Macrophages provide a first line of defense against microorganisms, and while some mechanisms to kill pathogens such as the oxidative burst are well described, others are still undefined or unknown. Here, we report that the Rab32 guanosine triphosphatase and its guanine nucleotide exchange factor BLOC-3 (biogenesis of lysosome-related organelles complex–3) are central components of a trafficking pathway that controls both bacterial and fungal intracellular pathogens. This host-defense mechanism is active in both human and murine macrophages and is independent of well-known antimicrobial mechanisms such as the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate)–dependent oxidative burst, production of nitric oxide, and antimicrobial peptides. To survive in human macrophages, Salmonella Typhi actively counteracts the Rab32/BLOC-3 pathway through its Salmonella pathogenicity island–encoded type III secretion system. These findings demonstrate that the Rab32/BLOC-3 pathway is a novel and universal host-defense pathway and protects mammalian species from various pathogens.

INTRODUCTION

Cells of our innate immune system, e.g., macrophages, are involved in the first line of defense against microorganisms. After phagocytosis, macrophages can eliminate most of the microorganisms they encounter by directing them in intracellular compartments where conditions are not compatible with microorganism life. A key strategy used by macrophages to kill microbes is the production of reactive oxygen species (ROS) through activation of the NADPH oxidase complex that is assembled on cellular membranes in response to infection (1). Other mechanisms, such as the production of nitric oxide, or cathelicidin-related antimicrobial peptide (Cramp), can also mediate bacterial killing (2). Despite the presence of a number of potent antimicrobial mechanisms, some microorganisms have evolved to become effective intracellular pathogens by escaping clearance and killing mechanisms present in macrophages and other immune cell types. For example, Salmonella enterica harbors two type III secretion systems that are responsible for the delivery of a battery of effectors that allow Salmonella to actively invade host cells, including macrophages, and survive in a specialized intracellular compartment known as the Salmonella-containing vacuole (SCV) (3–5). S. enterica is a genetically diverse bacterial species that includes hundreds of different serovars that can cause human and important veterinary diseases. S. enterica serovar Typhi (S. Typhi) is a human-restricted serovar that causes typhoid fever, a disease that affects ≈22 million people every year (6). Unlike many Salmonella serovars that can infect a broad range of hosts, S. Typhi naturally only infects humans (7). For example, it cannot establish an oral infection in laboratory mice (8).

Previously, we have shown that the inability of S. Typhi to infect mice depends, at least in part, on the fact that this pathogen cannot target the Rab32 GTPase in mouse macrophages (9). This GTPase and its guanine nucleotide exchange factor BLOC-3 (biogenesis of lysosome-related organelles complex–3) are central components of a pathway that regulate membrane trafficking to lysosome-related organelles in several specialized cell types (8, 9). The murine Rab32/BLOC-3 pathway is effectively neutralized by the murine-virulent S. enterica serovar Typhimurium (S. Typhimurium) through the delivery of two Salmonella Pathogenicity Island 2 (SPI-2) type III secretion effectors, GtgE and SopD2, that directly target Rab32 by acting as a protease and a GTPase-activating protein, respectively (8, 10). S. Typhimurium mutants defective for both these effectors are virtually avirulent in wild-type mice but are able to infect mice that are either deficient for Rab32 or BLOC-3 (10).

RESULTS

Our previous work suggested that the Rab32/BLOC-3–dependent pathway limits the infectivity of bacteria that have not evolved to neutralize it. Therefore, we investigated whether this pathway can control other pathogens that are known to persist intracellularly. When bone marrow–derived macrophages (BMDMs) from wild-type, Rab32, or BLOC-3–deficient mice were infected with Staphylococcus aureus, we observed a substantial increase in intracellular persistence in BMDMs deficient for the Hermansky-Pudlak Syndrome 4 (HPS4) protein, one of the two subunits of BLOC-3, or Rab32 when compared to wild-type BMDMs (Fig. 1A and fig. S1). In line with this, Rab32 is recruited to the vacuole containing S. aureus in wild-type but not HPS4-deficient BMDMs (Fig. 1B). Given that S. aureus is a Gram-positive bacterium, these data suggested that Rab32 and...
BLOC-3 are components of an antimicrobial pathway that is important for the clearance of a number of different bacterial pathogens. Therefore, we investigated whether this pathway can also limit infection by fungal pathogens. Wild-type and HPS4-deficient mice were infected with Candida albicans, and the fungal burden in kidneys, the main organ affected by this pathogen, was evaluated 72 hours post-infection (p.i.). BLOC-3–deficient mice exhibited a 14-fold increase in kidney fungal colony-forming units (CFUs) (Fig. 1C). These results indicate that the Rab32/BLOC-3–dependent pathway is critical for defending the host from both bacterial and fungal attacks.

To investigate potential mechanisms for the Rab32-dependent clearance, we infected BMDMs from wild type and mice defective in particular antimicrobial factors with S. Typhi. ROS are molecules that are toxic to many species that have not evolved strategies to neutralize them (11). Innate immune cells can assemble phagocytic NADPH oxidase on the phagosome to generate ROS to kill intracellular pathogens (12). BMDMs derived from NADPH oxidase-deficient mice (Phox−/−) clear S. Typhi similarly to wild-type BMDM, while an S. Typhi strain engineered to deliver the protease GtgE that cleaves Rab32 (8) is not killed so efficiently in either macrophage (Fig. 2A). Similarly, the production of nitric oxide radicals by the inducible nitric oxide synthase (iNOS) and Cramp, two important mechanisms that control pathogenic species, is not essential to clear S. Typhi in murine BMDMs (Fig. 2, B and C). These data indicate that the Rab32/BLOC-3–dependent pathway works independently of these well-characterized mechanisms of pathogen clearance and that another unknown mechanism underpins that ability of the Rab32 pathway to clear S. Typhi infections in murine cells.

The broad-host Salmonella serovar S. Typhimurium delivers two type III secretion effectors GtgE and SodP2 that confer the ability of isolates of this serovar to infect mice (8, 9, 13). S. Typhi lacks these two effectors and cannot infect mice but is able to survive in human macrophages and cause a systemic infection in humans. These facts could suggest that the Rab32/BLOC-3–dependent host-defense pathway is not fully active in human macrophages. However, the Rab32 and BLOC-3 genes are present in humans and genomewide association studies have shown that single-nucleotide polymorphisms in the Rab32 untranslated regions are associated with increased susceptibility to leprosy, a human bacterial infection caused by the intracellular bacterium Mycobacterium leprae (14, 15). This suggests that Rab32 could be part of a pathway critical to control some bacterial infection in humans. Two scenarios could explain these findings: (i) Rab32/BLOC-3 are not part of a host-defense pathway in humans or (ii) a Rab32/BLOC-3–dependent pathway is active in humans as an antimicrobial mechanism, but S. Typhi has evolved molecular strategies to evade it.

To assess whether the Rab32/BLOC-3 host-defense pathway is active as an antimicrobial pathway in humans, we investigated the requirement of Rab32 and BLOC-3 in controlling bacterial growth in human macrophages. We used an S. Typhi strain engineered to express the S. Typhimurium type III secretion effector GtgE, a specific protease that cleaves the three Rab GTPases, Rab32, Rab29, and Rab38 (8, 16). We infected human macrophage-like THP-1 cells with an S. Typhi wild-type isolate [ISP2825 (17)] or an isogenic strain engineered to express GtgE (S. Typhi::gtgE). GtgE delivery from S. Typhi results in the cleavage of human Rab32 (Fig. 3A), indicating that GtgE can target endogenous human Rab32, in agreement with the previous observation that GtgE cleaves ectopically expressed human Rab32 (8). When we infected human blood monocyte–derived primary macrophages, we observed that Rab32 localizes on the surface of the vacuoles containing wild-type S. Typhi but is mostly absent from the surface of the vacuoles containing S. Typhi::gtgE (Fig. 3, B and C).

We then investigated whether the removal of Rab32 from the bacterial vacuole has any effect on S. Typhi survival. GtgE expression confers S. Typhi a threefold replicative advantage in blood monocyte–derived primary macrophages at 24 hours p.i. (Fig. 4A), suggesting that one of the three Rab GTPases targeted by GtgE (8, 16) controls S. Typhi intracellular survival in human macrophages. As Rab38 mRNA is hardly detectable in either THP-1 or primary macrophages (fig. S2 and “The Human Protein Atlas”), we analyzed whether either Rab32 or Rab29 is responsible for the limitation of S. Typhi growth in human macrophages by knocking down either Rab29 or Rab32 from THP-1 cells (>70 and >80% knockdown, respectively; fig. S2). While depletion of Rab32 resulted in a significantly increased replication of S. Typhi (Fig. 4B), only a slightly reduced replication was observed when Rab29 was depleted. Together, these results indicate that Rab32 is critical to control S. Typhi infections in human macrophages.

We then used macrophages derived from human-inducible pluripotent stem cells (hiPSCs), a recently established model for the study of Salmonella infection (18, 19). First, we confirmed that, similarly to what was observed in THP-1 and primary macrophages, GtgE expression also confers an advantage to S. Typhi in hiPSC–derived macrophages (Fig. 4C). Next, we used CRISPR-Cas9 technology to generate hiPSCs deficient for HPS4. As shown in Fig. 4D,
Typhi has a replicative advantage in human macrophages. The survival and replication of Salmonella in human macrophages is dependent on the Rab32/BLOC-3 pathway. To test whether the human Rab32/BLOC-3–dependent pathway exerts broad antimicrobial activity, we infected macrophages derived from two independent clones of HPS4-deficient hiPSCs with S. aureus. As shown in Fig. 5A, HPS4 knockout results in ≈10-fold increased survival of S. aureus in human macrophages. However, in contrast to S. aureus (Fig. 5A) and other pathogens, such as Escherichia coli O157 (Fig. 5B), S. Typhi is not as efficiently cleared by wild-type human macrophages during infection but instead persist in the majority of infected cells (Figs. 5B and 4, A to D). Therefore, we hypothesized that S. Typhi actively counteracts the pathway controlled by Rab32/BLOC-3. Because the broad-host S. Typhimurium neutralizes this pathway through the action of effectors delivered by type III secretion systems, we tested whether S. Typhi survival in wild-type human macrophages is dependent on S. Typhi type III secretion systems. We observed that S. Typhi survival in hiPSC macrophages is dependent on its SPI-1 (Fig. 5C), but not on its SPI-2 type III secretion system (fig. S3). An SPI-1 type III secretion system mutant of S. Typhi (S. TyphiΔinvA) was unable to survive in macrophages derived from hiPSCs (Fig. 5C), in agreement with published results (20). S. TyphiΔinvA survived much better in HPS4-deficient macrophages (Fig. 5C), suggesting that the Rab32/BLOC-3 pathway is involved in S. Typhi killing and that S. Typhi needs this secretion system to counteract this pathway. To confirm that HPS4 removal did not result in a completely impaired bacterial killing, we infected HPS4-deficient macrophages with pathogenic E. coli O157. This pathogen is not able to survive in either wild-type or HPS4-deficient macrophages (Fig. 5D). These results indicate that S. Typhi is able to target the human Rab32/BLOC-3–dependent pathway likely through expression of the SPI-1 type III secretion system. As S. Typhi cannot neutralize the mouse Rab32/BLOC-3–dependent pathway, we suggest that S. Typhi targets a human-specific component of this pathway.

DISCUSSION

Overall, we show that Rab32 and BLOC-3 regulate host-defense activity against a variety of pathogens, including Gram-negative pathogens like S. Typhi. This Rab32/BLOC-3–dependent pathway is active as a host-defense pathway in human macrophages and can limit S. Typhi replication.
and Gram-positive bacterial pathogens and the fungal pathogen *C. albicans*. This activity is critical for the clearance of *S. aureus* and *S. Typhi* in mouse macrophages and for the clearance of *S. aureus* in human macrophages but is dispensable for *E. coli* killing. While neither the overall antimicrobial mechanism nor mechanisms controlled by Rab32 are yet defined, we show here that they do not require the production of ROS by the phagocytic NADPH oxidase, an ancient and broad antimicrobial mechanism active in macrophages. We also demonstrate that this activity does not require production of nitric oxide by iNOS and the macrophage antimicrobial peptide CRAMP.

In agreement with previous studies (21), we found that removal of Rab32 induces a general decrease in lysosome acidification in human macrophages (fig. S4). However, considering that *Salmonella* is resistant to the low phagosome pH in macrophages (22), it is not clear how this decrease in acidification can result in a substantial increase in intracellular persistence. It is also possible that Rab32 and BLOC-3 control different antimicrobial mechanisms; however, while this paper was under revision, a new study by Chen et al. (23) has shown that Rab32 is important for the delivery of itaconic acid to the SCV resulting in bacterial killing.

In contrast to *S. aureus*, *S. Typhi* uses its SPI-1 type III secretion system to evade this pathway in humans and persist in human macrophages. Not dissimilar from *S. Typhimurium*, which has evolved to deliver the protease GtgE and the Rab GAP SopD2 that act redundantly to inactivate the murine Rab32/BLOC-3 trafficking pathway, *S. Typhi* appears to have evolved a strategy to target the human Rab32/BLOC-3– dependent pathway that requires its SPI-1 type III secretion system. A possible reason for evolving a different strategy could rely on the fact that GtgE also targets Rab29, which is required for the efficient delivery of typhoid toxin from *S. Typhi*–infected cells (16). Therefore, we speculate that GtgE, although able to cleave the human Rab32, would not confer, overall, an advantage to *S. Typhi* because it would interfere with other pathogenic features of this bacterium, including the delivery of typhoid toxin. Similar to *S. Typhimurium*, *S. Typhi* may have evolved a number of
redundant effectors with different activities to target the Rab32/BLOC-3-dependent pathway. In conclusion, here, we reveal that Rab32 and BLOC-3 control a novel antimicrobial activity against a variety of infectious pathogens and demonstrate that \textit{S}. Typhi uses its SPI-1 type III secretion system to counteract this pathway and survive in human macrophages.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

The wild-type \textit{S} Typhi strain ISP2825 has been previously described (17). All the \textit{S}. Typhi deletion strains were constructed by standard recombinant DNA and allelic exchange procedures as previously described (24) and are listed in table S1. All the plasmids used in this study were constructed using standard recombinant DNA techniques and are listed in table S2. \textit{S}. Typhi \textsc{glmS}::\textit{Cm}::\textit{mCherry} and \textit{S}. Typhi::\textit{gtgE \textsc{glmS}::Cm::mCherry} that constitutively express \textit{mCherry} from a single chromosomal copy at the \textit{attTn7} site were generated by P22 transduction using phages obtained from the \textit{S}. Typhimurium SL1344 \textsc{glmS}::\textit{Cm::mCherry} \textsc{[a gift from L. Knodler; (25)]}. HPS4\textsuperscript{−−} (strain B6.C3-Pde6brd1 Hps4le/J) was purchased from the Jackson Laboratory.

**Cell culture**

THP-1 cells were maintained in RPMI 1640 medium (Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 10 mM Hepes (Invitrogen). The cells were maintained at a concentration between 0.1 and 1 million cells/ml. THP-1 differentiation was induced by adding 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 hours before infection. For intracellular growth experiments, THP-1 differentiated cells were treated with human interferon-\(\gamma\) (IFN-\(\gamma\)) (150 ng/ml) 24 hours before infection. Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) high glucose, 2 mM Glutamax (Invitrogen), and 10% FBS.

Blood was collected from healthy human volunteers, according to procedures approved by the Life Science and Medicine College Ethics Review Board of the University of Aberdeen (CERB/2016/11/1299). Peripheral blood monocyte–derived macrophages were prepared as described in (26) with some modifications. Briefly, 13 ml of blood was collected from each donor, diluted to 35 ml of Hanks’ balanced salt solution (HBSS; Invitrogen), and then loaded onto 15 ml of Lymphoprep (Stem Cell Technology) for the separation of the peripheral blood mononuclear cells. Isolated peripheral blood mononuclear cells were resuspended in DMEM containing...
10\% autologous human serum (freshly prepared from the same donor) and seeded on coverslips or tissue-treated plastic. Cells were plated at 5 \times 10^5 per well in 24-well plates. After 24 hours, the nonadherent cells were removed, fresh medium was added, and the cells were left for 7 to 9 days to differentiate.

Undifferentiated human-induced pluripotent stem cells line (KOLF2-C1) was maintained on a monolayer of mitotically inactivated mouse embryonic feeder cells in advanced DMEM/F12 medium, supplemented with 20\% knockout replacement serum (Invitrogen), 2 mM \( \gamma \)-glutamine, 0.055 mM \( \beta \)-mercaptoethanol (Sigma-Aldrich), and recombinant human fibroblast growth factor 2 (FGF2) (8 ng/ml; R&D Systems), as described previously (18). These cells were differentiated into macrophages as described in a previously published method (18).

C. albicans infection in wild-type or HPS4\(^{+/−}\) mice

C. albicans (strain SC5314) was serially grown overnight at 30°C with shaking. Yeast cells were washed in phosphate-buffered saline (PBS; Sigma-Aldrich), counted, and injected intravenously via the lateral tail vein. Animals were infected with 2 \times 10^5 CFUs. For analysis of fungal burdens in the kidneys, animals were euthanized 72 hours p.i. Kidneys were weighed, homogenized in PBS, and serially diluted before plating on to YPD (yeast extract, peptone, and dextrose) agar supplemented with penicillin/streptomycin (Invitrogen). Colonies were counted after incubation at 37°C for 24 to 48 hours.

CRISPR-Cas9 targeting of HPS4

Isogenic intermediate targeting vectors for HPS4 were generated using isogenic and haplotype-specific DNA by polymerase chain reaction (PCR) amplification of KOLF2-C1 genomic DNA (gDNA). First, a PCR fragment including homology arms and the critical exon of HPS4 was amplified from KOLF2-C1 gDNA using the following primers: f5F gccagtgaattcgatatacctgccttcttgaactgttttg and puc19_RV vector for 15 min at 50°C and transformed into Stellar competent cells (Takara Bio). Positive clones were verified by Sanger sequencing.

To deliver plasmids expressing gRNA, donor templates, and Cas9, 2 \times 10^6 KOLF2-C1 cells were nucleofected (AMAXA nucleofector 2B) with 2 \mu\g/ml of donor plasmid, 4 \mu\g/ml of hCas9 D10A (Addgene, plasmid #41816) (29), and 3 \mu\g/ml of gRNA plasmid DNA. Following nucleofection, cells were selected for up to 11 days with 0.25 \mu\g/ml puromycin. Individual colonies were picked into 96-well plates, expanded, and genotyped. Positive insertion of the cassette into the correct locus was confirmed by PCR using cassette-specific primers (CRISPR (CCA), GCGGAGATGGAGGGCGAGGC; and right CRISPR (CCT), TCAGCAACAGGGGCTCC (WGE IDs: 1181940311 and 1181940319, respectively).

Intracellular growth experiments

Overnight cultures of the different S. Typhi strains or S. aureus [strain SH1000 (30)] were diluted 1/20 in LB broth containing 0.3 M NaCl and grown for 2 hours and 45 min at 37°C. Cells were infected with the different strains of S. Typhi in HBSS at the desired multiplicity of infection. One-hour p.i. cells were washed three times with HBSS and incubated in growth medium supplemented with gentamicin (100 \mu\g/ml) for 30 min to kill extracellular bacteria. Cells were then washed with HBSS, and fresh DMEM containing gentamicin (5 \mu\g/ml) was added to avoid cycles of reinfection. At the indicated time points, the cells were washed twice in PBS, and the intracellular bacteria recovered lysing the cells in 0.1\% sodium deoxycholate (S. enterica) or 0.1\% Triton X-100 (Sigma-Aldrich) (S. aureus) in PBS were counted by plating serial dilutions on LB-agar plates.

Western blot

PMA-differentiated THP-1 cells were infected as described above and lysed in SDS-polyacrylamide gel electrophoresis loading buffer.
2.5 hours p.i. Western blot analysis was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences). The following antibodies were used for Western blot analysis: rabbit polyclonal anti-Rab32 (GeneTex; 1:1000 dilution) and donkey anti-rabbit IR Dye 800 (LI-COR Biosciences; 1:10,000 dilution).

**Rab32 and Rab29 knockdown in THP-1**

THP-1 cells were transduced with lentivirus expressing short hairpin RNA targeting Rab32 (TRCN0000299449, TRCN0000038685, TRCN00000381042, and TRCN0000036321, Sigma-Aldrich) or Rab32 (TRCN0000047746, Sigma-Aldrich). Twenty-four hours after transduction, cells were treated with puromycin (5 μg/ml) to kill the nontransduced cells and kept in culture for not more than 2 weeks. Seventy-two hours before the infection, the cells were treated with 100 nM PMA for 48 hours to induce differentiation. Twenty-four hours before the infection, the PMA was removed, and the cells were stimulated with human IFN-γ (100 ng/ml) and then finally infected with S. Typhi as described above.

**Immunofluorescence**

Bone marrow–derived mouse macrophages, human monocyte-derived macrophages, and wild-type or HPS4-deficient iPSDM were plated on glass coverslips (#1, Thermo Fisher Scientific) and fixed at the indicated times p.i. with 4% paraformaldehyde (PFA) for 10 min. Cells were then permeabilized for 20 min by incubating in 0.02% saponin (Sigma-Aldrich), 0.2% BSA (Sigma-Aldrich), and 50 mM NH4Cl (Sigma-Aldrich) in PBS and incubated for 1 hour with monoclonal mouse anti-CD68 (KP1, Invitrogen; 1:200 dilution). Alternatively, cells were permeabilized for 20 min by incubating in 0.2% Triton X-100 (Sigma-Aldrich), 0.2% BSA (Sigma-Aldrich), and 50 mM NH4Cl (Sigma-Aldrich) in PBS and incubated for 1 hour with a rabbit polyclonal anti-Rab32 (GeneTex; 1:200 dilution). Cells were then stained using the appropriate Alexa Fluor 488– or Alexa Fluor 555–conjugated secondary antibodies (Invitrogen). Images were acquired using either a Nikon (Eclipse T2) equipped with a CFI (chromatic aberration free infinity) Plan Apochromat 100× objective and a Prime 95B 25-mm complementary metal oxide semiconductor (CMOS) camera (Photometrics) or a PerkinElmer Spinning disk confocal equipped with an ORCA Flash 4.0 CMOS camera (Hamamatsu). Images were analyzed using the respective software (Nikon Elements or Volocity).

**Flow cytometry**

hiPSC-derived macrophages wild type or HPS4 deficient were plated on non–tissue culture–treated six-well plates (Thermo Fisher Scientific) and infected with S. Typhi glmS::Cm::mCherry or S. Typhi::gtge glmS::Cm::mCherry. At the indicated time p.i., the cells were detached using 500 μl of Versene (Invitrogen) and mixed with an equal volume of 4% PFA for 5 min. Fixed cells were then centrifuged and resuspended in 4% PFA for 5 min. The cells were then transferred in flow cytometry tubes, permeabilized for 15 min in PMZ-S (50 mM NH4Cl, 0.5% bovine serum albumin, 0.05% saponin), and then incubated for 1 hour with anti-CD68 (1:200) and then with anti-mouse Alexa Fluor 488. The samples were analyzed by flow cytometry (Fortessa, BD Biosciences) and FlowJo software.

**Statistical analysis**

CFU data in macrophages are presented as mean ± SEM. Differences between two groups were analyzed using the appropriate paired or unpaired Student’s t test. For C. albicans CFUs (Fig. 1C), the indicated P values were determined by one-way analysis of variance (ANOVA) test with Dunnett’s posttest. P values of 0.05 or less were considered to be statistically significant. Excel (Microsoft) and GraphPad Prism7 (GraphPad Software Inc.) were used to perform all statistical analyses.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/3/eabb1795/DC1

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**


In memoriam: This paper is part of Stefania Spanò's scientific legacy, and this work would have not been possible without her intelligence, vision, and persistence. A dreadful destiny has snatched her from us too early, but her discoveries and ideas are living and flourishing.

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The Rab32/BLOC-3–dependent pathway mediates host defense against different pathogens in human macrophages

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