiCheck-2 vector; *Eco*RV digestions were then used to identify recombinant clones, which were sequenced to confirm their identity (Potenza et al., 2016; Potenza et al., 2018). Control plasmid (indicated as I) for miR-377 target sequence was obtained by the same approach, with the exception that the cloned couple of oligonucleotides represented the inverted target sequence (5′-AAGTGTGTTTTCCCAT GAAAATT -3′).

2.2 | Cell cultures and transfections

Human colorectal adenocarcinoma cell lines, HT-29 and Caco-2, were cultured in RPMI 1640 and DMEM respectively, containing 10% fetal bovine serum, 2mM L-glutamine, 50 U/ml penicillin, and 100 mg/ml streptomycin; nonessential amino acids (0.1 mM) was added in the culture medium of Caco-2. The day before transfection, the cells were trypsinized and seeded in a medium without antibiotics in 12-well plates. Transfections were performed with cells at 80–90% of confluence by using 3 μl of Lipofectamine 2000 (Invitrogen) for 1 μg of nucleic acids, as described by the manufacturer. Cells were transfected with 0.2 μg of reporter constructs; miScript miR-377 mimic and its control with unrelated sequence AllStars Negative Control, miRCURY LNA miR-377 Inhibitor and its

miRCURY LNA miRNA Inhibitor Negative Control A (all from Qiagen) were transfected at 50 nM. After 6 h, the transfection mix was replaced with a complete medium. The analyses were performed 48 h after transfection.

2.3 | Luciferase assays and Western blot analysis

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. All the analyses were performed on three independent experiments, each in triplicate.

Total proteins from cultured cells were prepared in RIPA lysis buffer (Sigma Aldrich) containing a protease inhibitor cocktail (Sigma Aldrich). A total of 30 μg proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Invitrogen) following standard protocols. Antibody against T-cadherin and GAPDH (Invitrogen) was used for immunodetection according to the manufacturer's instructions. Protein bands were visualized by incubating the blots with the horseradish peroxidase-conjugated antirabbit antibody (Santa Cruz Biotechnology) and by using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions. Blot images were captured by ChemiDoc XRS (Bio-Rad) and protein bands were quantified by Image Lab software (Bio-Rad).

Comparison of data sets in the different experiments was performed by Student's *t* test and a value of $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Searching for microRNAs targeting T-cadherin

Alternative splicing of pre-mRNA from the *cdh13* gene results in multiple transcript variants annotated in RefSeq and encoding different isoforms, with four validated and sharing an identical 3′ UTR. On that 3′ UTR, a bioinformatics prediction of target sites for miRNAs was performed, a number of criteria were applied to select putative miRNAs and finally, T-cadherin sequence target sites were subject to validation tests for responsiveness to selected miRNAs.

The bioinformatics prediction was first performed on microRNA.org platform by miRanda and mirSVR tools; we selected miRNAs below the cut-off pairing value of -1, with a perfect miRNA-mRNA pairing matching of the seed region (2-8nt of miRNA), and then also predicted by Target scan, although classified as "poorly conserved"; we also selected miR-30 family (from a to e) since they were predicted as "conserved target sites" by Target scan. Finally, we further restricted results to miRNAs expressed in the colon or in cultured colorectal cell lines according to the literature. The analyses yielded twelve top-ranking target sites (Figure 1).

Those target sites were subjected to a validation test based on luciferase reporter constructs transfected in cell lines. Given that most patients develop CRC as a consequence of tumor progression from adenoma into COAD, we decided to use as experimental cell model HT-29 and Caco-2 cell lines, both derived from COAD. In detail, T-cadherin fragments containing predicted miRNA target sites were singularly cloned downstream of the *Renilla reniformis* luciferase (Rl) coding sequence carried by the psi-Check-2 vector. Only one reporter construct was prepared for miR-34a, miR-34c, and miR-449a, miR-449b, since their target sites resulted overlapped, and only one reporter construct was prepared for the conserved target site of miR-30 family. The expected results were the observation of a reduction in the luciferase activity if a given miRNA binds to the T-cadherin target sequence, in comparison to the activity of control, represented by psiCheck-2 vector transfection that did not contain any T-cadherin sequence. Under this experimental setting, the 3′ UTR T-cadherin sequence 1040–1062, potentially targeted by miR-377-3p (miR-377), inhibited significantly the expression of the reporter construct transfected in HT-29 cells, with a registered activity approximately 30% lower than that measured with parental vector (Figure 2). Similar results were obtained by reporter constructs transfection in Caco-2 cells (data not shown). The results indicated that miR-377 was able to interfere with the reporter gene expression by direct binding to 3′ UTR T-cadherin.

3.2 | Validation of T-cadherin as a miR-377 target

The silencing activity of miR-377 was further investigated by transfecting the reporter construct of the above experiments and a control construct carrying the inverted target sequence (now denominated WT and I, respectively) along with miR-377 mimic and its negative control molecule, Ctrl-miRNA, not matching the target sequence. The luciferase activity registered after WT construct transfection (WT + Ctrl-miRNA) was significantly lower than that obtained with the control I construct (I + Ctrl-miRNA or I + miR-377); as a consequence of the increased level of miRNA concentration due to mimic transfection (approximately 250-fold as evaluated by RT-QPCR, data not shown), an additional 25% reduction on the WT reporter construct was observed, leading to a total 51% inhibition in comparison to the I control for HT-29 cells; similar results were obtained by Caco-2 cells experiments (Figure 3). The results definitely indicated that the registered interfering activity toward the T-cadherin segment 1040–1069 can be attributed to miR-377.

However, the described experiments evaluated the interfering activity of miR-377 on the expression of a chimeric transcript composed of *Renilla* luciferase coding sequence and a segment of T-cadherin UTR. To determine whether miR-377 could downregulate endogenous T-cadherin expression, the cells were transfected with miRNA mimic and T-cadherin level was evaluated by Western blot analyses (Figure 4). The results showed a reduction of T-cadherin after miRNA mimic transfection in comparison to Ctrl-miRNA transfection, indicating that miR-377 was able to effectively silence

Human miRNA	CDH13 3′UTR position	MiRNA-mRNA pairing
miR-377-3p	1040-1062	miRNA 3' UGUUUUCAACGG-AA**ACACACU**A 5' :|||||| || ||||||||| mRNA 5' UUAAAAGUACCCUUUUGUGUGAA 3'
miR-543-5p	212-232	miRNA 3' UUCUUCACGUGGCG**CUUACAA**A 5' ||| | : || :|||||||| mRNA 5' CUGAA-UUUUCCCUGAAUGUUU 3'
miR-216b-5p	1506-1528	miRNA 3' AGUGUA-AACGGACG**UCUCUAA**A 5' |||| | | |||||||||| mRNA 5' GAACAUAUAGAAUGCAGAGAUUU 3'
miR-34a-5p	664-683	miRNA 3' UGUUGGUCGAUUCU**GUGACGG**U 5' ||||||| :| |||||||| mRNA 5' ACAACCAAUU--UACACUGCCA 3'
miR-34c-5p	660-683	miRNA 3' CGUUAGUCGAUU-GAU**GUGACGG**A 5' | || | || :||||||||| mRNA 5' GACUACAACCAAUUUACACUGCCA 3'
miR-449a-5p miR-449b-5p	663-683	******************* miRNA 3' CGGUCGAUUGUUAU**GUGACGG**A 5' miRNA 3' UGGUCGAUUGUUAU**GUGACGG**U 5' || | ||: |||||||||| mRNA 5' UACAACCAAU-UUACACUGCCA 3'
miR-30 family miR-30e-5p miR-30d-5p miR-30c-5p miR-30b-5p miR-30a-5p	67-89	*** ********** miRNA 3' -GAAGGUCAGUUCCU**ACAAAUG**U 5' 3' -GAAGGUCAGCCCCU**ACAAAUG**U 5' 3' CGACUCUCACAUCCU**ACAAAUG**U 5' 3' UCGAACUCACAUCCU**ACAAAUG**U 5' 3' -GAAGGUCAGCUCCU**ACAAAUG**U 5' : | ||||||| mRNA 5' AAAUCUGAAGAUUGCGGUUUACA 3'

FIGURE 1 Human microRNAs with potential binding sites on T-cadherin (CDH13). A computational analysis of CDH13 transcript by miRANDA and TargetScan yielded different potential miRNA binding sites, whose nucleotide numbering starts after the CDH13 stop codon. The predicted miRNAs are listed according to their alignment score (from the higher to the lower one); predicted miRNAs belonging to the same family are reported as aligned to each other (* indicates conserved nucleotide) and then paired with the target if the potential site was the same. The seed region of miRNAs is marked in bold

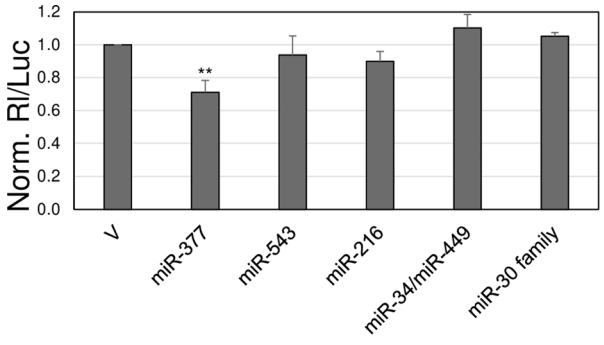

FIGURE 2 T-cadherin sequences screening for responsiveness to miRNAs in HT-29 cell line. The predicted T-cadherin target sequences of indicated miRNAs were cloned in the reporter vector psiCheck-2. One construct was used for miR-34a, miR-34c, miR-449a, and miR-449b (miR-34/miR-449) since their target sequences result overlapped; for the same reason, one construct was also used for miR-30 family. After 48 h of transfection, luciferase activities were recorded; the Renilla luciferase activity (RI) was normalized to the firefly luciferase activity (Luc), whose gene is also contained in the reporter vector and the uninhibited activity relative to the parental vector psiCheck-2 (V) was set to 1. **p-value <.01 at Student's t test

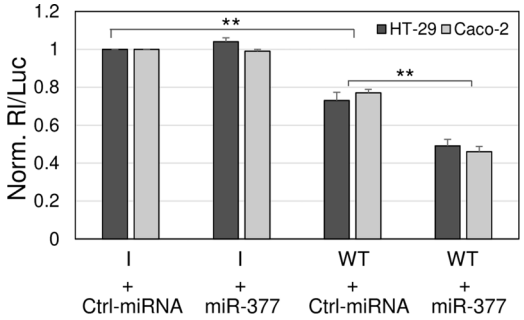

FIGURE 3 T-cadherin sequence validation as a target of miR-377. HT-29 and Caco-2 cells were transfected with the luciferase-based reporter plasmid psiCheck-2 containing the T-cadherin target sequence for miR-377 (WT) or a control DNA with an inverted target sequence (I), along with 50 nM miR-377 mimic (miR-377), or unrelated molecule used as a negative control, Ctrl-miRNA. **p < .01 at Student's t test

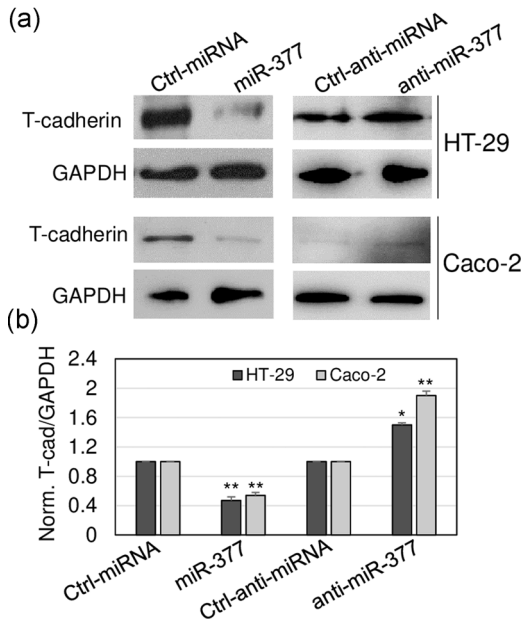

FIGURE 4 Interfering activity of miR-377 on T-cadherin expression. (a) Representative Western blot analyses of protein extracts at 48 h after transfection with miR-377 mimic and anti-miR-377 or their negative control molecules, Ctrl-miRNA and Ctrl-anti-miRNA, respectively. (b) Quantification of T-cadherin protein band signal normalized to that of GAPDH; signal intensity values determined for the control experiments (Ctrl-miRNA or Ctrl-anti-miRNA) were set at 1; values represent the mean ± *SD* of three independent experiment. *$p < .05$; **, $p < .01$ at Student's *t* test for indicated points relative to their control experiments

T-cadherin expression in cell cultures. Conversely, transfection of the miRNA inhibitor led to an increased expression of T-cadherin in comparison to Ctrl-anti-miRNA transfection in both HT-29 and Caco-2 cells (Figure 4).

4 | DISCUSSION

The study of APN dysfunctions in cancer so far has overlooked the role of T-cadherin as a potent APN-binding and anti-tumorigenic protein, although the emerging notion of strong downregulation of T-cadherin in many cancers, such as lung, breast, ovarian, cervical, prostate, liver, melanoma, and colorectal cancer, in contrast with the ubiquitary expression of AdipoR1/AdipoR2 (Andreeva & Kutuzov, 2010; Hebbard & Ranscht, 2014; Kawashima et al., 2017). In particular, loss of T-cadherin is correlated to increased tumorigenicity and cancer progression in vivo; consistently, re-expression of T-cadherin into cancer cells inhibits cell proliferation and invasiveness, increases susceptibility to apoptosis, and decreases tumor growth in xenograft models (Andreeva & Kutuzov, 2010). In the proposed model for APN-mediated inhibition of cancer pathways, in normal epithelial cells, APN is bound by T-cadherin and presented directly or indirectly to AdipoR1/R2 to suppress signaling pathways promoting cancer; in contrast, cancer cells downregulate T-cadherin, thus allowing the prevailing of pathways prompting the carcinogenesis (Hebbard & Ranscht, 2014). Given the relevance of T-cadherin in carcinogenesis, it is important to investigate the molecular mechanisms underlying its downregulation, also in terms of perspective therapeutic interventions. So far the role of aberrant methylation has been extensively documented in numerous cancer cell lines and cancers. In particular, different studies and a comprehensive meta-analysis suggested that T-cadherin promoter methylation plays an important role in the initiation and progression of CRC, it was strongly associated with CRC risk, it is associated with poor prognosis and correlated with the overall survival of patients (Duan et al., 2017; Hibi et al., 2004; Ren & Huo, 2012; Toyooka et al., 2002; Wang et al., 2012; Xu et al., 2004; Ye et al., 2017).

Our study investigated a possible contribution of miRNAs in downregulating T-cadherin, also taking into account their well-recognized role in colorectal carcinogenesis (Wai Hon et al., 2020). A bioinformatics prediction and application of a number of criteria detailed in the results yielded 12 miRNAs potentially targeting the 3′ UTR of T-cadherin (Figure 1). miR-377 resulted the miRNA targeting T-cadherin in colorectal adenocarcinoma cells (HT-29 and Caco-2 cell lines): luciferase reporter construct carrying the putative target sequence resulted inhibited by endogenously expressed miR-377 in comparison to control constructs; miR-377 mimic transfection augmented that luciferase reduction, validating the direct interaction between the miRNA and 3′ UTR sequence of T-cadherin (Figures 2 and 3). Finally, modulation of miR-377 by transfecting miR-377 mimic or its inhibitor resulted in a strong and significant reduction or increase of T-cadherin level, respectively, as revealed by Western blot analyses (Figure 4). With the aim to verify a correlation between miRNA and T-cadherin expression levels also in tumor tissues, we performed an inspection of the Encori platform (http://starbase.sysu.edu.cn) based on The Cancer Genome Atlas (TCGA) data, revealing a possible reverse correlation between miR-377 and T-cadherin expression in 450 COAD tissues patients, although with a weak Pearson correlation coefficient ($r = -0.011$), probably due to the very large heterogeneity of tumor stages and thus requiring further studies. The miRNA is impressively upregulated in the 450 COAD tissue patients in comparison to normal tissues (fold change = 154.2, data from the Encori platform) and could have a role in downregulating T-cadherin expression during carcinogenesis. Consistently, the oncogenic power of miR-377 was recently unveiled in the CRC context: it prompted cell proliferation by accelerating the G1-S phase transition; it promoted cell migration, invasion, and epithelial-mesenchymal transition (EMT) (Liu et al., 2018). Given the oncogenic activity of miR-377 and the oncosuppressive activity of T-cadherin in COAD context, the regulatory circuit highlighted in this study may add new insights into molecular mechanisms driving the multistep process of colorectal carcinogenesis, and perspectively it could be exploited to identify novel biomarkers and therapeutic targets, as well as for development of innovative therapeutic interventions based on the recovery of T-cadherin expression. Finally, the finding of a miRNA regulation acting on T-cadherin expression may also have implications in other biological contexts, since the emerging

multifaceted role of T-cadherin, an atypical member of cadherin superfamily, plasma membrane receptor of adiponectin, but lacking transmembrane and cytosolic domains. As an example, it could be interesting to investigate the regulatory circuit here unveiled also in obesity and metabolic disorders, where the adiponectin-receptor axis has a role and miR-377 was recently found to promote adipose tissue inflammation and insulin-resistance (Peng et al., 2017).

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DATA AVAILABILITY STATEMENT

Data available on request from the authors

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