miR-217 is an oncogene that enhances the germinal center reaction

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microRNAs are a class of regulators of gene expression that have been shown critical for a great number of biological processes; however, little is known of their role in immature B cells. Although the GC reaction is crucial to ensure a competent immune response, GC B cells are also the origin of most human lymphomas, presumably due to bystander effects of the immunoglobulin gene remodeling that takes place at these sites. Here we report that miR-217 is a positive modulator of the GC response that increases the generation of class-switched antibodies and the frequency of somatic hypermutation. We find that miR-217 downregulates the expression of a DNA damage response and repair gene network and in turn stabilizes Bcl-6 expression in GC B cells. Importantly, miR-217 overexpression also promotes mature B-cell lymphomagenesis; this is physiologically relevant as we find that miR-217 is upexpressed in aggressive human B-cell lymphomas.

Therefore, miR-217 provides a novel molecular link between the normal GC response and B-cell transformation. (Blood. 2014; 124(2):229-239)

Introduction

The GC reaction involves the clonal expansion of antigen-specific B lymphocytes and the generation of B-cell subclones with related antigen specificities, from which those expressing immunoglobulins with improved affinity for the antigen are positively selected.6 Molecularly, this is triggered by the so-called somatic hypermutation (SHM), which introduces point mutations on the variable region of the Ig molecule—responsible for antigen recognition. In addition, GCs are the home of the class switch recombination (CSR) reaction, a region-specific recombination reaction between 2 switch regions of the immunoglobulin heavy chain (IgH) locus that generates antibodies with different isotypes. SHM and CSR are both initiated by activation-induced deaminase (AID), which deaminates cytosines on the immunoglobulin locus.7 This initial lesion on DNA is subsequently processed by DNA repair and recombination factors to allow the fixation of a mutation, in the case of SHM, or the generation of a DNA double-strand break and a recombination reaction, in the case of CSR.8

The fact that inactivation of AID causes an immunodeficiency in humans9 highlights the importance of the GC reaction in the immune response. However, the generation of mutations and double-strand breaks associated with the GC reaction entails a risk for the genome

Key Points

- miR-217 enhances the GC response by dampening genotoxic-induced Bcl-6 degradation in GC B cells.
- miR-217 is an oncogene and its overexpression provides a model of miRNA-induced mature B-cell lymphomagenesis.
miR-217 is a positive regulator of the GC reaction and an oncogene in B cells. Using gain and loss of function in vivo models, we found that miR-217 is upregulated after in vitro and in vivo stimulation of mature B cells.230

E X P E R I M E N T was performed with 2 individual mice. (B) qRT-PCR of the (left) miR-217 precursor and (right) mature miR-217 in resting (open bars) or GC (filled bars) B cells from total RNA was converted to cDNA using random primers (Roche), reversed transcribed with SuperScript II (Invitrogen), and quantified by SYBR Green assay (Applied Biosystems). Data are means ± standard deviation from 2 independent experiments. Two-tailed Student t test; * P < .05. (C) Quantification of miR-217 expression in human resting (CD19+CD27+ IgD+) and GC (CD19+CD10+) B cells as measured by miRNA array hybridization (data were obtained from GSE29483, P = .003, 2-tailed Student t test). (D) Representative fluorescence-activated cell sorter (FACS) analysis of bone marrow from miR-217 TG, miR-217 KI, and mice whose GC B cells are depleted of miRNAs mount inefficient GC responses. However, there is little direct evidence for the role of individual miRNAs in this context, and to date, only miR-155 has been clearly associated with the GC response. Here we show that miR-217 is upregulated after in vitro and in vivo stimulation of mature B cells. Using gain and loss of function in vivo models, we found that miR-217 is a positive regulator of the GC reaction and an oncogene in GC B cells.

**Methods**

**miR-217 detection by miRNA microarray hybridization and quantitative reverse transcriptase-polymerase chain reaction**

RNA was extracted with Trizol (Invitrogen). Mouse miRNA microarray analysis was performed with paired samples of nonstimulated and stimulated B cells. For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) of the miR-217 precursor, total RNA was converted to cDNA using random primers (Roche), reversed transcribed with SuperScript II (Invitrogen), and quantified by SYBR Green assay (Applied Biosystems). Primers are detailed in supplemental Methods available on the Blood Web site. Mature miR-217 was quantified from total RNA with miR-217 miRCURY LNA primers (Exiqon). U6 and sno-142 (Exiqon) were used as normalization controls.

**T cell-dependent immunizations**

Groups of 7 to 9 littermate mice were immunized by footpad injection with 50 μg of 4-hydroxy-3-nitrophenylacetyl (NP) hapten conjugated to chicken γ globulin (NP-CGG; Biosearch Technologies) in complete Freund’s adjuvant. The proportion of cells is indicated in each gated in B220− cells. (E) Quantification of the proportions and absolute cell numbers of developing bone marrow B-cell subsets in control (open bars), (upper) miR-217−/− (filled bars), and (lower) miR-217+/+ mice (filled bars). The proportions of different B-cell subsets were quantified within the B220− population. The proportion of cells is indicated in each gated in B220− cells. (E)
Mice were euthanized 14 days after immunization for analysis of primary response or were reimmunized with 50 μg of NP-CGG in incomplete Freund’s adjuvant.

Somatic mutation analysis

For analysis of mutations at the Sm and JH4 regions, GC B cells were purified by cell sorting of pooled Peyer’s patches (4-6 animals per genotype). DNA was extracted, and the Sm and JH4 regions were PCR-amplified using specific oligonucleotides (supplemental Methods). Amplification products were purified and sequenced by next-generation sequencing (NGS) or cloned and sequenced by conventional Sanger sequencing.

RNAseq analysis and miRNA target prediction

GC (CD19+ Fas+ GL7+) B cells from pooled Peyer’s patches (4-6 animals per genotype) from control and miR-217TG mice were isolated by cell sorting. Total RNA was extracted with Trizol (Invitrogen) and sequenced by RNAseq. Differential expression analysis was done using the edgeR package from Bioconductor, and genes with \( P \leq 0.05 \) were considered for analysis. Pathway analysis of the significantly up- and downregulated genes in miR-217TG vs control GC B cells was performed by ingenuity pathway analysis software (Ingenuity Systems).

Significantly downregulated genes in miR-217TG GC B cells were analyzed for the presence of 835 different miRNAs target sites based on predicted and experimentally validated miRNA-mRNA interactions. Scores for every predictive algorithm were normalized, and the compound score of predictive and experimental databases was calculated. See supplemental Methods for more details.

miR-217 expression in Ramos cells, immunobloting, and cell cycle analysis

Ramos cells were transduced with miR-217 pmirNA or control vectors and selected by green fluorescent protein (GFP) expression by flow-activated cell sorting (FACS). Cells were treated for 6 hours with etoposide (Sigma-Aldrich) in the presence or absence of caffeine (Sigma-Aldrich), lysed in radioimmunoprecipitation assay buffer, and immunobloted with anti-Bcl-6 (N-3; Santa Cruz Biotechnology) or α-tubulin (Sigma-Aldrich) antibodies or fixed overnight with 70% ethanol and stained with 2.5 μg/mL 4’,6 diamidino-2-phenylindole for flow cytometry cell cycle analysis.

Human samples and animal procedures

The use of human samples was approved by the ethics committee of the Instituto de Salud Carlos III. The study was conducted in accordance with the Declaration of Helsinki.

**Results**

**miR-217 expression is upregulated in activated B cells**

To identify miRNAs that play a role during B-cell activation, we profiled miRNA expression in mature B cells during in vitro activation with lipopolysaccharide (LPS) + interleukin (IL)4, which promotes AID expression, SHM in the switch regions, and CSR. Microarray analysis showed that one of the few miRNAs whose expression is upregulated during this process is miR-217 (supplemental Figure 1A). Induction of precursor and mature miR-217 during B-cell activation in vitro was confirmed by qRT-PCR (Figure 1A). In addition, we generated GCs in vivo by immunizing mice with sheep red blood cells and conducted qRT-PCR on purified resting and GC B cells. miR-217 precursor and mature miR-217 expression increased by five- and eightfold, respectively, in GC cells (Figure 1B). Similarly, we found that miR-217 expression is increased in GC human B cells compared with resting B cells (Figure 1C). Together, these results show that miR-217 expression is induced during B-cell activation both in mouse and humans.

**Generation of B cell-specific miR-217 mouse models**

To investigate the role of miR-217 during B-cell activation, we generated 2 independent mouse models of B cell-specific miR-217 overexpression. In the first, 4 independent transgenic mouse lines were generated with a construct encoding the miR-217 precursor and a GFP reporter gene under the control of regulatory elements of the mouse κ light chain (Igκ) gene (miR-217TK+). In the second strategy, the miR-217 precursor/GFP construct was preceded by a transcriptional stop cassette flanked by LoxP sites and inserted within the endogenous Rosa26 locus (Rosa26miR217ki−/− mice) (supplemental Figure 1B; supplemental Methods); specific expression of miR-217 in B cells was achieved by crossing ROSA26miR217ki+/+ mice with CD19−Creκi+ mice (hereafter, miR-217KI mice). ROSA26miR217++/+ CD19−Creκi+ mice were used as controls. B cells from miR-217TK+ and miR-217KI mice showed full GFP and miR-217 expression in mature splenic B cells (supplemental Figure 1C-D). GFP expression was not detected in non-B-cell lineages, such as T, myeloid, or epithelial cells (data not shown). The proportions and absolute numbers of bone marrow and peripheral B cells in the miR-217TK+ and miR-217KI models were similar to those in wild-type littermate controls (Figure 1D-E), indicating that the miR-217TK+ and miR-217KI mouse models allow B cell-specific miR-217 overexpression without perturbing B-cell differentiation.

**miR-217 expression enhances the GC reaction**

To address the role of miR-217 during B-cell activation in GCs, we analyzed the B-cell response to a T cell-dependent antigen in miR-217TK+ mice. We first immunized miR-217TK+ and wild-type littermate controls with NP-CGG. As an immunization control, mice were injected with phosphate-buffered saline (PBS). The lymph nodes of NP-CGG-immunized wild-type mice contained GC B cells (Fas+GL7+) and B cells that had undergone CSR (IgG1+); but in miR-217TK+ mice, the response to NP-CGG immunization was greater, with 22% more GC B cells (P = .01) and 20% more IgG1+ B cells (P = .08; Figure 2A-B). A similarly enhanced GC response to NP-CGG immunization was found in miR-217KI mice (supplemental Figure 2A). We next analyzed the secondary B-cell immune response in control and miR-217TK+ mice. The enhancement of the GC reaction in miR-217TK+ mice was notably greater in secondary immunization assays, with 40% more Fas+GL7+ B cells and 60% more IgG1+switched B cells than controls (P = .019 and .018, respectively; Figure 2A,C). In addition, we found that the proportion of CD138+ plasma cells is larger in miR-217TK+ mice than in controls (P = .02; supplemental Figure 2B). To analyze the influence of miR-217 overexpression on the long-lived memory B-cell compartment, we quantified IgG1+ B cells in spleens from control and miR-217KI mice 1 year after immunization. The proportion of memory B cells was 40% higher in miR-217KI mice than in controls (P = .003; Figure 2D).

To further characterize the role of miR-217 in the GC reaction, we examined the extent of SHM in miR-217TK+ mice in vivo. We isolated Fas+GL7+ GC B cells from Peyer’s patches of control and miR-217TK+ mice and quantified the mutation frequency in an intronic DNA region downstream of the JH4 segment of the IgH locus, a region that accumulates mutations but cannot be subject to affinity maturation events. Conventional Sanger sequencing of the JH4 region downstream of the JH4 segment of the IgH locus showed that miR-217TK+ B cells have a higher mutation load than wild-type controls (control, 2.1 × 10−5/bp and miR-217TK+, 3.7 × 10−5/bp, P = .028; Figure 3A). Likewise, GC B cells from miR-217KI mice accumulated more mutations downstream of JH4 than did wild-type controls (supplemental Figure 2C). Similar results were obtained...
when we analyzed SHM frequency at the J_{H4} intronic region by NGS, which allows large numbers of mutations to be analyzed at very high depth.\(^{29}\) No mutations were detected in B cells isolated from AID\(^{-/-}\) mice, used as a negative control. We detected total mutation frequencies of \(1.7 \times 10^{-3}\) bp in control animals and \(3.7 \times 10^{-3}\) bp in miR-217\(^{TG}\) mice (\(P = 1.9 \times 10^{-5}\)), a 2.2-fold difference (Figure 3B). This difference was even higher (2.7-fold) when the analysis was limited to cytosines located in AID mu-switch regions (Figure 3B). This difference was even higher (2.7-fold) when the analysis was limited to cytosines located in AID mu-switch regions (Figure 3B). However, this difference was even higher (2.7-fold) when the analysis was limited to cytosines located in AID mu-switch regions (Figure 3B).

Inhibition of endogenous miR-217 impairs the GC reaction

To address whether endogenous miR-217 expression plays a physiological role in GCs, we generated bone marrow mouse chimeras in which endogenous miR-217 expression was inhibited by the expression of a miR-217 sponge (miR-217\(^{SPG}\)) construct (Figure 4A), where sequences complementary to miR-217 were cloned in tandem within the 3\(^{\prime}\) untranslated region of a reporter gene and thus function as competitive inhibitors for the binding of the miRNA to its endogenous binding sites.\(^{31,32}\) Bone marrow cells from wild-type mice were transduced with miR-217\(^{SPG}\) or control retroviruses and transferred into lethally irradiated CD45.1 congenic recipient mice and immunized 4 weeks later with NP-CGG. Chimeric mice injected with PBS were used as immunization negative controls. Spleen and lymph nodes from immunized miR-217\(^{SPG}\) mice contained fewer GC and IgG1 switch B cells than immunized control chimeric mice, and indeed, the proportions of these cell subsets in immunized miR-217\(^{SPG}\) mice were as low as in PBS-injected, nonimmunized animals (Figure 4B-C). We next analyzed SHM in Peyer’s patch GC B cells from miR-217\(^{SPG}\) and control chimeric mice by NGS. We found that miR-217\(^{SPG}\) GC B cells had a 25% lower mean mutation frequency than control B cells at the J_{H4} intronic region (\(P = 3.7 \times 10^{-5}\); Figure 4D). Very similar results were obtained when we analyzed the mutation load in a fragment of the \(\mu\) switch region (S\(_{\mu}\)) of IgH (\(P = 4 \times 10^{-4}\)), where mutations...
accumulate concomitantly with CSR (Figure 4D). These results thus show that inhibition of endogenous miR-217 attenuates the GC reaction and decreases the frequency of somatic mutations associated with both the SHM and the CSR reactions.

miR-217 regulates a DNA damage response and repair gene network and stabilizes Bcl-6 expression in GC B cells

To identify the genes regulated by miR-217 in activated B cells, we performed a genome-wide comparison of the gene expression profiles of miR-217TG and wild-type GC B cells by RNAseq. Comparison of GC B cells from Peyer’s patches of miR-217TG and wild-type mice identified 1740 differentially expressed genes with \( P \leq .05 \) (supplemental Table 1), 1236 of which were downregulated in miR-217TG vs control GC B cells. This set of downregulated genes was significantly enriched in predicted miR-217 targets (Figure 5A), suggesting that a high proportion of the downregulated genes identified in miR-217TG GC B cells are direct miR-217 targets. To identify the gene networks altered by miR-217 expression in GC B cells, we performed a bioinformatics analysis on the genes differentially expressed...
miR-217 regulates a DNA damage response (DDR) and repair gene network in GC B cells (P < 0.001; Figure 5B). Interestingly, this gene network contained 2 main hubs, RNA pol II and replication protein A, which are directly linked to AID activity. The miR-217-regulated gene network included a number of genes linked to the DDR (Nbs1 and Rad50 components of the MRN complex), DNA repair and CSR (Wrm, Lig4, and Xrcc2), and genes of the cohesin complex (SMC2, SMC3, PDS5B, and SA2) involved in chromatin cohesion, as well as in DNA replication, transcription, recombination, and repair; all of them were downregulated by miR-217.

Bcl-6 expression is regulated by the DDR through a signaling pathway that promotes Bcl-6 degradation. As we found that miR-217 downregulates the expression of a number of genes involved in DDR and repair in GC B cells, we hypothesized that miR-217 could stabilize Bcl-6 expression in these cells. To test this hypothesis, we transduced Ramos cells, a human BL GC B-cell line, with miR-217-GFP and control retroviral vectors and analyzed Bcl-6 expression on genotoxic stress induction. We first verified that genes linked to the DDR and downregulated by miR-217 in mouse GC B cells (Nbs1, Xrcc2, Lig4, and Pds5b) were also downregulated by miR-217 in human Ramos cells (Figure 5C). To determine if miR-217 impacts on the response to genotoxic stress, we analyzed the effect of etoposide treatment on the cell cycle of control- and miR-217-transduced Ramos cells. Expectedly, we found that etoposide-induced DDR promoted an increase in the fraction of cells in the S phase of the cell cycle, consistent with a replicative delay (Figure 5D). However, this S phase increase was milder in miR-217-transduced cells (Figure 5D), which suggested that miR-217 may contribute to bypass the DDR response induced by etoposide. Immunoblot analysis showed that etoposide treatment of control Ramos cells rapidly induced Bcl-6 degradation (Figure 5E), in agreement with previous results. However, Ramos cells overexpressing miR-217 were
had to be euthanized before 90 weeks; supplemental Figure 3A). miR-217 was classiﬁed as follicular lymphomas (FL), although we also observed other mature B-cell lymphomas, such as DLBCL and B-cell plasmacytomas (PCT) (Figure 6B-C; Table 1). To further characterize the origin of these lymphomas, we performed molecular analysis of their V(D)J rearrangements by PCR amplification and sequencing. We found that a fraction of the lymphomas yielded unique ampliﬁcation bands (Figure 6D), which were conﬁrmed to correspond with single rearrangements (Table 2), thus providing proof of their clonal origin. In addition, all of the rearrangements subject to this analysis contained mutations (SHM) in their V genes (Table 2) and an additional fraction showed Bcl-6 expression (Figure 6C; Table 1), conﬁrming their GO post-GC origin.

To examine the contribution of tumor suppressor pathways to the B-cell lymphomagenesis induced by miR-217, we analyzed the incidence of B-cell lymphoma in miR-217KI mice in the Ink4a/Arf−/− and p53−/− backgrounds. Ink4a/Arf−/− and p53−/− mice often developed histiocytic sarcomas (40%) and T-cell lymphomas (70%), respectively (data not shown). In addition, we found that roughly 20% of both control Ink4a/Arf−/− and p53−/− tumor suppressor-deﬁcient mice generated B-cell lymphomas. This incidence was not signiﬁcantly altered (supplemental Figure 3A). miR-217 overexpression increased B-cell lymphoma incidence to 43% (Figure 6E), although the mean survival of miR-217KI Ink4a/Arf−/− mice was not signiﬁcantly altered (supplemental Figure 3A). B-cell lymphomas in miR-217KI Ink4a/Arf−/− mice showed histopathological features of mature GC or post-GC B-cell lymphomas (supplemental Figure 3). These results suggest that the Ink4a/Arf, but not the p53, tumor suppressor pathway acts as a surveillance mechanism against the lymphomagenic events induced by miR-217 in mature B cells.

To determine if miR-217 expression levels are altered in mature B-cell lymphomas, we conducted a qRT-PCR analysis of miR-217 expression in a collection of BL and DLBCL samples. Expression of miR-217 was higher in BL and DLBCL than in control, P<0.0058; Figure 6A; Table 1). Histopathological evaluation of the spleens showed that a large proportion (33%) of miR-217KI mice developed B-cell lymphoma (P = 0.028 vs control mice; Figure 6B). Flow cytometry analysis of lymphomas from miR-217KI mice showed frequent alterations in the expression of the B-cell surface molecules B220, IgD, IgM, and Igk (supplemental Figure 3B; Table 1). We found that lymphomas in miR-217KI mice had features of mature GC or post-GC B-cell origin. Most (70%) were classiﬁed as follicular lymphomas (FL), although we also

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<th>Splenomegaly§</th>
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*Metal identification number.
†SBL, small B-cell lymphoma.
‡Lymphoma classification grade.
§Splenomegaly: spleens larger than 20 mm length.
††Phenotype of tumoral cells determined by morphological features, flow cytometry, and immunohistochemical stainings. N/A, not analyzed.
in that both show increased GC formation and develop long latency
or deletion in aggressive B-cell lymphomas, whereas ARF silencing
transforming activity of miR-217. This result is in agreement with the
plays the predominant role in protecting GC B cells against the
stress-induced degradation, suggesting that both molecules are part
repair genotoxic events on DNA, which in turn can increase the
show that miR-217 downregulates a network of genes that sense and
promotes B-cell lymphomagenesis. Interestingly, mice that over-
master transcriptional repressor that dampens the DDR in GC B cells
remodeling; (2) the intense proliferation of GC B cells subjects them
instability: (1) they are programmed to undergo AID-mediated gene
miR-217 regulates the expression of a gene network involved in
miRNAs modulate the expression of gene networks in a cell
context-specific manner. Here, RNAseq analysis showed that
miR-217 regulates the expression of a gene network involved in
DDR and repair, including Rad50, Nbs1, Wrm, Lig4, and XRCC2, as
well as a set of genes of the cohesin complex, all of which are down-
regulated by miR-217. GC B cells are intrinsically prone to genome instability: (1) they are programmed to undergo AID-mediated gene remodeling; (2) the intense proliferation of GC B cells subjects them to replicative stress; and (3) the GC reaction depends on Bcl-6, a master transcriptional repressor that dampens the DDR in GC B cells and whose deregulation generates B-cell lymphomas. Our data show that miR-217 downregulates a network of genes that sense and repair genotoxic events on DNA, which in turn can increase the tolerance of GC B cells to DNA damage, very much like Bcl-6. Notably, we found that miR-217 protects Bcl-6 from genotoxic stress-induced degradation, suggesting that both molecules are part of the same network that renders GC cells permissive to genomic instability and prone to malignant transformation.

Consistent with this idea, we found that miR-217 overexpression promotes B-cell lymphomagenesis. Interestingly, mice that overexpress miR-217 resemble Bcl-6-overexpressing IκB-HABCL6 mice in that both show increased GC formation and develop long latency mature B-cell lymphomas. We also found that Ink4a/Arf but not p53 loss sensitizes B cells to miR-217-promoted lymphomagenesis. Although it is possible that a protective role of p53 is masked by the early appearance of T-cell lymphomas in p53−/− mice, our data suggest that Ink4a rather than the Arf-p53 oncogenic stress pathway plays the predominant role in protecting GC B cells against the transforming activity of miR-217. This result is in agreement with the finding that Ink4a protein is frequently lost through gene methylation or deletion in aggressive B-cell lymphomas, whereas ARF silencing is a rarer event.

Deregulation of miRNA expression in human B-cell lymphomas has been extensively documented. In some instances, the functional relevance of miRNA deregulation has been tested in genetically modified mouse models. This is the case of Eq4-miR-155 transgenic mice, which developed acute lymphoblastic leukemia/high grade lymphoma or miR15/miR16 deficiency, which promoted chronic lymphocytic leukemia, among other examples. Evidence for miRNAs involved in GC or post-GC lymphomagenesis was thus far restricted to the combined action of the miR-17-92 miRNA cluster. Here we find that miR-217 overexpression in B cells promotes lymphomas of GC or post-GC origin, most likely by impinging on the regulation of the DDR and Bcl-6. Accordingly, we found increased levels of miR-217 in BL and DLBCL, 2 of the most aggressive lymphomas arising from GCs. Our results identify miR-217 as a novel molecular link between the GC response and B-cell transformation and provide an in vivo model of mature B-cell lymphomagenesis.

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### Authorship

**Contribution:** V.G.d.Y., N.B.-I., P.P.-D., and S.M.M. performed experiments; N.M., L.D.L., and M.A.P. collected and prepared human samples; D.F.R. constructed the original backbone for miR-217 transgene cloning; R.N.-C. and A.P.-M. performed bioinformatics analyses; M.C. did histopathological evaluation; V.G.d.Y., N.B.-I., and A.R.R. analyzed the data; V.G.d.Y. and A.R.R. designed experiments and wrote the manuscript; and A.R.R. supervised the research.

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References


miR-217 is an oncogene that enhances the germinal center reaction

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