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mTORC1 Directly Phosphorylates and Regulates Human MAF1▽

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mTORC1 is a central regulator of growth in response to nutrient availability, but few direct targets have been identified. RNA polymerase (pol) III produces a number of essential RNA molecules involved in protein synthesis, RNA maturation, and other processes. Its activity is highly regulated, and deregulation can lead to cell transformation. The human phosphoprotein MAF1 becomes dephosphorylated and represses pol III transcription after various stresses, but neither the significance of the phosphorylations nor the kinase involved is known. We find that human MAF1 is absolutely required for pol III repression in response to serum starvation or TORC1 inhibition by rapamycin or Torin1. The protein is phosphorylated mainly on residues S60, S68, and S75, and this inhibits its pol III repression function. The responsible kinase is mTORC1, which phosphorylates MAF1 directly. Our results describe molecular mechanisms by which mTORC1 controls human MAF1, a key repressor of RNA polymerase III transcription, and add a new branch to the signal transduction cascade immediately downstream of TORC1.

The PIKK family conserved TOR (target of rapamycin) kinase, originally discovered in *Saccharomyces cerevisiae* (9), is a central regulator of cell growth in response to nutrient availability and other environmental cues (see references 4 and 44 for reviews). TOR is part of two complexes, TORC1 and TORC2, of which the first is inhibited by low concentrations of the macrolide rapamycin. Inhibition of TORC1 by nutrient deprivation or rapamycin has broad consequences, among them the inhibition of ribosome biogenesis and protein translation. This inhibition is mediated in part through transcriptional repression of genes required for these processes such as the RNA polymerase (pol) I-transcribed large rRNA genes, the pol II-transcribed ribosomal protein genes, and a number of pol III-transcribed genes, including, for example, tRNA genes (4, 44).

In yeast, repression of pol III transcription in response to nutrient deprivation, rapamycin treatment, or other stresses such as DNA damage and secretory pathway defects requires the repressor Maf1 (38) (see 6 and 42 for reviews). The protein is regulated by phosphorylation/dephosphorylation events, which control nuclear/cytoplasmic transport as well as the pol III repression function of the protein. The two processes are, however, at least partially independent (20, 23, 28, 36, 40). Several kinases have been implicated, in particular PKA and Sch9, the second of which appears to be the main Maf1 kinase (11, 17, 20, 41). Recently, TORC1 was also described as a kinase that weakly phosphorylates yeast Maf1 on unknown residues within the nucleus (40).

Human MAF1, like yeast Maf1, is a phosphoprotein. It is unclear, however, whether human MAF1 is indispensable for repression of pol III transcription in response to various stresses, and neither the function of MAF1 phosphorylation nor the MAF1 kinases have been identified. Here we show that mammalian cells lacking the *MAF1* gene do not repress pol III transcription in response to serum starvation or inhibition of TORC1 by treatment with Torin1 or rapamycin. We identify the phosphosites of human MAF1, show that the phosphorylation state of these residues regulates the MAF1 pol III repression function, and identify mTORC1 as the MAF1 kinase.

MATERIALS AND METHODS

Mass spectrometry analysis. To analyze MAF1 phosphorylation sites, we generated a HeLa spinner cell line (referred to as the DBR28 cell line) expressing MAF1 tagged with Flag and His tags at its C terminus. We grew two samples of DBR28 cells as well as two samples of control spinner HeLa cells that did not express tagged MAF1. For each pair, one sample was exposed to 0.08% methyl methanesulfonate (MMS) for 40 min at 37°C, after which the cells were collected by centrifugation at 4°C. We then purified the doubly tagged MAF1 as described previously for pol III (10) and loaded the protein samples on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were rapidly stained with Coomassie blue, and each lane was excised as five equal-size acrylamide pieces. The proteins were digested with trypsin (Promega) as described previously (31, 43). Data-dependent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of extracted peptide mixtures after digestion with trypsin was carried out on a hybrid linear trap LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) interfaced to a nanocapillary high-performance liquid chromatography (HPLC) equipped with a C_{18} reversed-phase column. Collections of tandem mass spectra for database searching were generated from raw data with Mascot Distiller 2.1.1 and searched using Mascot 2.2 (Matrix Science, London, United Kingdom) against release 13.2 of the UNIPROT database (Swiss-Prot plus TrEMBL [www.expasy.org]) restricted to human taxonomy. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 ppm. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine were specified as variable modifications. The software Scaffold (version Scaffold-02_00_01; Proteome Software, Inc.) was used to validate MS/MS-based peptide (minimum of 90% probability [14]) and protein (minimum of 95% probability [22]) identifications and to perform data set alignment as well as parsimony analysis to discriminate homologous hits.

Northern blotting. A total of 10 μg of total RNA separated on a 6% denaturing polyacrylamide gel and transferred on Hybond N^+ membranes (Amersham) was probed with ^{32}P-end-labeled pre-tRNA Leu probes, as follows: MS3

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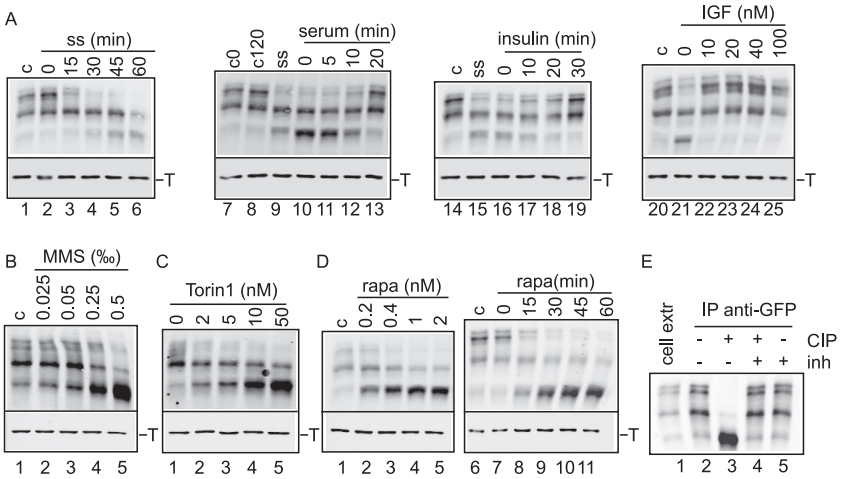

FIG. 1. MAF1 is dephosphorylated by treatments that inhibit mTOR. IMR90hTert cells stably expressing MAF1-EGFP were treated as indicated, and the samples were separated on a Phos-tag gel and immunoblotted with an anti-GFP antibody (top panels). The bottom panels show the same samples immunoblotted with an antitubulin antibody (T). (A) Cells were serum starved (ss) for 0 to 60 min (lanes 2 to 6) or 30 min (lanes 9 to 13, 15 to 19, and 21 to 25), after which serum was added back for 0 to 20 min (lanes 10 to 13), 100 nM insulin was added for 0 to 30 min (lanes 16 to 19), or different concentrations of IGF were added for 1 h (lanes 22 to 25). Lanes 1, 14, and 20, nontreated (c) cells; lanes 7 and 8, nontreated cells lysed at 0 and 120 min, respectively. (B) Cells were treated for 1 h with MMS at the indicated concentrations. Lane 1, untreated cells. (C) Cells were treated for 1 h with Torin1 at the indicated concentrations. (D) Cells were treated with rapamycin for 1 h at the indicated concentrations (lanes 2 to 5) or with 1 nM rapamycin for the indicated times (lanes 7 to 11). Lanes 1 and 6, nontreated cells. (E) MAF1-EGFP was immunoprecipitated from untreated cells and incubated with calf intestine phosphatase (CIP) for 30 min at 30°C either with or without phosphatase inhibitors (inh). Lane 1, cell lysate.

(5′TCC CCA GAC AGG GAA GCT AA3′), MS4 (5′CCC TCA GTA GAG GAA GCG AAC3′), MS5 (5′CAC CCG TAG GTA AGG CTT GTC A3′), MS6 (5′CCC TCA GAG CGA GGA AGC CAT AG3′), or antisense ^{32}P-labeled 7SK RNA.

Protein gels. Cells were lysed in Laemmli buffer (MAF1-enhanced green fluorescent protein [EGFP]). Alternatively, they were lysed in buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 0.5% Igepal CA-630, 1 mM dithiothreitol [DTT], protease inhibitors [Roche Complete EDTA free], phosphatase inhibitors [P2850; Sigma] and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), incubated for 20 min on ice, and centrifuged for 10 min at 10,000× *g*; MAF1 with a C-terminal His tag (MAF1-HT) was bound to nickel beads for 2 h at 4°C, and the beads were washed in buffer B (10 mM HEPES [pH 7.9], 10 mM KCl, 200 mM NaCl, and 0.5% Igepal 630) and resuspended in Laemmli buffer. MAF1-HT and MAF1-EGFP were separated on 8% and 6% SDS-polyacrylamide gels, respectively, containing 75 μM Phos-tag acrylamide (NARD Institute), and transferred onto Hybond ECL membranes (Amersham). The membranes were probed with anti-MAF1 (Sc-98715; Santa Cruz), anti-green fluorescent protein (anti-GFP) (A6455 and 11 814 460 001; Invitrogen and Roche, respectively), anti-myc (C3956; Sigma), antihemagglutinin (anti-HA) (12CA5), anti-S6K (9202; Cell Signaling), or antitubulin (Sc-32293; Santa Cruz) antibodies.

Kinase assays. Untransfected HeLa cells or cells transiently overexpressing HA-mTOR (wild type [wt] or kinase dead [KD]), myc-S6K, or myc-S6K- glutathione *S*-transferase (GST) (KD) were lysed by incubation for 10 min in buffer C (40 mM HEPES [pH 7.4], 150 mM NaCl, 0.3% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, protease inhibitors [Roche Complete EDTA free], 10 μg/ml aprotinin, 10 mM NaF, 10 mM glycerol-2-phosphate, 0.5 mM sodium vanadate, 1 mM DTT). The extract was centrifuged for 10 min at 10,000 × *g* and used for immunoprecipitation for 2 h at 4°C with anti-HA (A2095; Sigma) beads or with anti-myc (C3956; Sigma), anti-Raptor, or anti-Rictor (13) bound to protein A-Sepharose (GE Healthcare). The beads were then incubated for 30 min at 30°C with recombinant MAF1-HT of GST-URI in buffer D (25 mM HEPES [pH 7.4], 50 mM NaCl, 500 μM ATP, and 10 μCi [γ-^{32}P]ATP) containing 1 mM $MnCl_2$ (mTOR) or 1 mM $MgCl_2$ (S6K). Where indicated, 1 μg FKBP12 (R&D Systems) and 100 nM rapamycin (553211; Calbiochem) were added. Samples were separated on a 12% polyacrylamide gel, and the gel was then Coomassie blue stained and analyzed with a phosphorimager. Alternatively, MAF1-HT was purified on nickel beads as described above, separated on a Phos-tag gel, and transferred to a membrane which was immunoblotted and analyzed by a phosphorimager.

Transfections and retroviral infections. Transient transfections were performed by the calcium phosphate method. To generate stable cell lines, a vector carrying puromycin resistance was cotransfected, and cell clones were selected with puromycin. Murine embryonic fibroblasts (MEFs) were isolated from *Maf1* knockout (KO) or control mice (our unpublished data) and transformed or not with the simian virus 40 (SV40) large T antigen. Nontransformed cells were infected with retroviral vectors containing different MAF1-Flag-HT versions and kept under puromycin selection. Experiments were performed within 3 weeks after infection.

RESULTS

Human MAF1 dephosphorylation correlates with repression of pol III transcription. We and others have shown that human MAF1 is a phosphoprotein that is dephosphorylated upon treatment of cells with rapamycin or methyl methanesulfonate (MMS) or upon serum deprivation (7, 27). To extend these observations, we generated permanent IMR90hTert cell lines expressing a MAF1-EGFP fusion protein. We used SDS-polyacrylamide gels containing Phos-tag to separate phosphorylated forms of the fusion protein (15, 16) and visualized them by immunoblotting with anti-EGFP antibodies. As shown in the top panels of Fig. 1A to D, MAF1-EGFP migrated as a series of slowly migrating bands (lanes labeled with "c"). Upon serum starvation (Fig. 1A, lanes 1 to 6) or treatment with MMS (Fig. 1B), Torin1 (Fig. 1C), which specifically inhibits both mTORC1 and mTORC2 (35), or rapamycin (Fig. 1D), which at the concentrations used inhibits only mTORC1, these slowly migrating bands collapsed into one main, fast-migrating band. Upon readdition of serum, insulin, or insulin-like growth factor 1 (IGF-1) to serum-starved cells, slower-migrating bands again became prominent (Fig. 1A, lanes 10 to 13, 16 to 19, and 21 to 25). We then immunoprecipitated the fusion protein and treated it with calf intestine phosphatase (CIP). As shown in

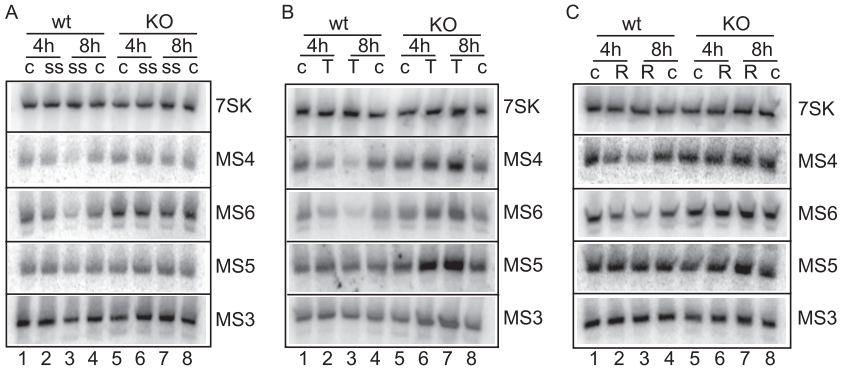

FIG. 2. Pol III inhibition depends on *Maf1*. Wild-type (wt) or *Maf1* KO MEF cells were treated as indicated, and 10 μg of total RNA was analyzed by Northern blotting with probes against mature 7SK and different tRNA Leu precursors (MS3, -4, -5, and -6). (A) Cells were either serum starved (ss) or left untreated (c) for the indicated times; (B and C) cells were left untreated (c) or treated with Torin1 (T) or rapamycin (R) for the indicated times.

Fig. 1E, the slow-migrating bands collapsed into the fastest-migrating band upon treatment with CIP but not with CIP plus phosphatase inhibitors (compare lanes 2 to 5), indicating that the slowly migrating bands correspond to phosphorylated forms of MAF1.

The effects of the treatments described above on pol III transcription were monitored in murine embryonic fibroblasts (MEFs) as well as in MEFs lacking the *Maf1* gene (*Maf1* KO MEFs) (W. Hodroj, P. Cousin, and N. Hernandez, unpublished results). We measured the levels of some tRNA precursors, which provide a measure of ongoing transcription, and those of 7SK RNA, which was stable over the length of the experiment and served, therefore, as an internal control. As shown in Fig. 2A, in wild-type (wt) MEFs, 7SK RNA remained constant for 8 h after serum deprivation, but the MS4 and MS6 leucine tRNA precursors decreased, especially after 8 h, suggesting transcription repression (lanes 1 to 4). This decrease was MAF1 dependent, as it did not occur in *Maf1* KO MEFs (Fig. 2A, lanes 5 to 8). The MS3 and MS5 precursors were less or not affected. We observed the same pattern in MEFs and *Maf1* KO MEFs treated with Torin1 or rapamycin (Fig. 2B and C), except that, for unknown reasons, all leucine precursors were slightly increased upon Torin1 treatment in *Maf1* KO MEFs (Fig. 2B, lanes 6 and 7).

These results show that MAF1 is phosphorylated in actively growing cells and rapidly dephosphorylated upon various stresses. The maintenance of the phosphorylated state depends on mTOR, as treatment with two highly specific mTOR inhibitors resulted in dephosphorylation. Dephosphorylation correlated with a MAF1-dependent decrease in selected leucine tRNA precursors, suggesting transcription inhibition of specific pol III genes. This is consistent with MAF1 dephosphorylation mediating the repression of some pol III genes upon stress and, in particular, upon mTOR inhibition.

MAF1 phosphorylation sites. To determine MAF1 phosphorylation sites *in vivo*, we first generated a HeLa cell line expressing Flag- and His-tagged MAF1. Tagged MAF1 affinity purified from this cell line either untreated or treated with MMS was analyzed by mass spectrometry. As shown in Table 1, the following five residues were found to be phosphorylated at least three times: S60, S68, S75, T212, and S214. In all cases except for that of T212, which was more often phosphorylated after MMS treatment, phosphorylation decreased after MMS treatment. The most frequent phosphorylations were the S68

TABLE 1. MAF1 phosphorylation sites identified by mass spectrometry

Phosphorylated residue	Phosphorylation results obtained by mass spectrometry			
	Without MMS		With MMS	
	No. of phosphorylated peptides/ no. of sequenced peptides[a]	% phosphorylated[b]	No. of phosphorylated peptides/ no. of sequenced peptides[a]	% phosphorylated[b]
T64	1/77	1	1/32	3
S65	1/77	1	1/32	3
S68	24/77	31	4/32	12
S60-S65	1/77	1	0/32	0
S60-S68	5/77	6	0/32	0
S70	1/77	1	0/32	0
S75	14/39	36	3/20	15
T212	0/29	0	3/21	14
S214	3/29	10	1/21	5

[a] The number of times the specific residue was phosphorylated out of the number of times the relevant peptide was sequenced in cells either untreated or treated with MMS.
[b] Percentage relative to the number of times the peptide was sequenced.

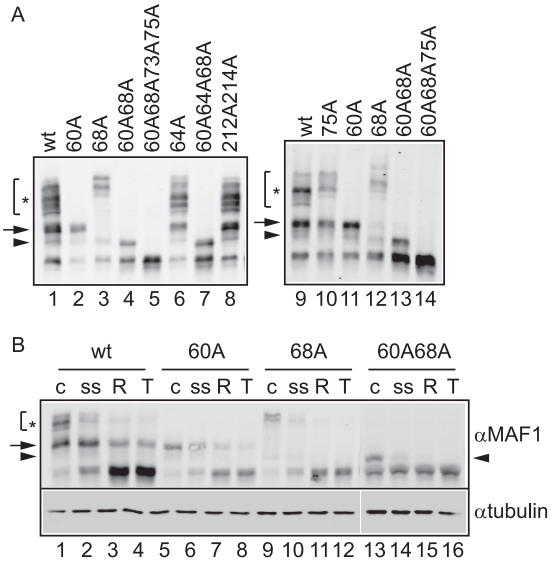

FIG. 3. MAF1 phosphorylation on S60, S68, and S75 depends on mTORC1 activity. (A) MAF1-HT transiently expressed in HeLa cells was affinity purified, electrophoresed on a Phos-tag gel, and revealed with an anti-MAF1 antibody. (B) IMR90hTERT cells stably expressing either wt, S60A, S68A, or S6068A MAF1-EGFP were either left untreated (c), serum starved (ss) for 1 h, or treated with 1 nM rapamycin (R) or 50 nM Torin1 (T) for 1 h and lysed. (Top) Proteins were separated on Phos-tag gels and revealed by immunoblotting with an anti-GFP antibody. Bands corresponding to phosphorylated serine 75 (arrowhead), 68 (arrow), or a group of bands depending on phosphorylated serine 60 (bracket, with the strongest band marked by an asterisk) are indicated. (Bottom) Immunoblot of the same samples probed with an anti-tubulin antibody. α, anti.

and S75 phosphorylations and the S60-S68 double phosphorylation, thus identifying these residues as the predominant phosphosites becoming dephosphorylated upon MMS-induced cellular stress. Of note, this analysis could not determine whether S75 phosphorylation occurred concomitantly with S60 and S68 phosphorylation because the peptides were generated by trypsin digestion, and there is a trypsin cleavage site at a lysine at position 74 that separates the S60-S68 and S75 residues onto two different peptides. Our identification of S60, S68, and S75 as prominent MAF1 phosphorylation sites is consistent with large-scale phosphoproteomics studies identifying these same residues (3) or just S75 (34) (19) as phosphoresidues (http://www.phosphosite.org).

We then engineered vectors expressing versions of MAF1 with a C-terminal His tag (MAF1-HT) in which various phosphorylated residues were mutated to alanines. These constructs were transiently transfected into HeLa cells, and the MAF1 phosphorylation patterns were examined on Phos-tag gels. As shown in Fig. 3A, the higher resolution obtained with the His-tagged protein than with the MAF1-EGFP fusion protein (see above) allowed the detection of up to nine bands with the wt construct. This pattern changed upon mutation of various serines to alanines. Thus, the second band from the bottom (Fig. 3A, arrowheads) was lost whenever S75 was mutated (lanes 5, 10, and 14) but not upon mutation of any other residue (lanes 2 to 4, 6 to 8, and 11 to 13), suggesting that it corresponds to MAF1 phosphorylated at S75. Interestingly, this band became stronger upon mutation of both S60 and S68 (Fig. 3A, lanes 4, 7, and 13). Perhaps S75 is often phosphorylated together with S60 and S68, resulting in slower-migrating bands that collapse into the singly phosphorylated S75 band upon mutation of these residues. The third band from the bottom (Fig. 3A, arrows) was lost whenever S68 was mutated (lanes 3 to 5, 7, and 12 to 14) but not upon mutation of any other residue (lanes 2, 6, 8, 10, and 11), suggesting that it corresponds to MAF1 phosphorylated at S68. All bands above the third band (Fig. 3A, brackets) strictly depended on the presence of S60, as they all disappeared upon mutation of S60 (lanes 2, 4, 5, 7, 11, 13, and 14). However, consistent with the mass spectrometry analysis in which we never found S60 phosphorylated alone, none of these upper bands was uniquely sensitive to mutation of S60, suggesting that they correspond to combinations of several phosphorylations. In particular, the strongest of these upper bands, labeled with an asterisks in Fig. 3A, disappeared upon mutation of S60, became much weaker upon mutation of S68 (lanes 3 and 12), and became slightly weaker upon mutation of S75 (lane 10), suggesting that it is a mixture of MAF1 phosphorylated on S60 and S68 together with a smaller amount of MAF1 phosphorylated on S60 and S75.

Mutation of either T64 or T212 and S214 did not change the band pattern (Fig. 3A, compare lanes 1, 6, and 8), suggesting that the amounts of MAF1 phosphorylated at these sites are too small to be detected. We cannot exclude, however, that phosphorylation on these sites does not alter the migration of the protein in Phos-tag gels. Mutation of S73, which was not identified as a phosphosite by mass spectrometry, did not modify the band pattern when introduced either alone (not shown) or in combination with other mutations (Fig. 3A, compare lanes 5 and 14). Importantly, mutation of all three residues, S60, S68, and S75, left only the fastest-migrating band corresponding to dephosphorylated MAF1 (and, perhaps, to MAF1 phosphorylated on the minor T64, S65, S212, or T214 site) (compare Fig. 3A, lane 14, to Fig. 1E, lane 3).

We then examined whether these individual bands depend on mTOR activity in cells stably expressing MAF1-EGFP mutant constructs. The MAF1 bands remaining after mutation of S60, S68, or both S60 and S68 became weaker or disappeared after either serum starvation or rapamycin or Torin1 treatment (Fig. 3B, top; the bottom panel shows an anti-tubulin immunoblot of the same samples). Thus, MAF1 is phosphorylated mostly on serines 60, 68, and 75 in an mTOR-dependent manner.

MAF1 with S60, S68, and S75 mutated to alanines is hyperactive. To evaluate the functional significance of S60, S68, and S75 phosphorylation, we transduced *Maf1* KO MEFs with vectors expressing either wt MAF1 or MAF1 with S60, S68, and S75 mutated to alanines (60A68A75A mutant) or aspartates (60D68D75D mutant) and monitored the effect on the levels of leucine tRNA precursors both before and after stress. As shown in Fig. 4A and quantified in Fig. 4B, transduction of wt and mutated MAF1 resulted in a decrease of tRNA Leu precursors even in the absence of stress (Fig. 4A, lanes 1 to 4, and B, bars labeled "ctrl"). Significantly, 60A68A75A MAF1 was the most active repressor, reducing MS6, the most responsive gene, to 36%. In fact, we could even observe a small (about 10%) but reproducible repression of 7SK RNA. In contrast, both wt MAF1 and 60D68D75D MAF1 were less active re-

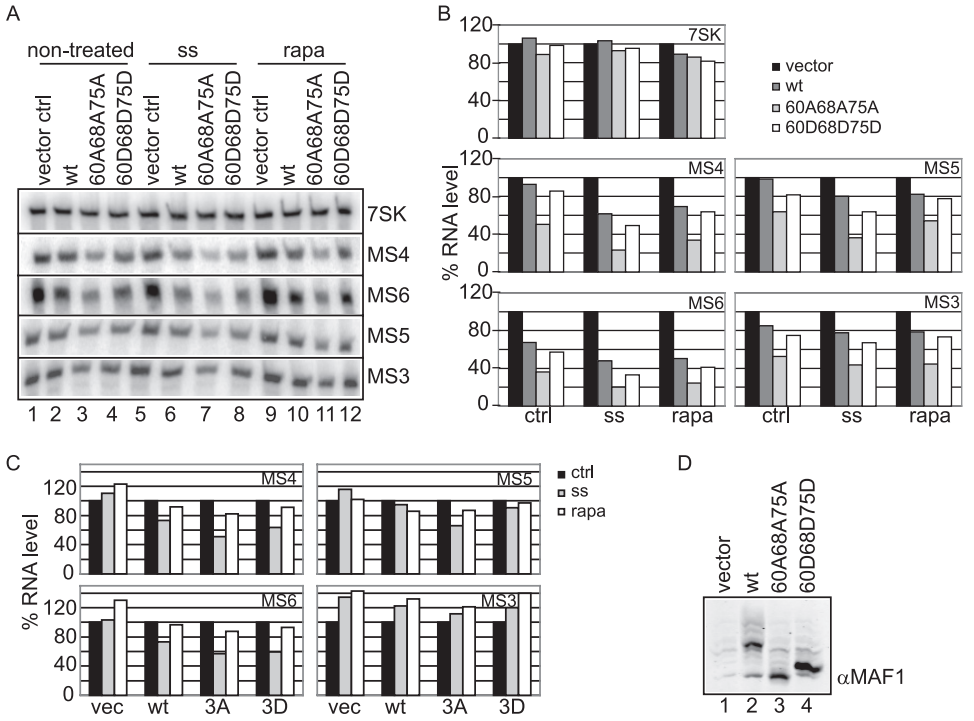

FIG. 4. Expression of 60A68A75A MAF1 decreases the level of tRNA Leu precursors. (A) Total RNA of *Maf1* KO MEFs transduced with vectors either empty (lane 1, vector control [ctrl]) or expressing the proteins indicated above the lanes was probed for different tRNA Leu precursors (MS3, -4, -5, and -6) and 7SK RNA. Cells were either left untreated (lanes 1 to 4), serum starved for 8 h (lanes 5 to 8), or treated with 2 nM rapamycin for 8 h (lanes 9 to 12). (B) Quantification of the signals in panel A, with the vector control set at 100%. (C) Quantification of the signals in panel A, with the nontreated control cells set at 100%. 3A, 60A68A75A MAF1; 3D, 60D68D75D MAF1. (D) Immunoblot analysis of affinity-purified MAF1 from the transduced cells, probed with an anti-MAF1 antibody.

pressors, with 60D68D75D MAF1 seemingly slightly more active than the wt protein.

We then submitted transduced cells to serum starvation or rapamycin treatment. The results, shown in Fig. 4A and B, are summarized in Fig. 4C, in which for each MAF1 version, we set the amounts of the various leucine precursors observed before stress at 100%. We observed a further decrease in pol III transcription for all tRNA Leu precursors except that for MS3. This further decrease was largest after serum starvation but was also clearly detectable after rapamycin treatment. In each case, it was most pronounced for the 60A68A75A mutant, whereas the wt and 60D68D75D were quite similar to each other. As an example, after serum starvation, the MS4 precursor was decreased by 27%, 49%, and 37% in cells expressing wt, 60A68A75A, and 60S68D75D MAF1 proteins, respectively.

The levels of the MAF1 proteins in transduced MEFs were analyzed by immunoblotting. As shown in Fig. 4D, KO MEFs transduced with empty vector showed no detectable MAF1 (lane 1). In contrast, in cells transduced with MAF1-expressing vectors, we detected phosphorylated wt MAF1 (Fig. 4D, lane 2), and 60A68A75A or 60D68D75D migrating as unphosphorylated proteins (compare lanes 3 and 4 to lane 2; note that the 60D68D75D mutant migrated slower than the 60A68A75A mutant, probably as a result of the different amino acid sequences). Importantly, the levels of wt and mutant proteins were quite similar, with the 60D68D75D protein being slightly more expressed, perhaps explaining its slightly larger effect compared to that of the wt protein.

The effects of wt and mutated MAF1 proteins on pol III transcription were tested in at least three separate experiments. Although the absolute repression levels varied, we consistently observed the strongest repression with the 60A68A75A mutant protein, followed by the 60D68D75D mutant and the wt protein. Together, these results indicate that mutation of the phosphosites to alanines results in a MAF1 protein capable of repressing pol III transcription more efficiently than wt or 60D68D75D MAF1 in the absence of stress. Significantly, the 60A68A75A mutant protein directs a further reduction in tRNA precursors after stress. This is an important result, which, as discussed further below, points to the existence of a stress-regulated cofactor required for full MAF1 activity.

MAF1 phosphorylation and repression of pol III transcription do not require S6 kinases 1 and 2. In yeast cells, Maf1 is phosphorylated by Sch9, a kinase corresponding to metazoan S6 kinase 1 (S6K1) (11, 17, 41). To explore the role of S6K1 in human MAF1 phosphorylation, we transiently transfected HeLa cells with vectors expressing Myc-tagged S6K1 or Myc-GST-tagged S6K1 carrying an inactivating (kinase-dead [KD]) mutation (2). The wt or KD S6 kinases were purified by immunoprecipitation with anti-Myc tag antibodies and incubated with recombinant MAF1 with a C-terminal His tag (MAF1-HT) or GST-URI, a known S6K1 substrate, in the presence of [γ-^{32}P]ATP (5). As shown in Fig. 5A, URI was efficiently

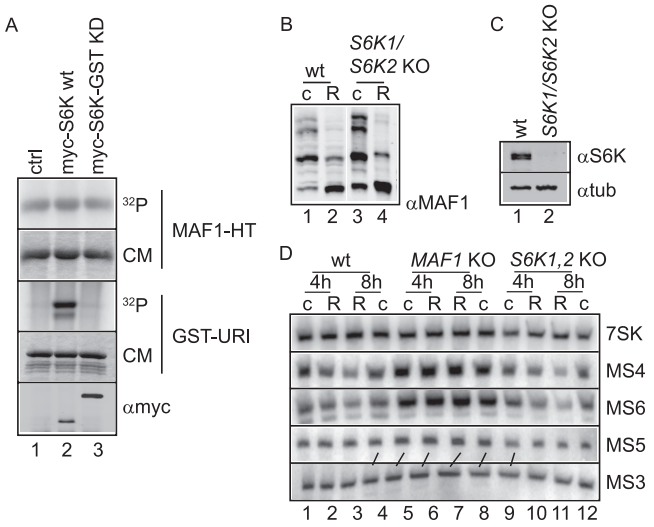

FIG. 5. S6K cannot phosphorylate MAF1. (A) Active myc-S6K (wt) or inactive myc-S6K-GST (KD) was immunoprecipitated from transiently transfected HeLa cells (ctrl, cells transfected with empty vector) and incubated with recombinant MAF1-HT or GST-URI in the presence of Mg^{2+} and [γ-^{32}P]ATP. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and the gel was first stained with Coomassie blue (CM) and then analyzed with a phosphorimager (^{32}P). In the bottom panel, the presence of S6K1 was revealed with an anti-myc antibody. (B) Wt or *S6K1 S6K2* KO MEFs were transiently transfected with a vector expressing MAF1-HT and treated or not (c) with 2 nM rapamycin (R) for 1 h. MAF1-HT was affinity purified, separated on a Phos-tag gel, and revealed by immunoblotting with an anti-MAF1 antibody. (C) Wt and *S6K1 S6K2* KO MEF cells were probed with anti-S6K1 and anti-tubulin antibodies. (D) The indicated cells were treated or not (c) with 2 nM rapamycin (R) for 4 or 8 h. RNA was analyzed by Northern blotting, with probes against the indicated RNAs.

phosphorylated by wt, but not KD, S6K1 (compare lanes 2 and 3 in the GST-URI panel labeled "^{32}P"). In the case of MAF1, we observed background phosphorylation with the mock immunoprecipitate and the KD S6 kinase, but the radiolabeling of MAF1 was not increased in the presence of wt S6 kinase (Fig 5A, MAF-HT panel labeled "^{32}P"), consistent with the possibility that MAF1 is not a substrate of wt S6K1.

We then transfected MAF1-HT into control MEFs or MEFs lacking S6 kinase. Mammalian cells have two S6 kinases that are more than 80% homologous in the kinase and linker domains, S6K1 and S6K2 (8, 29, 32). To ensure a complete lack of S6K activity, we used cells deleted for both genes (25). Proteins were separated on a Phos-tag acrylamide gel, and MAF1 was visualized with an anti-MAF1 antibody. As shown in Fig. 5B, the MAF1 banding patterns observed in control wt and *S6K1 S6K2* KO cells were similar in untreated cells (lanes 1 and 3) and showed the same reduction in phosphorylated species after treatment with rapamycin (Fig. 5B, lanes 1 to 4; Fig. 5C shows that the *S6K1 S6K2* double KO cells indeed lacked S6K1). This suggests that *in vivo*, S6 kinase activity is not required to maintain proper MAF1 phosphorylation. Further, repression of pol III transcription after rapamycin treatment of cells was unaffected in *S6K1 S6K2* KO cells compared with that in wt MEFs (Fig. 5D, MS4 and MS6 panels, compare lanes 1 to 4 with lanes 9 to 12), indicating that it is still dependent on the TOR pathway. In contrast and as expected, it was abolished in *Maf1* KO MEFs (Fig. 5D, lanes 5 to 8). (Note that in this experiment, the basal levels of the leucine tRNA precursors MS4 and MS6 observed in *Maf1* KO MEFs are higher than those in wt cells. We have observed this repeatedly in *Maf1* KO MEFs immortalized by the 3T3 protocol rather than by transfection with the SV40 early region, as used for the MEFs shown in Fig. 1. A possibility is that in SV40-transformed cells, basal pol III transcription is operating at maximum levels, and therefore, loss of MAF1 cannot have an additional effect.) The results described above suggest that human MAF1 is not efficiently phosphorylated by S6K1 *in vitro* and that neither S6K1 nor S6K2 is required for proper MAF1 phosphorylation *in vivo*.

MAF1 is directly phosphorylated by mTORC1 kinase. In yeast, TORC1 itself has been described as a Maf1 kinase (40), although it may be a minor kinase relative to Sch9 (41). To determine whether mTORC1 might phosphorylate MAF1 in mammalian cells, we transfected HeLa cells with a vector expressing HA-tagged versions of either wt mTOR or KD mTOR (2). mTOR-containing complexes were then immunopurified with an anti-HA antibody and tested for phosphorylation of recombinant MAF1 in the presence of [γ-^{32}P]ATP. To ensure specificity, we performed the kinase reaction in the presence of manganese, as mTOR is one of the rare kinases that preferentially uses manganese rather than magnesium (30). In contrast to S6 kinase, HA-mTOR (but not KD HA-mTOR) efficiently phosphorylated MAF1, as revealed by the radioactive signal obtained (Fig. 6A, compare lanes 2 and 3). Similarly, endogenous mTORC1 immunoprecipitated with an anti-RAPTOR antibody phosphorylated MAF1 much more efficiently than the control immunoprecipitated with preimmune serum (Fig. 6A, compare lanes 4 and 5). As RAPTOR is part of mTORC1 but not mTORC2, this suggested specific phosphorylation by mTORC1. To confirm this, we used rapamycin, which when complexed with the FKBP12 protein, specifically binds to and inhibits mTORC1 but not mTORC2 (see reference 13 for an example). MAF1 phosphorylation was strongly decreased upon addition of both rapamycin and the FKBP12 protein, further confirming phosphorylation by mTORC1 (Fig. 6A, compare lanes 7 and 8).

To determine the sites of mTOR phosphorylation *in vitro*, we subjected the various MAF1-HT mutants to phosphorylation by an anti-HA-mTOR, an anti-RAPTOR, or an anti-RICTOR immunoprecipitate and separated them on Phos-tag gels. The proteins were then transferred onto a nitrocellulose membrane and analyzed by a phosphorimager. In the case of the HA-mTOR immunoprecipitate, we also loaded on the gel extracts from HeLa cells expressing MAF1-HT and MAF1-HT S60A, which were then revealed with anti-MAF1 antibodies to serve as markers. The results are shown in Fig. 6B, top. The bands corresponding to MAF1 phosphorylated on S75, on S68, and to forms depending on S60 phosphorylation are marked with arrowheads, arrows, and brackets, respectively, as described above for Fig. 3A. In addition, the band likely corresponding to a mixture of MAF1 phosphorylated on S60 and S68 and on S60 and S75 is marked by an asterisk. The bottom panels show the input recombinant Maf1 proteins used for the kinase reactions in the top panels.

When wt MAF1 was incubated with the anti-RICTOR

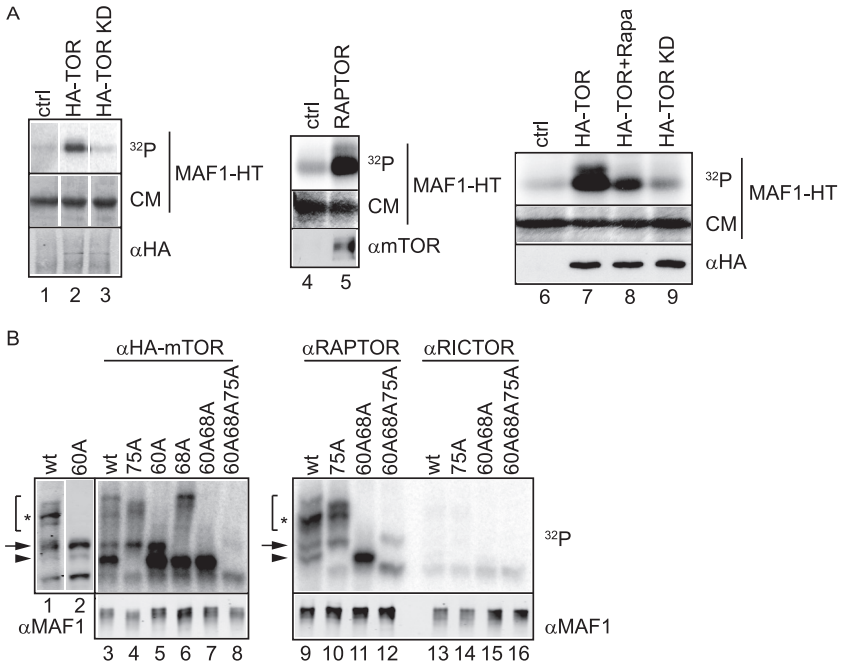

FIG. 6. mTORC1 can phosphorylate MAF1 *in vitro* on S60, S68, and S75. (A) Active HA-mTOR, inactive HA-mTOR (KD), or RAPTOR was immunoprecipitated from transiently transfected HeLa cells (ctrl, empty vector) and incubated with recombinant MAF1-HT in the presence of Mn^{2+} and [γ-^{32}P]ATP. Lane 8, 100 nM rapamycin and 1 μg of FKBP12 were added to the *in vitro* kinase reaction. The samples were processed as described in the legend to Fig. 5A. (Bottom) The presence of mTOR was revealed with anti-HA or anti-mTOR antibodies as indicated. (B, top) Wt and mutant MAF1-HT were incubated with anti-HA-mTOR (lanes 3 to 8), anti-RAPTOR (lanes 9 to 12), or anti-RICTOR (lanes 13 to 16) immunoprecipitates in the presence of Mn^{2+} and [γ-^{32}P]ATP and processed as described in the legend to Fig. 5A, except that after the kinase reaction, the various MAF1-HT proteins were affinity purified and separated on a Phos-tag gel (lanes 3 to 16). Lanes 1 and 2, transiently expressed MAF1-HT wt and 60A were loaded and revealed by anti-MAF1 immunoblotting. (Bottom) Immunoblots performed with an anti-Maf1 antibody showing the input recombinant MAF1 proteins used for the kinase reactions in the top panels separated on a 12% gel.

immunoprecipitate, no significant radioactive signal was obtained, confirming that TORC2 does not phosphorylate MAF1 (Fig. 6B, top, lanes 13 to 16). In contrast, with both the anti-HA-mTOR and the anti-RAPTOR immunoprecipitates, we obtained patterns of radioactive phosphorylated bands remarkably similar to those observed in the marker lanes by immunoblotting. With HA-mTOR, the strongest band corresponded to S75 phosphorylation (Fig. 6B, top, arrowhead, compare lane 3 to MAF1 markers in lanes 1 and 2) and the second strongest to S68 phosphorylation (top, arrow, compare lanes 3 and 5 to lanes 1 and 2). Additional bands dependent on S60 were also obtained, most of which comigrated with bands in the control (Fig. 6B, top, bracket, compare lane 3 to lane 1). With the anti-RAPTOR immunoprecipitate, the pattern was even closer to that observed by immunoblotting, with strong bands corresponding to MAF1 phosphorylated on S75 (arrowhead), S68 (arrow), and to the mixture of MAF1 proteins phosphorylated on S60 and S68 and on S60 and S75 (asterisk). The slight differences in relative intensities of the various phosphorylated forms in the marker lanes (lanes 1 and 2) and the radioactive samples may stem from detection of the markers by immunoblotting versus detection by radioactivity for the other samples. The differences observed between the anti-HA-mTOR and anti-RAPTOR immunoprecipitates may stem from the different antibodies used for the immunoprecipitation, which may alter the accessibility of certain MAF1 sites to the catalytic site of the kinase. Whichever the case, the results show that mTOR, as part of mTORC1, phosphorylates human MAF1 *in vitro* on the very same residues that are phosphorylated *in vivo*. Thus, human MAF1 is a direct mTORC1 target, and S60, S68, and S75 are mTORC1 phosphorylation sites.

DISCUSSION

We show that human MAF1 is dephosphorylated upon serum deprivation, treatment with MMS, or treatment with the mTOR inhibitors rapamycin or Torin1. These treatments all lead to repression of pol III transcription in mammalian cells (7, 27; this work), and this effect is completely MAF1 dependent, as it is not observed in *MAF1* KO MEFs. We observe, however, that different tRNA precursors are repressed at different levels. This could reflect different tRNA precursor stabilities, but it could also indicate that human MAF1 does not inhibit pol III transcription uniformly. This last possibility would be unexpected in view of the genome-wide analyses performed in yeast, indicating a very general Maf1 occupancy increase on all pol III transcription units after stress (23, 28). It would be consistent, however, with the observation of Ciesla et al. (1) that yeast Maf1 differentially regulates various tRNAs. Thus, it is conceivable that in both human and yeast cells, MAF1 selectively represses a subset of pol III genes.

We find that human MAF1 is phosphorylated on at least five residues, of which three, S60, S68, and S75, are the main sites. These sites are located between the conserved A and B do-

mains (26) of human MAF1, in a sequence highly conserved in vertebrates as well as in *Drosophila melanogaster* and *Anopheles gambiae* but not in *S. cerevisiae*. Yet, in yeast, all identified phosphosites lie between the A and B domains. Thus, the presence of phosphosites in the region separating the A and B domains has been conserved from yeast to humans, even though the specific amino acid sequence has not been conserved.

To evaluate the significance of the 60, 68, and 75 phosphosites on the pol III repression activity of MAF1, we carefully evaluated the ability of wt and mutated MAF1 proteins to repress tRNA leucine levels in MEFs devoid of endogenous Maf1. We observed that in the absence of stress, the 60A68A75A MAF1 mutant behaves like an activated MAF1, repressing pol III transcription by up to 40 to 50% of the levels observed with wt MAF1 or the 60D68D75D mutant. This is a large effect compared to that observed in yeast, where a Maf1 with seven serines mutated to alanines reduced tRNA transcription by about 10% relative to wt Maf1 (11). This large effect is consistent with the 60A68A75A mutant protein mimicking activated, dephosphorylated MAF1 and 60D68D75D mimicking, to some extent, a permanently phosphorylated MAF1. On the other hand, however, the mutant MAF1 proteins are able to respond to stress by a slight further reduction in pol III transcription. This effect is MAF1 dependent, as we observe no pol III repression in MAF1 KO MEFs, and it is most prominent for the 60A68A75A mutant that mimics the dephosphorylated state. We envision two possible explanations. First, we may not have mutated all the MAF1 phosphosites that prevent pol III repression. Second, there may be a cofactor that is required for full pol III repression and that is regulated by mTORC1. The activity of such a factor must be absolutely dependent on MAF1, since it cannot repress pol III transcription in *Maf1* KO MEFs. Such a factor could correspond to a MAF1 inhibitor that would have to be inactivated to allow full MAF1 repression activity after stress or to a MAF1 activator that would function together with MAF1 to repress pol III transcription after stress.

The results described above are consistent with a recent study published while this work was under review (33), in which those authors identified S75 as a human MAF1 phosphosite and showed that a quadruple mutation of S60, T64, S68, and S75 to alanines gives rise to a hyperactive MAF1 protein. Of note, the quadruple mutant, like our 60A68A75A mutant, still represses pol III transcription in response to amino acid deprivation (33), indicating that additional mutation of T64 does not completely inactivate MAF1 regulation, consistent with our observation that T64 is a minor phosphorylation site.

Yeast Maf1 can be phosphorylated by PKA (20) and by Sch9 (11, 41) largely on the same residues. Moreover, it can be weakly phosphorylated on unknown residues by TORC1 (40). As Sch9 appears to be the main yeast Maf1 kinase (11, 41), we explored whether S6K1, which is considered the human homologue of yeast Sch9 and is directly activated by mTOR phosphorylation (12, 39), could phosphorylate human MAF1. To our surprise, we found that S6K1 is not an efficient MAF1 kinase *in vitro* and that MAF1 remains normally phosphorylated in MEFs lacking both S6K1 and S6K2. These results do not exclude the possibility that S6K1 or S6K2 normally phosphorylated MAF1 and that some other kinase takes over MAF1 phosphorylation in *S6K1 S6K2* double KO cells. However, this seems highly unlikely, since in the *S6K1 S6K2* double KO cells, MAF1 is still dephosphorylated upon rapamycin treatment, indicating that the activity responsible for Maf1 phosphorylation in these cells is still dependent on the TOR signaling pathway. Thus, our results strongly suggest that S6K1 is not a main Maf1 kinase under the conditions tested.

On the other hand, both HA-mTOR and anti-RAPTOR immunoprecipitates, but not an anti-RICTOR immunoprecipitate, phosphorylated recombinant MAF1 in the presence of manganese in a rapamycin-sensitive reaction, virtually excluding any activity of a contaminating kinase. Furthermore, the main phosphorylation target sites *in vitro* corresponded to the sites phosphorylated *in vivo*. Together with the sensitivity of MAF1 phosphorylation to low doses of rapamycin and to Torin1, this strongly suggests that MAF1 is directly phosphorylated by mTORC1 *in vivo*. The MAF1 S75 site does not conform to the (S/T)P motif of some known mTOR-phosphorylated residues (21). Together with the S6K1 T389 mTOR-phosphorylated site, which is located in a hydrophobic sequence (24), this indicates a broad specificity for mTOR phosphorylation. The identification of MAF1 as a direct mTORC1 substrate adds a new major branch to the signal transduction cascade immediately downstream of mTORC1.

How do our results in human cells fit with the picture emerging from yeast studies? In yeast, Maf1 phosphorylation contributes to the regulation of Maf1 cellular localization. Phosphorylation by PKA and Sch9 contributes to excluding Maf1 from the nucleus, at least in the *S288C/FM391* strain (11, 20, 40). In contrast, Maf1 phosphorylation by TORC1 is thought to take place on chromatin in nucleoli and to contribute to Maf1 regulation by exclusion of Maf1 from chromatin (18, 40). There is so far no compelling published evidence that human MAF1 undergoes cytoplasmic-nuclear shuttling, and human MAF1 does not contain a clear nuclear localization signal. Together with the results described above, this raises the possibility that regulation of MAF1 localization by the Sch9 homologue S6K1 is not a prominent phenomenon in mammalian cells. On the other hand, mTOR has recently been reported to bind to pol I and pol III genes in mammalian cells (33, 37), and we have shown here that (i) human MAF1 is directly and efficiently phosphorylated by mTORC1 and (ii) mutation of the phosphorylated residues to alanines activates the pol III transcription-repressing function of MAF1 both before and after stress. Collectively, these data suggest that of the two steps of Maf1 inactivation observed in yeast, namely, nuclear exclusion mediated in large part by Sch9 and PKA and chromatin exclusion involving TORC1, the second step is by far the most important one with regard to pol III regulation in human cells and, most likely, in yeast cells as well.

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