Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 14, Issue of April 4, pp. 11731–11734, 2003

Reversal of Senescence in Mouse Fibroblasts through Lentiviral Suppression of p53*[S]

Received for publication, January 17, 2003
Published, JBC Papers in Press, January 27, 2003,
DOI 10.1074/jbc.C300023200

Annette M. G. Dirac‡ and René Bernards§

From the Division of Molecular Carcinogenesis, and Center for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Senescence is generally defined as an irreversible state of G_1 cell cycle arrest in which cells are refractory to growth factor stimulation. In mouse embryo fibroblasts (MEFs), induction of senescence requires the presence of *p19^{ARF}* and *p53*, as genetic ablation of either of these genes allows escape from senescence and leads to immortalization. We have developed a lentiviral vector that directs the synthesis of a p53-specific short hairpin transcript, which mediates stable suppression of p53 expression through RNA interference. We show that suppression of p53 expression in senescent MEFs leads to rapid cell cycle re-entry, is associated with loss of expression of senescence-associated genes, and leads to immortalization. These data indicate that senescence in MEFs is reversible and demonstrate that both initiation and maintenance of senescence is p53-dependent.

Most primary mammalian cells have a limited ability to proliferate in tissue culture (1, 2). After a variable number of cell divisions, primary cells will undergo what is believed to be an irreversible form of growth arrest in the G_1 phase of the cell cycle and become refractory to further growth factor stimulation (3–5). In this state of growth arrest, referred to as senescence, cells adopt a typical large and flat morphology and express a number of senescence-associated markers, including senescence-associated β-galactosidase, plasminogen activator inhibitor-1 (PAI-1),[1] and p21^{cip1} (5).

The triggers for the induction of senescence differ between mouse and human cells. In cultured rodent fibroblasts senescence is thought to result from stress signals generated in response to the inadequate tissue culture environment. This includes supraphysiological oxygen tension, lack of proper extracellular matrix, and the liberal administration of bovine growth factors (Refs. 6 and 7 and reviewed in Refs. 5 and 8). Indeed, under more gentle and more defined culture conditions, primary mouse cells can be convinced to proliferate for extended periods of time *in vitro* (9). Tissue culture stress signals induce expression of a number of anti-proliferative genes, including p16^{INK4A} and p19^{ARF} (10, 11). The induction of p19^{ARF} appears more relevant than the induction of p16^{INK4A}, as mouse embryo fibroblasts (MEFs) genetically deficient for p19^{ARF} are resistant to induction of senescence and readily become immortal (12), whereas p16^{INK4A}-deficient MEFs senesce normally (13, 14). Likewise, MEFs lacking the downstream effector of p19^{ARF}, p53, are immortal (15), whereas MEFs lacking the downstream effector of p16^{INK4a}, pRb, are mortal (16). However, MEFs lacking all three pRb family members, pRb, p107 and p130 are immortal (17, 18). These data indicate that the *Rb* family proteins not only act upstream of the p19^{ARF}-p53 pathway, through regulation of *p19^{ARF}* by E2F (19), but also downstream by rendering cells insensitive to p53 signaling (20).

Expression of oncogenes, such as an activated RAS oncogene, can further enhance tissue culture stress signals and induce rapid onset of senescence, referred to as "premature senescence" (21). Oncogenic RAS stimulates many of the same anti-proliferative genes that are induced by spontaneous senescence, including p19^{ARF} and p16^{INK4A}, and again only ablation of the p19^{ARF}-p53 pathway allows escape from oncogene-induced premature senescence to cause oncogenic transformation (12, 21). These observations have led to the suggestion that premature senescence is part of a fail-safe mechanism that protects cells from oncogenic transformation (22).

Senescence in human cells differs from senescence in rodent cells in that most primary human cells lack the catalytic component of telomerase, hTERT. As a consequence, *in vitro* propagation of primary human cells is associated with erosion of the chromosome ends, the telomeres, leading to DNA damage-like anti-proliferative signals when telomeres become critically short (23–25). Consequently, most human cells require expression of telomerase to overcome this barrier to immortality. However, similar to rodent cells, primary human cells (especially those of epithelial origin) also suffer from "tissue culture stress" and often arrest long before their telomeres are critically short (8, 26). This tissue culture stress response of primary human epithelial cells appears to depend on p16^{INK4a} rather than on p14^{ARF} (4, 27). However, several diploid human fibroblasts can be immortalized by hTERT expression, suggesting differential sensitivity of primary human cells to tissue culture stress.

It has been proposed that the stress-induced replicative arrest induced by tissue culture stress should be referred to as "stasis" (for "stimulation and stress induced senescence"), whereas the term "senescence" should be used for cells that undergo replicative arrest as a result of DNA-damage signals emanating from short telomeres. The different names for both forms of senescence suggest that the mechanisms that underlie both processes are distinct. However, the signaling pathways involved and the phenotypic consequences of both forms of replicative arrest are related. For this reason, the study of rodent cell stasis is likely to be relevant for our understanding of the signaling mechanisms that underlie replicative aging of primary human cells in culture.

We describe here a novel vector system to study the genes that are required to maintain senescence. We created a virus

* This work was supported by a grant from the Center for Biomedical Genetics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains a movie.

‡ Supported by a long term fellowship of EMBO.

§ To whom correspondence should be addressed. Tel.: 31-20-512-1952; Fax: 31-20-512-1954; E-mail: r.bernards@nki.nl.

[1] The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; FVB, Friend virus B-strand; MEF, mouse embryo fibroblast; CMV, cytomegalovirus; GFP, green fluorescent protein; WT, wild type; LTR, long terminal repeat.

Supplemental Material can be found at:
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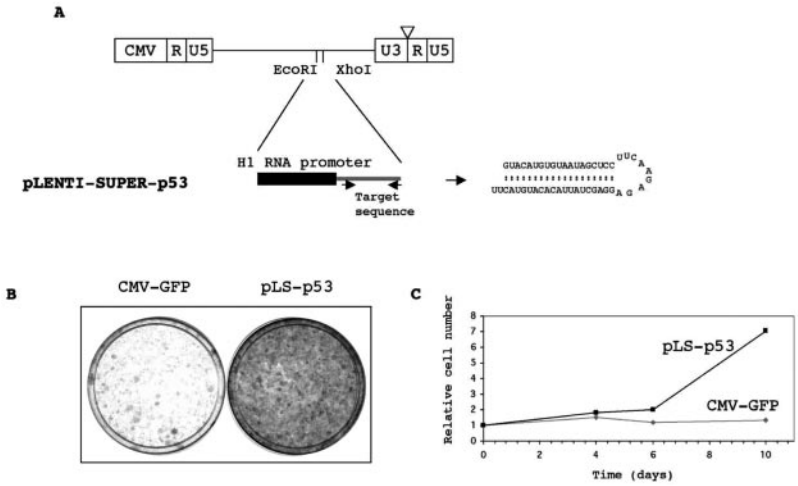

FIG. 1. **A lentiviral vector that mediates RNA interference.** *A*, a schematic overview of the lentiviral RNA interference vector pLENTI-SUPER-p53 (*pLS-p53*). A DNA fragment containing the H1 promoter and an oligonucleotide insert targeting murine p53 were transferred from pRETRO-SUPER (32) to HIV-SC (29) by digestion of both vectors with *Eco*RI and *Xho*I, followed by ligation of the H1-p53 DNA fragment into HIV-CS. This vector contains a deletion in the U3 region indicated by a triangle that silences the enhancer of the LTR, efficiently shutting off LTR-driven transcription after proviral integration. The predicted short hairpin RNA targeting murine p53 is shown. *B*, passage 3 FVB wild type MEFs were infected with either HIV-CS-CG (*CMV-GFP*) lentivirus or LENTI-SUPER-p53 virus, respectively. Fortyeight hours after infection 5×10^4 cells were seeded in 10-cm dishes for colony formation assays and stained after 14 days. *C*, 48 h after infection 1.5×10^3 cells were seeded per well in six-well dishes. At varying time intervals cells were fixed and stained with crystal violet, which was then solubilized with 10% acetic acid and quantified at OD_{590} as a relative measure of cell number. CMV-GFP and pLS-p53 curves are marked in *gray* and *black*, respectively.

that can infect post-mitotic cells and direct the synthesis of short hairpin transcripts that mediate post-transcriptional gene silencing through RNA interference. We used this vector system to ask if senescence is a reversible process.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The murine p53-specific short hairpin oligonucleotides were first cloned in pRETRO-SUPER (28). pRETRO-SUPER vector was digested with *Bgl*II and *Hin*dIII, and the annealed oligonucleotides targeting murine p53, 5′-gatccccGTACATGTGTAATAGCTCCttcaagagaGGAGCTATTACACATGTACtttttggaaa-3′ and 5′-agcttttccaaaaGTACATGTGTAATAGCTCCtctcttgaaGGAGCTATTACACATGTACggg-3′, were ligated with the vector, yielding pRETRO-SUPER-p53. The 19-mer p53 targeting sequence in the oligonucleotide is indicated in capital letters. The lentiviral transfer vector HIV-CS-CG (29) was digested with *Eco*RI and *Xho*I to remove the CMV-GFP sequence. The cassette containing the H1 promoter and the p53 target sequence was excised from pRETRO-SUPER-mp53 with *Eco*RI and *Xho*I and ligated into HIV-CS to yield pLENTI-SUPER-p53.

Cell Culture, Lentiviral Production, and Infection—Wild type Friend virus B-strand (FVB) MEFs, ST.*Hdh*^{Q111} mouse striatum cells (30), and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For production of lentivirus, 293T cells were transfected by the calcium-phosphate method using 10 μg transfer vector HIV-CS-CG or pLENTI-SUPER-p53, 3.5 μg of VSVg envelope vector pMD.G, 2.5 μg of RSV-Rev, and 6.5 μg of packaging vector pCMVDR8.2 (29). Lentiviruses were harvested 24 and 48 h after transfection and filtered through a 0.45-μM filter. ST.*Hdh*^{Q111} cells were shifted to 39 °C 14 days prior to lentiviral infection. WT MEFs were cultured to passage 9–10 whereupon cells were counted every 3–4 days 14 days prior to lentiviral infection. The senescent phenotype was also investigated by acidic β-galactosidase staining at the time of infection (31). 1.8×10^5 senescent WT MEFs in 6 cm dishes were infected with lentivirus for at least 12 h in the presence of 0.8 μg/ml polybrene and were then allowed to recover for 48 h before reseeding for colony formation assays and growth curves. 0.5×10^5 or 1×10^5 cells were seeded in 10 cm dishes for colony formation assays. Cells were fixed and stained with superstain (50% methanol, 10% acetic acid, 0.1% Coomassie Blue) 16 days after seeding. For growth curves 1.5×10^3 cells were seeded per 3.5-cm dish, at 3-day intervals cells were fixed with 0.5% formaldehyde, stained with 0.1% crystal violet, followed by re-solubilization in 10% acetic acid. The OD_{590} was quantified as a relative measure of cell number.

Western Blot Analysis—Whole cell extracts were separated on 12% SDS-PAGE gels and transferred to polyvinylene diflouride membranes (Millipore). Visualization was done using enhanced chemiluminescence (Amersham Biosciences, Inc.) Antibodies used were M-156 (Santa Cruz) against p16^{INK4a}, ab80-50 (Abcam) against 19^{ARF}, F-5 (Santa Cruz) against p21, Ab-7 (Oncogene) against p53, and P30620 (Transduction Laboratories) against PAI-1.

Time-lapse Microscopy—5×10^4 senescent MEFs were seeded in 3.5-cm dishes and infected with lentivirus. Time-lapse microscopy was initiated 34 h after infection in a temperature and CO_2-controlled chamber using 10× phase contrast. Frames were taken every 20 min over a period of 38 h.

RESULTS

We have recently described a vector, pSUPER, which mediates highly specific and persistent RNA interference through stable expression of short hairpin RNAs (28). We generated a lentiviral derivative of this vector by cloning the H1 RNA short hairpin gene expression cassette targeting murine p53 from pRETRO-SUPER (32) into the self-inactivating lentiviral vector pHIV-CS (29). We named this vector pLENTI-SUPER-p53 (Fig. 1*A*). As a control, we used a lentiviral vector that expresses GFP (HIV-CS-CG (29)).

Loss of p53 in primary mouse embryo fibroblasts is associated with acquisition of an immortal phenotype (15). To test whether the lentiviral p53 knockdown vector was capable of inducing a functional inactivation of p53 in MEFs, we infected early-passage primary MEFs with LENTI-SUPER-p53 virus or with control GFP lentivirus and asked whether p53 knockdown caused immortalization. Some 30–40% of control lentivirus-infected cells were GFP-positive, indicating that the primary MEFs were efficiently infected by the lentiviral vectors (data not shown). Fig. 1, *B* and *C*, show that infection with LENTI-SUPER-p53, but not with control GFP lentiviral vector, caused efficient immortalization of the infected primary MEFs, indicating that the LENTI-SUPER-p53 virus mediates functional inactivation of p53 expression (see also Fig. 3*A*).

We next asked whether suppression of p53 expression by lentiviral gene transfer in senescent cells would allow re-entry into the cell cycle. We employed two cell systems to address this question. First, we used conditionally immortalized STHdh^{Q111} neuronal cells derived from mouse embryonic striatum. These cells are conditionally immortalized due to the presence of a temperature-sensitive allele of SV40 T antigen (30). STHdh^{Q111} cells proliferate indefinitely at the permissive temperature (32 °C), but rapidly and synchronously become post-mi-

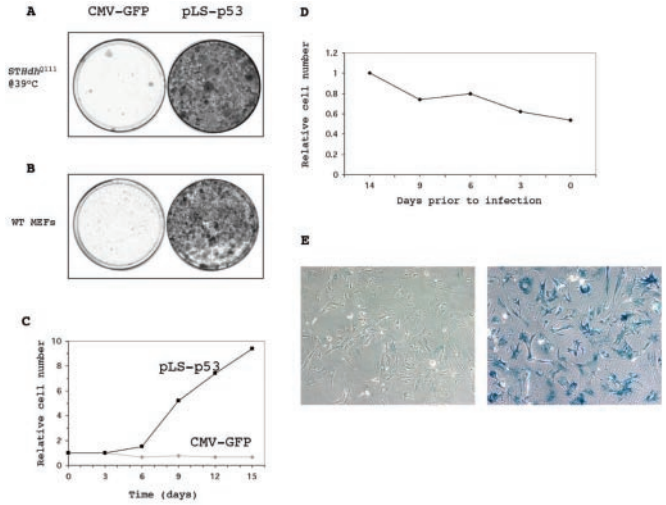

FIG. 2. **Lentivirus-mediated p53 suppression reverses senescence.** *A*, $STHdh^{Q111}$ cells were shifted to the non-permissive temperature of 39.5 °C at which T antigen is inactive and were kept for 14 days to assure that all cells were senescent prior to infection with CMV-GFP or pLS-p53 lentivirus. 5×10^4 cells were seeded for colony formation assays, and dishes were stained 2 weeks later. *B*, senescent MEFs infected with CMV-GFP or pLS-p53 lentivirus were seeded at 1×10^5 cells per 10-cm dish, and dishes were stained 16 days after seeding. *C*, 48 h after infection with CMV-GFP (*gray*) or pLS-p53 (*black*), 1.5×10^3 cells were seeded per well in six-well dishes. At 3-day intervals cells were fixed and stained with crystal violet and quantified by determining OD_{590} as a relative measure of cell number. *D*, WT MEFs were cultured to passage 9. Fourteen days prior to lentiviral infection cells were counted, and equal numbers of cells were replated every 3 days. *E*, immediately prior to lentiviral infection, passage 5 and senescent WT MEFs were subjected to acidic β-galactosidase staining (31). Cells were fixed with 0.5% gluteraldehyde and incubated with staining solution overnight at 37 °C.

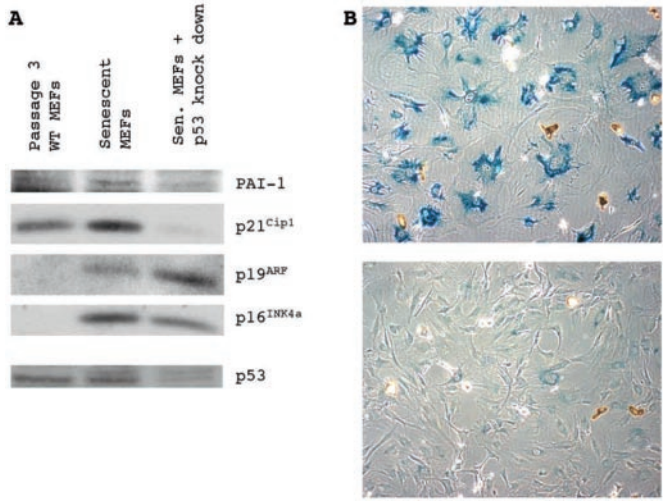

FIG. 3. **Senescence markers in reverted WT MEFs.** *A*, Western blots of senescence markers in passage 3 (*lane 1*), senescent (*lane 2*), and WT MEFs reverted from senescence by knockdown of p53 that were harvested 4 weeks after lentiviral infection (*lane 3*). *B*, acidic β-galactosidase staining performed on senescent and reverted WT MEFs (as described in the legend to Fig. 2).

totic and adopt a senescent morphology when shifted to the non-permissive temperature (39.5 °C) at which T antigen is inactive (33). We used $STHdh^{Q111}$ cells that had been maintained at 39.5 °C for 2 weeks to assure that the entire population was senescent and then infected the senescent cells with the LENTI-SUPER-p53 virus or control GFP lentivirus and maintained the infected cells at 39.5 °C for 2 weeks. Fig. 2*A* shows that knockdown of p53 led to re-entry into the cell cycle

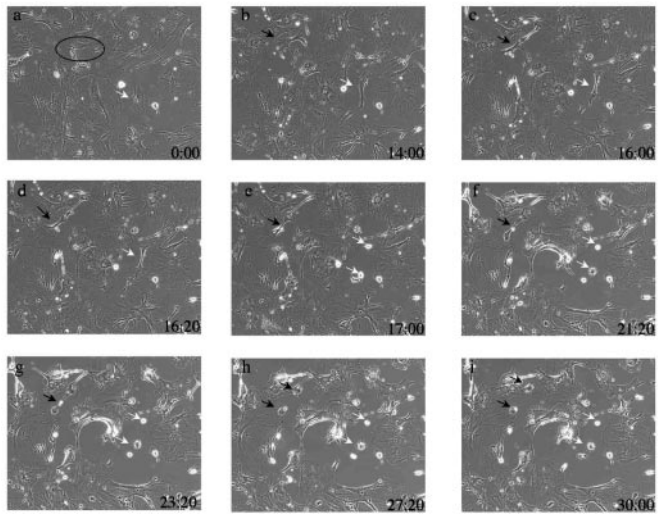

FIG. 4. **Time-lapse microscopy of senescent MEFs following knockdown of p53.** Selected frames from a 38-h recording period of senescent WT MEFs infected with LENTI-SUPER-p53 are shown. Time points are indicated in the *lower right corner* of the individual frames. A cell undergoing successful division is indicated with a *black ring* and *black arrows*, while a division immediately followed by apoptosis is indicated with *white arrows*.

and allowed continued proliferation, indicating that the senescence-like growth arrest of $STHdh^{Q111}$ cells at the non-permissive temperature can be reversed by suppression of p53.

Next we asked whether p53 knockdown would allow cell cycle re-entry in senescent primary MEFs. We cultured primary MEFs of FVB genotype until the cells no longer proliferated (Fig. 2*D*) and expressed high levels of the senescence-associated markers acidic β-galactosidase, PAI-1, $p21^{cip1}$, $p19^{ARF}$ and $p16^{INK4a}$ (Figs. 2*E* and 3*A*). All cells in the culture showed a flat senescent morphology and stained intensely for acidic β-galactosidase (Fig. 2*E*), indicating that these cells were quantitatively senescent. This notion is also supported by the growth curves of these late-passage MEFs, which showed a constant decline in cell number over time (Fig. 2*D*), indicative of the absence of spontaneously immortalized cells in the culture. Fig. 2, *B* and *C*, show that lentiviral knockdown of p53 in these senescent primary MEF cultures triggered a marked degree of proliferation. Importantly, cell cycle re-entry was associated with loss of expression of several of the senescence-associated markers, including PAI-1, $p21^{cip1}$, and acidic β-galactosidase (Fig. 3, *A* and *B*) and senescence-reverted cells continued to proliferate for several weeks without any signs of senescence, suggesting that they had become immortal (Fig. 2*B* and data not shown).

In principle, the observed proliferation following lentiviral knockdown of p53 could originate from cells that were not truly senescent in the culture. It was therefore important to follow the cultures of senescent MEFs in time after lentiviral infection. Fig. 4 shows a series of time-lapse photomicrographs of senescent MEFs after lentiviral knockdown of p53, which together indicate that cells with a completely flat and senescent morphology round up and divide within 48 h after infection with the p53 knockdown virus (Fig. 4, cells marked by *black arrows*). However, not all cell divisions are productive as many cells divide initially, but die by apoptosis during division or just after completion of cell division (Fig. 4, cells marked by *white arrows*). Assessed by time-lapse photography and colony formation efficiencies (Fig. 2*B*), ~0.5–1% of infected cells divide successfully. A complete movie of the senescent MEFs after infection with the p53 knockdown vector is provided as supplementary material. No division or apoptosis could be observed

following infection with control lentivirus encoding GFP (data not shown). We conclude that cells with all the hallmarks of fully senescent cells rapidly re-enter the cell cycle after p53 knockdown. We conclude that p53 is not only required to initiate senescence, but is also required, at least in MEFs, to maintain senescence.

DISCUSSION

Using a lentiviral vector system that silences gene expression, we provide evidence that suppression of p53 expression in senescent MEFs leads to a reversion of the senescent state and causes immortalization. Several lines of evidence support the notion that the MEFs were fully senescent at the time of infection with the lentiviral p53 knockdown vector. First, the cells had stopped proliferating in the presence of growth factors, indicating that they were senescent and refractory to growth factor stimulation, rather than quiescent and still responsive to growth factors (Fig. 1*D*). Second, they uniformly manifested a senescent morphology and expressed the senescence-associated markers acidic β-galactosidase, PAI-1, $p21^{cip1}$, $p19^{ARF}$ and $p16^{INK4a}$ (Figs. 2*E* and 3*A*). When cells emerged from senescence as a result of p53 knockdown, the cells behaved phenotypically as p53 null MEFs in that they were immortal and had low levels of $p21^{cip1}$ and high levels of $p19^{ARF}$ (10, 12, 15). Importantly, the cells that emerged from senescence by p53 knockdown maintained high levels of $p16^{INK4a}$ (Fig. 3*A*). As $p16^{INK4a}$ expression is induced during senescence in a p53-independent fashion (10), these data indicate that the signaling pathways that led to the induction of senescence are still operational in senescence-reverted MEFs. This provides further evidence that the cells that re-entered cell cycle by p53 knockdown were indeed fully senescent at the time of infection with the p53 knockdown virus.

Our data are in agreement with earlier experiments performed in senescent human diploid fibroblasts. Thus, ablation of p53 function by microinjection of p53 antibody in primary human fibroblasts allowed at least temporary reversal of senescence and re-entry into the cell cycle (34). However, inactivation of p53 in human fibroblasts delays, but does not abrogate, replicative senescence, indicating that p53 inactivation alone is not sufficient to mediate stable reversion of senescence in primary human fibroblasts and requires also induction of hTERT expression (35, 36). An essential feature of the lentiviral vector system described here is that suppression of gene expression is persistent, allowing the study of long term consequences of gene inactivation in post-mitotic cells. The LENTI-SUPER vector should therefore be a useful tool to investigate which genes are continuously required to maintain a post-mitotic state in cells that have exited the cell cycle.

Acknowledgments—We thank Dr. Inder Verma for the self-inactivating lentiviral vector, Lauran Oomen for invaluable assistance with the time-lapse microscopy, Thijn Brummelkamp and Roderick Beijersbergen for discussions, and Katrien Berns for critical reading of the manuscript.

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