Angiogenin cleaves tRNA and promotes stress-induced translational repression

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stress-induced phosphorylation of eIF2α inhibits global protein synthesis to conserve energy for repair of stress-induced damage. Stress-induced translational arrest is observed in cells expressing a non-phosphorylatable eIF2α mutant (S51A), which indicates the existence of an alternative pathway of translational control. In this paper, we show that arsenite, heat shock, or ultraviolet irradiation promotes transfer RNA (tRNA) cleavage and accumulation of tRNA-derived, stress-induced small RNAs (tiRNAs). We show that angiogenin, a secreted ribonuclease, is required for stress-induced production of tiRNAs. Knockdown of angiogenin, but not related ribonucleases, inhibits arsenite-induced tRNA production and translational arrest. In contrast, knockdown of the angiogenin inhibitor RNH1 enhances tRNA production and promotes arsenite-induced translational arrest. Moreover, recombinant angiogenin, but not RNase 4 or RNase A, induces tRNA production and inhibits protein synthesis in the absence of exogenous stress. Finally, transfection of angiogenin-induced tiRNAs promotes phospho-eIF2α–independent translational arrest. Our results introduce angiogenin and tiRNAs as components of a phospho-eIF2α–independent stress response program.

Introduction

The survival of mammalian cells exposed to adverse environmental conditions requires a radical reprogramming of protein translation (Yamasaki and Anderson, 2008). Stress-induced translational arrest of mRNAs encoding “housekeeping” proteins is triggered by a family of eIF2α kinases that reduce the availability of eIF2α–GTP–tRNAMet ternary complexes required for translation initiation (Anderson and Kedersha, 2008). Under these conditions, translation of a subset of mRNAs encoding upstream open reading frames (uORF; e.g., ATF4) is selectively enhanced, a consequence of uORF “read-through” (Lu et al., 2004). The reprogramming of protein translation is part of an integrated stress response that promotes the survival of mammalian cells analogous tRNA-derived fragments comprise a small subset of PIWI-associated RNAs (piRNAs), which suggests that tiRNA anticodon cleavage may lead to the assembly of specific RNP complexes (Brennecke et al., 2007). Supplemental Material can be found at:
/content/suppl/2009/03/30/jcb.200811106.DC1.html
/content/suppl/2009/04/06/jcb.200811106.DC2.html

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Abbreviations used in this paper: MEF, mouse embryo fibroblast; piRNA, PIWI-associated RNA; tiRNA, transfer RNA-derived stress-induced RNA.

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medium (Fett et al., 1985). The secretion of angiogenin is enhanced by hypoxia, which indicates that it may be a component of a stress response program (Hartmann et al., 1999; Nakamura et al., 2006). Angiogenin binds to receptors on the surface of endothelial cells that facilitate its internalization and transport to the nucleolus (Hu et al., 1997; Hatzis and Badet, 1999; Wiedlocha, 1999). Remarkably, promotion of new blood vessel growth is dependent on its ribonuclease activity (Shapiro and Vallee, 1987). Although the RNA targets required for angiogenesis are unknown, in vitro studies have shown that tRNAs are preferred targets (Saxena et al., 1992). Angiogenin also promotes ribosomal RNA transcription and cellular proliferation (Tsuji et al., 2005), which suggests that it has multiple functions. We have discovered that angiogenin is a stress-activated ribonuclease that cleaves tRNA and inhibits protein translation. Our results introduce angiogenin and tRNA-derived stress-induced RNAs (tiRNAs) as previously unappreciated components of the mammalian stress response.

Results and discussion

Extracts prepared from human U2OS cells exposed to arsenite-induced oxidative stress, heat shock, or UV irradiation were separated on a denaturing gel and developed with SYBR gold to visualize stress-induced small RNAs (Fig. 1 A). In stressed cells, two discrete bands corresponding to RNAs centered around 30 and 40 nucleotides were observed. Northern blotting using cDNA probes complementary to the 5’ end of tRNA Met (Fig. 1 A, bottom, NB) and the 5’ and 3’ ends of tRNA Met, tRNA Gly, and tRNA Pro (Fig. 1 B; 5S RNA is included as a loading control) confirms that the stress-induced RNAs are produced by tRNA cleavage. The size of these fragments requires that cleavage occur, as in T. thermophila (Lee and Collins, 2005), in or near the anticodon loop. These tiRNAs appear rapidly (within 20 min) in cells subjected to arsenite-induced oxidative stress, and persist for at least 11 h in cells allowed to recover from stress (Fig. S1 A). The phosphorylation and dephosphorylation of eIF2α over this time course provides a marker of stress and recovery from stress (Fig. S1 A). Arsenite-induced tiRNAs are observed in several different primate cell lines (Fig. S1 B). Importantly, tiRNAs are not observed in cells undergoing etoposide- or caffeine-induced apoptosis (Fig. S1 C), which indicates that tiRNA production is not a nonspecific consequence of cell death.

To determine the potential for tiRNAs to mediate phospho-eIF2α–independent translational arrest, we compared their

Figure 1. Stress-induced production of tiRNA. (A) U2OS cells treated with sodium arsenite (SA; 500 µM, 2 h), heat (42°C, 2 h), or UV irradiation (200 J/m², 12 h) were extracted with Trizol, and total RNA (10 µg) was separated on a 15% TBE-urea gel before processing with SYBR gold. The gel was also transferred to membrane and hybridized to a biotin probe complementary to the 5’ end of tRNA Met (NB). (B) Northern blotting analysis of RNA extracted from U2OS cells cultured in the absence (–) or presence (+) of sodium arsenite (500 µM, 2 h). Blots were hybridized to cDNAs complementary to the 5’ or 3’ fragments of the indicated tRNAs (bottom) or 5S RNA as a loading control (top). (C) MEFs derived from wild type (wt) or eIF2α (S51A) mutant (mut) mice were cultured in the absence (–) or presence (SA) of sodium arsenite (500 µM) for the indicated times before Trizol extraction. RNA and 5’ tRNA fragments were quantified by Northern blotting (top) and SYBR gold staining (middle). Phospho- and total eIF2α were quantified by immunoblotting (IB; bottom).
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induction in mouse embryo fibroblasts (MEFs) derived from wild-type or eiF2α (S51A) mutant mice (Scheuner et al., 2001). The expression of mature tRNA\textsuperscript{Met} is similar in wild-type and mutant (mut) cells in the absence or presence of arsenite (Fig. 1 C, SA). In both wild-type and mutant MEFs, tRNAs are much less abundant than tRNAs. Although densitometric analysis may be misleading because of the relative overexpression of tRNA\textsuperscript{Met}, the calculated ratios of tRNA\textsuperscript{Met}/tRNA\textsuperscript{Met} are always <0.1, which indicates that depletion of tRNA is unlikely to contribute to the functional effects of tRNA cleavage. Interestingly, the induction of tRNA\textsuperscript{Met} is significantly greater in mutant cells compared with wild-type cells, indicating that phospho-eIF2α is not required for, and may inhibit, tRNA production. We also quantified the production of tRNAs in U2OS cells treated with control or heme-regulated initiation factor 2-α kinase (HRI)-specific siRNAs (Fig. S2). Knockdown of HRI, the eiF2α kinase activated by arsenite, increases the arsenite-induced production of tRNAs. Collectively, these results indicate that the induction of tRNAs does not require phospho-eIF2α. Moreover, phospho-eIF2α may suppress the induction of tRNA.

Ribonucleases that target the anticodon loop of tRNA are found in both prokaryotes and eukaryotes (Ardelt et al., 1991; Kaufmann, 2000; Suhasini and Sirdeshmukh, 2006). In Xenopus oocytes, a tRNA anticodon nuclease designated onconase has been found to inhibit protein synthesis and promote the death of selected tumor cells (Ardelt et al., 1991; Suhasini and Sirdeshmukh, 2006). The toxic effects of onconase are observed at doses that do not markedly deplete cellular tRNAs, which suggests that activation of the ribotoxic stress response may contribute to its toxic effects (Iordanov et al., 2000). Angiogenin is an onconase-related ribonuclease that selectively cleaves tRNA in mammalian cells (Saxena et al., 1992). To determine whether angiogenin is required for the stress-induced production of tRNA, we used siRNA to knock down angiogenin expression before quantifying arsenite-induced tRNA production and stress-induced translational repression.

Transfection of a Dharmacon SMART pool targeting angiogenin reduces the expression of angiogenin mRNA (quantified using quantitative RT-PCR) to 63 ± 8% (n = 5) of the control level. Under these conditions, the arsenite-induced production of tRNA is reduced to 55 ± 6% (n = 3) of the control level (Fig. 2 A, lane 3; representative of three independent experiments). This result was confirmed using an angiogenin-specific siRNA that more efficiently reduces angiogenin expression (Tsuji et al., 2005). This reagent reduces the expression of angiogenin mRNA (quantified using quantitative RT-PCR) to 27% ± 12 (n = 3) of the control level, and production of tRNAs to 36 ± 8% (n = 3) of the control level (Fig. 2 A, lane 4; representative of three independent experiments). To determine the effect of angiogenin on stress-induced translational repression, U2OS cells were treated with the indicated siRNAs, then pulsed with [\textsuperscript{35}S]methionine-containing medium in the absence or presence and pulsed with [\textsuperscript{35}S]methionine-containing medium for 60 min before protein extraction. [\textsuperscript{35}S]Methionine incorporation (mean ± SD, n = 3–5) is normalized to that observed in cells treated with control siRNA (designated 100%). **, P = 0.04 (n = 5).
of sodium arsenite (Fig. 2 B, SA; 100 µM for 1 h) for 30 min. In all cases, [35S]methionine incorporation was normalized to that of cells treated with control siRNA. This analysis reveals that reduced expression of angiogenin significantly inhibits arsenite-induced translational repression (Fig. 2 B, compare lane 4 to lanes 5 and 6). These results are consistent with a role for angiogenin-induced tiRNAs in stress-induced translational repression, although cleavage of mRNA and/or rRNA could also contribute to inhibition of protein synthesis.

In S. cerevisiae, oxidative stress-induced tRNA cleavage is mediated by the RNaseT2 orthologue RNY1 (see Thompson et al., on p. 43 of this issue). In U2OS cells, knockdown of RNase T2 does not prevent arsenite-induced tiRNA production (Fig. S3), which indicates that yeast and humans use different enzymes to cleave tRNA. Knockdown of either RNase L or RNase Z (ELAC2) does not prevent arsenite-induced tiRNA cleavage (Fig. S3), which indicates that stress-induced tRNA cleavage is not a general property of ribonucleases.

We found that the angiogenin inhibitor RNH1 (Naddeo et al., 2005) regulates the production of tiRNA in U2OS cells. Targeted knockdown of RNH1 induces the production of tiRNAs in the absence or presence of arsenite-induced oxidative stress (Fig. 3 A, lanes 2 and 4, respectively). This result suggests that angiogenin is constitutively expressed but held in an inactive state by RNH1. In cells subjected to arsenite-induced oxidative stress, knockdown of RNH1 enhances tiRNA production and promotes stress-induced translational silencing (Fig. 3 B, compare lanes 3 and 4). In unstressed cells, tiRNAs induced by RNH1 knockdown do not inhibit protein synthesis under the assayed conditions (Fig. 3 B, compare lanes 1 and 2). This may be due to a requirement for stress- or secreted angiogenin–induced cofactors. Further experiments will be needed to clarify this point.

Because angiogenin is a stress-induced secreted protein that is taken up by adjacent cells, we tested the ability of purified recombinant angiogenin to induce the production of tiRNAs in U2OS cells. Wild-type angiogenin, but not an inactive mutant that has been implicated in the pathogenesis of amyotrophic lateral sclerosis (P112L; Fig 4 A, MUT; Wu et al., 2007), induces the production of tiRNA in U2OS cells (Fig. 4 A).
The angiogenin-related ribonucleases RNase 4 and RNase A do not induce the production of t\textit{i}RNA under these conditions (Fig. 4 B). Wild-type angiogenin, but not an inactive mutant (ANG-H13A; Shapiro et al., 1986), or RNase 4, significantly inhibits global protein synthesis in U2OS cells (Fig. 4 C). These results are consistent with a role for angiogenin in both stress-induced t\textit{r}RNA cleavage and stress-induced translational arrest.

Endogenous t\textit{i}RNAs corresponding to 5’ and 3’ t\textit{R}NA fragments were purified from angiogenin-treated cells and transfected into U2OS cells. Pulse labeling with [\textit{\textsuperscript{35}}S]methionine reveals that 5’ t\textit{i}RNAs, but not 3’ t\textit{i}RNAs or synthetic control RNAs (sequences corresponding to PIWI-associated RNAs; see Materials and methods) significantly inhibit protein synthesis (Fig. 5 A). The combination of 5’ and 3’ t\textit{i}RNAs also inhibits translation, indicating that 3’ t\textit{i}RNA does not inhibit the activity of 5’ t\textit{i}RNA. Importantly, the ratio of transfected t\textit{i}RNA/t\textit{R}NA (Fig. 5 B) is similar to the ratio of endogenous t\textit{i}RNA/t\textit{R}NA observed in arsenite-treated cells (Fig. 2 A). Similar results were obtained when 5’ and 3’ t\textit{i}RNAs were transfected into wild-type (Fig. 5 C: SS) and S51A mutant MEFs (Fig. 5 C, AA), which indicates that inhibition of protein synthesis does not require phosphorylation of eIF2\textalpha, ruling out a primary role for PKR in this process. Autoradiographic analysis of [\textit{\textsuperscript{35}}S]-labeled proteins reveals that 5’ t\textit{i}RNAs inhibit global protein synthesis (Fig. 5 D).

Angiogenin is a secreted protein that functions in the acute phase response induced by inflammatory stimuli (O\textsuperscript{\textregistered}lson et al., 1998). We have shown that: (1) recombinant angiogenin induces the production of t\textit{i}RNAs in U2OS cells (while this manuscript was under review, similar results were reported by Fu et al. [2009]), (2) recombinant angiogenin inhibits protein synthesis in U2OS cells, (3) knockdown of angiogenin inhibits arsenite-induced t\textit{i}RNA production and translational repression, (4) knockdown of RNH1 enhances t\textit{i}RNA production and promotes arsenite-induced translational repression, and (5) transfection of purified, endogenous 5’ but not 3’ t\textit{i}RNAs inhibits protein synthesis in U2OS cells as well as wild-type and S51A mutant MEFs. Collectively, these results strongly implicate angiogenin and t\textit{i}RNAs in a process of stress-induced translational repression.

The finding that t\textit{R}NA fragments possess posttranscriptionally added “CCA” residues and lack leaders, trailers, and introns suggests that t\textit{i}RNAs are derived from mature t\textit{R}NAs (Lee and Collins, 2005; Thompson et al., 2008; Fu et al., 2009). We have found that neomycin, a drug that prevents recombinant angiogenin from entering the nucleus, has no effect on angiogenin-induced t\textit{R}NA cleavage, which suggests that angiogenin

or presence of sodium arsenite (SA; lane 2; 500 \textmu M, 1 h), recombinant wild-type angiogenin (WT; 1 \textmu g/ml, lane 3), or recombinant mutant angiogenin (MUT, 1 \textmu g/ml, lane 4) for 1 h before Trizol extraction, separation by 15% TBE-urea gel, and CYBR gold staining. The positions of t\textit{R}NAs and t\textit{i}RNAs are indicated on the left. (B) U2OS cells were treated with the indicated ribonucleases (1 \textmu g/ml) for 1 h prior to processing as described in A. (C) U2OS cells were treated with the indicated ribonucleases (1 \textmu g/ml) for 30 min in the presence of [\textit{\textsuperscript{35}}S]methionine-containing medium before protein extraction. [\textit{\textsuperscript{35}}S] counts in cells cultured in media alone [Cont] were normalized to 100%. Results are the means ± SD (n = 3). *, P < 0.02.

**Figure 4.** Effect of recombinant angiogenin on t\textit{R}NA production and protein synthesis. (A) U2OS cells were cultured in the absence (–; lane 1) or presence of sodium arsenite (SA; lane 2; 500 \textmu M, 1 h), recombinant wild-type angiogenin (WT; 1 \textmu g/ml, lane 3), or recombinant mutant angiogenin (MUT, 1 \textmu g/ml, lane 4) for 1 h before Trizol extraction, separation by 15% TBE-urea gel, and CYBR gold staining. The positions of t\textit{R}NAs and t\textit{i}RNAs are indicated on the left. (B) U2OS cells were treated with the indicated ribonucleases (1 \textmu g/ml) for 1 h prior to processing as described in A. (C) U2OS cells were treated with the indicated ribonucleases (1 \textmu g/ml) for 30 min in the presence of [\textit{\textsuperscript{35}}S]methionine-containing medium before protein extraction. [\textit{\textsuperscript{35}}S] counts in cells cultured in media alone [Cont] were normalized to 100%. Results are the means ± SD (n = 3). *, P < 0.02.
likely to be 5’ monophosphates. In contrast, the 5’ ends of 3’ tiRNAs are likely to be hydroxyl groups. As miRNAs and piRNAs have 5’ monophosphates, this modification may promote the stability and function of small RNAs. The 3’ ends of 5’ tiRNAs are likely to be 2’, 3’ cyclic phosphates, as angiogenin cleavage leaves this moiety at the 3’ ends of cleaved RNAs (Rybak and Vallee, 1988). This cyclic phosphate residue may be resolved to 2’ phosphate or 3’ phosphate groups. Whether this moiety is

cleaves mature, cytoplasmic tiRNAs (unpublished data). The finding that 5’ but not 3’ tiRNAs inhibit protein synthesis reveals a functional difference between these tiRNA fragments. This may be a consequence of tiRNA fragment size: smaller 5’ fragments (∼30 nucleotides) may bind to a protein cofactor more efficiently than larger 3’ fragments (∼40 nucleotides). Another important consideration is the nature of the 5’ and 3’ ends of the tiRNA fragments. The 5’ ends of 5’ tiRNAs, like tRNA, are likely to be 5’ monophosphates. In contrast, the 5’ ends of 3’ tiRNAs are likely to be hydroxyl groups. As miRNAs and piRNAs have 5’ monophosphates, this modification may promote the stability and function of small RNAs. The 3’ ends of 5’ tiRNAs are likely to be 2’, 3’ cyclic phosphates, as angiogenin cleavage leaves this moiety at the 3’ ends of cleaved RNAs (Rybak and Vallee, 1988). This cyclic phosphate residue may be resolved to 2’ phosphate or 3’ phosphate groups. Whether this moiety is
Materials and methods

Cell culture and treatment

U2OS cells were cultured in DME (Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 U/ml antibiotics (penicillin), and 100 µg/ml streptomycin. Lipofectamine 2000 (Invitrogen) and Optimum medium (Invitrogen) were used for transfection of tiRNAs and siRNAs. The wild-type (SS) and S51A knock-in (AA) MEFs, a gift from D. Scheuner and R. Kaufman (University of Michigan Medical Center, Ann Arbor, MI), were cultured in DME with 10% fetal calf serum and antibiotics. For stress induction, the indicated doses of sodium arsenite were added. U2OS cells were harvested in 400 µl of lysis buffer (2% SDS/20 mM Hepes, pH 7.4) and sonicated, and the protein was precipitated by the addition of 5% dialyzed fetal bovine serum (Thermo Fisher Scientific) for 30–60 min, replaced with fresh labeling medium containing ~150–250 µCi of [35S]methionine per well (EasyTag EXPRESS 35S Protein Labeling Mix; PerkinElmer) and incubated for 30 min. Recombinant ribonuclease or arsenite was added together with the 35S labeling mix and incubated for 30 min or 60 min, respectively, before processing. After washing with PBS twice, cells were harvested in 400 µl of lysis buffer (2% SDS/20 mM Hepes, pH 7.4) and sonicated, and the protein was precipitated by the addition of 60% acetone. The proteins were resuspended in lysis buffer, and 10 µl of each sample in Ecosint H (National Diagnostics) was counted using a liquid scintillation counter (Beckman Coulter). Protein concentration was determined by Protein Assay BCA Protein Assay kit (Pierce).

Antibodies and reagents

Antibodies against phospho-eIF2α or total eIF2α were obtained from Assay Designs or Santa Cruz Biotechnology, Inc., respectively. Polyclonal rabbit anti-RN1H1 was obtained from Proteintech Group, Inc.

tiRNA isolation and transfection

U2OS cells (6.0 × 107) were treated with 0.5 µg/ml recombinant angiogenin or 500 µM sodium arsenite for 90 min before extraction with Trizol. 2 µg of total RNA was separated using four sets of 15% TBE-urea acrylamide gels. Gel fractions containing 5′-tiRNA and 3′-tiRNA visualized using SYBR Gold nucleic acid gel stain (Invitrogen) were crushed and soaked in 20 ml of 0.5 M sodium pyrophosphate, pH 7.4, and sonicated, and the protein was precipitated by the addition of 5% dialyzed fetal bovine serum (Thermo Fisher Scientific) for 30–60 min, replaced with fresh labeling medium containing ~150–250 µCi of [35S]methionine per well (EasyTag EXPRESS 35S Protein Labeling Mix; PerkinElmer) and incubated for 30 min. Recombinant ribonuclease or arsenite was added together with the 35S labeling mix and incubated for 30 min or 60 min, respectively, before processing. After washing with PBS twice, cells were harvested in 400 µl of lysis buffer (2% SDS/20 mM Hepes, pH 7.4) and sonicated, and the protein was precipitated by the addition of 60% acetone. The proteins were resuspended in lysis buffer, and 10 µl of each sample in Ecosint H (National Diagnostics) was counted using a liquid scintillation counter (Beckman Coulter). Protein concentration was determined by Protein Assay BCA Protein Assay kit (Pierce).

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siRNA treatment

For angiogenin knockdown, HRI, RNH1, RNaseL, ELAC2, and RNase T2 siGENOME SMART pools for each molecule were purchased from Thermo Fisher Scientific. The individual angiogenin siRNA sequence was 5′-GAC UUGCUAUUCUUGAGUUU-3′. U2OS cells were transfected with 40 nM of siRNA using Lipofectamine 2000 12 h after plating cells. On the next day, the cells were replated, and a second siRNA transfection was performed; after which cells were cultured for 24 h. Total RNA was isolated using Trizol after a 90-min incubation with 500 µM sodium arsenite.

RNA oligos

crtrNA1 (pIR58620, control RNA): 5′-UGUGAGUCACCGUGGAGGAGAACGUCUC-3′; crtrNA2 (pIR006650, control RNA): 5′-UGAGGUGUGUUGUGCUCLUUAGCUCUC-3′; crtrNA3 (pIR016792, control RNA): 5′-CCUCUCAGAGUGUGGUAGUUAGCUCUCG-3′.

DNA oligos

SY173, probe for 5′-trNAVal: 5′-GGGCCCAGCACGCTTCCGCTGGCGCACCAGTCCG-3′; SY167, probe for 3′-trNAVal: 5′-TAGACAGAGGATGTTGACATTCGACCAAC-3′; SY176, probe for 5′-trNAVal: 5′-GAC-3′; SY164, 3′-half of trNA12B-GlyGcc: 5′-GCCGGCGACATCCGACCAACGCCC-3′; SY180, probe for 5′-trNAVal: 5′-CCCGCCCGGAATGCAACGGGCCC-3′, SY186, probe for 3′-trNAVal: 3′ half of trNA12B-GlyGcc: 5′-CCCGCCCGGAATGCAACGGGCCC-3′; SY181, probe for 3′-trNAVal: 3′ half of trNA11-ProAgG: 5′-CTCTCCTGGGCTTAACCCCGGGGACCTTCCG-3′; SY161, probe for 5′-trNAArg: 5′-CC-3′.
Online supplemental material

Fig. S1 shows arsenite-induced production of tiRNAs. Fig. S2 shows that phospho-eIF2α is a negative regulator of tiRNA production. Fig. S3 shows the effect of endonucleases on tiRNA production. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200811106/DC1.

We thank the Anderson laboratory for helpful discussions and advice. We acknowledge Dr. Kaneyuki Iuchimochi for helpful discussions and Dr. Randal Kaufman for providing S51A mutant MEFs. This work was supported by National Institutes of Health grants AI065858, AI033600, and AR0514732.

Submitted: 20 November 2008
Accepted: 6 March 2009

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