Activation of the Glutamate Receptor GRM1 Enhances Angiogenic Signaling to Drive Melanoma Progression

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Abstract
Glutamate-triggered signal transduction is thought to contribute widely to cancer pathogenesis. In melanoma, overexpression of the metabotropic glutamate receptor (GRM)-1 occurs frequently and its ectopic expression in melanocytes is sufficient for neoplastic transformation. Clinical evaluation of the GRM1 signaling inhibitor riluzole in patients with advanced melanoma has demonstrated tumor regressions that are associated with a suppression of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways. Together, these results prompted us to investigate the downstream consequences of GRM1 signaling and its disruption in more detail. We found that melanoma cells with enhanced GRM1 expression generated larger tumors in vivo marked by more abundant blood vessels. Media conditioned by these cells in vitro contained relatively higher concentrations of interleukin-8 and VEGF due to GRM1-mediated activation of the AKT–mTOR–HIF1 pathway. In clinical specimens from patients receiving riluzole, we confirmed an inhibition of MAPK and PI3K/AKT activation in posttreatment as compared with pretreatment tumor specimens, which exhibited a decreased density of blood vessels. Together, our results demonstrate that GRM1 activation triggers proangiogenic signaling in melanoma, offering a mechanistic rationale to design treatment strategies for the most suitable combinatorial use of GRM1 inhibitors in patients. Cancer Res; 74(9): 2499–509. ©2014 AACR.

Introduction
Melanoma is the most aggressive form of skin cancer and its incidence is on the rise worldwide (1). Although surgically curable at early stages, metastatic melanoma is relatively refractory to current therapies and has a poor prognosis (2). Cytotoxic chemotherapeutic agents such as dacarbazine have response rates of only 7% to 12% with few long-term survivors. Agents, such as vemurafenib, have shown promising early results (3), but the majority of patients who initially respond eventually progress and die (4). Combinations of Raf inhibitors with other small-molecule inhibitors have shown some improvement over single-agent therapy (5) and combination immunotherapies, such as anti-CTLA4 or anti-PD1 antibodies, have shown improved response rates and some improvement in survival in patients with advanced melanoma, but the majority of patients still do not respond or recur after initial response (6). Clearly, new therapeutic targets are needed in this patient population.

Activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathways are important early steps in melanomagenesis (7). Activating mutations in NRAS or BRAF combined with a gain-of-function of murine metabotropic glutamate receptor (GRM)-1 occurs frequently (8). Our group was the first to discover the expression of these cell surface receptors in spontaneous melanoma (9). Activation of GRM1 results in the release of glutamate from melanocytes is sufficient for neoplastic transformation. Clinical evaluation of the GRM1 signaling inhibitor (mGRM1) in mouse melanocytes is sufficient to induce spontaneous melanoma in vivo with 100% penetrance (10, 12) and expression of mGRM1 in an immortalized (but not transformed) melanocytic cell line (melan A), results in transformation, rendering it capable of producing tumors in immunocompetent syngenic mice (11). Recently, ectopic expression of the other group I GRM, mGRM5, has been shown to also produce melanoma in a second transgenic model (13).

A gain-of-function of murine metabotropic glutamate receptor 1 (mGRM1) in mouse melanocytes is sufficient to induce spontaneous melanoma in vivo with 100% penetrance (10, 12) and expression of mGRM1 in an immortalized (but not transformed) melanocytic cell line (melan A), results in transformation, rendering it capable of producing tumors in immunocompetent syngenic mice (11). Recently, ectopic expression of the other group I GRM, mGRM5, has been shown to also produce melanoma in a second transgenic model (13).

These transgenic mouse studies prompted us to examine human melanoma for expression of the human form of this receptor, GRM1. Of 25 human melanoma cell lines tested, 23 express GRM1 (9). We also found that 60% to 80% of human melanoma specimens express GRM1 mRNA and protein, while normal skin and melanocytes from the same patients did not (9). Activation of GRM1 results in the release of glutamate from
neurons and melanoma cells, setting up paracrine feedback loops that enhance GRM1 activation and signal transduction (9, 14). In preclinical studies, we found that induction of GRM1 signaling in vitro and in vivo results in a G2/M cell-cycle arrest and subsequent apoptosis in human melanoma. GRM1 inhibition also results in decreased human melanoma xenograft growth (9).

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a potent inhibitor of glutamate release by GRM1-expressing cells and is currently the only U.S. Food and Drug Administration (FDA)-approved agent for amyotrophic lateral sclerosis (15). Using riluzole, we have translated our laboratory findings into the clinic through phase 0 (16) and phase II (17) trials in patients with advanced melanoma. In our phase 0 trial, administration of oral riluzole resulted in suppression of MAPK and PI3K/AKT signaling, and involution of tumor in 34% of patients, independent of BRAF and NRAS mutational status. We also found an increase in the number of apoptotic cells in posttreatment tumor samples (16). In the phase II trial of single-agent riluzole, similar evidence of biologic activity was seen that correlated with initial stable disease in 30% of patients (17), consistent with our preclinical findings (18).

Logical clinical trial design requires a better understanding of how GRM1 signaling affects melanomagenesis and disease progression. We began by enhancing the expression of GRM1 in three human melanoma cell lines; a low GRM1-expressing subclone derived from UACC903, the metastatic line C8161 that has moderate GRM1 expression, and a related line, C81-61, (derived from the same patient as C8161) that does not express GRM1. We introduced exogenous full-length GRM1 cDNA into these lines to increase the expression of GRM1 and found that this did not increase in vitro proliferation of UACC903 or C8161 cells, though the normally slowly proliferating C81-61 cells did have a moderate increase in proliferation. What we did find was a marked increase in tumor growth and blood vessel formation when enhanced GRM1-expressing cells were used to produce xenografts. We hypothesized that increasing GRM1 signal transduction triggered an increase in the production of proangiogenic factors. Examination of the parental and enhanced GRM1-expressing cells confirmed this hypothesis revealing that AKT, mTOR, and hypoxia-inducible factor-1α (HIF-1α) participated in regulating the secretion of interleukin (IL)-8 and VEGF secondary to enhanced GRM1 expression.

Incorporation of GRM1 into xenografts resulted in a G2/M cell-cycle arrest and subsequent apoptosis in human melanoma. GRM1 inhibition also results in decreased human melanoma xenograft growth (9).

Angiogenesis and apoptosis antibody arrays
The human angiogenesis (Panomics, Affymetrix) and the human apoptosis (R&D Systems) antibody array assays were performed using conditioned media from UACC903-G2 and UACC903-V1 cells following the manufacturers’ protocol. Stable clones UACC903-G2, UACC903-G4, C8161-G21, C81-61-G6, and C81-61-G7 that express GRM1 at significantly higher levels than the parental lines were propagated under selection with 10 μg/mL blasticidin. Stable clones transfected with empty vector were produced as controls and designated UACC903-V1, C8161-V2, and C81-61-V3.

Tumor induction in nude mice, in vivo drug treatment, and vessel imaging
Cells from each line were grown to 80% confluence, trypsinized, counted, and resuspended in ice-cold PBS. A total of 10⁶ cells in 0.1 mL PBS were injected subcutaneously into each flank of nude mice (nu/nu), with each line injected into 5 mice. Tumors were measured twice a week (19). The mice were treated with either riluzole (20 mg/kg), MK-2206 (60 mg/kg), rapamycin (20 mg/kg), or YM-1 (30 mg/kg) by intraperitoneal injection every other day for 2 weeks starting when the tumors reached approximately 6 to 10 mm³. Tumor blood vessels were visualized by injecting 200 μL of high-molecular weight (average 2,000,000 MW) FITC-dextran (Sigma) into the mice 10 minutes before sacrifice. Xenografts were removed, fixed in 10% formalin, thinly sliced, and photographed under UV illumination.

Conditioned media
Conditioned media were generated by seeding 10⁶ cells from each cell line in 1 mL serum-free RPMI in tissue-culture wells for 20 hours.

Materials and Methods
Animal studies were approved by the Institutional Animal Care and Use Committee of Robert Wood Johnson Medical School (Piscataway, NJ; protocol I11-007). Human tumor samples were obtained from patients enrolled on a phase II trial approved by our Institutional Review Board. Informed, signed consent for the protocol therapy and the use of the tumor samples was obtained from all patients.

Cell culture and cell proliferation assay
Melanoma lines were maintained in RPMI-1640 (Invitrogen) containing 10% FBS. SVEC4–10-immortalized endothelial cells were grown in Dulbecco’s Modified Eagle Medium containing 10% FBS. Primary endothelial cells (HMVECnd) were grown following the manufacturer’s protocol (Invitrogen). All cells were maintained in a humidified incubator at 37°C with 5% CO₂. Cell viability was ascertained using MTS reagent (Promega) following the manufacturer’s protocol.
Endothelial cell migration assay
A similar 24-well/PET membrane inserts set up was used for this assay. A total of 1 × 10^5 HMVECnd endothelial cells in 250 μL serum-free RPMI were seeded into the upper wells, whereas the lower wells contained 750 μL serum-free RPMI and either 7.5 × 10^5 UACC930-V1, UACC930-G2, or UACC930-G4 cells. All other procedures were performed in the same fashion as the tumor cell migration assay. The endothelial cell migration assay was repeated using a transformed endothelial cell line (2 × 10^5 SVEC4–10 cells in 0.25 mL serum free RPMI medium per upper well).

IL-8 and VEGF ELISA
ELISAs (Invitrogen) were used to measure IL-8 and VEGF levels in conditioned media. A total of 2 × 10^5 cells were seeded in triplicate in a 96-well plate, each well had 200 μL serum-free RPMI containing dimethyl sulfoxide, MK-2206 (2 μmol/L), or aminoavone (50 μmol/L). After 9 hours, the media were replaced with fresh serum-free RPMI with matching treatments and the cells were incubated for another 11 hours. Conditioned medium was collected for ELISA, fresh RPMI (100 μL with serum), and 20 μL of MTS reagent was added to the wells, and the plates were incubated at 37°C for one hour followed by measurement of cell viability.

Inducible HIF-1 transcription reporter activity assay
Of note, 100 μL of resuspended UACC930-V1, UACC930-G2, and UACC930-G4 cells (0.5-1 × 10^5 cells) were seeded in triplicate in a 96-well plate and transfected with inducible HIF-1 transcription reporter lentiviral particles (Qiagen) as per the manufacturers’ protocol. Reporter gene expression level was measured using dual-glo luciferase assay system (Promega), using the manufacturer’s protocol.

Western blot analysis
Protein lysates from clinical tumor samples and cultured cells were prepared, and immunoblotting was performed, as previously described (18, 20). Cells were incubated with each inhibitor for 18 hours in 24-well plates to assess changes in signal transduction. Inhibitors used include riluzole (10 μmol/L), AF (50 μmol/L), or MK-2206 (10 μmol/L).

Statistical analysis
Heterogeneous variance models (21) were used to account for the different variances among groups. The comparisons of treatment groups with controls were conducted with Dunnett’s adjustment (22) to account for multiplicity in multiple comparisons. Adjusted P values were provided when multiple comparisons were performed.

Results
Enhanced expression of GRM1 in human melanoma cells promotes cell migration and activates AKT and MAPK signaling
The melanoma cell lines that express GRM1 at significantly higher levels than the parental cell lines (UACC930-G2, UACC930-G4, C8161-G21, C81-61-G6, and C81-61-G7) were found to have increased AKT and extracellular signal–regulated kinase (ERK) activation compared with controls (Fig. 1A–C), consistent with our previous findings that GRM1 activates the MAPK and AKT pathways (9, 18). Increased expression of GRM1 in UACC930 and C8161 did not significantly alter in vitro proliferation (Fig. 1D and E). In contrast, expression of GRM1 in C81-61 (C81-61-G6 and C81-61-G7) cells led to increase proliferation in vitro, compared with the control C81-61-V3 cells (Fig. 1F).

We used a Boyden Chamber assay to examine how increased expression of GRM1 affects migration of melanoma cells. We found that UACC930-G2 and UACC930-G4 cells had increased migration compared with UACC930-V1 control cells using 0.5% FBS as the chemoattractant (Fig. 1G). These data support our previous findings that inhibiting GRM1 signaling with riluzole or a noncompetitive inhibitor Bay36-7620 decreased the migration of GRM1-expressing melanoma cells (18).

Increased GRM1 expression in melanoma cells leads to larger xenografts with more abundant blood vessels
UACC930-G2, UACC930-G4, and C8161-G21 xenografts grew faster than UACC930-V1 or C8161-V2 xenografts (Fig. 2A and B). The control C81-61-V3 cells did not produce xenografts though the GRM1-expressing C81-61-G7 (G7) cells did (Fig. 2C). It is unlikely that the larger UACC930-G2, UACC930-G4, and C8161-G21 xenografts were produced by faster cell proliferation as these cell lines proliferated slightly slower in vitro than the controls (Fig. 1D and E). There was also an absence of hemorrhagic necrosis in UACC930-G2, UACC930-G4, and C8161-G21 xenografts, while UACC930-V1 and C8161-V2 xenografts showed extensive hemorrhagic necrosis (Fig. 2D). These results suggest that enhanced GRM expression increased angiogenesis in vivo. This hypothesis was supported by the appearance of significantly more blood vessels in the UACC930-G2, UACC930-G4, and C8161-G21 xenografts than UACC930-V1 and C8161-V2 xenografts (Fig. 2E).

Increased expression of GRM1 results in increased IL-8 and VEGF secretion
We performed an endothelial cell migration assay to determine whether conditioned media produced by cells with enhanced GRM1 expression would act as a chemoattractant for endothelial cells. Significantly more migration of primary human endothelial cells (HMVECnd; Fig. 3A and B) and transformed endothelial cells (SVEC4–10; data not shown; ref. 23) was detected using conditioned medium from UACC930-G2 and UACC930-G4 cells as the chemoattractant, compared with conditioned medium from UACC930-V1, suggesting that higher levels of proangiogenic factors were being produced by the cells with enhanced GRM1 expression.
To determine which angiogenic factors were modulated by increased expression of GRM1, we performed an angiogenesis antibody array assay using serum-free conditioned media from UACC903-V1 and UACC903-G2 cells. We found increases in IL-8 and VEGF in UACC903-G2 medium compared with UACC903-V1 medium (Fig. 3C). We confirmed that all of the human melanoma cell lines with enhanced GRM1 expression released more IL-8 and VEGF into the medium compared with controls by ELISA (Fig. 3D–I). We conclude that increased expression of
GRM1 in melanoma cells results in increased IL-8 and VEGF expression.

**Enhanced expression of GRM1 increases the activation of HIF-1α**

We treated UACC903-G2 and UACC903-G4 cells with an inhibitor of glutamate release, riluzole, in an *in vitro* cell viability assay. UACC903-G2 and UACC903-G4 cells were more sensitive to riluzole compared with control UACC903-V1 cells (Fig. 4A). Previously, we found that the reduced number of viable cells after riluzole treatment was due to an increase in apoptotic cells (9, 18). We therefore performed an apoptosis antibody array assay using protein lysates from cultured UACC903-V1 and UACC903-G2 cells. We found differences in the protein levels of cIAP-1, claspin, TRAILR1, TRAILR2, FADD, HIF-1α, HO-2/HMOX2, HSP70, phospho-p53 (S15), phospho-p53 (S392), SMAC/Diablo, and survivin (Supplementary Fig. S1) confirmed by Western blotting (data not shown).

One of these factors, HIF-1α, is a transcription factor known to regulate VEGF and IL-8 expression (24). HIF-1α transcriptional activity can be activated by PI3K/AKT/mTOR signaling (25) and because HIF-1α expression is increased in UACC903-G2 cells compared with UACC903-V1 cells, it may be at least partially responsible for the angiogenesis-enhancing effects of GRM1. Another molecule upregulated in UACC903-G2 cells was survivin, an anti-apoptotic molecule whose transcription is also regulated by HIF-1α (26). Immunoblotting protein lysates from UACC903-V1, UACC903-G2, and UACC903-G4 cells showed that HIF-1α, p-mTOR, p-70S6K (a downstream target of mTOR), and survivin, were all upregulated in cell lines with...
increased GRM1 expression (Fig. 4B and C). To confirm that increased expression of HIF-1α resulted in an increase in its transcriptional activity, we transiently transfected an inducible HIF-1α luciferase-reporter construct into UACC903-V1, UACC903-G2, and UACC903-G4 cells. We found that HIF-1α transcriptional activity was significantly higher in UACC903-G2 and UACC903-G4 cells as compared with UACC903-V1 cells (Fig. 4D). We found a similar increase in HIF-1α expression and transcriptional activity in C8161-G21, C81-61-G6, and C81-61-G7 cells as compared with C8161-V2 and C81-61-V3 cells (data not shown). We next treated C8161-V2, C8161-G21, UACC903-V1, UACC903-G2, and UACC903-G4 cells with inhibitors targeting the GRM1/AKT/mTOR/HIF-1α pathways and looked for alterations in IL-8 and VEGF expression. Regardless of the inhibitors used, levels of HIF-1α and secreted IL-8 and VEGF were all reduced (Fig. 4E and F and Supplementary Fig. S2). These results demonstrated that upregulated HIF-1α expression and transcriptional activity in response to an increase in GRM1 expression in human melanoma cells are likely mediated via direct activation of the PI3K/AKT/mTOR pathway.
GRM1/AKT/mTOR/HIF-1α signaling promotes xenograft vessel formation

Xenografts generated using UACC903-G4 cells and treated by intraperitoneal injection every other day with riluzole (20 mg/kg), MK-2206 (60 mg/kg), rapamycin (20 mg/kg), or YC-1 (HIF-1α inhibitor, 30 mg/kg; ref. 27), all showed significant inhibition of xenograft growth compared with vehicle-treated controls (Fig. 5A). Treated UACC903-G4 xenografts showed a significant decrease in blood vessel growth compared with vehicle-treated xenografts and residual vessels in treated xenografts were decreased in quantity and highly fragmented (Fig. 5B). Signaling analysis revealed that HIF-1α expression in UACC903-G4 xenografts was much higher than in UACC903-V1 xenografts and decreased significantly with riluzole, MK-2206, rapamycin, or YC-1 treatment (Fig. 5C). We repeated these xenograft experiments using the C8161-G21 cell line and found that these cells formed larger xenografts than the control C8161-V2 cells (Fig. 2B) and were exquisitely sensitive to anti-GRM1 (riluzole) or anti-HIF-1α (YC-1) treatment (Fig. 5D). Therefore, larger tumors with more abundant blood vessels seen in xenografts produced from melanoma cell lines with enhanced GRM1 expression (Fig. 2) are partially due to increased activation of the PI3K/AKT pathway and can be inhibited by agents targeting GRM1, AKT, mTOR, and HIF-1α. These data support our hypothesis that the AKT/mTOR/HIF-1α signaling pathway is downstream of GRM1 and functions
to promote blood vessel formation as a consequence of GRM1 signal transduction.

**Response to riluzole corresponds to a decrease in activation of the AKT/mTOR/HIF-1α pathway and decreased CD31 and survivin in clinical melanoma samples**

We conducted a phase II trial of single-agent riluzole in patients with advanced melanoma. All 13 patients enrolled on the trial were GRM1 positive (by immunohistochemistry) and treated with the maximum FDA-approved dose of riluzole (100 mg b.i.d.). Although no RECIST (Response Evaluation Criteria in Solid Tumors) responses were seen (17), stable disease at first restaging was seen in 46% of previously progressing patients. We collected pre- and posttreatment tumors from eight patients and three of these eight patients were among those with initial stable disease. Immunoblotting results comparing pre- and posttreatment tumor samples show a reduction in pERK, pAKT, CD31, survivin, HIF-1α, and p-mTOR levels only in the posttreatment samples from these three patients (1, 5 and 11; Fig. 6). CD31 is highly expressed by endothelial cells and is used as a marker of vessel density (28). Decreased CD31 expression suggests that riluzole may have inhibited angiogenesis in these patients. These results demonstrate that signaling molecules whose activity is modulated in response to increased expression of GRM1 in our preclinical models are also modulated in melanoma samples from patients treated with an inhibitor of GRM1 signal transduction.

**Discussion**

Constitutive activation of the MAPK and PI3K/AKT pathways is found in the vast majority of melanomas and agents that target these pathways have been developed (29). However, regulation of signal transduction through these pathways is more complicated than originally thought (30, 31). Driver mutations such as V600E BRAF are likely early events in the development of melanoma but are not necessary for continued progression of the disease as evidenced by its loss in patients who recur after initial response to vemurafenib (30, 31). It seems that after initial transformation subsequent mutations, feedback loops, changes in the tumor microenvironment, and other factors add layers of genotypic and phenotypic complexity to the neoplasm that requires a more complex treatment approach. Ectopic expression of seven transmembrane...
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G-protein–coupled receptors (GPCR) seems to be one of the subsequent changes found in many different cancers (32).

GRM1 is a GPCR belonging to the GRM family that has eight different isoforms that bind glutamate as their natural ligand (33). In the central nervous system (CNS), l-glutamate is a major excitatory neurotransmitter and activates both ionotropic and GRMs. GRM1 and GRM5 belong to group I and activate PLC-β, resulting in intracellular Ca²⁺ release and protein kinase C (PKC) activation (33).

Our discovery that GRM1 is expressed by the majority of human melanomas adds to our understanding of the multiple mechanisms of the MAPK and AKT pathway activation in this disease (9, 10, 18). Interestingly, we are not the first investigators to suggest that suppression of glutamatergic signaling with riluzole decreases the expression of VEGF and inhibits angiogenesis. Yoo and colleagues reported in two different studies that riluzole can inhibit VEGF-induced endothelial cell proliferation and block vascular retinopathy induced by hyperglycemia (36, 37). Retinopathy resulting from overgrowth of blood vessels is common in diabetic patients and premature infants and is likely caused by an increase in VEGF expression brought on by tissue hypoxia and stimulation of insulin-like growth factor receptors (38). Yoo and colleagues demonstrated that increased VEGF expression, endothelial cell proliferation, and angiogenesis seen in preclinical models of retinopathy could be inhibited by riluzole. They hypothesized that riluzole inhibited PKC-βIII activation and did not link its effects to the expression of GRMs. As part of the CNS, retinal tissue does express GRM1 and other GRMs (39) and riluzole likely exerts its effects on angiogenesis in the retina through inhibition of glutamatergic signaling, supporting our findings of a similar effect in melanoma.

IL-8 has been reported to function as an autocrine/paracrine factor stimulating cell growth, enhancing metastasis, and promoting angiogenesis in melanoma (40). Uptregulation of IL-8 and VEGF is involved in the resistance of melanomas to chemotherapeutic agents such as dacarbazine (41). The roles of VEGF in solid tumors have been well validated and therapies targeting VEGF/VEGFR are approved for clinical use in...
patients with colon, renal, non–small cell lung, and other types of human cancers (42). Our prior research demonstrated high levels of VEGF, VEGF-R1, VEGF-R2, and VEGF-B3 expression in melanoma (43), and a recently published phase II study of bevacizumab and temozolomide versus nab-paclitaxel and carboplatin in patients with advanced melanoma showed promising results with the addition of the antiangiogenic agent, though the tolerability of the regimen was a concern (44). It is also possible that resistance to therapies that target VEGF, such as bevacizumab, in patients with advanced melanoma could be secondary to simultaneous upregulation of IL-8 with continued angiogenesis even as VEGF activity is inhibited (42).

Our finding that activation of the AKT/mTOR/HIF-1α pathways and downstream upregulation of VEGF and IL-8 is consistent with previous reports (24, 25, 28). However, it has also been reported that voltage-gated sodium channels are involved in invasion and metastasis in both prostate cancer and triple-negative breast cancer, and riluzole can inhibit the effects of these ion channels in cancer cells (45). Therefore, we cannot exclude the possibility that the effects of riluzole in melanoma may be partly due to the inhibition of sodium channels. We are currently conducting studies to test this hypothesis.

Collectively, our findings support the hypothesis that GRM1 plays important roles in melanoma development and progression. These data will be useful in the design of further preclinical studies and future clinical trials combining GRM1 blockade with agents inhibiting components of the MAPK, PI3K/AKT, mTOR/HIF-1α, and other cellular pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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