Decreased RNA-binding protein IGF2BP2 downregulates NT5DC2, which suppresses cell proliferation, and induces cell cycle arrest and apoptosis in diffuse large B-cell lymphoma cells by regulating the p53 signaling pathway

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Received April 16, 2021; Accepted June 23, 2022

DOI: 10.3892/mmr.2022.12802

Abstract. Diffuse large B-cell lymphoma (DLBCL) remains difficult to treat clinically due to its highly aggressive characteristics. Insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) and 5'-nucleotidase domain-containing 2 (NT5DC2) have been suggested as potential regulators in numerous types of cancer. The present study aimed to determine whether downregulation of IGF2BP2 and NT5DC2 suppresses cell proliferation, and induces cell cycle arrest and apoptosis in DLBCL cells by regulating the p53 signaling pathway. The expression levels of IGF2BP2 and NT5DC2 in DLBCL cells were determined by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Transfection of cells with IGF2BP2 overexpressing plasmids and NT5DC2 interference plasmids was performed, and the efficacy of transfection was confirmed by RT-qPCR and western blot analysis. The viability, proliferation, cell cycle progression and apoptosis of DLBCL cells were analyzed by Cell Counting Kit-8 assay, 5-bromo-2-deoxyuridine staining and flow cytometry. RNA pull-down and immunoprecipitation assays were used to verify the binding of IGF2BP2 and NT5DC2. The expression levels of apoptosis, cell cycle and p53 pathway-associated proteins were determined by western blotting. The results revealed that NT5DC2 expression was increased in DLBCL cell lines and was the highest in OCI-Ly7 cells. IGF2BP2 expression was also increased in OCI-Ly7 cells and IGF2BP2 bound to NT5DC2. Knockdown of NT5DC2 suppressed cell viability and proliferation, induced cell cycle arrest and promoted apoptosis in DLBCL cells, which was reversed by upregulation of IGF2BP2. In addition, knockdown of NT5DC2 increased the expression of p53 and p21, but suppressed the expression of proliferating cell nuclear antigen, CDK4 and cyclin D1; these effects were reversed by upregulation of IGF2BP2. In conclusion, knockdown of NT5DC2 suppressed cell viability and proliferation, induced cell cycle arrest and promoted apoptosis in DLBCL cells by regulating the p53 signaling pathway and these effects were reversed by upregulation of IGF2BP2.

Introduction

Malignant lymphomas are a group of malignant hematological tumors originating in lymph nodes or other lymphoid tissue and include Hodgkin's lymphoma and non-Hodgkin lymphoma (NHL) (1). Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of NHL worldwide (2). Currently, in Europe, the incidence of DLBCL is ~3.8/100,000 individuals (3). DLBCL is an aggressive and highly heterogeneous hematological tumor (4), which accounts for 80% of invasive lymphomas (5) and can lead to diffuse destruction of normal lymph node structure (4). At present, there is no effective clinical treatment for patients with DLBCL; thus, novel treatment methods for DLBCL are needed.

Previous studies on the mechanism of 5'-nucleotidase domain-containing 2 (NT5DC2) in certain types of malignancy have shown that knockout of NT5DC2 can inhibit angiogenesis and recruitment of tumor-associated macrophages to suppress colorectal cancer progression (6). NT5DC2 inhibition by regulating p53 signaling has also been reported to suppress non-small cell lung cancer metastasis (7). NT5DC2 upregulation is associated with poor overall and relapse-free survival of hepatocellular carcinoma (HCC) and is an independent prognostic factor for overall survival (8). Furthermore, NT5DC2 may promote liver cancer cell proliferation by stabilizing EGF...
receptor (9) and causes tumorigenicity of glioma stem cells by increasing Fyn expression (10). However, to the best of our knowledge, there are no studies on the value of NT5DC2 for the treatment of DLBCL.

The IGF2BP family consists of three members, namely IGF2BP1, 2 and 3, which bind directly to and stabilize the mRNA of their target gene (11,12). IGF2BP2 serves an important role in myogenesis (13,14), maintenance of glioblastoma stem cells (15,16), and invasion and migration of cancer cells (primarily HCC) (17). Previous studies have shown that IGF2BP2 enhances HCC proliferation in vitro and in vivo via an N6-methyladenosine-flap structure-specific endonuclease 1-dependent mechanism (18). Upregulation of IGF2BP2 in pancreatic cancer via multiple mechanisms has also been reported to promote tumor cell proliferation through activation of the PI3K/Akt signaling pathway (19). However, the functions of RBP IGF2BP, and the interactions between IGF2BP2 and NT5DC2 in DLBCL remain to be explored.

The present study aimed to investigate whether downregulation of IGF2BP2 and NT5DC2 suppresses cell proliferation, and induces cycle arrest and apoptosis in DLBCL cells by regulating the p53 signaling pathway.

Materials and methods

Cell culture. Human B lymphocytes (GM12878) and DLBC cell lines (SU-DHL-8, OCI-Ly8 and OCI-Ly7) were purchased from BeNa Culture Collection; Beijing Beina Chunglian Institute of Biotechnology. GM12878 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (P/S). OCI-Ly8 and OCI-Ly7 cells were cultured in IMDM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% P/S. SU-DHL-8 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), containing 20% FBS and 1% P/S. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Cell transfection. The pGPU6/GFP/Neo vector (Shanghai GenePharma Co., Ltd.) was used to construct a vector containing short hairpin RNA (shRNA) targeting NT5DC2. In addition, an IGF2BP2 genomic fragment was cloned into a pcDNA3.1 vector (Shanghai GenePharma Co., Ltd.) to overexpress IGF2BP2. OCI-Ly7 cells (4x10⁵ cells/well) were seeded in a 6-well plate and cultured until they reached 70-80% confluence. Subsequently, OCI-Ly7 cells were transfected with 5 μg shRNA-NT5DC2#1/2, with non-targeting sequences (shRNA-NC) as a negative control, or 4 μg overexpression (Oe)-IGF2BP2, with empty vector (Oe-NC) as a negative control, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. Following 48 h transfection, the transfection efficiency was validated by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis; the cells were collected for subsequent experiments 48 h after transfection.

RT-qPCR analysis. Total RNA was extracted from transfected cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was generated using PrimeScript™ RT Reagent kit (Takara Bio, Inc.) according to the manufacturer’s protocol. RT-qPCR was performed with SYBR Premix Ex Taq™ II kit (Takara Bio, Inc.) using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification conditions were as follows: 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec, and a final extension step at 72°C for 10 min. The following primer sequences were used: NT5DC2, forward 5′-GAC TGT GG-3′ and reverse 5′-AAG TGA GGT GTC TAC TTT TA-3′; IGF2BP2, forward 5′-GAA TCT CCG CAG CTG TTT GA-3′; and GAPDH, forward 5′-GCT GTT GTA GGC GGA AAG GTG CT-3′ and reverse 5′-AGT GAT GGC AT GACT GTT GG-3′. The relative expression levels of NT5DC2 and IGF2BP2 were calculated using the 2^ΔΔCq method (20) and normalized to GAPDH.

Western blot analysis. Total protein was extracted from human B lymphocyte and DLBC cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified with SDS-PAGE (Beyotime Institute of Biotechnology) on 12% gels and transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% non-fat milk for 2 h at room temperature and then incubated with the following primary antibodies at 4°C overnight: Anti-NT5DC2 (1:1,000; cat. no. orb312336; Biorbyt Ltd.), anti-CDK4 (1:1,000; cat. no. orb48321; Biorbyt Ltd), anti-CDK6 (1:1,000; cat. no. orb538814; Biorbyt Ltd.), anti-cyclin D1 (1:1,000; cat. no. ab34175; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab96495; Abcam), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-cleaved caspase 3 (1:1,000; cat. no. #9661; Cell Signaling Technology, Inc.), anti-cytochrome c (cyto-c; 1:1,000; cat. no. #4272; Cell Signaling Technology, Inc.), anti-cleaved poly-ADP ribose polymerase (PARP; 1:1,000; cat. no. ab32561; Abcam), anti-IGF2BP2 (1:2,000; cat. no. ab124930; Abcam), anti-p53 (1:1,000; cat. no. orb99409; Biorbyt Ltd.), anti-p21 (1:1,000; cat. no. orb38089; Biorbyt Ltd.), anti-PCNA (1:1,000; cat. no. ab92552; Abcam) and anti-GAPDH (1:2,500; cat. no. ab9485; Abcam). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Finally, protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) and the expression levels of each protein were semi-quantified using AlphaImager™ 2000 Imaging System (ProteinSimple).

Cell counting kit-8 (CCK-8) assay. Transfected OCI-Ly7 cells (1x10⁵ cells/well) were seeded into a 96-well plate, which was placed in a humidified incubator at 37°C for 24 h. At 24, 48 and 72 h, 10 μl CCK-8 reagent was added to each well and the 96-well plate was then incubated at 37°C for 2 h according to the manufacturer's protocol (Beyotime Institute of Biotechnology). Finally, the OD value at 450 nm was determined using a microplate reader.

5-Bromo-2-deoxyuridine (BrdU) staining. Transfected OCI-Ly7 cells (5x10⁵ cells/well) were seeded on a pre-treated glass coverslip in a 24-well plate. Subsequently, 200 μl BrdU
(Beyotime Institute of Biotechnology) was added to the cells and incubated at 37°C in the presence of 5% CO₂ for 24 h. The transfected OCI-Ly7 cells were then fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with PBS containing 0.1% Tween-20 and blocked with 2% BSA (Sigma Aldrich; Merck KGaA) at room temperature for 2 h. The transfected OCI-Ly7 cells were then incubated with primary antibody against BrdU (1:500; cat. no. ab6326; Abcam) for 1 h at 4°C and with a secondary antibody (cyane 3-conjugated goat anti-mouse IgG; cat. no. A0521; 1:500; Beyotime Institute of Biotechnology) for 30 min at room temperature, then stained with 20 µg/ml DAPI at 37°C for 30 min. After washing with PBS, BrdU/DAPI-positive cells were observed under a fluorescence microscope (magnification, x200).

**Flow cytometry.** Transfected OCI-Ly7 cells (1x10⁶ cells/well) were seeded into a 96-well plate and cultured for 48 h at 37°C. For cell cycle analysis, transfected OCI-Ly7 cells were collected and fixed with 70% ethanol at 4°C overnight. After washing with PBS, the transfected OCI-Ly7 cells were incubated with 1 mg/ml RNase A for 20 min at 37°C, followed by staining with propidium iodide (PI) and 1% Triton X-100 for 20 min at 4°C. The experimental data were collected using a flow cytometer (BD FACScan; Becton, Dickinson and Company). For apoptosis analysis, cells were washed with PBS and resuspended with 100 µl binding buffer, followed by addition of 10 µl PI/FITC-Annexin V (Roche Diagnostics) and incubation at room temperature for 1 h in the dark. Flow cytometry (BD FACScan; Becton, Dickinson and Company) was used to detect apoptosis within 1 h. The cell cycle distribution and apoptosis of transfected OCI-Ly7 cells were analyzed by Cell Quest acquisition and analysis software (V6.0; BD Biosciences).

**RNA immunoprecipitation (RIP) assay.** An EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-704; MilliporeSigma) was employed to perform RIP assay following the manufacturer's instructions. A total of 2x10⁶ OCI-Ly7 cells were incubated with 100 µl RIP Lysis Buffer (MilliporeSigma) for 5 min at 4°C and centrifuged at 40,000 x g at 4°C for 10 min to obtain the cell lysate. Subsequently, 50 µl protein A/G magnetic beads conjugated with 5 µg anti-IGF2BP2 (cat. no. ab117809; 2 µg/mg; Abcam) or IgG antibody (cat. no. ab72730; 2 µg/mg; Abcam) were added to 800 µl cell lysate and incubated overnight at 4°C. After being washed with washing buffer six times, protease K was then used to isolate the immunoprecipitated RNA at 55°C for 30 min. The expression levels of NT5DC2 in the immunoprecipitated RNA were detected by RT-qPCR analysis as aforementioned.

**RNA pull-down assay.** An RNA pull down kit (cat. no. P0202; Geenseed) was synthesized in vitro by Shanghai GenePharma Co., Ltd., labeled with biotin using the RNA 3' End Desthiobiotinylination kit (Thermo Fisher Scientific, Inc.) and incubated with streptavidin magnetic beads (Thermo Fisher Scientific, Inc.). Cellular lysates (50 µl) were incubated with NT5DC2 or control probes overnight at 4°C. The magnetic beads (30 µl) were mixed with RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.), incubated overnight at 4°C and centrifuged at 1,000 x g for 1 min at 4°C. After washing with RIP wash buffer (50 mM Tris; pH 7.5; 300 mM NaCl and 0.1% NP-40) three times, the magnetic beads were boiled in SDS buffer to denature the protein bound to RNA. The protein expression levels of IGF2BP2 were determined by western blot analysis as aforementioned. The sequences of NT5DC2 and non-targeting control were as follows: NT5DC2, forward 5'-TAATACGACTCACTATAGGG CAGCGTATCGAGA-3' and reverse 5'-TACGCG GTGACTGAAAG-3'; non-targeting control, forward 5'-TAAATACGACTCACTATAGGG CAGCGTATCGAGA-3' and reverse 5'-AGTGATGCGATGACTGTGG-3'.

**Biostatistics.** StarBase v2.0 (https://starbase.sysu.edu.cn/) was used to predict the potential binding of NT5DC2 with IGF2BP2.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.) and data are presented as the mean ± standard deviation of ≥3 repeats. Statistical differences between two or multiple groups were determined using unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test, respectively. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**NT5DC2 is upregulated in DLBC cells and knockdown of NT5DC2 inhibits proliferation of DLBC cells.** The mRNA and protein expression levels of NT5DC2 in DLBC cells were increased compared with those in GM12878 cells. mRNA and protein expression of NT5DC2 was highest in OCI-Ly7 cells compared with the other DLBC cell lines (Fig. 1A).

The mRNA and protein expression levels of NT5DC2 were decreased in DLBC cells transfected with shRNA-NT5DC2#1/2 compared with in the untransfected group, and were lower in the shRNA-NT5DC2#2 group compared with in the shRNA-NT5DC2#1 group (Fig. 1B). Therefore, shRNA-NT5DC2#2 was used in the subsequent experiments. Knockdown of NT5DC2 suppressed the viability (Fig. 1C) and proliferation (Fig. 1D) of OCI-Ly7 cells. These findings indicated that NT5DC2 silencing may have a suppressive role in DLBC cell proliferation.

**Knockdown of NT5DC2 induces DLBC cell cycle arrest and apoptosis.** Knockdown of NT5DC2 induced DLBC cell cycle arrest, as indicated by an increased population of cells in G₀/G₁ phase and a decreased population of cells in S phase (Fig. 2A).

The expression levels of cell cycle-associated proteins (CDK4, CDK6 and cyclin D1) were significantly decreased in the shRNA-NT5DC2 group (Fig. 2B). In addition, knockdown of NT5DC2 promoted OCI-Ly7 cell apoptosis (Fig. 2C), decreased Bcl-2 expression, and increased expression of Bax, cleaved caspase 3, cyto-c and cleaved PARP (Fig. 2D). These findings suggested that NT5DC2 depletion stimulated cell cycle arrest and apoptosis in DLBC.
IGF2BP2 is upregulated in DLBC cells and is the RBP of NT5DC2. Based on starBase, the potential interaction between IGF2BP2 and NT5DC2 was predicted. The mRNA and protein expression levels of IGF2BP2 were increased in OCI-Ly7 cells compared with in GM12878 cells (Fig. 3A). The interaction between IGF2BP2 and NT5DC2 was confirmed by RIP and RNA pull-down assays (Fig. 3B and C).

The mRNA and protein expression levels of IGF2BP2 were increased in OCI-Ly7 cells transfected with Oe-IGF2BP2 (Fig. 3D). Furthermore, the mRNA and protein expression levels of NT5DC2 were decreased in OCI-Ly7 cells transfected with shRNA-NT5DC2; this was reversed by IGF2BP2 overexpression (Fig. 3E). These findings indicated that IGF2BP2 was the RBP of NT5DC2.

Upregulation of IGF2BP2 reverses the regulatory effect of NT5DC2 knockdown on the proliferation and apoptosis of DLBC cells. Upregulation of IGF2BP2 enhanced the viability (Fig. 4A) and proliferation (Fig. 4B) of OCI-Ly7 cells transfected with shRNA-NT5DC2. Upregulation of IGF2BP2 also promoted cell cycle progression of OCI-Ly7 cells transfected with shRNA-NT5DC2, as indicated by a decreased number of cells in the G0/G1 phase and an increased number of cells in the S phase (Fig. 4C).

The apoptosis of OCI-Ly7 cells transfected with shRNA-NT5DC2 was decreased by upregulation of IGF2BP2 (Fig. 4D). In addition, upregulation of IGF2BP2 promoted Bcl-2 expression, and suppressed the expression levels of Bax, cleaved caspase 3, cyto-c and cleaved PARP in OCI-Ly7 cells.
transfected with shRNA-NT5DC2 (Fig. 4E). Collectively, IGF2BP2 elevation may reverse the effects of NT5DC2 knockdown on DLBC cell proliferation and apoptosis.

**Discussion**

The incidence of DLBCL in China has been increasing at a rate of ≥25,000 cases/year (21), and the mortality rate of malignant lymphoma was estimated at 7.07/100,000 in Spain...
in 2018 (22). Although rituximab and anthracycline-based combination chemotherapy regimens have the potential to cure DLBCL, 30–40% of patients still have poor prognosis due to refractory or recurrent disease, as well as severe side effects and development of drug resistance due to long-term chemotherapy (23). Therefore, it is necessary to identify novel effective treatments for patients with DLBCL.

NT5DC2 is a member of the NT5DC family, which has a highly similar sequence to 5'-nucleotidase, cytosolic II (NT5C2). Mutant NT5C2 has been reported to be associated...
with proliferation, metastasis and chemotherapy resistance of acute lymphoblastic leukemia cells (24,25). The present results indicated that NT5DC2 was increased in DLBCL, cells and knockdown of NT5DC2 markedly suppressed the viability and proliferation of OCI-Ly7 cells in vitro. These findings were consistent with previous studies, which showed that NT5DC2...
is oncogenic in numerous types of tumor, such as HCC, glioblastoma, leiomyosarcoma and colorectal cancer (6,9,10,26). The occurrence and development of tumors are associated with dysregulation of the cell cycle and cell cycle arrest is one of the primary causes of the apoptosis of tumor cells (27). In the present study, knockdown of NT5DC2 increased the G<sub>0</sub>/G<sub>1</sub> phase ratio and decreased the S phase ratio in OCI-Ly7 cells, which suppressed the viability and proliferation of OCI-Ly7 cells. The cell cycle is regulated by multiple proteins, including cell cycle-promoting (cyclin D1, cyclin D3, CDK4 and CDK6) and -arresting proteins (p18, p21 and p27) (9). The present data showed that the expression levels of cyclin D1, CDK4 and CDK6 was downregulated in NT5DC2-knockdown OCI-Ly7 cells, which led to cell cycle arrest.

Apoptosis, also known as type-I programmed cell death, is an active process of programmed cell death regulated by genes, including Bcl-2 and Bax, under physiological or pathological conditions (28). Apoptosis in different tissues shows common characteristics, such as morphological (formation of apoptotic bodies) and biochemical changes (ectropion of phosphatidylserine in the membrane and DNA fragmentation) (29). The present study revealed that knockdown of NT5DC2 induced the apoptosis of OCI-Ly7 cells, which was accompanied by decreased expression levels of anti-apoptotic proteins (Bcl-2) and increased expression levels of pro-apoptotic proteins (Bax, cleaved caspase 3, cyto-c and cleaved PARP); these effects were reversed by IGF2BP2 overexpression.

The signaling pathway regulated by the tumor suppressor gene p53 is inhibited in numerous types of cancer, such as colon, breast, lung and esophageal cancer (30). In vitro and in vivo studies have revealed that reactivation of p53 may eliminate tumors initiated by transforming events independent of p53 (31,32). The tumor suppressor protein p53 serves a key role in tumors, and its dysfunction is associated with the pathogenesis of B-cell malignancy, including DLBCL (33,34). Drakos et al (35) found that the activated p53 signaling pathway led to cell cycle arrest and apoptosis in DLBCL cells. Evasion of apoptosis of DLBCL cells occurs via p53 inactivation, and an effective and precise therapeutic strategy for DLBCL has been observed using the mouse double minute 2 homolog-p53 inhibitor APG-115 (36). The present study reported that p53 expression levels were markedly upregulated in NT5DC2-knockdown OCI-Ly7 cells, whereas the opposite results were detected in cells transfected with Oe-IGF2BP2, which was consistent with the findings reported in a previous study (7).

There are certain limitations in the present study. Firstly, the present study only investigated the role of NT5DC2 in DLBCL in vitro; in vivo data to verify these findings should be collected in future work. Secondly, further clarification is required to identify the detailed and specific regulatory mechanism underlying NT5DC2/p53 signaling-mediated DLBCL. To the best of our knowledge, there have been no studies on drugs targeting NT5DC2 for clinical application. Further investigation of the molecular role of NT5DC2 is required for the clinical application of NT5DC2 as a therapeutic target.

In conclusion, the present study demonstrated that knockdown of NT5DC2 suppressed proliferation, and induced cell cycle arrest and apoptosis in DLBCL cells by regulating the p53 signaling pathway, which was reversed by IGF2BP2 overexpression. Therefore, IGF2BP2 and NT5DC2 may be promising targets to improve treatment efficacy in DLBCL.
Acknowledgements

Not applicable.

Funding

The present study was supported by the Scientific Research Fund of Heilongjiang Provincial Education Department (grant no. 2018-KYYWF-0932); the North Medicine and Functional Food Characteristic Subject Project in Heilongjiang Province (grant no. 2018-TSXXK-02) and the Innovation and Entrepreneurship Training Program Project of College Students in Heilongjiang Province (grant no. 201910222086).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

HQ and CW were involved in designing the experiments. YC, YW, CL, DZ and YY performed the experiments, analyzed the data and interpreted the results. YC and YW drafted the manuscript, and HQ and CW revised the manuscript. HQ and CW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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