Inhibition of NR4A1 Promotes ROS Accumulation and IL24-Dependent Growth Arrest in Rhabdomyosarcoma

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Abstract

Nuclear receptor 4A1 (NR4A1, Nur77) is overexpressed in rhabdomyosarcoma (RMS), and inactivation of NR4A1 (siNR4A1) or treatment with the NR4A1 antagonist 1,1-bis (3-indoly)-1-(p-hydroxy-phenyl)methane (DIM-C-pPhOH) has antiproliferative and proapoptotic effects on RMS cells. However, the mechanism by which NR4A1 inhibition exerts these effects is poorly defined. Here, we report that NR4A1 silencing or inhibition resulted in accumulation of reactive oxygen species (ROS) and ROS-dependent induction of the tumor suppressor–like cytokine IL24 in RMS cells. Mechanically, NR4A1 was found to regulate the expression of the proreductant genes thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1), which are downregulated in RMS cells following NR4A1 knockdown or inhibition. Silencing TXNDC5 and IDH1 also induced ROS accumulation and IL24 expression in RMS cells, suggesting that NR4A1 antagonists mediate their antiproliferative and apoptotic effects through modulation of proreductant gene expression. Finally, cotreatment with the antioxidant glutathione or IL24-blocking antibody reversed the effects of NR4A1 inhibition, demonstrating the importance of both ROS and IL24 in mediating the cellular responses.

Implications: Overall, these data elucidate the mechanism by which NR4A1 inhibition functions to inhibit the proliferation, survival, and migration of RMS cells.

Introduction

Rhabdomyosarcoma (RMS) is a cancer primarily seen in children and adolescents and represents >50% of soft tissue sarcomas in this younger age group. The prognosis of patients with RMS is dependent on multiple factors including age of the patient, the site of the tumor, and the tumor size, weight, and degree of metastasis from the tumor site (1–3). Prognosis also differs among patients classified into the major tumor types, namely embryonal RMS (ERMS) and alveolar RMS (ARMS), which is characterized by expression of genes resulting from the fusion of PAX3 and PAX7 with FOXO1 (PAX3-FOXO1 and PAX7-FOXO1; refs. 4, 5). The PAX3-FOXO1 fusion gene is the critical prognostic marker for ARMS patients with metastatic disease, with an estimated overall 4-year survival of 8% compared with 75% survival rate of patients with PAX7-FOXO1–expressing tumors (6–8). Five-year survival rates can be as high as 83% in younger patients (age 1–4 years) with localized disease (3); however, among childhood and adolescent cancers, the decline in mortality has been minimal with patients with RMS. Results of PAX3-FOXO1 knockdown or overexpression studies demonstrate the functional importance of this fusion gene in maintaining the aggressive ARMS cancer cell phenotype and this is due, primarily, to the pro-oncogenic PAX3-FOXO1-regulated genes (9–11). Moreover, patients treated with cytotoxic drug therapy for childhood cancers exhibit a high rate of chronic disease incidence (95.5%) in their 40s (12, 13), indicating that there is a critical need for development of innovative, less toxic therapies for treating patients with RMS.

Development of new mechanism-based drugs for treatment of RMS is ongoing and includes ROS-inducing anticancer agents, which have been identified in genomic studies and are effective tumor growth inhibitors in RMS patient-derived xenografts (14–18). The nuclear orphan receptor NR4A1 is overexpressed in tumors from patients with RMS and in other solid tumors (19–27), and studies in the laboratory have been investigating the functions of NR4A1 in RMS and solid tumor-derived cancer cell lines by RNAi (23–25, 27–32). The results indicate that NR4A1 is pro-oncogenic and in RMS, NR4A1 regulates cell proliferation, survival, and migration/invasion and associated genes. The bis-indole derived compound 1,1-bis(3-indoly)-1-(p-hydroxyphenyl) methane [DIM-C-pPhOH/CDIM8] acts as an NR4A1 antagonist and inhibits NR4A1-regulated pro-oncogenic responses and genes (19–27). Analysis of changes in gene expression after knockdown of NR4A1 or PAX3-FOXO1 or treatment with the NR4A1 antagonist DIM-C-pPhOH identified IL24 as a gene induced by all three treatments (33). In this study, we demonstrate that silencing of NR4A1 by RNAi or treatment with DIM-C-pPhOH resulted in ROS-dependent induction of IL24, which is due to downregulation of two proreductant NR4A1-regulated genes, namely thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1). Results of this study demonstrate a novel pathway for inducing expression...
of the tumor suppressor–like IL24 directly in RMS cells. Like most cancer cell lines, IL24 expression in RMS cells is low but is inducible. IL24 exhibits a broad spectrum of anticancer activities in multiple cancer cell lines and is being developed for clinical applications as a biotherapeutic (34–36). Results of this study illustrate a novel small-molecule approach for delivering IL24 in tumors.

Materials and Methods

Cell lines, antibodies, and cell proliferation

RD and Rh30 rhabdomyosarcoma cell lines were initially purchased from ATCC and authenticated in 2014 (Promega Powerplex 18D) at Duke University DNA Analysis Laboratories. Mycoplasma contamination was routinely monitored and cell lines were Mycoplasma-free. Cells were maintained at 37°C in the presence of 5% CO2 in DMEM/Ham’s F-12 medium with 10% FBS with antibiotic or RPMI1640 Medium with 10% FBS and antibiotic, respectively, that was purchased from GenDepot. β-Actin antibody DMEM, RPMI1640 Medium, and 36% formaldehyde were purchased from Sigma-Aldrich. Hematoxylin was purchased from Vector Laboratories. Sp1 antibody was purchased from Abcam and Glutathione (GSH) reduced free acid was purchased from Millipore. TXNDC5 and IDH1 antibodies were purchased from Genetex. PAX3-FOXO1A antibodies were purchased from Millipore. TXNDC5 and IDH1 antibodies were purchased from Abcam and Glutathione (GSH) reduced free acid was purchased from GenDepot. FBS with antibiotic or RPMI1640 Medium with 10% FBS and antibiotic, respectively, that was purchased from GenDepot. FBS with antibiotic or RPMI1640 Medium with 10% FBS and antibiotic, respectively, that was purchased from GenDepot.

Results of this study illustrate a novel small-molecule approach for delivering IL24 in tumors.

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Western blot analysis

RD and Rh30 rhabdomyosarcoma cells (3.0 × 10^5/well) were seeded in DMEM/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped FBS and were allowed to attach for 24 hours. Cells were seeded and subsequently treated with varying concentration of DIM-C-pPhOH for 24 hours or with 100 nm of respective siRNA for 72 hours. Western blots were determined with Immobilon Western Chemiluminescent Substrates (Millipore) to develop images captured on a Kodak Image Station 4000 MM Pro (Molecular Bioimaging) as described previously (30–33).

siRNA interference assay

RD and Rh30 rhabdomyosarcoma cells were seeded (1.2 × 10^5/well) in 6-well plates in DMEM/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped FBS and left to attach for 24 hours. Knockdown was carried out using Lipofectamine 2000 reagent according to the manufacturer’s protocol. Small inhibitory RNAs and GL2 (nonspecific oligonucleotide) were prepared and purchased from Sigma-Aldrich. The siRNA complexes used in the study are as follows: siGL2-5’, CGU ACG CGG AAU ACU UCG A; siNR4A1, SASI_Hs02_00333289; siTXNDC5, SASI_Hs01_00211116; siIDH1, SASI_Hs01_00340497; siIL-24, SASI_Hs01_00097938.

Generation and measurement of ROS

Cellular ROS levels were ascertained using the cell-permeable probe CM-H2DCFDA [5-(and-6)-chloromethyl-2’/3’ dichlorodihydrofluorescein diacetate ester] from Invitrogen. CM-H2DCFDA is nonfluorescent until cleavage of the acetyl groups by intracellular esterases and oxidation that transpires within the cell. Following treatment of the cells for 12 hours with DIM-C-pPhOH (±5 mmol/L GSH 3 hours prior to treatment) or 72 hours with siRNAs, cells plated on a 6-well culture plate were trypsinized, neutralized, then loaded with 10 μmol/L of probe for 20 minutes, washed once with serum-free medium, and then ROS was measured by flow cytometry using Accuri’s C6 Flow Cytometer (Accuri).

Statistical analysis

Statistical significance of differences between the treatment groups was determined by Student t test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a P value less than 0.05 was considered statistically significant. All statistical tests were two-sided.

Results

It was previously reported that inactivation of NR4A1 by RNAi or treatment with a C-DIM/NR4A1 antagonist resulted in down-regulation of PAX3-FOXO1 and induction of IL24 (33); down-regulation of PAX3-FOXO1 by RNAi also resulted in the induction of IL24. These results suggest that inactivation of NR4A1 using the antagonist DIM-C-pPhOH (CDM8) would proceed via NR4A1 → PAX3-FOXO1 → IL24. Results in Fig. 1A show that
Figure 1.
Induction of IL24 by NR4A1 antagonist. A, Rh30 cells were treated with CDIM8 in the absence (−) or presence of IL24 knockdown (siIL-24), and whole-cell lysates were analyzed by Western blots. B and C, Rh30 cells were treated with 20 μmol/L CDIM8 for different times or for 24 hours (B) and whole-cell lysates were analyzed by Western blots (C). D, ARMS cells were transfected with pCMV-IL24 expression plasmid and after 24 hours, whole-cell lysates were analyzed by Western blots. E, Tumor lysates from athymic nude mice bearing Rh30 cells as xenografts (39) and treated with corn oil (control) or adenoviral–IL24 were analyzed by Western blots. Results are expressed as means ± SD for at three replicate determinations for each treatment group (E) and significant (P < 0.05) effects (⁎) are indicated.
DIM-C-pPhOH (CDIM8) induced IL24 and downregulated PAX3-FOXO1 and PAX3-FOXO1-regulated genes (NMyc, RASSF4, MyoD1, Gremlin, DAPK) in wild-type Rh30 cells; however, this was not observed in cells where IL24 is silenced by RNAi and quantitation of the bands is illustrated in Supplementary Fig. S1A. These results suggest that in Rh30 cells, CDIM8-mediated downregulation of PAX3-FOXO1 is IL24-dependent and in cells treated with CDIM8 IL24 is upstream from PAX3-FOXO1. This observation is supported by the time course study illustrated in Fig. 1B showing that DIM-C-pPhOH rapidly induced IL24 (within 3 hours), and this was followed by downregulation of PAX3-FOXO1 (after 3–6 hours). Moreover, treatment of Rh30 cells with CDIM8 also modulates expression of several IL24-regulated genes (Fig. 1C; refs. 34–36), and similar effects were observed for other NR4A1 ligands (Supplementary Fig. S1B; ref. 37). Further evidence that IL24 is upstream from PAX3-FOXO1 is illustrated in Fig. 1D, which shows that overexpression of IL24 in three ARMS cell lines (Rh30, Rh18, and Rh41) downregulated PAX3-FOXO1 and PAX3-FOXO1-regulated genes. Moreover, further analysis of tumor lysates from a previous mouse xenograft study (33) showed that administration of adenosiral IL24 directly into the tumor decreased expression of PAX3-FOXO1 and PAX3-FOXO1-regulated genes (Fig. 1E). These results confirm that IL24 decreases expression of PAX3-FOXO1, suggesting that induction of IL24 by silencing NR4A1 or by treatment with CDIM8 is the major pathway for downregulation of PAX3-FOXO1.

The role of IL24 in mediating the downstream effects of DIM-C-pPhOH or knockdown of NR4A1 (siNR4A1) on regulating PAX3-FOXO1 expression and inhibiting cell growth, survival, and invasion was investigated in Rh30 cells. Figure 2A shows that CDIM8 or siNR4A1-mediated induction of IL24 and the subsequent decreased repression of PAX3-FOXO1 are reversed in cells cotransfected with siIL24. Moreover, CDIM8 and siNR4A1-mediated inhibition of Rh30 cell growth (Fig. 2B), induction of Annexin V staining (Fig. 2C), and inhibition of Rh30 cell migration (Fig. 2D) were also attenuated in cells cotransfected with siIL24.

Because previous studies show that IL24 is induced by reactive oxygen species (ROS; ref. 38) and siNR4A1 and CDIM8 also induce ROS in RMS cells (28), we therefore investigated the role of CDIM8/siNR4A1–induced ROS in mediating induction of IL24. CDIM8 and NR4A1 silencing induced ROS in Rh30 cells and this response was attenuated in cells cotreated with GSH (Fig. 3A). This same treatment also induces IL24 and decreases PAX3-FOXO1 expression in Rh30 cells, and these responses are also attenuated after cotreatment with GSH (Fig. 3B). This same treatment also induces IL24 and decreases PAX3-FOXO1 expression in Rh30 cells, and these responses are also attenuated after cotreatment with GSH (Fig. 3B). The role of siNR4A1- and CDIM8-induced ROS on cell proliferation (Fig. 3C), Annexin V staining (Fig. 3D), and cell migration (Fig. 3E) was confirmed because the siNR4A1/CDIM8–mediated responses were all significantly attenuated after cotreatment with GSH. These results suggest that siNR4A1/CDIM8–mediated induction of ROS (Fig. 3) and subsequent induction of IL24 (Figs. 1 and 2) play a major role in treatment-related inhibition of Rh30 cell migration and invasion and induction of apoptosis.

Because induction of ROS and IL24 by DIM-C-pPhOH or NR4A1 silencing is PAX3-FOXO1 independent, we therefore investigated the importance of this pathway in RMS (RD) cells that do not express PAX3-FOXO1. Treatment of RD cells with siNR4A1 or CDIM8 induced ROS (Fig. 4A), IL24 (Fig. 4B), inhibited growth (Fig. 4C), induced Annexin V staining (Fig. 4D), and inhibited RD cell migration (Fig. 4E), and all of these responses were significantly attenuated after cotreatment with GSH. Induction of IL24 (Fig. 4F), inhibition of cell growth (Fig. 4G), induction of Annexin V staining (Fig. 4H), and inhibition of cell migration (Fig. 4I) by CDIM8 or siNR4A1 were also attenuated by simultaneous knockdown of IL24 (siIL24). This indicates that the NR4A1 (silencing or inactivation) → ROS → IL-24 is common to both ERMS (RD) and ARMS (Rh30) cell lines and is an important contributor to the anticancer activity observed after inactivation of NR4A1.

TXNDC5 and IDH1 are NR4A1-regulated genes (28–31) that maintain levels of cellular reductants, and therefore, we further investigated the role downregulation of these genes by CDIM8/siNR4A1 in the induction of ROS and IL24. Results in Fig. 5A and B show that NR4A1 silencing or treatment with CDIM8 decreased expression of TXNDC5 and IDH1 in RD and Rh30 cells, and this response was not blocked after cotreatment with GSH, suggesting that this response was ROS-independent due to decreased expression of these gene products. In contrast, knockdown of TXNDC5 (Fig. 5C) and IDH1 (Fig. 5D) by RNAi induced ROS and this response was attenuated in RD and Rh30 cells cotreated with GSH. These results suggest that induction of ROS by CDIM8 or by NR4A1 silencing is due, in part to their effects on decreasing expression of the proreductant genes TXNDC5 and IDH1.

Because induction of ROS is important for enhancing expression of IL24 and its subsequent inhibition of cell growth, survival, and migration, we examined the effects of knockdown of TXNDC5 and IDH1 on these same parameters in RD and Rh30 cells. Transfection of RD and Rh30 cells with siTXNDC5 (Fig. 6A) or siIDH1 (Fig. 6B) increased expression of IL24, and this was abrogated after cotreatment with GSH. Using this same protocol, we also observed that siTXNDC5 and siIDH1 decreased RD and Rh30 cell proliferation (Fig. 6C), induced Annexin V staining (Fig. 6D), and decreased invasion (Fig. 6E), and these responses were inhibited by cotreatment with GSH.

The role of NR4A1-mediated downregulation of TXNDC5 and IDH1 as intermediates in generating ROS-dependent induction of IL24 was investigated in RD and Rh30 cells transfected with oligonucleotides targeting TXNDC5 (siTXNDC5; Fig. 7A) and IDH1 (siIDH1; Fig. 7B). Both oligonucleotides induced IL24 expression and in Rh30 cells, PAX3-FOXO1 expression was decreased in Rh30 cells and these responses were blocked in cells cotransfected with siIL24. Results in Fig. 6C–E demonstrate that siTXNDC5/siIDH1–mediated inhibition of cell growth, survival (induced Annexin V staining), and migration was ROS-dependent (inhibited by GSH). Using this same experimental approach, we show that the effects of siTXNDC5/siIDH1 on cell proliferation, Annexin V staining and migration of RD and Rh30 cells (Fig. 7C–E) were also blocked by silencing of IL24. Thus, a major pathway associated with the anticancer activity of CDIM8/NR4A1 antagonists involves initial decreased transcription of TXNDC5 and IDH1, induction of ROS, and induction of the tumor suppressor-like cytokine IL24, which triggers downstream inhibition of RMS cell growth and migration and induction of apoptosis (Fig. 7F).

Discussion

IL24 [or melanoma differentiation associated gene-7 (MDA-7)] is a member of the IL10 family of cytokines secreted by immune
IL24 was initially identified and cloned from terminally differentiated human metastatic melanoma cells (34–36). Expression of MDA-7/IL24 protein is low or absent in the majority of cancer cells compared with their normal counterpart; however, it can be induced by appropriate treatments. Adenoviral-IL24 has been used in preclinical studies to investigate the anticancer activities of this biotherapeutic alone and in combination therapies using several different models (34, 35, 39, 40). Fisher and...
colleagues demonstrated that ROS-inducing agents, including HDAC inhibitors, overcame resistance to IL24-mediated therapy in cancer cell lines (34–36, 39–41), and the HDAC inhibitors trichostatin and butyrate induced IL24 in melanoma cells (42). This observation is highly relevant for RMS because genomic analysis of patients with ERMS "identified oxidative stress as a pathway of therapeutic relevance for RMS" and this was confirmed using ROS-inducing HDAC inhibitors (17).

Figure 3.
Role of ROS in mediating the anticancer activities of NR4A1 silencing or CDIM8. A, Rh30 cells were transfected with siNR4A1 or treated with CDIM8 in the presence or absence of 5 mM GSH and induction of ROS was determined by fluorescence using the cell-permeable probe as outlined in the Materials and Methods section. B, Cells were treated as described in A and effects on protein expression (from whole-cell lysates) were determined by Western blots. Rh30 cells were treated as described in A and effects on cell proliferation (C), Annexin V staining (D), and migration (E) were determined as outlined in the Materials and Methods section. Results are expressed as means ± SD for three replicate determinations for each treatment group (A, C, D + E) and significant (P < 0.05) effects (*) and reversal of these responses (**) are indicated.

Analysis of RNA sequencing data after treatment of Rh30 cells with DIM-C-pPhOH or knockdown of NR4A1 and PAX3-FOXO1 showed that all three treatments induced IL24 and we characterized induction of IL24 after PAX3-FOXO1 knockdown (33). These observations raised some interesting but unresolved questions, namely in cells transfected with siNR4A1 or treated with the receptor antagonist DIM-C-pPhOH is IL24 upstream or downstream from PAX3-FOXO1 and if it is upstream then what is the
Figure 4.
Role of ROS and IL24 in mediating the anticancer activities of NR4A1 inactivation by siNR4A1 or CDIM8 in RD cells. A, RD cells were transfected with siNR4A1 or treated with CDIM8 in the presence or absence of 5 mmol/L GSH, and ROS was determined using the cell-permeable fluorescent probe as outlined in the Materials and Methods section. RD cells were treated as described in A and effects on protein expression (B), cell proliferation (C), Annexin V staining (D), and cell migration (E) were determined as outlined in the Materials and Methods section. F, RD cells were transfected with siNR4A1 or treated with CDIM8 in the presence or absence of siIL24 (cotransfected) and whole-cell lysates were analyzed by Western blots. RD cells were treated as outlined in F and effects on cell proliferation (G), Annexin V staining (H), and cell migration (I) were determined as outlined in the Materials and Methods section. Results are expressed as means ± SD for three replicate determinations for each treatment group (A, C, D, E, G + H) and significant (P < 0.05) effects (*) and reversal of these responses (**) are indicated.
mechanism of IL24 induction by NR4A1. Results illustrated in Figs. 1 and 2 demonstrate that CDIM8-mediated downregulation of PAX3-FOXO1 was IL24-dependent and the IL24 was upstream from PAX3-FOXO1. Moreover, the inhibition of cell growth and migration and induction of apoptosis by CDIM8 or siNR4A1 in Rh30 and RD (ERMS) cells was also inhibited by si-IL24, suggesting that induction of IL24 is a major pathway associated with the anticancer activities of the NR4A1 antagonist CDIM8 and related compounds (Supplementary Fig. S1B; refs. 34–36, 39–41, 24, 25, 27–33, 37). Previous studies with

Figure 5. Regulation of TXNDC5 and IDH1 by NR4A1 and the effects of knockdown of TXNDC5 and IDH1 on ROS. RD and Rh30 cells were transfected with siNR4A1 (A) or treated with 20 mmol/L CDIM8 (B) in the presence or absence of GSH and after 24 hours, whole-cell lysates were analyzed by Western blots. RD and Rh30 cells were transfected with siTXNDC5 (C) or siIDH-1 (D) to knock down the TXNDC5 and IDH-1 genes, respectively, and induction of ROS was determined using the fluorescent cell-permeable probe. Results are expressed as means ± SD for three replicate determinations for each treatment group (C + D) and significant (P < 0.05) effects (*) and reversal of these responses (**) are indicated.
Knockdown of TXNDC5 and IDH-1 induced ROS-dependent anticancer activities. RD and Rh30 cells were transfected with siTXNDC5 (A) or siIDH-1 (B) in the presence or absence of 5 mmol/L GSH and after 24 hours, whole-cell lysates were analyzed by Western blots. RD and Rh30 cells were transfected with siTXNDC5 or siIDH-1 in the presence or absence of GSH and the effects on cell proliferation (C), Annexin V staining (D), and cell migration (E) were determined as outlined in the Materials and Methods section. Results are expressed as means ± SD for three replicate determinations for each treatment group (C, D, E) and significant (P < 0.05) effects (*) and reversal of these responses (**) are indicated.
Figure 7.
IL24-induced anticancer activities are due to silencing of TXNDC5 and IDH-1. RD and Rh30 cells were transfected with siTXNDC5 (A) or siIDH-1 (B) in the presence or absence of siIL24 and whole-cell lysates were analyzed by Western blots. RD and Rh30 cells were treated as described in A and B in the presence or absence of cotransfected siIL24 and the effects on cell proliferation (C), Annexin V staining (D), and cell migration (E) were determined as outlined in the Materials and Methods section. F, Model describing the anticancer activities of NR4A1 antagonists. Results are expressed as means ± SD for three replicate determinations for each treatment group (C, D + E) and significant (P < 0.05) effects (*) and reversal of these responses (**) are indicated.
NR4A1 Antagonists Induce IL24 in RMS

Thus, results of this study demonstrated that NR4A1 antagonists represent a novel class of drugs that induce IL24 in RMS and represent an alternative to adenoviral delivery for inducing IL24 expression in tumors from patients with RMS (35, 36, 39). In addition, we also demonstrate that the mechanism of ROS-induced IL24 involves inactivation of NR4A1, which results in the induction of oxidative stress due to the decreased expression of the reductant genes TXNDC5 and IDH1 (Fig. 7F). Because RMS tumors and particular ERMS are sensitive to inducers of oxidative stress, results of this study and previous reports (27, 28) support clinical applications of NR4A1 antagonists for RMS chemotherapy and ongoing studies are focused on developing more potent bis-indole derived compounds for treatment of this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E. Hedrick, K. Mohankumar, S. Safe
Development of methodology: E. Hedrick, K. Mohankumar, S. Safe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Hedrick, K. Mohankumar, A. Lacey, S. Safe
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Mohankumar, S. Safe
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