The Level of **TWIST1** expression determines the response of colon cancer cells to mitogen-activated protein kinases inhibitors

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**INTRODUCTION**

Colorectal cancer (CRC) is one of the deadliest diseases worldwide.[1] The overall prognosis for patients with early stages (I and II) CRC is relatively good, while patients with advanced CRC are at increased risk of unfavorable outcome.[2] Longstanding observations showed that adjuvant chemotherapy improves the overall survival of patients with stage III and IV CRC.[3,4] For years, the most widely used chemotherapeutic agent in patients with CRC was 5-fluorouracil (5FU).[4] However, developing drug resistance affects the effectiveness of chemotherapy.

**Abstract**

**Background/Aim:** Currently, it has been proposed that combination of 5-fluorouracil (5FU) with inhibitors of the mitogen-activated protein kinases (MAPKs) signaling pathway might enhance the efficacy of 5FU-based chemotherapy in colon cancer. Our study aimed to investigate an impact of **TWIST1** silencing on the sensitivity of cancer cells to 5FU and selected MAPK inhibitors.

**Materials and Methods:** The suppression of **TWIST1** expression in human colon cancer HT29 and HCT116 cell lines was achieved by transduction with lentiviral vector carrying the **TWIST1** silencing sequence (pLL3.7-sh**TWIST1**). The statistical calculation was performed with analysis of variance or Dunnett’s test for comparison to control group. Paired Student’s *t*-test was performed when two groups were analyzed.

**Results:** Suppression of **TWIST1** reduced the proliferation rate of colon cancer cells and enhanced their sensitivity to 5FU and MAPKs inhibitors. The sensitivity of HT29 cells to examined compounds was more dependent on **TWIST1** expression level compared to HCT116 cells. The most noticeable effect of **TWIST1** suppression on sensitivity of both colon cancer cell lines to combined treatment of 5FU and the MAPKs inhibitors was observed for inhibitors of p38α/β and JNK1-3. We also noted that the suppression of **TWIST1** significantly sensitized both cell lines to combined treatment of 5FU and Rac inhibitor.

**Conclusions:** Our observations point to **TWIST1** expression level as a marker of colon cancer sensitivity to combined treatment of 5FU and MAPKs inhibitors.

**Keywords:** 5-Fluorouracil, colon cancer, mitogen-activated protein kinases inhibitors, **TWIST1**

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In the recent decade, several genes and signaling pathways that determine the sensitivity to chemotherapeutics were identified (reviewed in Holohan et al.[8]). Multiple observations indicate that dysregulation of mitogen-activated protein kinase (MAPK) pathway due to oncogenic mutations of genes encoding the key components of this signaling cascade contributes to the pathogenesis of various tumors, and are implicated in tumor growth and resistance to anticancer therapies.[6,7] Recently, several therapies based on the use of selective inhibitors that block this pathway entered the clinical trials.[8,9] It has been observed that some of these inhibitors, e.g., trametinib (MEK inhibitor), increase the sensitivity of colon cancer cells to 5FU.[10]

Transition of epithelial cells to a mesenchymal phenotype [epithelial–mesenchymal transition (EMT)] is another adaptive mechanism that renders the tumor cells resistant to chemotherapy.[11–13] Several studies have shown that TWIST, a transcription factor belonging to the family of basic-helix-loop-helix proteins (bHLH), is the major EMT inducer and factor that permits the release of cancer cells from the primary tumor.[14,15] Moreover, it has been reported that overexpression of TWIST1/2 in cancer cells was associated with resistance to some chemotherapeutic agents.[16]

Our present study aimed to investigate the impact of TWIST1 expression level on sensitivity of colon cancer cells to 5FU and some inhibitors of MAPK pathway.

MATERIALS AND METHODS

Reagents
Penicillin, streptomycin, Dulbecco’s Modified Eagle’s Medium (DMEM), SB 203580, GW 5074, PD 98059, JNK-IN-8, and manumycin A were obtained from Sigma-Aldrich (St. Louis, MO, USA). Geldanamycin, SB 202190, SP 600125, EHT 1864 were obtained from Tocris Bioscience (Northpoint, UK).

Lentivirus transduction-driven TWIST1 knockdown
The knockdown of TWIST1 was performed as described previously.[17] Briefly, the TWIST1 silencing sequence (shRNA) was subcloned into the pL.L3.7 lentiviral vector (Addgene, Cambridge, MA, USA). The pL.L3.7 vector with scrambled oligo was used as a negative control. Packaging cells (HEK 293T) were cotransfected with either empty pL.L3.7 vector or pL.L3.7-shTWIST1, an envelope plasmid pMD2.G, and a packaging plasmid psPAX2 with X-tremeGENE 9 (Roche, Mannheim, Germany), according to the manufacturer’s protocol. The culture medium was used as the source of lentiviral particles for HCT116 and HT29 cells transfection. The cancer cells were cultured with the infectious medium until at least 80% of the population became green fluorescent protein (GFP)-positive.

Cells and culture conditions
Human colon carcinoma HCT116 and HT29 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained under standard conditions (5% CO₂–95% air, 98% humidity, and 37°C) in DMEM supplemented with penicillin (10 U/ml), streptomycin (100 g/ml), and 10% fetal bovine serum (Gibco). Cells were transferred to a fresh medium twice weekly.

Cells treatment
After passaging, the cells were plated in 24-well plates at a density of 0.1 × 10⁶/ml/well and were cultivated overnight. The medium was then removed and replaced with a medium containing appropriate drug at a desired concentration. The control cells were maintained in a medium containing equal amount of solvent (0.01% dimethyl sulfoxide) used to dissolve the chemical compound. The relative effective concentration of 5FU for 50% response (EC₅₀) was estimated by means of the four-parameter logistic nonlinear regression model using GraphPad Prism v 6.07 software.

Cell viability
The number of viable cells was determined by Trypan Blue dye exclusion and by determining the mitochondrial dehydrogenase activity using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is converted to formazan in living cells.[18] The absorbance of converted dye was measured in a microplate reader (1420 multilabel Counter VICTOR3) at 570 nm with background subtraction at 690 nm. The results were expressed as a percentage relative to untreated control cells.

RNA extraction and mRNA level determination
Total RNA was isolated using a High Pure RNA Isolation Kit (Roche Applied Science) according to the manufacturer’s instruction. The gene expression level was determined by one-step real-time polymerase chain reaction (RT-PCR) performed in LightCycler 2.0 with Path-ID Multiplex one-step RT-PCR Kit (Applied Biosystems) and appropriate Universal ProbeLibrary Probe for Human (Roche Applied Science). Transcript levels were normalized to that of the β-actin gene (ACTB) except TWIST1 transcript, which was normalized to TATA-binding protein gene (TBP) transcript. The primer sequences, TaqMan probes, and cycling conditions used are listed in Table 1.
**TWIST1** protein determination

The qualitative assessment of **TWIST1** was performed by Western blot as described previously.[17] Rabbit anti-**TWIST1** (Abcam, Cambridge, MA, USA) (2.5 µg/ml), and goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO, USA) were used as a primary and secondary antibody, respectively. For detection of β-actin (reference), mouse anti-β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) was used.

**Statistical analysis**

The statistical calculation was performed with analysis of variance or Dunnett's test for comparison with control group. Paired Student's t-test was performed when two groups were analyzed. P values <0.05 were considered as significant.

**RESULTS**

**Suppression of TWIST1 in colon cancer HT29 and HCT116 cells**

Examination of **TWIST1** expression in colon cancer cell lines showed that in HCT116 cells the level of **TWIST1** expression was almost three times higher than in HT29 cells. The relative **TWIST1** transcript level (**TWIST1**/**TBP**) averaged 2.21 ± 0.34 and 5.89 ± 0.76 in HT29 and HCT116 cells, respectively [Figure 1a]. We noted that **TWIST1** silencing procedure reduced the **TWIST1** transcript level by 110- and 45-fold in HT29 **sh** **TWIST1** and HCT116 **sh** **TWIST1** cells, respectively. Western blot analysis showed that the **TWIST1** protein level in both cell lines transfected with vector carrying the silencing sequence that significantly reduced as compared to cells transfected with a vector carrying scrambled oligo (parental cells) [Figure 1b].

**Suppression of TWIST1 reduced the proliferation rate of colon cancer HT29 and HCT116 cells**

We observed that the proliferation rates of HT29 and HCT116 parental cells were similar. Downregulation of **TWIST1** expression resulted in a significant reduction of the growth rate of both cell lines, however, the growth retardation induced by **TWIST1** silencing was more prominent in HT29 **sh** **TWIST1** cells [Figure 2].

**Suppression of TWIST1 enhances the sensitivity of colon cancer HT29 and HCT116 cells to inhibitors of MAPK signaling**

To define the key components of MAPK signaling that are crucial for survival and proliferation of HT29 and HCT116 cells, we used specific inhibitors. The exact target of a particular compound is indicated on the scheme presented in Figure 3. The concentrations of employed inhibitors were in accordance with previous reports.[19-22]

The sensitivity of HCT116 cells to most inhibitors examined was not dependent on **TWIST1** expression level, except that for JNK and Rac inhibitors. In the presence of Rac inhibitor (EHT1864) or JNK inhibitors (SP600125, JNK-IN-8) significantly higher reduction of viable cells was observed in cultures of HCT116 cells with suppressed expression of **TWIST1** (HCT116 **sh** **TWIST1**) compared to parental HCT116 cells [Figure 4b]. Conversely, the sensitivity of HT29 cells to examined compounds was more dependent on **TWIST1** expression level compared to HCT116 cells. Inclusion of manumycin A in the cell culture medium, a selective and strong inhibitor of Ras farnesyl transferase, affected significantly the HT29 cell viability, but only very weak effect on HCT116 cells viability was observed [Figure 4a]. Inclusion into HCT116 cells culture, the Raf1 kinase inhibitors (geldanamycin, GW5074) resulted in a moderate reduction of viable cells number (by 15–25%) irrespective of **TWIST1** expression level. Conversely, these inhibitors in HT29 cells produced more pronounced effects, which were dependent on **TWIST1** expression level [Figure 4a]. PD98059, an inhibitor of MEK, only marginally (~20%) affected the viability of HT29 cells and had no effect on number of HCT116 cells. In the presence of p38α/β inhibitors (SB202190, SB203580), the number of viable cells decreased significantly in cultures of both cell lines, but this phenomenon was not dependent on the level of **TWIST1** expression [Figure 4].

**Suppression of TWIST1 expression affects the expression level of genes encoding proteins involved in MAPK signaling**

The cellular response to inhibitors of MAPK pathways could depend on the expression level of genes encoding proteins involved in MAPK signaling. Therefore, we examined the effect of **TWIST1** silencing in colon cancer HT29 and HCT116 cells on the expression level of genes encoding the key proteins of ERK1/2, JNK, and p38α/β module of MAPK signal transduction pathways. Our measurements showed that the expression level of **K-ras** decreased ~5-fold in HT29 **sh** **TWIST1** cells, but no change in **K-ras** transcript level could be observed in HCT116 **sh** **TWIST1** cells [Figure 5]. Conversely, the expression level of gene that encodes other members of Ras superfamily, the **RAC1** was not changed in HT29 **sh** **TWIST1** cells [Figure 5]. There are three isoforms of Rac proteins encoded by **RAC1**, **RAC2**, and **RAC3** genes located on chromosomes 7, 22, and 17, respectively. In HT29 cells the transcript level of **RAC2** and **RAC3** was below the detection limit, whereas in HCT116 cells all three transcripts were detected. In HCT116 **sh** **TWIST1** cells, the expression of **RAC1**, **RAC2**, and **RAC3** decreased
by 6-, 3-, and 2.5-fold, respectively [Figure 5]. We noted that the expression level of gene encoding RAF1 kinase, which is the initiating kinase of the ERK1/2 module, decreased ~2-fold in the cells with suppressed TWIST1. On the contrary, the expression level of MAP2K1 gene encoding MEK1 kinase was not affected by TWIST1.

The transcript level of MAPK8 gene encoding JNK1 kinase decreased 1.6- and 2.5-fold in HT29 shTWIST1 and HCT116 shTWIST1 cells, respectively. We observed that the expression level of MAPK14 gene encoding p38α/β kinase decreased 3- and 1.6-fold in HT29 shTWIST1 and HCT116 shTWIST1 cells, respectively. The functions of MAPKs are mediated by family members of MAPK-activated protein kinases (MAPKAPKs). This family comprises the p90 ribosomal S6 kinases (RSKs), mitogen- and stress-activated kinases (MSKs), MAPK-interacting kinases (MNKs), and MAPK-activated kinases (MKs). Our analyses revealed that in HCT116 cells the transcript level of RPS6KA3 gene encoding RSK2, and RPS6KA2 gene encoding RSK3 was beyond the detection limit. In HT29 cells the suppression of TWIST1 expression resulted in remarkable increase of RPS6KA3 gene expression and decreased expression of RPS6KA2 gene. In HT29 cells we have detected two transcript variants of RPS6KA3 gene. The level of RPS6KA3v1 increased ~10-fold and the level of RPS6KA3v2 decreased 2-fold in HT29 shTWIST1 cells [Figure 5]. The expression level of MKNK2 encoding MNK2 kinase was not altered in HCT116 shTWIST1 cells, whereas in HT29 shTWIST1 cells expression of this gene decreased by 1.8-fold. The two transcript variants of MAPKAPK2 gene encoding MK2 kinase decreased in cells with suppressed expression of TWIST1, although the extents of transcripts level diminution were significantly larger in HT29 shTWIST1 cells.

Suppression of TWIST1 enhances the sensitivity of colon cancer HT29 and HCT116 cells to 5FU cytotoxicity

Cultivation of HCT116 and HT29 cells with various concentrations of 5FU showed different sensitivity of

| Gene transcript | Primers | TaqMan probe |
|-----------------|---------|--------------|
| TWIST1          | GGACATCATGGACTTCTCTATT | Universal ProbeLibrary Probe # 6 (Roche) |
| MAP2K1          | TTTTGAGAATTAGACATGTGGTCTT | Universal ProbeLibrary Probe #7 (Roche) |
| KRAS            | AGGGCTGACATCTCTGGCGCTC | Universal ProbeLibrary Probe # 44 (Roche) |
| RAC1            | ATTTTCAGGTTGGCTCCCTGCA | Universal ProbeLibrary Probe # 77 (Roche) |
| RAC2            | GCTGCTCCTCAACACAGAGC | Universal ProbeLibrary Probe # 7 (Roche) |
| RAC3            | CATCAAGTGCTTGTTGCTG | Universal ProbeLibrary Probe # 60 (Roche) |
| MAPK8           | GGAGGACCCCTCTTCCTTTA | Universal ProbeLibrary Probe # 89 (Roche) |
| RAF1            | CATTGACAGAGCAGCATGATG | Universal ProbeLibrary Probe # 56 (Roche) |
| MAPK14          | GGACCCTCCTCTAGCTCTTGCAG | Universal ProbeLibrary Probe # 77 (Roche) |
| RPS6Ka3v1       | GTGCCGAGGTCTCTCTCTGT | Universal ProbeLibrary Probe # 4 (Roche) |
| RPS6Ka3v2       | CAGTTGCTGGAATCTGAAAAA | Universal ProbeLibrary Probe # 39 (Roche) |
| RPS6Ka2         | CAGTTGCTGGAATCTGAAAAA | Universal ProbeLibrary Probe # 69 (Roche) |
| MKNK2           | AAGACACCTTGCCCACTCC | Universal ProbeLibrary Probe # 2 (Roche) |
| MAPKAPK2v1      | GGCAATCAAGGCAAGATT | Universal ProbeLibrary Probe # 2 (Roche) |
| MAPKAPK2v2      | CAACAAAGTCCCTCAAAACC | Universal ProbeLibrary Probe # 88 (Roche) |
| TBP             | Universal ProbeLibrary Reference | Universal ProbeLibrary Reference Gene Assay Roche, Human TBP Gene Assay |
| ACTB            | Universal ProbeLibrary Reference | Universal ProbeLibrary Reference Gene Assay Roche, Human ACTB Gene Assay |

Reverse transcription: 48 ºC (10 min), 95 ºC (10 min). Amplification: 95 ºC (10 s), 60 ºC (45 s). TWIST1 expression was normalized to transcript of TATA-binding protein gene. The human genes are listed according to HUGO Gene Nomenclature Committee.
these cell lines to cytotoxic effect of 5FU. The relative effective concentration of 5FU for 50% response (EC$_{50}$) averaged 0.17 ± 0.02 and 0.61 ± 0.05 μM for HT29 and HCT116 cells, respectively. TWIST1 silencing procedure increased significantly the cancer cells sensitivity to 5FU [Figure 6]. The 5FU EC$_{50}$ decreased to 0.016 ± 0.001 and 0.12 ± 0.02 μM for HT29 shTWIST1 and HCT116 shTWIST1 respectively. In order to determine whether inhibitors of MAPKs signaling enhance the efficacy of 5FU, we assessed the cytotoxicity of both kinds of compounds individually and in combination using both parental and HT29 shTWIST1 and HCT116 shTWIST1 cell lines. The combined manumycin A and 5FU treatment resulted in a significant synergistic enhancement in cytotoxicity in parental and HCT116 shTWIST1 cell lines. Similar effects were observed in parental HT29 cells, but not in HT29 shTWIST1 cells. In HT29 shTWIST1 cells manumycin A alone exerted strong cytotoxic effect, resulting in reduction of viable cells number by 90% [Figure 4]. The Rac inhibitor (EHT 1864) exerted strong and additive to 5FU cytotoxic effect in HCT116 but not in HT29 cells. Moreover, the combined cytotoxicity of EHT 1864 and 5FU was enhanced in HCT116 cells with suppressed TWIST1. We observed that an inhibitor of MEK1/2 (PD 98059) alone had no effect on both parental and HCT116 shTWIST1 cells growth and viability [Figure 4], while the combined 5FU and PD 98059 treatment significantly potentiated the 5FU cytotoxicity. Such an additive effect of 5FU and PD98059 was observed in parental HT29 cells but not in HT29 shTWIST1 cells. The higher enhancement of 5FU cytotoxicity in both parental and HCT116 shTWIST1 cells was noted for combined 5FU and JNK-IN-8 (an inhibitor of JNK1-3) treatment [Figure 7b]. Whereas, combination of these two compounds did not enhance the 5FU effect on HT29 cells. The cytotoxic effect of 5FU was potentiated by an inhibitor of p38α/β kinase (SB203580) in HT29 and HCT116 parental cell lines. However, this effect was only moderately enhanced in HCT116 shTWIST1 and was not observed in HT29 shTWIST1 cells [Figure 7].

**DISCUSSION**

Over the last decade, tremendous progress has been made in the development of molecular targeted therapy for human cancers. This resulted, for some cancers, in a significant improvement of patients’ survival.[23,24] It has been observed that cancer cells sensitivity to several agents depended on the presence of $K$-ras mutation.[25] This could explain the relative resistance of HCT116 cells harboring $K$-ras mutation[25] to most agents used in our study except Rac and JNK inhibitors. A previous study documented the involvement of Ras–Rac–MEK signaling pathway in colon carcinogenesis.[26] Our observations indicate that HCT116 cell line is dependent on Rac–MEK–JNK signaling. The increasing sensitivity of HCT116 shTWIST1 cells to Rac and JNK inhibitors could be related to decreased expression of genes encoding Rac and JNK proteins. We have observed that in HCT116 cells with lowered TWIST1, the levels of $RAC1$, $RAC2$, $RAC3$, and MAPK8 expression decreased significantly. This scenario did not occur in HT29 cells. HT29 cell line expresses wild K-ras but harbors activating mutation of BRAF (V600E).[22] In our study, the HT29 cells showed relatively high sensitivity to inhibitors of Ras and RAF. Moreover, the effects of these compounds increased
significantly in the HT29 cells with suppressed TWIST1. The high effect of TWIST1 suppression on the HT29 cells sensitivity to inhibitors of Ras–Raf–MAPK signaling might be associated with EMT. Several studies demonstrated that TWIST is a crucial EMT inducer (reviewed in Je et al. [27]). Therefore, it might be assumed that cells with decreased TWIST1 protein level gain more epithelial phenotype. Importantly, evidence accumulated over the last decade indicates that EMT is not a binary process, and cells may display a hybrid epithelial–mesenchymal phenotype (reviewed in Jolly et al. [28]).

Figure 3: Simplified scheme depicting the core components of MAPK signaling pathway and its pharmacological modulation at different levels.

Figure 4: The effect of TWIST1 suppression on the sensitivity of HT29 (a) and HCT116 (b) cells to MAPKs inhibitors. The parental cells and shTWIST1 cells were cultured in the presence of given compound at concentrations indicated in figure for 48 hours, and the cell viability was determined by MTT assay. The control cells (no additives) were maintained in a medium containing an equal amount of solvent (0.01% DMSO) used to dissolve drugs. The results are expressed as a percentage (mean ± SD, n=5) relative to control cells (no additives). *P < 0.05

Figure 5: Effect of TWIST1 suppression on the expression level of some MAPKs. The expression levels of genes encoding: MAP2K, KRAS, RAC1, RAC2, RAC3, MAPK8, RAF1, MAPK14, RPS6KA2, RPS6KA3, MKNK2 and MAPKAPK2 were determined by qPCR. Data were normalized to ACTB mRNA level and are presented as fold-change (mean ± SD, n=4) relative to parental cells (transduced with scrambled vector). *P < 0.05 vs parental cell.
that EMT reduces the Ras dependency of cancer cells, whereas upon MET the Ras dependency is gained. The high dependency of the HT29 shTWIST1 cells on Ras was associated with high sensitivity of these cells to inhibitors of RAF-1 kinase. These observations indicate that, unlike the HCT116 cells, the HT29 cells proliferation depends on Ras–Raf pathway, and suppression of TWIST1 sensitizes this cell line to inhibitors of this signaling pathway.

Recently, several clinical trials showed that combined BRAF and MEK inhibition improved the outcome of patients with BRAF V600-mutant metastatic melanoma. However, such treatment in a subset of BRAF-mutant colorectal patients yielded significantly lower efficacy than what has been achieved in BRAF-mutant melanoma patients. It was suggested that in CRC harboring mutated BRAF combined targeting of BRAF and other, than MEK, therapeutic target would increase the clinical response. Our study showed that colon cancer cells with mutated BRAF and suppressed TWIST1 become highly sensitive to RAS inhibition.

5FU-based adjuvant chemotherapy became the standard of care for patients with colon cancer. Inclusion of oxaliplatin and irinotecan to 5FU-based treatment significantly improved the efficacy of the chemotherapy. Currently, it has been proposed that targeting the signaling proteins that are responsible in reactivating RB protein might enhance the efficacy of 5FU-based chemotherapy in colon cancer. The study on HT29 cell line showed that the combination of 5FU with MEK inhibitor trametinib enhanced the efficacy of 5FU. In our present study, we also observed that the inhibitor of MEK sensitized the HT29 and HCT116 cell lines to the cytotoxicity of 5FU; however, these effects were not dependent on TWIST1 expression level. Previously we have reported that suppression TWIST1 enhanced the sensitivity of colon cancer cells to 5FU. In the present study, the most noticeable effect of TWIST1 suppression on sensitivity of colon cancer cell lines to combined treatment of 5FU and the MAPKs inhibitors was observed for inhibitors of p38α/β and JNK1-3. We also noted that the suppression of TWIST1 significantly sensitized both cell lines to combined treatment of 5FU and Rac inhibitor. Due to the central position of Rac in intracellular signaling that
induces cell transformation and tumor growth, this protein became the molecular target for anticancer therapies.\[33,34\] Our observation on increased sensitivity of cancer cells with low level of TWIST1 to Rac inhibitor might be used for selection of patients who respond positively to this kind of drug.

**CONCLUSION**

We conclude that the TWIST1 expression level correlates with the sensitivity of colon cancer cells to cytotoxic effect of 5FU. Moreover, cells with low level of TWIST1 are highly sensitive to combined treatment of 5FU and various MAPKs inhibitors. We assume that determination of TWIST1 expression may prove to be a useful tumor marker in selecting patients who may benefit from combined treatment of 5FU and some MAPKs inhibitors.

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**Conflicts of interest**

There are no conflicts of interest.

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