The Cancer/Testis Antigen Melanoma-Associated Antigen-A3/A6 Is a Novel Target of Fibroblast Growth Factor Receptor 2-IIIb through Histone H3 Modifications in Thyroid Cancer

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Abstract

Purpose: Fibroblast growth factor (FGF) signals play fundamental roles in development and tumorigenesis. Thyroid cancer is an example of a tumor with nonoverlapping genetic mutations that up-regulate mitogen-activated protein kinase. We reported recently that FGF receptor 2 (FGFR2) is down-regulated through extensive DNA promoter methylation in thyroid cancer. Reexpression of the FGFR2-IIIb isoform impedes signaling upstream of the BRAF/mitogen-activated protein kinase pathway to interrupt tumor progression. In this analysis, we examined a novel target of FGFR2-IIIb signaling, melanoma-associated antigen-A3 and A6 (MAGE-A3/6).

Experimental Design: cDNA microarray analysis was done on human WRO thyroid cancer cells transfected with FGFR2-IIIb or empty vector. Identified gene target was confirmed by reverse transcription-PCR and Western blotting. Gene regulation was examined by treatment of WRO cells with the methylation inhibitor 5'-azacytidine followed by methylation-specific PCR and reverse transcription-PCR and by chromatin immunoprecipitation.

Results: Gene expression profiling identified the cancer/testis antigen MAGE-A3/6 as a novel target of FGFR2-IIIb signaling. MAGE-A3/6 regulation was mediated through DNA methylation and chromatin modifications. In particular, FGF7/FGFR2-IIIb activation resulted in histone 3 methylation and deacetylation associated with the MAGE-A3/6 promoter to down-regulate gene expression.

Conclusions: These data unmask a complex repertoire of epigenetically controlled signals that govern FGFR2-IIIb and MAGE-A3/6 expression. Our findings provide insights into the interrelationship between novel tumor markers that may also represent overlapping therapeutic targets.

Thyroid cancer is a common endocrine malignancy and is increasing in incidence in North America (1). The majority, derived from follicular epithelial cells, represent a model of malignant transformation from benign adenomas and well-differentiated carcinomas to poorly differentiated thyroid carcinoma and the rare but rapidly lethal undifferentiated thyroid carcinoma (2). This spectrum of progression has been linked with a pattern of cumulative intragenic defects that correlates with tumor differentiation, aggressiveness, and metastatic potential (2). Gene rearrangements involving the RET proto-oncogene or activating point mutations along the Ras/BRAF pathway account for the majority of these carcinomas (2). These intragenic abnormalities are considered to be early events in thyroid carcinogenesis; cancer behavior and progression are further modified by dysregulation of growth factors and their cognate receptors.

Fibroblast growth factors (FGF) and FGF receptors (FGFR) have been implicated in the progression of endocrine neoplasms including thyroid carcinoma (3, 4). FGFs, a family of 23 known heparin-binding proteins, signal through four high-affinity tyrosine kinase receptors (FGFR1-FGFR4; refs. 4, 5). Each receptor has three immunoglobulin-like extracellular domains; alternative splicing of the third immunoglobulin-like domain of FGFR1 to FGFR3 results in two major transcripts referred to as IIIb and IIic isoforms. These isoforms display distinct FGF binding with differing functional properties (4, 6).

We have shown previously that FGFR expression is dysregulated in human thyroid tumors and cell lines (3). FGFR2 is consistently detected in normal thyroid tissues; its expression was diminished in thyroid tumors and in six carcinoma cell lines (3), due to down-regulation by DNA promoter methylation (7). Moreover, whereas FGFR1 promotes thyroid cell growth, FGFR2-IIIb displays a protective role against cancer progression in transformed thyroid carcinoma cells (7). These findings prompted us to investigate putative targets of FGFR2-IIIb, the major isoform of FGFR2 expressed in epithelial cells, through gene expression profiling. The current data unmask a novel relationship with the melanoma-associated antigen-A3 and A6 (MAGE-A3/6). Further, we show that these two genes are governed by common epigenetic mechanisms.
Table 1. Primers used in RT-PCR, MSP, and ChIP

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<td>F: 5'-GGATCAAGACGAGTTGAAAG-3'</td>
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<td>199</td>
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</table>

Abbreviations: F, forward primer; R, reverse primer; M, methylation; U, unmethylation.

Materials and Methods

Cell lines and cell culture. Human thyroid carcinoma-derived cell lines, WRO, NPA, and DRO (provided by Dr. J. Fagin, University of Cincinnati, Cincinnati, OH, originally established by Dr. G. Julliard, University of California at Los Angeles, Los Angeles, CA, TPC-1 (provided by Dr. S. M. Jhiang, Ohio State University, Columbus, OH), KTC-1 (established by Dr. J. Kurebayashi, Kawasaki Medical School, Okayama, Japan; ref. 8), and 8505C (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, streptomycin sulfate (100 units/ml), and penicillin (100 μg/ml). Primary human thyroid specimens were obtained at the time of surgery informed consent and Institution Review Board Approval.

Vector constructs and stable cell transfection. Full-length human FGFR2-IIIb cDNA (established by Drs. M. Terada and T. Yoshida, National Cancer Institute, Tokyo, Japan) was inserted into pcDNA1/Neo expression vector (Invitrogen) and a control empty vector (pcDNA1/Neo) were kindly provided by Dr. F. Radvanyi (Centre National de la Recherche Scientifique, Paris, France). The expression vectors were transfected into cells using LipofectAMINE (Invitrogen). Stable clones were selected and maintained in a growth medium containing 1 mg/mL geneticin (Life Technologies) as described previously (7). Alteration of FGFR2 expression was confirmed by Western blotting.

Growth factor stimulation. After overnight starvation in serum-free medium, cells were treated with varying doses of FGF7 (Sigma), each with 10 units/ml heparin (Sigma) in serum-free medium for 24 h at 37°C. Identical volume of vehicle served as control.

Oligonucleotide microarray analysis. Total RNA was extracted from cultured cells with Trizol (Invitrogen) and purified using an RNeasy kit (Qiagen, Inc.). Array hybridization to the Affymetrix U133Plus2 was conducted at The Center of Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada). RNA from two independent FGFR2-IIIb-transfected clones and two independent control clones transfected with vector alone (pcDNA) was subjected to in vitro transcription, labeling, and hybridization using standard Affymetrix protocols. Hybridized chips were washed and scanned on an Affymetrix GeneChip 3000 confocal scanner. Raw microarray data were analyzed by ArrayAssist software (Iobion) using the PLIER algorithm. Genes were considered to be differentially expressed if the signal changed at least 2-fold (or signal log 2 ratio 1). The four data sets (two for FGFR2-IIIb and two for pcDNA controls) were analyzed using gene expression and statistical tools in Spotfire’s DecisionSite software package. To identify gene targets that had significantly altered gene expression in FGFR2-IIIb- or transfected clones versus pcDNA, groups were compared with each other using the Student’s t test. Target genes were further restricted on filtering for a minimum 2-fold up or down change in signal log ratio, respectively. Gene targets were then characterized and graphically summarized based on similar expression profiles using Hierarchical and K-Means Clustering. These clustering algorithms were able to further elucidate intrinsic grouping of the significant genes based on two different statistical methods (hierarchical and exclusive clustering). 4

RNA extraction and reverse transcription-PCR analysis. Total RNA was isolated from cultured cells and frozen human thyroid tissue using Trizol. cDNA was generated using the Taqman Reverse Transcription Reagent kit (Applied Biosystems). PCR primers to amplify FGFR2-IIIb, MAGE-A3/6, and PGK-1 (as an internal control) are listed in Table 1.

Amplicons were designed to span introns to exclude genomic DNA contamination. Amplification was done using HotStarTaq DNA polymerase kit (Qiagen). PCR conditions were as follows: (a) 95°C for 15 min; (b) 30 cycles of 94°C for 30 s, 56°C or 38°C for 30 s, and 72°C for 1 min; (c) 72°C for 10 min; and (d) 4°C hold. Negative controls omitting reverse transcriptase and positive controls were included in each PCR.

Protein isolation and Western blotting analysis. Thyroid tissues were homogenized using a polytron homogenizer in radioimmunoprecipitation assay lysis buffer with proteinase inhibitors (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 12 μg/ml aprotinin, and 1 mmol/L sodium orthovanadate). Cultured cells were lysed in a radioimmunoprecipitation assay lysis buffer with proteinase inhibitors. Protein concentration of samples and bovine serum albumin standard were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories) by measurement at A 595.

Equal amounts of protein (40 μg) solubilized in sample buffer were separated on 10% SDS polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked in TBS containing 0.5% Tween 20 plus 5% nonfat dried milk for 1 h at room temperature and probed with primary antibodies at 4°C overnight.

4 http://www.biomedcentral.com/1471-2105/5/103
Primary antisera or monoclonal antibodies were used at the specified dilutions: anti–MAGE-A3 (1:500; Abgent) and anti-actin (1:1,000; Sigma). The antibody to MAGE-A3 also detects MAGE-A6 because of the high protein sequence homology. Membranes were washed three times for 10 min each in TBS containing 0.5% Tween 20 and incubated with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (1:2,000; Santa Cruz Biotechnology). Incubation with antibody was carried out overnight at 4°C with agitation. Negative controls omitted antibody or used an anti-IgG antibody. For PCR analysis, the histone-DNA cross links of eluates were reversed at 65°C, and the immunocomplexes were digested with protease K for 1 h at 50°C, and DNA was purified by phenol extraction and used for PCR amplification. PCRs were designed to amplify the corresponding CpG island region including noncoding exon 1 of MAGE-A3 genomic DNA (Table 1). Experiments were done on three independent occasions with product intensities quantified by scanning densitometry (Quantity One software; Bio-Rad).

### Results

**Establishment of stable clones of FGFR2-IIIb–expressing thyroid carcinoma cells.** We hypothesized that FGFR2 displays a protective role against cancer progression in transformed thyroid carcinoma cells because FGFR2 protein, and specifically FGFR2-IIIb, was the main FGFR consistently detected in normal thyroid tissues and diminished in the majority of human thyroid carcinoma cell lines (3, 7). To explore the implication of FGFR2 signaling in thyroid cancers, WRO cells, which do not endogenously express FGFR2, were forced to express FGFR2 by stable transfection of full-length human MAGE-A3/6 in Thyroid Cancer

**Table 2. Microarray analysis of FGFR2-IIIb target genes**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>U133Plus2 probe ID</th>
<th>Value (fold)</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
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NOTE: Ratio 1 indicates the fold of expression for each gene as determined by microarray analysis of pcDNA/WRO clone 1 versus FGFR2b/WRO clone 1. Ratio 2 indicates the fold of expression of pcDNA/WRO clone 1 versus FGFR2b/WRO clone 2. Ratio 3 indicates the fold of expression of pcDNA/WRO clone 2 versus FGFR2b/WRO clone 1. Ratio 4 indicates the fold of expression of pcDNA/WRO clone 2 versus FGFR2b/WRO clone 2.
FGFR2-IIIb cDNA. Western blotting showed strong expression of FGFR2 in FGFR2-IIIb/WRO cells, whereas pcDNA/WRO cells were negative (Fig. 1A). The doublet migration of FGFR2 is consistent with variable NH2-terminal glycosylation (11). Two independent clones of each transfected were used for further studies.

Oligonucleotide microarray analysis of thyroid cancer cell response to FGFR2-IIIb expression. To identify factors that are targeted by FGFR2-IIIb expression in thyroid cancer cells, cDNA microarray analysis was done comparing pcDNA/WRO cells (two independent clones, 1 and 2) versus FGFR2-IIIb/WRO cells (two independent clones, 1 and 2). Although expression of the majority of spotted genes was not significantly altered, we found that MAGE-A3 and MAGE-A6 were down-regulated by 76-fold and 60-fold, respectively (Table 2).

Reverse transcription-PCR (RT-PCR) was done to validate the oligonucleotide microarray findings. MAGE-A3 and MAGE-A6 are highly homologous with 98% alignment of nucleotide and 95% alignment of protein sequences and are considered functionally equivalent. Thus, we used common PCR primers targeted for both MAGE-A3 and MAGE-A6. MAGE-A3/6 gene expression was undetectable in normal thyroid specimens but was strongly expressed by wild-type WRO cells and pcDNA/WRO clones, whereas mRNA for both MAGE-A genes was undetectable in FGFR2-IIIb/WRO cells (Fig. 1B). We further confirmed the alteration of MAGE-A expression by Western blotting: MAGE-A3/6 protein was readily detectable in pcDNA/WRO but was down-regulated in response to expression of FGFR2-IIIb in WRO cells (Fig. 1C).

Reciprocal expression of MAGE-A3/6 and FGFR2-IIIb in normal thyroid and thyroid carcinoma cell lines. We determined the expression profiles of MAGE-A3/6 in relation to FGFR2-IIIb expression in transformed and nontransformed thyroid follicular cells. MAGE-A3/6 was detected by RT-PCR in four of six thyroid carcinoma cell lines; in contrast, only a faint signal was found in normal thyroid tissue specimens (Fig. 2A). We reported previously that FGFR2 protein is expressed in normal thyroid and is undetectable in seven of eight human thyroid carcinoma cell lines (TPC-1, NPA, 8505C, WRO, MRO, DRO, and ARO; only KTC-1 cells express FGFR2; refs. 3, 7). Papillary thyroid carcinoma-derived KTC-1 cells express FGFR2-IIIb (Fig. 2A). These same cell lines showed that MAGE-A3/6 expression is reciprocally expressed with undetectable levels of expression in normal thyroid tissue. In contrast, thyroid cancer cell lines were more likely to show MAGE-A3/6 expression. Thus, seven of eight thyroid carcinoma cell lines that were MAGE-A3/6 positive were negative for FGFR2-IIIb expression.

**FGF7/FGFR2 modulates MAGE-A3/6 expression in thyroid carcinoma cell lines.** Based on gene expression profiling, we asked whether stimulation of endogenous FGFR2-IIIb signaling modulates MAGE-A3/6 expression in thyroid carcinoma cells. We selected KTC-1 cells as a model of detectable FGFR2-IIIb and MAGE-A3/6 expression (Fig. 2A). Consistent with the reduced expression of MAGE-A3/6 induced by FGFR2-IIIb forced expression in WRO cells, FGF7 stimulation to engage endogenous FGFR2-IIIb in KTC-1 cells effectively down-regulated MAGE-A3/6 expression (Fig. 2B).

**MAGE-A3/6 expression is governed by epigenetic regulation in thyroid carcinoma cell lines.** Having determined that FGFR2-IIIb signaling can modulate MAGE-A3/6 expression in thyroid carcinoma cells, we examined the underlying mechanism of MAGE-A expression that is regulated by FGF signaling. As shown in Fig. 3A, a large CpG island is located in the promoter region surrounding the noncoding exon 1 of human MAGE-A3 DNA (9). Methylation-specific PCR (MSP) and ChIP primers designed for the MAGE-A3 promoter also coamplify the MAGE-A6 promoter, which is indistinguishable from MAGE-A3 promoter CpG islands. MSP analysis showed amplification of CpG-methylated MAGE-A3 genomic DNA and no amplification of unmethylated DNA in normal thyroids (Fig. 3B). Reciprocally, unmethylated PCR products of MAGE-A3/6 were observed in five of six thyroid carcinoma cell lines. A parallel assessment of the FGFR2 promoter also showed a reciprocal pattern with that associated with MAGE-A3/6. Namely, CpG methylation of the FGFR2 promoter was evident in tumor cell lines with contrasting lack of methylation in normal thyroid (Fig. 3B). These combined MSP and expression analyses suggested a possible role for DNA methylation in modulating the reciprocal expression of FGFR2 and MAGE-A3/6 in normal and neoplastic cells.

To examine whether epigenetic modifications can regulate MAGE-A3/6 expression, we treated WRO cells with the DNA-demethylating agent (AZA) or the HDAC inhibitor (TSA) or both. Compared with vehicle treatment, AZA treatment reduced
the MSP product (Fig. 3C). However, AZA treatment also resulted in reduction of the unmethylated product (Fig. 3C). Thus, to clarify the net effect of AZA treatment on MAGE-A3/6 expression, we next examined the effect of this DNA-demethylating agent, which we have shown previously does not significantly affect FGFR1 expression (7), on MAGE A3/6 mRNA expression (Fig. 3D). This confirmed that AZA treatment alone can induce MAGE-A3/6 expression and to a greater effect

Fig. 3. Epigenetic regulation of MAGE-A3/6 in thyroid carcinoma cells. A, the human MAGE-A3 promoter region encompassing the 5'-untranslated region and noncoding exon 1 contains two CpG islands as indicated. CG sites are denoted by vertical bars immediately below the graph. MSP examined the methylation status of the gene promoter. ChIP analysis examined histone-modifying effects on gene inactivation. Sequence numbering follows Genbank accession no. NT011726. MSP and ChIP primers also amplify the MAGE-A6 promoter region, which is indistinguishable due to marked homology with MAGE-A3 promoter sequence. B, MSP analysis. The CpG methylation status of the MAGE-A3/6 and FGFR2 promoters were examined in normal thyroid (six independent samples) and thyroid carcinoma cell lines (six cell lines as indicated). In normal thyroids, the methylation-specific amplicon for MAGE-A3/6 is detected in all samples without amplification of the unmethylated PCR product. Conversely, five of the six carcinoma cell lines are positive for unmethylated MAGE-A3/6 genomic product. Note the reciprocal methylation status of MAGE-A3/6 compared with that noted with FGFR2. C, MSP analysis. Treatment with the DNA-demethylating agent AZA decreases the methylated (M) but also reduces the unmethylated (U) amplicon of MAGE-A3/6 in WRO cells. D, RT-PCR confirms that AZA treatment restores MAGE-A3/6 expression in WRO cells (Exp.1). Although TSA treatment alone has no effect on MAGE-A3/6 expression (Exp.2), HDAC inhibition by TSA enhances the effect of AZA compared with either treatment alone (Exp.3) as shown by the densitometric assessments immediately below.
in the presence of the HDAC inhibitor TSA (Fig. 3D). These findings suggested a role for epigenetic control through DNA methylation as well as possibly histone deacetylation in governing MAGE-A3/6 expression.

FGFR2-IIIb expression and FGF7 ligand activation modulate histone H3 associated with MAGE-A3/6 promoters in thyroid carcinoma cells. To determine the state of histone modifications surrounding the MAGE-A3/6 promoter region, we did ChIP assays. As shown in Fig. 4A (top), WRO cells expressing FGFR2-IIIb revealed evidence of diminished histone H3 acetylation associated with the MAGE-A3/6 promoter compared with pcDNA-transfected control WRO cells. Further activation of FGFR2-IIIb using its selective ligand FGF7 resulted in progressive deacetylation of histone H3 associated with MAGE-A3/6 promoter (Fig. 4B, top). Moreover, examination of histone H3 methylation revealed enhancement of this modification in response to FGFR2-IIIb expression or FGFR2-IIIb activation through FGF7 treatment (Fig. 4A and B, bottom).

Discussion

The current study discloses the cancer/testis antigen MAGE-A3/6 as a novel target of FGFR2-IIIb in thyroid carcinoma cell lines. Gene expression profile and CpG methylation status showed a reciprocal relationship between MAGE-A3/6 and FGFR2-IIIb in normal and neoplastic human thyroid tissues. Moreover, we directly showed that FGFR2-IIIb–generated signals participate in epigenetic down-regulation of MAGE-A3/6.

The FGFR2-IIIb isoform is typically restricted to epithelial cells, whereas FGFR2-IIic is characteristic of mesenchymal lineages (12, 13). Targeted disruption of FGFR2-IIIb causes agenesis of the lungs, anterior pituitary, thyroid, teeth, and limbs (14). Consistent with these observations, FGFR2-IIIb was readily detected in normal thyroid tissues while it is strikingly down-regulated in most thyroid carcinoma cell lines through hypermethylation of CpG dinucleotides at the 5'-end of the FGFR2 gene. Down-regulation of FGFR2 has also been reported in several human neoplasms, including astrocytomas, bladder and prostatic carcinomas, pituitary adenomas, and thyroid carcinomas (3, 6, 14, 15). Moreover, forced FGFR2-IIIb expression significantly retards cancer cell proliferation (15–17) and enhanced FGFR2-IIIb signaling imposes on the BRAF/mitogen-activated protein kinase pathway to modulate thyroid carcinoma cell behavior (7). In this study, we identified MAGE-A3/6 as a putative target of FGFR2-IIIb by gene profiling in thyroid carcinoma cells.

MAGE-A3 and MAGE-A6 genes are members of the MAGE-I family that includes the MAGE-A, MAGE-B, and MAGE-C subfamilies (18). The MAGE-I family consists of a large number of chromosome X–clustered genes, which are expressed nearly exclusively in testicular germ cells, placenta, and various malignant tumors, and hence the designation as cancer/testis antigens (18–22). Our current data confirm a previous report that MAGE-A3/6 is expressed in thyroid carcinomas but not in normal thyroid tissues (23). Although restrictive expression of MAGE-A3/6 suggests a putative target of immunotherapy for thyroid cancers, their functions in thyroid cancer remain to be
the phosphorylation of Ser10 and Ser28 on histone H3 is one of multiple downstream targets, and eventually, mitogen-activated protein kinase signaling results in phosphorylation as well as histone modifications of histone H3 (28). In particular, stimulation of Ras/Raf/MAPK signaling imposes on histone modifications to the MAGE-A3/6 promoter. Chromatin modification by mitogen-activated protein kinase signaling has gained attention recently (28). In particular, stimulation of Ras/Raf/mitogen-activated protein kinase signaling results in phosphorylation of multiple downstream targets, and eventually, the phosphorylation of Ser10 and Ser28 on histone H3 is one early downstream event (28). Mitogen-activated protein kinase-mediated histone H3 phosphorylation has been linked with immediate-early gene induction (29–31). Our current and previous data suggest that FGFR signaling epigenetically modulates MAGE-A gene expression in thyroid cancers. Gene expression and CpG methylation status of FGFR2-IIIb and MAGE-A3/6 were reciprocal in normal and thyroid carcinoma cells. In addition, FGFR2-IIIb, a putative tumor-retarding factor (7), can participate in epigenetic modifications of MAGE-A3/6 genes and lead to their down-regulation.

Epigenetic gene silencing of other tumor suppressor genes, including E-cadherin, PTEN, and RASSF1A, and of differentiation-related genes, such as thyroid-stimulating hormone receptor and the sodium-iodide symporter, is known in thyroid cancers (2, 32). In addition, AZA or HDAC inhibition restores thyroid-specific gene expression including sodium-iodide symporter in dedifferentiated carcinoma cells; this approach has been proposed for differentiation-inducing therapy (33, 34). However, our current study shows that the demethylating agent can up-regulate MAGE-A3/6 gene expression, at least in thyroid carcinoma cells. These observations suggest that further studies should be pursued to determine the potential application of demethylating agents alone or in combination with other classes of antitumor agents, in the pharmacotherapy of thyroid cancer.

In conclusion, our data identify a reciprocal expression profile of FGFR2-IIIb and MAGE-A3/6 in thyroid carcinomas and normal thyroid tissue. Whereas FGFR2-IIIb plays a well-recognized tumor-suppressive role, MAGE-A3/6 is considered to harness growth-promoting functions (24, 25). Our data highlight MAGE-A3/6 as a novel downstream target of FGFR2-IIIb. These findings underscore the complex network of epigenetic changes and their actions in modulating signalings of opposing functions. Clearly, pharmacotherapeutic approaches based on epigenetic mechanisms will require selective targeting for more effective disease control.

References

24. Monte M, Simonatto M, Peche LY, et al. MAGE-A


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