Supporting Information

Kantidakis et al. 10.1073/pnas.1005188107

SI Materials and Methods

Antibodies. The mTOR antibodies were obtained from Santa Cruz Biotechnology (sc-8319), Cell Signaling (2972), Calbiochem (OP97), and Sigma-Aldrich (T2949). Raptor antibodies were purchased from Bethyl Laboratories (A300-506A and -553A). Phosphospecific antibody against S75 of Maf1 (2260) was raised by Eurogentec using peptides corresponding to residues 69–80 of human Maf1. The actin (sc-1615), HA (sc-7392), c-Jun (sc-1694), TFIIA (sc-25365), TFIIIC110 (sc-81406), TFIIIC63 (sc-134082), and UBF (sc-9131 and sc-13125) antibodies were purchased from Santa Cruz Biotechnology. The tubulin antibody (T9026) and nonimmune IgGs (I5006 and I5381) were from Sigma-Aldrich. The S6K antibody (9202) was from Cell Signaling. Alexa Fluor 488 (A11029 and A11034), Alexa Fluor 594 (A11032 and A11037), and GFP (A6455) antibodies were from Invitrogen. Antibodies 1166, 1167, and 1767 to Maf1 (1), 3208 to TFIIIC110 (2), Ab7 to TFIIIC220 (3), 128 to Brf1 (4), MTBP-6 to TBP (5) and 1900 to pol III (6) have been described previously.

Cell Lines and Culture. Cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. HA-TFIIIC110–inducible HeLa Tet-On cells (2) were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% doxycycline-free FCS (Clontech), 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, 100 μg/mL G418, and 100 μg/mL hygromycin. Expression of HA-TFIIIC110 was achieved by the addition of 1 μg/mL doxycycline for 24 h. Where indicated, cells were treated with 100 nM rapamycin (Calbiochem) for 4 h.

Mutagenesis and Transfections. Single amino acid mutagenesis primers (Invitrogen)—5′-GCCCCAGCAGACTCAGCAAAGCCCAAGGCGGTG-3′ and 5′-CACCGCCTTGGGCTTTGCTGAGTCTGCTGGGGC-3′ for Maf1 S75A, 5′-GCCCCAGCAGACTCAGCAAAGACCAAGGCGGTG-3′ and 5′-CACCGCCTTGGTCTTTGCTGAGTCTGCTGGGGC-3′ for Maf1 S75D, and 5′-CCACTCCGAGGTCACAATTGACATGGAGATCCT-3′ and 5′-AGGATCTCCATGTCAATTGTGACCTCGGAGTGG-3′ for TFIIIC63 F109I—were designed with the QuikChange Primer Design Program (Stratagene), and mutations were created using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The generated mutants were verified by sequencing. Cells were transfected with plasmid DNA using lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and harvested 24 h later.

Cell Lysis and Western Blot Analysis. Cells were rinsed twice with ice-cold PBS and harvested in a lysis buffer similar to that used previously (7) (40 mM Hepes [pH 7.4], 2 mM EDTA, 120 mM NaCl, and 0.3% CHAPS containing 1× EDTA-free Halt proteases and phosphatases inhibitor mixture [Thermo Scientific]), and left to lyse 20 min in ice. The cell lysates were then passed through a 26G syringe three times, and the soluble fractions were collected after centrifugation at 17,000 × *g* for 15 min. Western blot analysis was performed as described previously (8) using antibodies 1167 for total Maf1, 2260 for phosphoS75 Maf1, 2972 and T2949 for mTOR, A300553A for raptor, sc-81406 for TFIIIC110, and sc-134082 for TFIIIC63.

Immunoprecipitation. For immunoprecipitation, cell lysates containing 300 μg of total protein were incubated with 1 μg of antibody for 2 h at 4 °C. Then 50 μL of a 50% slurry of protein A or G Sepharose (Sigma-Aldrich) or 30 μL of DYNAL protein A or protein G magnetic beads (Invitrogen) were added, and the incubation was continued for 1 h at 4 °C. DNase I treatment was performed with 20 U DNase1 (Roche), as described previously (1). The immunoprecipitates were washed five times with lysis buffer, and then denatured by the addition of SDS-loading buffer and boiling for 10 min. The antibodies A300506A for raptor, 3208 for TFIIIC110, and 2972 and T2949 for mTOR were used for immunoprecipitation.

Kinase Assays. Recombinant Maf1 (200 ng) was incubated with 64 ng of active mTOR (residues 1362–2549; Millipore) in 25 mM Hepes (pH 7.5), 50 μM ATP, 10 mM $MgCl_2$, 1mM EDTA, 0.01% Tween-20, and 0.15 MBq γ^{32}-[P] ATP for 30 min at 30 °C. Reactions were stopped by the addition of SDS loading buffer and incubation at 80 °C for 10 min. Reactions were analyzed by SDS/PAGE gel and transfer onto nitrocellulose membrane, followed by autoradiograpy and Western blot.

Kinase assays with endogenous mTOR were performed as described previously (7), using mTOR immunoprecipitated with antibody 2972 from HeLa cells and recombinant human Maf1 (1) as substrate.

RNA, RT-PCR, and qPCR. RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's specifications. cDNA was synthesized using 0.2 μg of RNA, random hexanucleotide mix (Roche), and SuperScript III (Invitrogen), according to the manufacturers' recommendations. RT-PCR for ARPP P0, TFIIB, 5S, and tRNA transcripts was performed as described previously (9–11). Real-time qPCR was performed using the Chromo4 system (Biorad) with Dynamo SYBR green qPCR kit mastermix (Finnzymes), according to the manufacturers' recommendations. The primers used were the same as those described previously for RT-PCR. The cycling parameters for pre-$tRNA^{Tyr}$ and pre-$tRNA^{Leu}$ cDNA were 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 68 °C for 1 min, 72 °C for 1 min (with a plate read after each cycle), and a final round of 72 °C for 10 min. The amplification parameters for TFIIB mRNA and 5S rRNA cDNA were the same, with the exception of annealing at 55 °C. The signal values of pol III transcripts were normalized to those of TFIIB or ARPP P0 and represented in graphs as the average fold with SD. *P* values were calculated using the unpaired two-tailed *t* test.

ChIP. ChIP assays were performed as described previously (12). The immunoprecipitated DNA was purified by phenol-chloroform extraction or using a PCR purification kit (Qiagen), according to the manufacturer's instructions. The mTOR sc-8319 and T2949 antibodies were used for chromatin immunoprecipitation. The primers and cycling parameters for the PCR amplification have been described previously (13). Serial dilutions were used to establish that PCR values were within a linear range. The signal values were normalized to 1% of total input and represented in graphs as the average fold with SD. *P* values were calculated using the unpaired two-tailed *t* test.

Immunofluorescence. For immunofluorescence assays, 5×10^5 cells per well were plated in six-well plates containing sterile coverslips. The cells were left to grow in culture conditions overnight, and the next day were fixed with 4% paraformaldehyde for 15 min, followed by three washes with 0.1% Triton X-100 in PBS (5 min each) and blocking with 10% FCS and 0.5% BSA in PBS for 1 h at room temperature. The cells were then incubated with a 1:100 dilution of antibody in 10% FCS and 0.5% BSA in PBS,

at 4 °C overnight. The next day, the coverslips were washed three times (5 min each) with 10% FCS and 0.5% BSA in PBS and incubated with secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594; 1:100 dilution) in 10% FCS and 0.5% BSA in PBS for 1 h at room temperature. After two more washes with 10% FCS and 0.5% BSA in PBS and one wash with 0.1% Triton X-100 in PBS, the coverslips were applied on glass slides with Vectashield containing DAPI (Vector Laboratories). The signals were visualized using an Olympus BX51 FL microscope with a DP71 color camera and a 100× objective (Plan-Neofluar; Zeiss). The images were collected using Cell ^ F software (Olympus).

PLA. PLAs were performed using the Duolink In Situ PLA Kit (Olink Bioscience). First, 5×10^4 cells/chamber were plated in eight-well glass chamber slides (Lab-Tek) and left in culture conditions overnight. The next day, the cells were treated as those prepared for immunofluoresence, with the exception that the primary antibodies for mouse (mTOR-OP97 or UBF sc-13125) and rabbit (TFIIIC-3208, TFIIIC-Ab7, GFP-A6455, UBF-sc-9131, or Maf1-1166) were incubated together (1:100 dilution each) on the cells at 4 °C overnight. The next day, the PLA assay was performed according to the manufacturer's instructions. The signals were visualized using an Olympus BX51 FL microscope with a DP71 color camera and a 100× objective (Plan-Neofluar; Zeiss). The images were collected using Cell ^ F software (Olympus) and thresholded using Adobe Photoshop CS2 (Adobe Systems), as described previously (14). For quantification, the images of ∼100–500 cells per experiment were captured by a 40× or 20× objective (Plan-Neofluar; Zeiss), and the signals were counted using Blobfinder version 3.2 (15), as recommended by the kit manufacturer (Olink Bioscience). The graphs represent the average number of signals per cell, and the error bars represent the SD from three independent experiments.

Subcellular Fractionation. Cells were washed twice with PBS, scraped in PBS, and centrifuged at 500 × *g* for 5 min at 4 °C. The pellet was resuspended in 1 mL of hypertonic buffer [20 mM Hepes (pH 7.0), 10 mM KCl, 1 mM DTT, 0.1% Triton X-100, 20% glycerol, 2 mM PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin], and the cell suspension was subjected to 10 slow strokes in a Dounce homogenizer. After centrifugation at 800 × *g* for 5 min at 4 °C, the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in 100 μL of cold extraction buffer [20 mM Hepes (pH 7.0), 10 mM KCl, 1 mM DTT, 0.1% Triton X-100, 20% glycerol, 2 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 420 mM NaCl], and the suspension was placed on a spinning wheel for 20 min at 4 °C. After centrifugation at 16,000 × *g* for 10 min at 4 °C, the supernatant was collected as the nuclear fraction.

Phosphate Labeling. HeLa cells transfected with empty pcDNA3 vector or vector encoding HA-tagged Maf1 were labeled for 3 h with 0.3 mCi/mL of [^{32}P] orthophosphate in phosphate-free medium in the presence of DMSO or 100 nM rapamycin. Cells were then harvested and the HA-tagged Maf1 was immunoprecipitated, as described previously (16).

1. Goodfellow SJ, et al. (2008) Regulation of RNA polymerase III transcription by Maf1 in mammalian cells. *J Mol Biol* 378:481–491.
2. Innes F, Ramsbottom B, White RJ (2006) A test of the model that RNA polymerase III transcription is regulated by selective induction of the 110-kDa subunit of TFIIIC. *Nucleic Acids Res* 34:3399–3407.
3. Shen Y, Igo M, Yalamanchili P, Berk AJ, Dasgupta A (1996) DNA binding domain and subunit interactions of transcription factor IIIC revealed by dissection with poliovirus 3C protease. *Mol Cell Biol* 16:4163–4171.
4. Cairns CA, White RJ (1998) p53 is a general repressor of RNA polymerase III transcription. *EMBO J* 17:3112–3123.
5. Pruzan R, Chatterjee PK, Flint SJ (1992) Specific transcription from the adenovirus E2E promoter by RNA polymerase III requires a subpopulation of TFIID. *Nucleic Acids Res* 20:5705–5712.
6. Fairley JA, Scott PH, White RJ (2003) TFIIIB is phosphorylated, disrupted and selectively released from tRNA promoters during mitosis in vivo. *EMBO J* 22:5841–5850.
7. Sancak Y, et al. (2007) PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* 25:903–915.
8. White RJ, Gottlieb TM, Downes CS, Jackson SP (1995) Mitotic regulation of a TATA-binding protein–containing complex. *Mol Cell Biol* 15:1983–1992.
9. Daly NL, et al. (2005) Deregulation of RNA polymerase III transcription in cervical epithelium in response to high-risk human papillomavirus. *Oncogene* 24:880–888.
10. Grandori C, et al. (2005) c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* 7:311–318.
11. Winter AG, et al. (2000) RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. *Proc Natl Acad Sci USA* 97:12619–12624.
12. Kantidakis T, White RJ (2010) Dr1 (NC2) is present at tRNA genes and represses their transcription in human cells. *Nucleic Acids Res* 38:1228–1239.
13. Kenneth NS, et al. (2007) TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. *Proc Natl Acad Sci USA* 104:14917–14922.
14. Söderberg O, et al. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 3:995–1000.
15. Allalou A, Wählby C (2009) BlobFinder, a tool for fluorescence microscopy image cytometry. *Comput Methods Programs Biomed* 94:58–65.
16. Johnston IM, et al. (2002) CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. *Mol Cell Biol* 22:3757–3768.

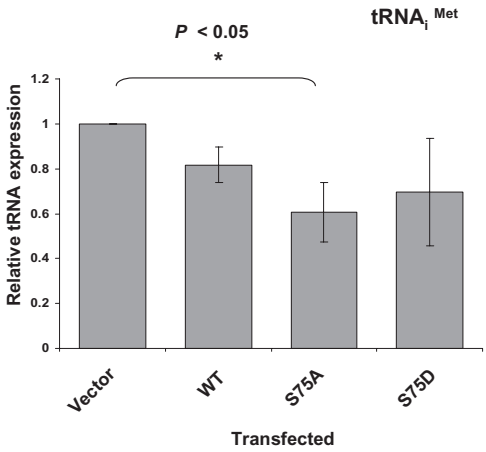

Fig. S1. Expression of tRNA$_i^{Met}$ assessed by real-time qRT-PCR following transfection with empty vector or vector encoding WT, S75A, or S75D Maf1. Data represent levels of tRNA$_i^{Met}$ after normalization to TFIIB mRNA, with empty vector assigned a value of 1.0. $n = 3$

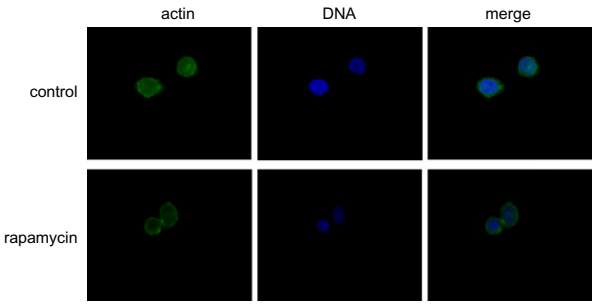

Fig. S2. Indirect immunofluoresence with antibody against actin in HeLa cells treated with vehicle or rapamycin.

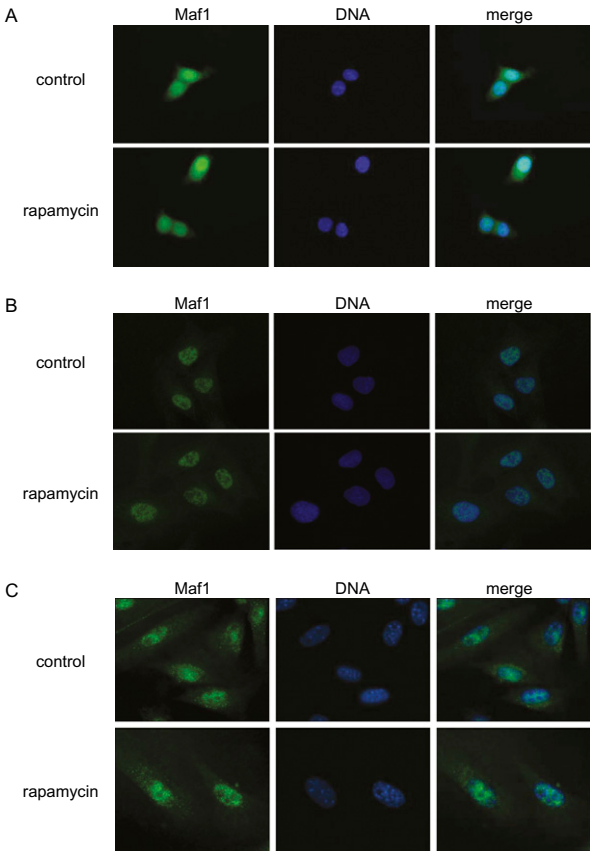

Fig. S3. Indirect immunofluoresence with antibody against Maf1 in human MCF-7 breast cancer (*A*), human U2OS osteosarcoma (*B*), and mouse A31 fibroblast (*C*) cells treated with vehicle or rapamycin.

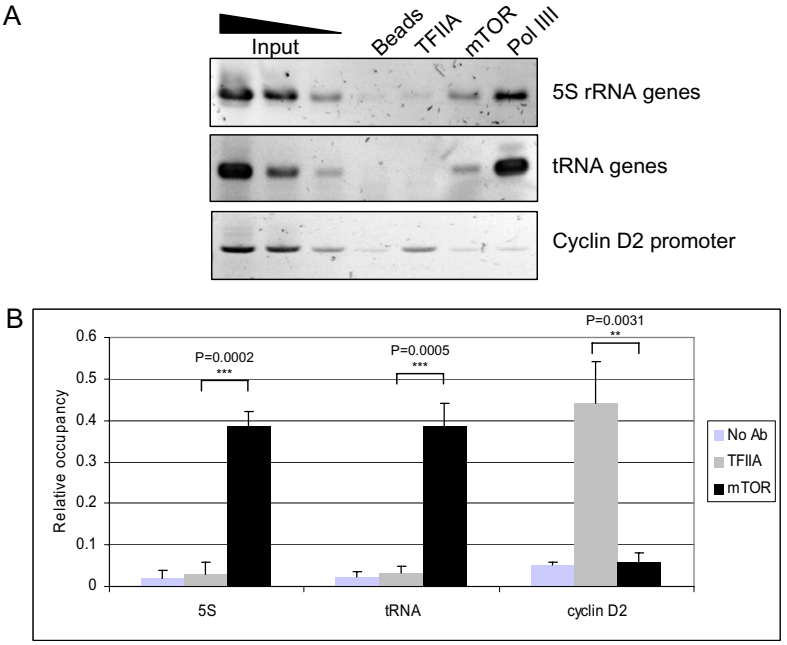

Fig. S4. Endogenous mTOR is found at tRNA and 5S rRNA genes in A31 fibroblasts. (*A*) ChIP assays showing mTOR occupancy at tRNA and 5S rRNA genes in A31 cells. The mTOR sc-8319 antibody was used. Antibody to pol III provided a positive control, and antibody to TFIIA and beads without antibody served as negative controls. (*B*) Quantification of signals from *A* and two additional independent experiments using antibodies to TFIIA and mTOR or beads without antibody, as indicated.

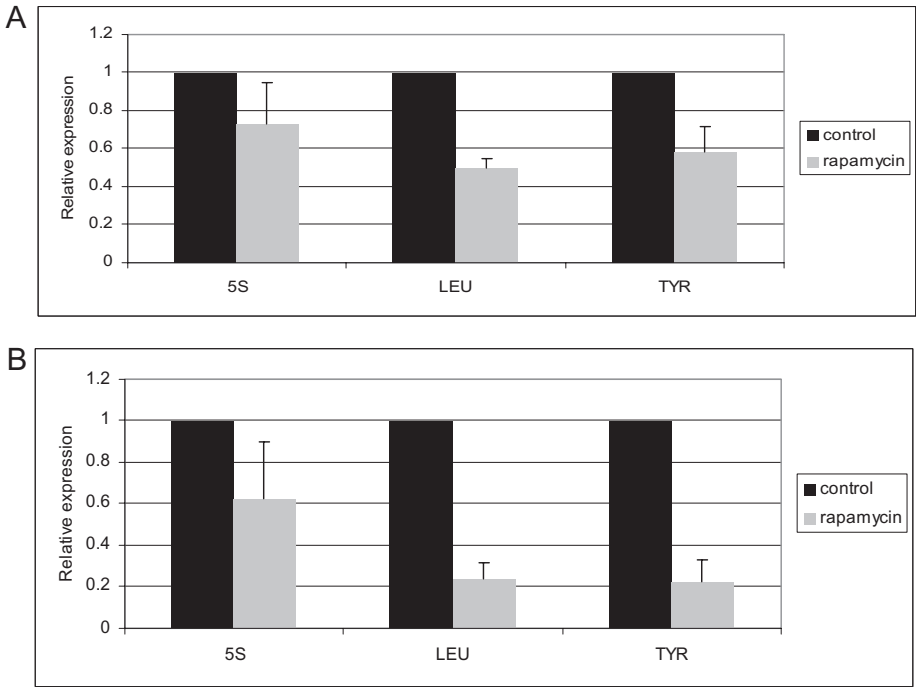

Fig. S5. Rapamycin treatment of HeLa and U2OS cells results in down-regulation of pol III transcripts. HeLa cells (*A*) or U2OS cells (*B*) were treated with vehicle or rapamycin, and expression of 5S rRNA (5S), pre-tRNA^{Leu} (LEU), and pre-tRNA^{Tyr} (TYR) transcripts was assessed by real-time qRT-PCR. Quantitation is presented of results from three independent experiments after normalization to TFIIB mRNA. Expression in vehicle-treated control cells was assigned a value of 1 in each case.

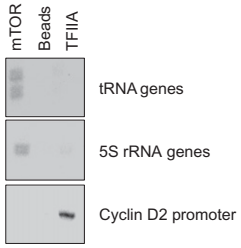

Fig. S6. Endogenous mTOR is found at tRNA and 5S rRNA genes in A31 fibroblasts. This ChIP assay used mTOR antibody T2949, with antibody to TFIIA and beads without antibody serving as negative controls.

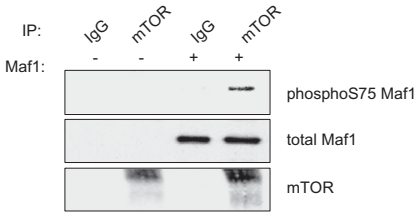

Fig. S7. Kinase activity immunoprecipitated from HeLa cells with rabbit IgG or mTOR antibody was used to phosphorylate bacterially expressed recombinant Maf1. Panels show Western blots using antibodies against phosphorylated S75 of Maf1, total Maf1, and mTOR.

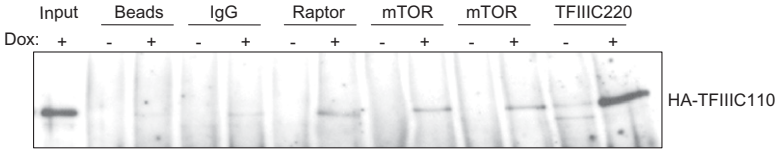

Fig. S8. TFIIIC associates with mTOR. Shown are Western blots using HA antibody of immunoprecipitates from HeLa cells expressing doxycycline-inducible HA-tagged TFIIIC110 grown in the presence (+) or absence (–) of doxycycline. Immunoprecipitations used beads without antibody, nonimmune IgG, and antisera to raptor, TFIIIC220, and two different antisera to mTOR.

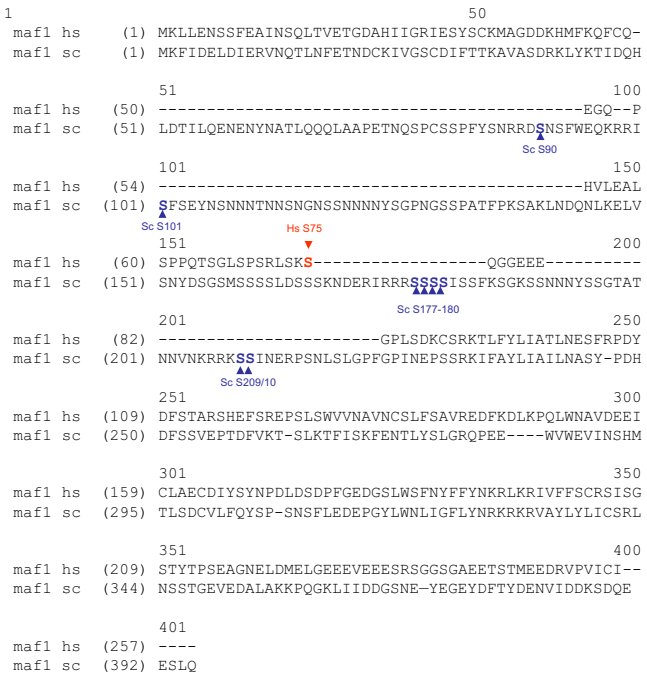

Fig. S9. Sequence alignment of human (hs) and *S. cerevisiae* (sc) Maf1 proteins. Phosphoacceptor sites known to regulate yeast Maf1 are shown in blue; none of these are conserved in the human protein. The S75 phosphoacceptor identified in this study in human Maf1 is indicated in red.