Inhibition of tRNA Gene Transcription by the Immunosuppressant Mycophenolic Acid

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Running head: MPA inhibits tRNA transcription

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Abstract

Mycophenolic acid (MPA) is the active metabolite of mycophenolate mofetil, a drug that is widely used for immunosuppression in organ transplantation and autoimmune diseases, as well as anticancer chemotherapy. It inhibits inosine monophosphate dehydrogenase, a rate-limiting enzyme in *de novo* synthesis of guanidine nucleotides. MPA treatment interferes with transcription elongation resulting in a drastic reduction of pre-rRNA and pre-tRNA synthesis, the disruption of the nucleolus, and consequently cell cycle arrest. Here, we investigated the mechanism whereby MPA inhibits RNA polymerase III (Pol III) activity, both in yeast and mammalian cells. We show that MPA rapidly inhibits Pol III by depleting GTP. Although MPA treatment can activate p53, this is not required for Pol III transcriptional inhibition. The Pol III repressor MAF1 is also not responsible for inhibiting Pol III in response to MPA treatment. We show that upon MPA treatment, the levels of selected Pol III subunits decrease, but this is secondary to transcriptional inhibition. ChIP experiments show that Pol III does not fully dissociate from tRNA genes in yeast treated with MPA, even though there is a sharp decrease in the levels of newly transcribed tRNAs. We propose that in yeast, GTP depletion may lead to Pol III stalling.

Introduction

Mycophenolate mofetil (MMF) is a highly effective immunosuppressive prodrug that is used widely and in a range of clinical contexts, including organ transplantation, autoimmune disease and cancer therapy (1, 2). Its active metabolite, mycophenolic acid (MPA), inhibits inosine monophosphate dehydrogenase (IMPDH). IMPDH catalyses the NAD-dependent oxidation of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP), which is a rate-limiting step in the *de novo* guanosine nucleotide synthesis pathway. This pathway utilizes glucose and amino acids to generate GTP (2). The clinical relevance of MPA is based on the fact that inhibition of IMPDH impacts especially on B and T lymphocytes, which depend singularly on the *de novo* pathway for purine synthesis, instead of using the salvage pathway (3). T and B lymphocytes play a key role in acute and chronic antigen-dependent transplant rejection (4). It has now become clear, however, that myeloid cells such as monocytes, dendritic cells and macrophages also play an important role in this process (4, 5).

In the yeast *Saccharomyces cerevisiae,* there are four paralogous genes encoding IMP dehydrogenases (*IMD1-IMD4*). Because *IMD1* is very close to the telomere, and it contains a frameshift insertion, it is considered to be a pseudogene (6). *IMD2* and, to a lesser extent *IMD4,* are induced in the presence of guanidine nucleotides-depleting drugs. Interestingly, when overexpressed, only *IMD2* confers resistance to these drugs (6, 7). In humans and other mammals, two isoforms of the *IMPDH* gene exist, *IMPDH1* and *IMPDH2*. The products of these genes are kinetically indistinguishable and are highly similar, being 84% identical at the protein level in humans. Whereas *IMPDH1* is constitutively expressed at low levels in virtually all tissues, *IMPDH2* is inducible and generally expressed in highly proliferative cells (8).

IMPDH inhibitors, 6-azauracil (6-AU) and MPA, reduce GTP levels and in doing so lead to transcription elongation defects by limiting a transcription substrate (9). Transcription in eukaryotic cells is directed by at least three different multimeric RNA polymerases (Pols). Pol I is responsible for synthesis of ribosomal RNA (rRNA). Pol II transcribes messenger RNAs (mRNA) and also most small nuclear RNAs (snRNA) and micro RNAs (miRNA). Pol III synthesises tRNA, 5S rRNA, 7SL RNA and a subset of small noncoding RNAs required for the maturation of other RNA molecules (e.g. U6 snRNA). Nucleotide depletion differentially impacts the three RNA polymerases and their RNA products levels. Treatment of yeast cells by 6-AU leads to the rapid cessation of Pol I and Pol III activity, whereas Pol II seems to be less affected, probably owing to the lower rate of transcription (10). In mammalian cells, GTP depletion by MPA also specifically leads to Pol I and Pol III inhibition (11). Therefore, nucleotide depletion leads to imbalances between precursors of mRNA, rRNA and tRNA. The consequence of nucleotide depletion, both in yeast and mammalian cells, is a nucleolar stress and cell cycle arrest. In mammalian cells, the cell cycle arrest is induced by p53, which is activated as a result of free L5 and L11 ribosomal proteins binding to Mdm2 E3 ubiquitin ligase, which normally targets p53 for degradation (11).

Pol III in yeast is negatively regulated by a general repressor, Maf1 (12). Maf1 integrates multiple signalling pathways and inhibits Pol III in response to nutrient limitation or stress conditions. Interestingly, in yeast, all so far tested stress conditions that repress Pol III activity, do so through Maf1 (13, 14). Maf1 is also conserved in higher eukaryotes where it plays a similar role in regards to Pol III (for review see (14) and references therein). However, in these organisms, Pol III is also directly inhibited by p53 and RB and activated by c-Myc, mTORC and ERK (15-18). Moreover, Pol III transcription has been shown to be directly activated by NF-κB, a key transcription factor mediating inflammatory signals (19). It is,

however, unknown whether inhibition of Pol III activity by MPA is an effect of one or more signalling pathways that impinge on Pol III.

Here we confirm previous observations that MPA inhibits Pol III activity in mammalian cells and show that it also occurs in yeast. We further explore this mechanistically by assaying Pol III association with tRNA genes. We show that in mammalian cells, both tRNA levels and Pol III binding to tRNA genes rapidly decrease upon MPA treatment. Strikingly, in yeast, the rapid decrease of tRNA levels is not fully followed by a dissociation of Pol III from its templates, which may be a result of Pol III stalling. Furthermore, the observed downregulation of Pol III subunits levels and p53 induction in a mouse macrophage cell line are also irrelevant to a drop in tRNA transcription. Finally, we show that the decrease of Pol III activity upon MPA treatment does not depend on Maf1, either in yeast or mammalian cells. Notably, to our best knowledge, this is a first report showing that a stress factor does not involve Maf1 to repress Pol III transcription in yeast.

Materials and Methods

Cell culture. Cells were cultured in a humidified incubator with 5% CO_2 at 37°C. Murine RAW264.7, U2OS human osteosarcoma cell line, human colorectal cancer HCT116 and HCT116 $p53^{-/-}$ cells were grown in DMEM supplemented with 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum (FBS), unless otherwise stated. When indicated, cells were treated with 10 μM MPA (Sigma, Cat. No. M3536) and rapamycin (BioShop Canada, Cat. No. RAP004).

Yeast culture. Yeast *Saccharomyces cerevisiae* strains used in this study are listed in the Supplementary Table 1. Yeast were grown on synthetic complete medium without uracil, SC-ura (2% glucose, 0.67% yeast nitrogen base contained 20 μg/ml of all the amino acids

required for growth except for uracil). Where indicated growth medium was supplemented with 50 or 100 µg/ml MPA dissolved in ethanol. To allow growth in the medium without uracil, MW4415 and AC40-GFP C160-HA strains were transformed with an empty plasmid harboring *URA3* gene, pFL44L (20).

Transfections. RAW264.7 cells were plated on 10 cm dishes at a density of 2.5 x 10^6. For Maf1 knockdown, cells were transfected with 25 nM small interfering RNA (siRNA) or 4 µg pcDNA Maf1-HA plasmid (21) using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's recommendations. The following day, the cells were split into 6 cm dishes. The treatment experiments were performed 2 days after transfections. The siRNAs used were Maf1 (Qiagen, Cat. No. SI01307327) and AllStars negative-control siRNA (Qiagen, Cat. No. 1027280). U2OS cells were transfected with pcDNA Maf1-HA as described previously (19).

Protein extracts and Western blotting. Mammalian cells. Cells were washed with ice-cold PBS and harvested by scraping directly into buffer (100 mM NaCl, 50 mM HEPES [pH 7.9], 1 mM EDTA, 5% glycerol, 0.05% NP-40, 0.1% SDS). Extracts were sonicated in Bioruptor (Diagenode) and spun for 10 min at 14,000 RPM at 4°C. Supernatants were collected and protein concentration was assessed using Bio-Rad protein assay. Proteins were precipitated from a volume of extract corresponding to 100 µg protein with an equal volume of 10% trichloroacetic acid (TCA). Samples were spun for 10 min at 14,000 RPM at 4°C, and the pellet was washed with cold acetone, dried, and resuspended in Laemmli buffer (pH 8.8), followed by incubation for 15 min at 55°C with shaking. Yeast cells. The protein extraction method was described earlier (22). Twenty µg of protein was resolved on a SDS-polyacrylamide gels transferred to a PVDF membrane and incubated with antibodies. The antibodies used are listed in the Supplementary Table 2.

142

143 *Co-immunoprecipitation.* Co-immunoprecipitation of Pol III subunits with AC40-GFP was
144 performed as described previously (23).

145

146 *RNA isolation from mammalian cells and cDNA synthesis.* Total RNA was isolated from cells
147 using TRI Reagent (MRC) according to the manufacturer's instructions. Eighty nanograms of
148 RNA was used for cDNA synthesis using a QuantiTect reverse transcriptase kit (Qiagen). To
149 increase the efficiency of tRNAs' cDNA synthesis, oligonucleotides specific to the 3' end of
150 tRNA were added to the reaction mixture, each at the final concentration 1 μM. The
151 oligonucleotides sequences are listed in the Supplementary Table 3.

152

153 *RNA isolation from yeast cells, Northern hybridization and cDNA synthesis.* RNA isolation
154 and nonradioactive Northern blotting was performed exactly as described previously (24). For
155 Northern hybridization, DIG-labelled oligonucleotides were used, and are listed in the
156 Supplementary Table 4. Band intensities from Northern blot images were quantified using the
157 MultiGauge v3.0 software (Fujifilm). cDNA synthesis from yeast RNA was performed as
158 above with the exception that 50 ng of RNA was used.

159

160 *ChIP from mammalian cells.* On the day before, 5 x 10^6 cells was seeded for each time point.
161 Cell cross-linking was done by adding 37% formaldehyde to a final concentration of 1% for 7
162 min at room temperature, followed by the addition of glycine to a final concentration of 125
163 mM. Cells were lysed in 1 ml chromatin immunoprecipitation (ChIP) buffer (150 mM NaCl,
164 5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 1 mM sodium butyrate, 50 mM Tris-HCl [pH
165 8.0]) containing 1.5 x cOmplete protease inhibitor cocktail (Roche). The lysate was sonicated
166 25 times for 15 s with 30 s intervals in a Diagenode Bioruptor set to maximum power. For

each IP, 100 µl of extract was used, and 50 µl of extract was used as input. For IPs, extract was diluted five times with ChIP buffer, and 3 µg of POLR3D antibody (A302-296A, Bethyl Laboratories) or, as control, normal IgG rabbit antibody (#2729, Cell Signaling Technology) was added. Following overnight incubation at 4°C with rotation, 50 µl of protein G magnetic beads (Dynal Life Technologies) was added, and the mixtures were further incubated for 6 h. Beads were washed twice with ChIP buffer, once with LiCl buffer (250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40, 10 mM Tris-HCl [pH 8.0]), once with high-salt buffer (500 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl [pH 8.0]) and one more time with ChIP buffer. All the washes were performed at room temperature for 3 min. Elution of DNA was performed by adding 100 µl of elution buffer (200 mM NaCl, 5 mM EDTA, 0.5% SDS, 25 mM Tris-HCl [pH 7.5]) and incubating for 20 min at 65°C with shaking. Eluates were treated with RNase A (Invitrogen) for 30 min at 37°C and pronase (Roche) for 60 min at 37°C. Chromatin was decrosslinked by overnight incubation at 65°C. DNA was purified using QIAquick PCR Purification Kit (QIAGEN) according to manufacturer instructions.

ChIP from yeast cells. Yeast ChIP experiments were performed as described previously (25). The input and immunoprecipitated samples were assayed by quantitative PCR to assess the extent of protein occupancy at different genomic regions. Occupancies of Pol III at tRNA genes were calculated by determining the immunoprecipitation efficiency that is the amount of PCR product in the immunoprecipitated sample divided by the amount of PCR product in the input sample multiplied by 100. Occupancy values were reduced by occupancy on untranscribed fragment of chromosome V (ARS504), which served as a negative control. The PCR primers are listed in Supplementary Table 5.

Molecular and Cellular Biology

Quantitative PCR. Quantitative PCR was performed on a Roche LightCycler 480 System using a 3-min incubation at 95°C, followed by 40 cycles of 20 s at 95°C, 30 s at 61°C, and 20 s at 72°C (with a plate read after each cycle). A melting curve analysis was performed for each sample after PCR amplification to ensure that a single product with the expected melting curve characteristics was obtained. Each sample was loaded in triplicate. Each plate contained cDNA dilutions for the standard curve, a non-reverse transcriptase control, and a no template control. PCR efficiencies were between 90% and 100%. Data were processed in LightCycler 480 Software and then analyzed in Excel (Microsoft). Data are expressed in arbitrary units calculated from standard curve where the highest cDNA concentration was set to 1. The primer sequences are listed in the Supplementary Table 5.

Clonogenic assay. Cells were harvested and counted using haemocytometer. 5.0 x 10^4 and 7.5 x 10^4 cells were re-plated on 6-well plates in triplicate for each condition. Next day the cells were treated with MPA at 10 μM or 0.1% ethanol (as a control) for 4 or 8 hours. Then the medium was removed, cells were rinsed with PBS and fresh medium without the drug was added. Plates were left in the incubator until cells have formed large colonies. Then medium was removed, cells were rinsed with PBS, fixed and stained with a crystal violet solution (0.05% w/v crystal violet, 1% formaldehyde, 1x PBS, 1% methanol). The cells were subsequently rinsed with PBS, dried at room temperature and photographed.

Data modeling. To compare the kinetics of the decrease in tRNA levels and Pol III dissociation from template genes, first non-linear regression method has been used to estimate the parameters of the exponential decay function $y = a \times e^{-\lambda t}$. This function was chosen as the visual inspection of plotted data suggested that both signals follow exponential decay. To estimate the parameters, a non-linear least squares approach was employed using nls()

function in R (26). Then, given the models, ANOVA was performed to test whether there is a significant difference between curves.

Results

MPA induces rapid inhibition of Pol III activity in eukaryotic cells

MPA inhibits Pol I and Pol III transcription *in vivo* leading to p53-dependent cell cycle arrest in cancer cells (11). Given the relevance of macrophages in transplant rejection, we tested whether MPA affects Pol III activity also in this type of cells. Pol III activity was followed by analysing the levels of primary transcripts of selected tRNA genes by RT-qPCR. Due to lack of modifications, the primary tRNA transcripts are preferential substrates for reverse transcriptase. As shown in Figure 1A, MPA rapidly decreases the expression of tRNA in the RAW264.7 macrophage cell line. To test if the observed effect of MPA on tRNA synthesis results from IMPDH inhibition and is not an effect on other cellular component(s), RAW264.7 cells were additionally treated with either guanosine 5′-monophosphate (GMP) or both MPA and GMP. Whereas GMP has no effect or modestly increases tRNA levels by itself, it clearly prevents the decrease in tRNA levels upon MPA treatment (Figure 1A). Moreover, addition of GMP after MPA treatment fully rescued the tRNA levels (Figure 1B).

Given that nucleotide depletion by 6-AU inhibits Pol III activity (10), we also tested the effect of MPA on tRNA synthesis in yeast. MPA triggers a rapid decrease of tRNA levels and within 60 min of treatment the pre-tRNA species are almost undetectable by Northern blotting (Figure 2A and B). It has been shown that the inhibitory effect of MPA on the cell cycle in yeast can be reversed by addition to the culture medium of guanine, which is converted to GMP by Hpt1 enzyme (27). Consistent with this, we observed rescue of tRNA

241 synthesis in MPA-treated cells after addition of guanine to the culture media (Figure 2C and
242 D). After 3 hours treatment of yeast cells with MPA, partial recovery of tRNA transcription
243 was observed (Figure 2A and B), which correlated with the induction of the *IMD2* mRNA
244 (Figure 2E). Possibly the elevated Impdh restored the levels of cellular GTP leading to
245 resumption of Pol III activity even in the presence of MPA in the culture medium. In contrast,
246 in RAW264.7 macrophages tRNA levels remain very low upon prolonged, 8 h, MPA
247 treatment with no accompanied change in IMPDH1 and only a slight, statistically
248 insignificant, increase in IMPDH2 mRNA levels (Figure 3). Such a prolonged treatment leads
249 to cell death (see below).

250 Overall, these data suggest that MPA induces very rapid cessation of Pol III activity
251 both in yeast and mammalian cells (see also Figure 4C and Figure 8B) and this effect is most
252 likely the result of inhibition of GMP synthesis.

253

254 ***Maf1 is not involved in tRNA regulation upon MPA treatment***

255 Maf1 is a negative regulator of Pol III in all eukaryotic cells investigated (14). Maf1
256 itself is regulated by phosphorylation and its dephosphorylated form is known to inhibit Pol
257 III (14). To study the role of MAF1 in the decrease of tRNA levels upon MPA treatment in
258 the RAW264.7 macrophage cell line, we first looked at its phosphorylation status by
259 resolution of protein extracts on Phos-tag gels (Figure 4A). Slower migrating bands,
260 corresponding to phosphorylated forms of MAF1 that are observed in untreated cells,
261 disappear when cells are treated with rapamycin, which is known to block MAF1
262 phosphorylation and inhibit Pol III (28-30). In contrast, MPA treatment has no effect on
263 MAF1 phosphorylation status in RAW264.7 macrophages. Similarly, while MPA efficiently
264 decreased tRNA levels in the human osteosarcoma cell line U2OS (Figure 4C), it had no
265 effect on MAF1 phosphorylation in these cells (Figure 4D). It is, however, possible that MPA

induces only modest phosphorylation changes to MAF1, which may be imperceptible by Phos-tag gels and still have an effect on Pol III activity. We therefore knocked down MAF1 in RAW264.7 macrophages using siRNA. As shown in Figure 4B, siRNA treatment resulted in the downregulation of Maf1 mRNA. Concomitantly, we observed increased tRNA levels, suggesting the effectiveness of Maf1 knock-down. Addition of MPA to the cells led to a decrease in tRNA levels both in the non-targeting and Maf1-targeting siRNA-treated cells (Figure 4B). Thus, depletion of Maf1 does not prevent inhibition by MPA.

The potential role of Maf1 in the inhibition of Pol III activity by MPA was also tested using the yeast model. To this end, the kinetics of Pol III inhibition were compared in wild-type and *maf1Δ* mutant cells. Time-course MPA treatment followed by Northern blot analysis demonstrated fast and strong reduction in the levels of various pre-tRNAs in both wild-type and *maf1Δ* cells (Figure 5A). The inhibition of tRNA synthesis was a little less prominent in cells with *MAF1* inactivation (Figure 5B). A drop in pre-tRNA levels can result from two processes, a decrease of pre-tRNA synthesis and their processing into mature tRNAs. It has been shown previously that in the absence of Maf1, the processing of tRNA precursors is delayed (31, 32). Thus, the slightly slower kinetics of the decrease in pre-tRNA levels in yeast *maf1Δ* mutant treated with MPA could be explained by the slower tRNA maturation.

Overall, the results suggest that Maf1 is not required for Pol III inhibition upon MPA treatment either in yeast or a mouse macrophage cell line.

Downregulation of Pol III subunits levels as a consequence of Pol III inhibition by MPA

Inhibition of transcription may lead to Pol III degradation (33). The multistep pathway described for defective yeast Pol III, inactivated by mutations or through decreased expression, involves sumoylation and ubiquitylation of selected subunits. This triggers disassembly of Pol III followed by proteosomal degradation of its largest subunit, C160 (33).

Ubiquitylation and proteosomal degradation of C160 was also observed as a consequence of Pol III repression upon physiological stress, which pertains to most of the polymerase complexes that dissociate from templates (24). Surprisingly, the interactions between Pol III subunits in the complexes that were retained under stress conditions become even more tight (24).

Here we tested the levels and interaction between selected Pol III subunits upon treatment of yeast cells with MPA (Figure 6). MPA elicited a substantial (~5-fold) decrease in C160 protein levels. Whether the C160 subunit is directed for degradation by the ubiquitin/proteasome system upon MPA treatment remains to be tested. The levels of C82 and C53 also decreased, but to a lesser extent than C160. The levels of the AC40 subunit seemed to be relatively stable. Thus, MPA treatment affects the levels of Pol III subunits to various extents (Figure 6A). It is unlikely that the decrease of C160 levels is a causative event in Pol III inhibition upon MPA treatment, as the decrease in pre-tRNA levels precedes C160 degradation (Figure 6B). The entire Pol III complex was immunopurified using GFP-specific antibody and GFP-tagged AC40 subunit as bait. The interactions between selected Pol III subunits were examined by co-immunoprecipitation followed by Western blot. Clearly, MPA treatment does not change the levels of co-immunoprecipitated C160, C82 and C53 subunits as compared to the respective controls (Figure 6C), suggesting that in the retained Pol III complexes the interactions between subunits are preserved.

We subsequently tested the levels of selected Pol III subunits in RAW264.7 macrophages treated with MPA. In contrast to yeast, MPA has no influence on POLR3A, POLR3D or POLR1D protein levels over 4 hours of treatment in these macrophages (Figure 7A), although transcription by Pol III was inhibited by approximately 90% (Figure 1). Levels of these Pol III subunits drop slightly, however, after 8 hours MPA treatment, which also correlates with the onset of cell death, as manifested by decreased cell survival in clonogenic

316 assays, poly ADP-ribose polymerase (PARP) cleavage and p53 activation (Figure 7B, C and
317 D). However, these decreases are too slow to account for the rapid Pol III transcriptional
318 inhibition in RAW264.7 macrophages under these conditions.
319
320 ***p53 is not required for the decrease of tRNA levels upon MPA treatment***
321 p53 is a key tumour suppressor which has been shown to negatively regulate
322 transcription by Pol III in mammalian cells (34). p53 has also been shown to be strongly
323 induced in human cells upon MPA treatment (35).
324 Since we saw cell death and p53 activation after 8 h of MPA treatment (Figure 7), we
325 reasoned that the p53 response may occur more rapidly and contribute to Pol III inhibition in
326 RAW264.7 macrophages. Indeed, p53 activation occurs much earlier and its levels begin to
327 rise within 1 hour after MPA treatment (Figure 8A). However, given that we see decreased
328 tRNA levels after only 30 minutes of MPA treatment (Figure 1), it is unlikely that p53 is
329 responsible for Pol III inhibition in these conditions. This is further supported by two lines of
330 evidence. First, in the colorectal cancer cell line HCT116 lacking p53, MPA treatment
331 induces similarly rapid tRNA transcription inhibition as in p53-positive HCT116 cells (Figure
332 8B). Second, in p53-positve HCT116 cells, p53 upregulation is not apparent within 4 h of
333 MPA treatment and a substantial p53 induction was observed only after 8 h of MPA treatment
334 (Figure 8C). As expected, the p53 induction was not observed in the control p53-null cells at
335 any time point tested (Figure 8C, bottom panel).
336 Finally, the cell death itself does not contribute to a rapid Pol III inhibition by MPA in
337 RAW264.7 macrophages, as we do not observe its onset within 4 hours of MPA treatment, as
338 measured by PARP cleavage and clonogenic survival assays (Figure 8A and D). In
339 conclusion, our data support no role for p53 in Pol III inhibition upon MPA treatment in
340 mammalian cells.

341

342 ***Pol III remains partially associated with tDNA templates upon MPA treatment in yeast***

343 To gain a deeper insight into the MPA effect on Pol III transcription, we assessed its
344 impact on Pol III association with tDNA using ChIP assays. MPA treatment induces quick,
345 but only partial dissociation of yeast Pol III from its template genes. However, the
346 dissociation appeared to be less rapid than the decrease in tRNA levels. Moreover, the
347 substantial portion of Pol III remained associated with tRNA genes after two hours of MPA
348 treatment (Figure 9A). The kinetics of Pol III dissociation clearly lag behind the drop in pre-
349 tRNA levels (Figure 9B). Perhaps, in yeast, MPA treatment, by depleting GTP, prevents
350 transcription elongation without apparent dissociation of Pol III from templates, probably
351 causing its stalling *in vivo*.

352 In contrast to yeast, the majority of Pol III complexes in RAW264.7 macrophages are
353 released from template genes upon MPA treatment (Figure 10A). Moreover, no significant
354 difference between the kinetics of tRNA transcription inhibition and Pol III dissociation is
355 observed by plotting together the data obtained from RT-qPCR and ChIP assays and fitting
356 the exponential decay function into it (Figure 10B). Thus, it seems that in a macrophage cell
357 line upon MPA treatment, either Pol III stalling is not occurring or stalled Pol III is rapidly
358 removed by a mechanism that is not present in yeast.

359

360 **Discussion**

361 Here we investigated the effects of MPA treatment on pre-tRNA synthesis both in
362 yeast and mammalian cells, with a special focus on macrophages, an important constituent of
363 the innate immune system. We observed that the cessation of tRNA synthesis by MPA is very
364 rapid in both systems. The decrease in tRNA levels upon MPA treatment in RAW264.7

365 macrophages and yeast was clearly rescued upon addition of GMP and guanine, respectively.
366 We excluded Maf1 and p53 as major regulatory contributors to the observed inhibition of Pol
367 III activity upon MPA treatment. Moreover, the downregulation of selected Pol III subunits
368 by MPA seems to be a consequence but not a cause of the decreased Pol III activity. We thus
369 conclude that the direct cause of Pol III inhibition is diminution of guanine nucleotides *per se*.
370 In MPA-treated yeast, Pol III does not dissociate completely from tDNA templates and the
371 substantial fraction of Pol III complexes remain associated with chromatin. These complexes
372 may correspond to DNA-bound, perhaps stalled Pol III transcription units. It is noteworthy
373 that prolonged MPA treatment results in the induction of the *IMD2* mRNA, possibly restoring
374 GTP levels and allowing activation of Pol III complexes associated with tRNA genes (Figure
375 2).

376 Pol III is tightly regulated in response to various growth and stress conditions, and
377 while several different mechanisms are involved in this process, the prominent role is played
378 by the repressor Maf1 (14, 16). Our data, however, clearly showed that in yeast lacking Maf1
379 and in RAW264.7 macrophages with knocked-down MAF1 mRNA, MPA potently inhibits
380 Pol III activity. Similarly, tRNA transcription in HCT116 cells remains sensitive to MPA in
381 the absence of p53, another Pol III repressor. Furthermore, the p53 induction in RAW264.7
382 and HCT116 cells occurs much later than the actual cessation of pre-tRNA synthesis. We
383 considered that a decrease in the levels of Pol III subunits may contribute to the response of
384 cells to MPA. We indeed observed downregulation of selected subunits; however, both in
385 yeast and RAW264.7 macrophages, this again occurs later than the pre-tRNA decrease and is
386 therefore likely to be secondary to the decreased Pol III activity. This is consistent with our
387 previously published results showing that in yeast upon metabolic stress, the downregulation
388 of the largest Pol III subunit, C160, is delayed in regards to Pol III activity inhibition (24). We

thus concluded that neither Maf1, p53 nor the decrease in Pol III levels are primarily responsible for MPA-induced Pol III inhibition.

In yeast, Pol III dissociates from tRNA genes, but the kinetics clearly lag behind the drop in pre-tRNA levels. Furthermore, in these conditions, Pol III does not dissociate completely even during prolonged treatment. This is strikingly different to the response in yeast cells upon metabolic shift from fermentation to medium with a non-fermentable carbon source, where decreases in tRNA levels and Pol III dissociation kinetics are comparable (24). We speculated that signalling event(s) triggered by this metabolic shift, which includes Maf1 dephosphorylation (36), actively impinge(s) on Pol III, whereas MPA treatment results in GTP depletion and perhaps Pol III stalling due to the lack of one of the substrates. Pol III may be especially prone to the stalling due to GTP depletion as it has been shown that incorporation of G by Pol I is slower than other residues, and that *in vivo* Pol I pauses just before it (37). Our observation is also consistent with the results of *in vitro* transcription studies where a ternary Pol III complex was formed with SUP4 tDNA as a template in the presence of NTPs without GTP. The SUP4 tRNA does not contain guanine nucleotides within its first 17 nucleotides, which allows for formation of the partial nascent transcript even in the absence of GTP. Importantly, such a ternary complex is stable and transcription can be resumed upon GTP addition (38). Furthermore, by co-immunoprecipitation experiments performed with extracts of MPA-treated cells, we observed stable interactions between subunits of the remaining undegraded Pol III complexes. These complexes may correspond to the DNA-bound, stalled Pol III transcription units.

Given that MPA efficiently inhibits tRNA transcription in both wild-type and *maf1Δ* yeast cells, it seems that the nucleotide depletion acts independently of Maf1, most likely directly on Pol III. This contrasts with the downregulation of Pol III induced by metabolic stress such as shift to glycerol media at elevated temperature. In this case, Maf1 is

414 indispensable for repressing Pol III activity, and in the cells depleted of this protein, tRNA
415 downregulation does not occur (Figure 11). Thus, MPA is the first reported stress-inducing
416 agent that does not require Maf1 to repress Pol III.
417
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Figure legends

Figure 1. **MPA decreases tRNAs levels in macrophage cell line through interfering with GMP nucleotides synthesis pathway. A**. RAW264.7 murine macrophage cell line was treated either with 10 µM MPA, 50 µM GMP or both for the indicated period of time. **B**. Similarly as in A, RAW264.7 cells were treated either with 10 µM MPA or 50 µM GMP alone for indicated period of time, or cells were first treated with MPA for 1 h and then GMP was added for another hour (MPA+GMP sample). Total RNA was isolated, reverse-transcribed and tRNA levels were measured using qRT-PCR. All samples were normalised to

535 the geometric mean of the ARPP, GAPDH and ACTB mRNAs. N = 3. The error bars
536 represent the standard deviation.

537

538 Figure 2. **MPA inhibits tRNA synthesis in yeast cells. A. Rapid decrease of tRNA**
539 **synthesis by MPA is reactivated upon prolonged treatment.** Wild-type yeast cells were
540 grown in the minimal medium without uracil to the exponential growth phase at 30°C and
541 then treated with 50 µg/ml MPA for the indicated period of time. RNA was extracted from
542 cells and subjected to Northern blot. The blot was hybridized with the following probes:
543 tL(CAA), tW(CCA), tY(GUA), tK(UUU), tL(UAG). 5.8S rRNA was used as a loading
544 control. **B. Kinetics of tRNA synthesis inhibition by MPA.** Quantification of pre-tRNA
545 from Northern blots from A. N=3. **C. Guanine rescues the low levels of tRNA in MPA-**
546 **treated yeast cells.** Wild-type yeast cells were grown in the minimal medium without uracil
547 to the exponential growth phase at 30°C and then left untreated (NT) or treated with 100
548 µg/ml MPA or 0.3 mM guanine (gua) for the indicated period of time. MPA treated cell
549 cultures were split in two and after 1 h 0.3 mM guanine was added to one sample for another
550 hour (MPA+gua). RNA was extracted from cells and subjected to Northern blot as in A. **D.**
551 Quantification of pre-tRNA from Northern blots from C. N=2. **E. Treatment of yeast with**
552 **MPA results in *IMD2* mRNA induction.** Wild-type yeast cells were grown in the minimal
553 medium without uracil to the exponential growth phase at 30°C and then treated with 100
554 µg/ml MPA for the indicated period of time. RNA was extracted from cells and *IMD2* mRNA
555 levels were assessed by RT-qPCR. All samples are normalised to the geometric mean of the
556 ACT1, ALG9 and TDH1 mRNAs. N=3. The error bars represent the standard deviation.

557

558 Figure 3. **The effects of prolonged MPA treatment on RAW264.7 macrophage cell line.**
559 RAW264.7 murine macrophage cell line was treated with either EtOH (MPA vehicle) or 10

560 μM MPA for 8 hours. Total RNA was isolated and indicated tRNA and mRNA levels were
561 measured using qRT-PCR. All samples are normalised to the geometric mean of the ARPP,
562 GAPDH and ACTB mRNAs. N = 3. Error bars represent the standard deviation. Asterisk
563 indicates p-value < 0.05.

564

565 Figure 4. **Maf1 is not involved in Pol III inhibition upon MPA treatment in macrophage**
566 **and tumour cell lines. A.** RAW264.7 cells transduced with vector expressing His-Flag-
567 tagged version of MAF1 were left untreated or treated with 10 μM MPA for indicated period
568 of time or 100 nM rapamycin for 1 hour. Protein extracts were prepared and resolved on
569 Phos-tag SDS-PAGE gel to visualise phosphorylated forms of MAF1. **B.** RAW264.7 murine
570 macrophage cell line was transfected with either non-targeting siRNA or siRNA pool
571 targeting Maf1. The cells were then left untreated or treated with 10 μM MPA for the
572 indicated period of time. Total RNA was isolated, reverse-transcribed and Maf1 mRNA and
573 the levels of indicated tRNAs were measured using qRT-PCR. All samples were normalised
574 to the geometric mean of the ARPP, GAPDH and ACTB mRNAs. N = 3. The error bars
575 represent the standard deviation. **C.** U2OS human osteosarcoma cells were either left
576 untreated or treated with 10 μM MPA for the indicated period of time. Total RNA was
577 isolated and tRNA levels were measured using qRT-PCR. All samples are normalised to the
578 geometric mean of the ARPP, ACTB mRNAs and 18S rRNA. N = 2. The error bars represent
579 the standard deviation. **D.** U2OS cells transfected with HA-tagged version of MAF1 were
580 treated either with DMSO, indicated concentrations of MPA for indicated period of time or 20
581 nM rapamycin for 1 h. Protein extracts were prepared and resolved on Phos-tag SDS-PAGE
582 gel as in A.

583

584 Figure 5. **Inhibition of tRNA synthesis by MPA treatment of yeast is Maf1-independent.**
585 Wild-type and *maf1Δ* yeast cells were grown in the minimal medium without uracil to the
586 exponential growth phase at 30°C and then treated with 100 µg/ml MPA for the indicated
587 period of time. RNA was extracted from cells and subjected to Northern blot analysis. Blot
588 was hybridized with the following probes: tL(CAA), tW(CCA), tY(GUA), tK(UUU),
589 tL(UAG). 5.8S rRNA was used as a loading control. **A.** A representative Northern blots. **B.**
590 Quantification of pre-tRNA levels. Pre-tRNA levels were normalized to 5.8S rRNA and a
591 sample from time point zero was set to 1. N=3. Error bars represent the standard deviation.

592

593 Figure 6. **Modulation of Pol III subunits levels as a consequence of Pol III inhibition by**
594 **MPA. A.** Wild-type yeast cells expressing HA-tagged C160 subunit were grown in the
595 minimal medium without uracil to the exponential growth phase at 30°C and then treated with
596 100 µg/ml MPA for 2 hours. (Top) Representative Western blot showing the levels of
597 indicated Pol III subunits and Pgk1, which was used as a loading control. (Bottom)
598 Quantification of Pol III subunits levels. The mean subunit expression is shown normalized to
599 the Pgk1 protein and time zero sample is set to 1. N=3. Error bars represent the standard
600 deviation. **B**. The plot comparing the kinetics of tRNA decrease and C160 levels
601 downregulation upon MPA treatment. The data of selected tRNA levels from Figure 5 were
602 used. The data related to C160 levels were taken from Leśniewska et al. (24), and were
603 obtained from the same strain MW4415, grown and treated similarly as in A. The error bars
604 were omitted for the sake of figure clarity. **C**. Wild-type yeast cells expressing HA-tagged
605 C160 and GFP-tagged AC40 subunits were grown in the minimal medium without uracil to
606 the exponential growth phase at 30°C and then left untreated or treated either with EtOH or
607 100 µg/ml MPA for 2 hours. Cellular extracts were incubated with magnetic beads coated
608 with anti-GFP antibody. After extensive washes, immunoprecipitated proteins were eluted

609 and analysed by Western blotting using antibodies against GFP, HA tag, C82 and C53 (Top).
610 (Bottom) Estimation of binding of C160, C82, C53 to AC40. The binding of the subunits in
611 MPA treated cells was calculated relative to their binding in EtOH-treated cells, which was
612 set to 1. N=3. Error bars represent the standard deviation.

613

614 Figure 7. **Prolonged MPA treatment induces cell death and modest Pol III subunits**
615 **downregulation in RAW264.7 macrophage cell line.** RAW264.7 murine macrophage cell
616 line was treated with either EtOH or 10 μM MPA for up to 4 hours (A) or 8 hours (B). **A** and
617 **B.** Representative Western blots showing the levels of indicated proteins. The samples were
618 run on 8 or 15% acrylamide gel. Actin was used as a loading control. **C.** Quantification of Pol
619 III subunits levels from B. The mean subunit expression is shown normalized to Actin and the
620 time zero sample is set to 1. N=5. Error bars represent the standard deviation. **D.** Clonogenic
621 survival assay. Seventy five or fifty thousands of RAW264.7 cells were plated, allowed to
622 attach overnight and then treated as in B. After treatment the cells were washed with fresh
623 media and allowed to grow for another 3 days, followed by staining with crystal violet.

624

625 Figure 8. **p53 is not required for decrease of tRNA levels upon MPA treatment. A.**
626 RAW264.7 murine macrophage cell line was left untreated or treated with 10 μM MPA for
627 the indicated period of time. (Left) Representative Western blots showing the levels of
628 indicated proteins. Actin was used as a loading control. (Right) Quantification of p53 levels.
629 The mean p53 expression is shown normalized to Actin. N=3. Error bars represent the
630 standard deviation. **B, C.** p53 positive (p53 +/+) and p53 null (p53 -/-) HCT116 colorectal
631 cancer cells were treated with 10 μM MPA for the indicated period of time. **B.** Total RNA
632 was isolated and the levels of indicated mRNAs were measured using qRT-PCR. All samples
633 are normalised to the geometric mean of the ARPP, GAPDH and ACTB mRNAs and non-

634 treated (NT) sample is set to 1. N=3. **C.** Representative Western blots showing the levels of
635 indicated proteins. Actin was used as a loading control. N=2. **D.** Clonogenic survival assay.
636 Seventy five or fifty thousands of RAW264.7 cells were plated, allowed to attach overnight
637 and then treated with 10 μM MPA for 4 h. After treatment the cells were washed with fresh
638 media and allowed to grow for another 3 days, followed by staining with crystal violet.

639

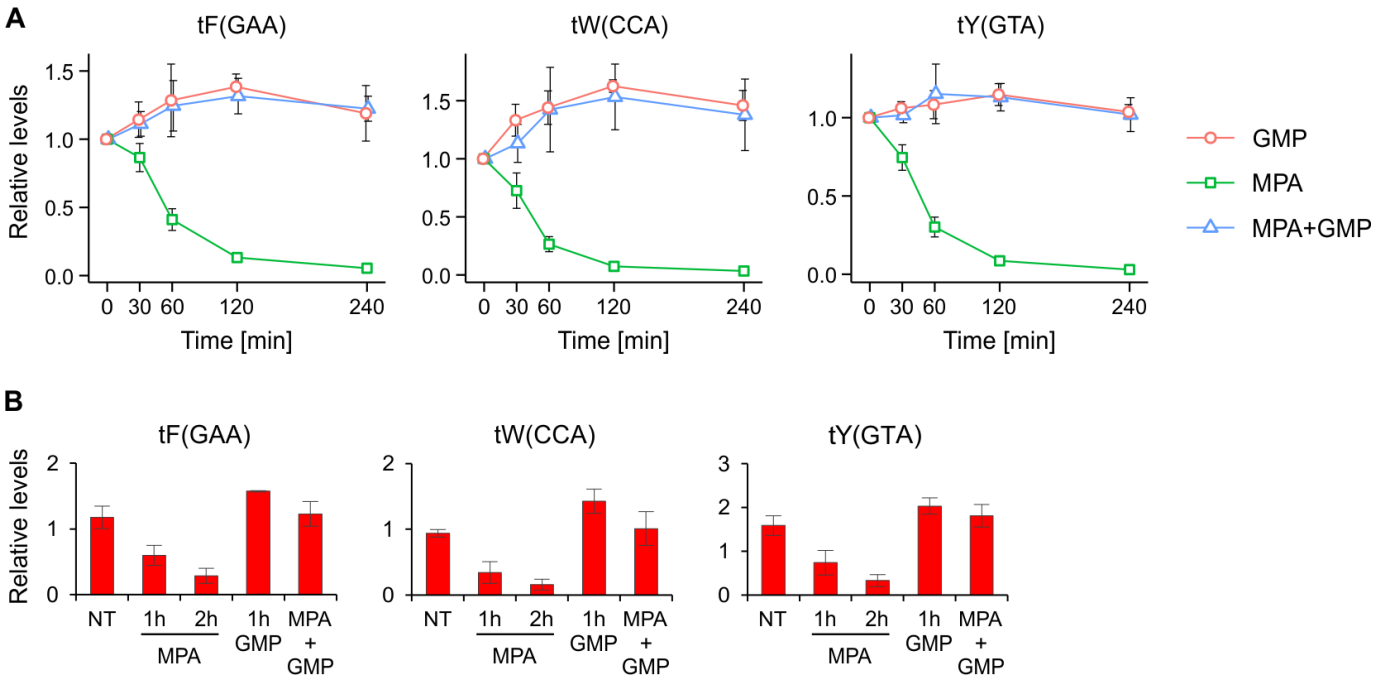
640 Figure 9. **A substantial amount of Pol III complexes is retained on tRNA genes upon**
641 **MPA treatment of yeast. A.** Wild-type yeast cells expressing HA-tagged C160 subunit were
642 grown in the minimal medium without uracil to the exponential growth phase at 30°C and
643 then treated with 100 μg/ml MPA for the indicated period of time. Pol III binding to the
644 indicated tRNA genes was assessed by ChIP assay. Cross-linked chromatin was
645 immunoprecipitated with antibodies against HA followed by RT-qPCR. N=3. **B.** (Top) The
646 exponential decay function has been fitted to data from the quantified Northern blots (Figure
647 5) and ChIP experiments using non-linear least squares fits method. ANOVA has been
648 performed to test whether there is a significant difference between curves. (Bottom) The
649 model formula and the table with ANOVA results.

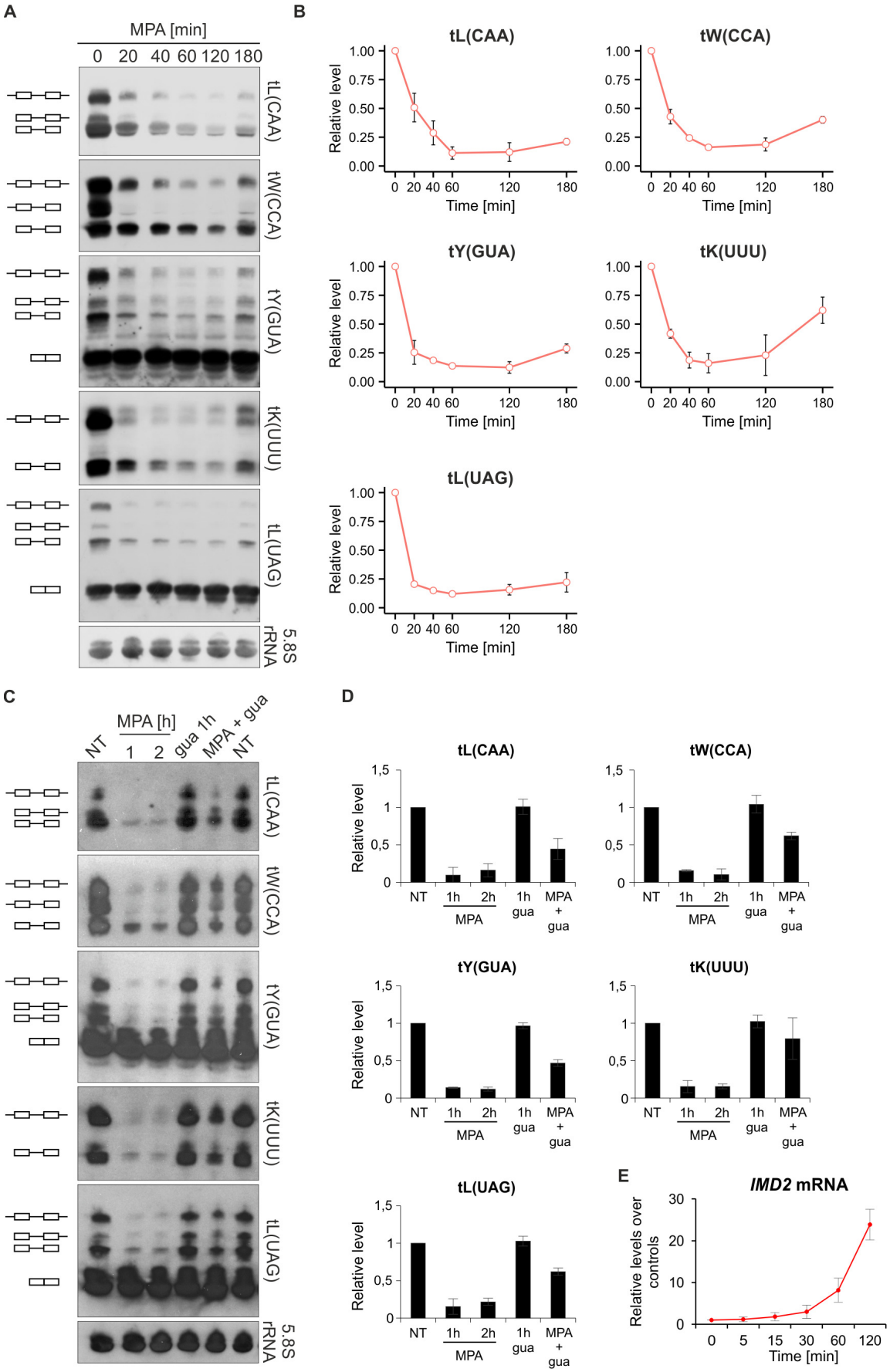
650

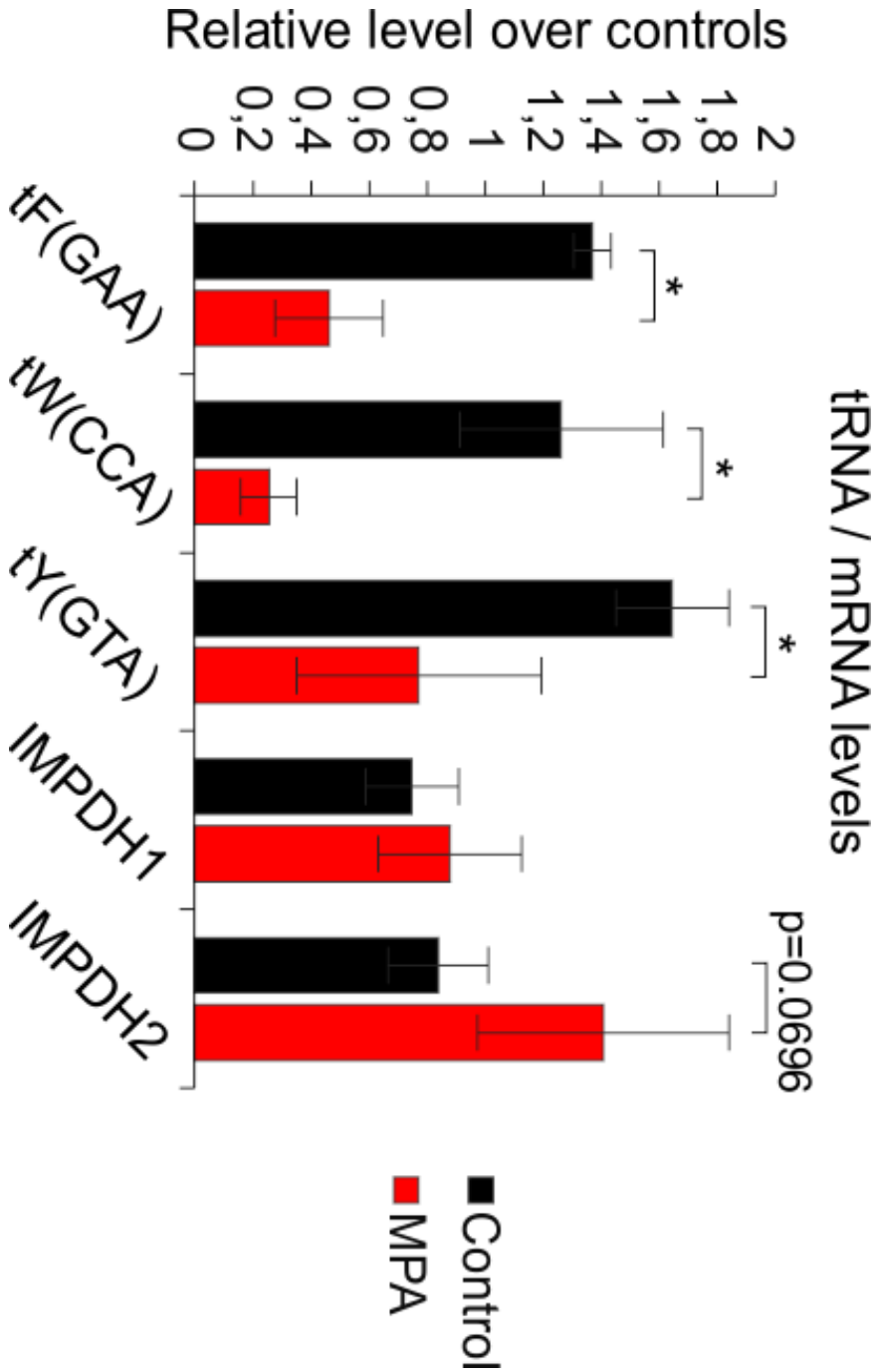
651 Figure 10. **In RAW264.7 macrophage cell line, decrease in tRNA levels is accompanied**
652 **by Pol III dissociation from DNA templates. A.** RAW264.7 murine macrophage cell line
653 was left untreated or treated with 10 μM MPA for the indicated period of time. Pol III binding
654 to the indicated tRNA genes was assessed by ChIP assay. Cross-linked chromatin was
655 immunoprecipitated with antibodies against POLR3D followed by RT-qPCR. N = 3. The
656 error bars represent the standard deviation. **B.** (Top) The exponential decay function has been
657 fitted to data from RT-qPCR (Figure 1A) and ChIP experiments using non-linear least squares

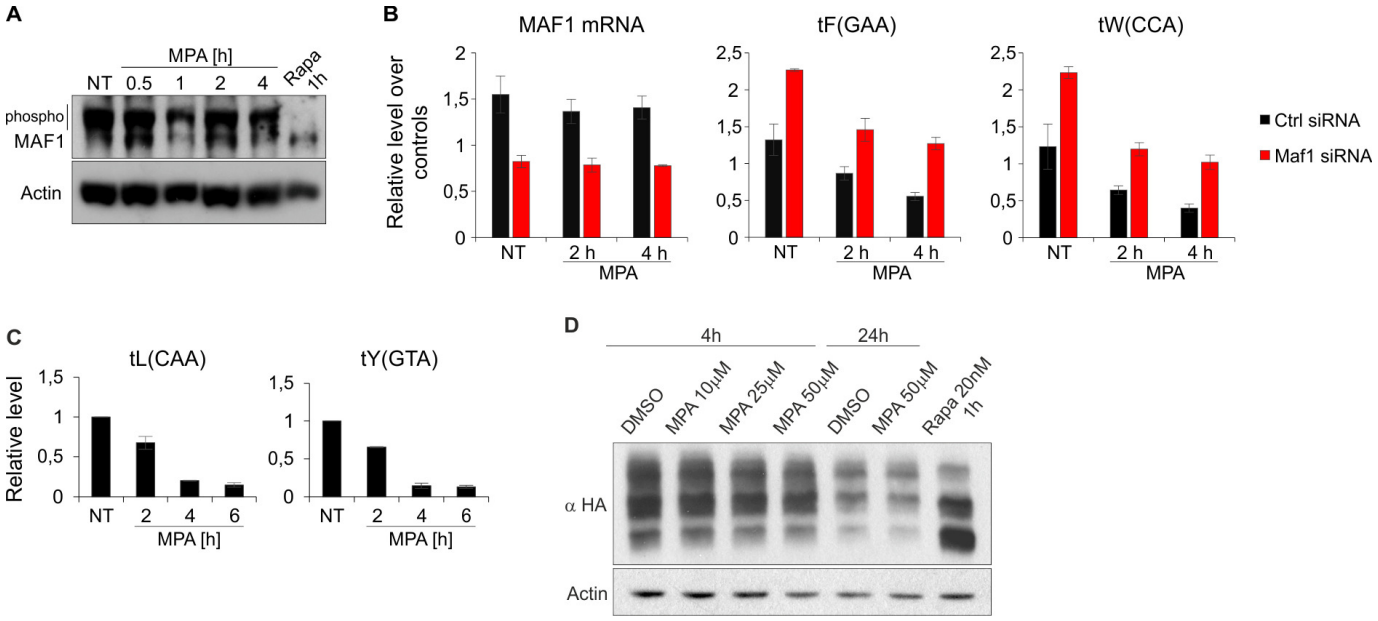
fits method. ANOVA has been performed to test whether there is a significant difference between curves. (Bottom) The model formula and the table with ANOVA results.

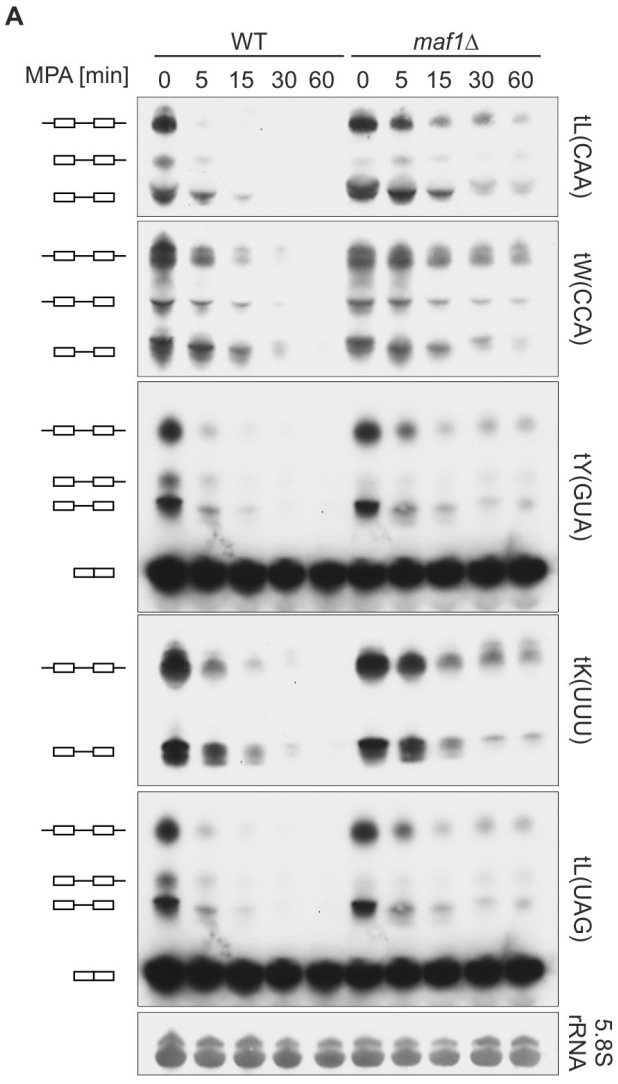
Figure 11. **In yeast cells depleted of Maf1, tRNA transcription is regulated differentially in response to metabolic shift and MPA.** The levels of indicated tRNAs from wild-type and *maf1Δ* yeast cells non-treated and treated with MPA for 1 h (data taken from Figure 5) were plotted together with the data from yeast cells shifted to glycerol media at 37°C for 2 h (data taken from Cieśla et al. (25)). Control samples from both wild-type and *maf1Δ* yeast cells were set to 1.

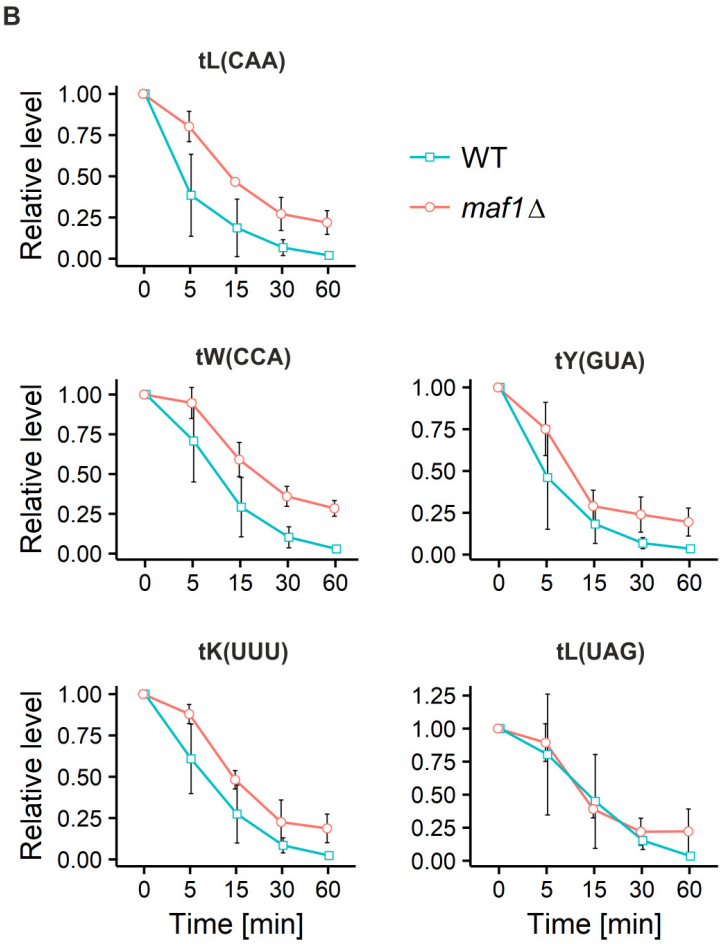


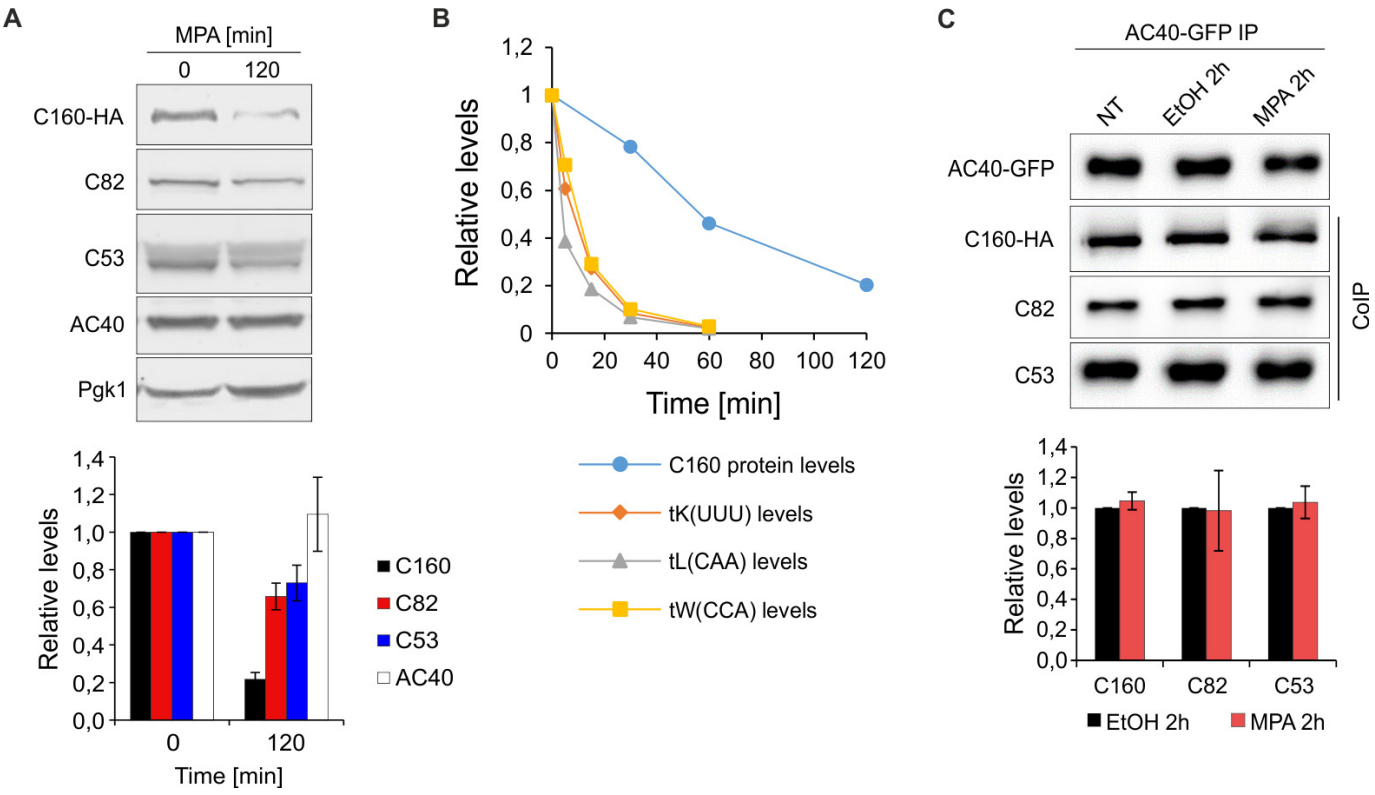


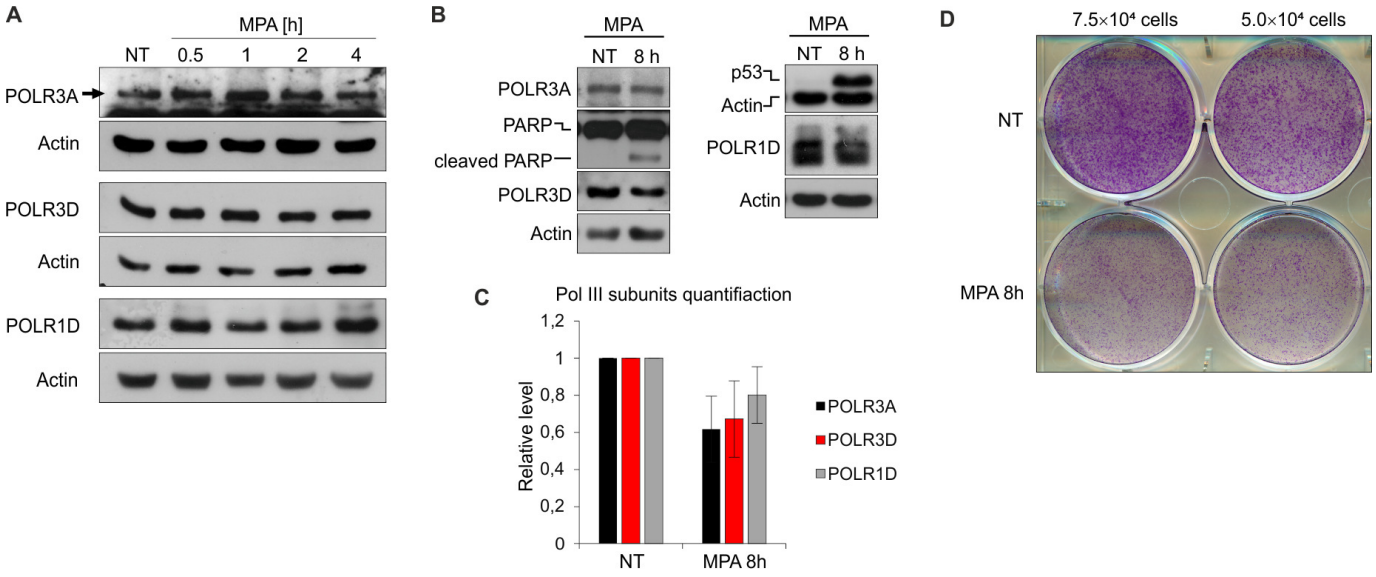


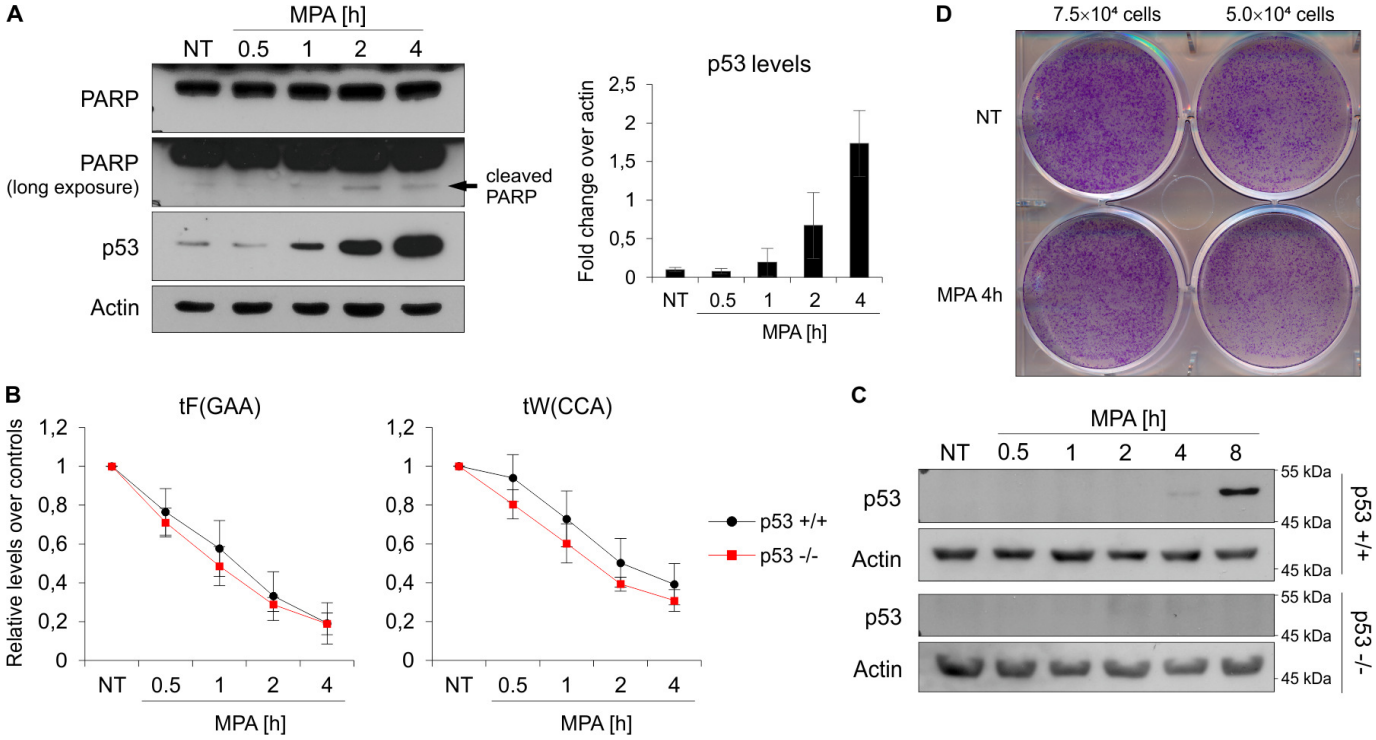


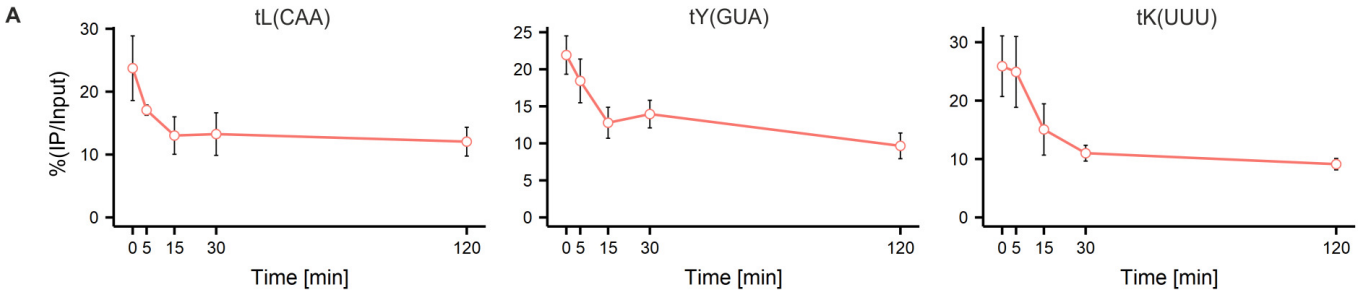


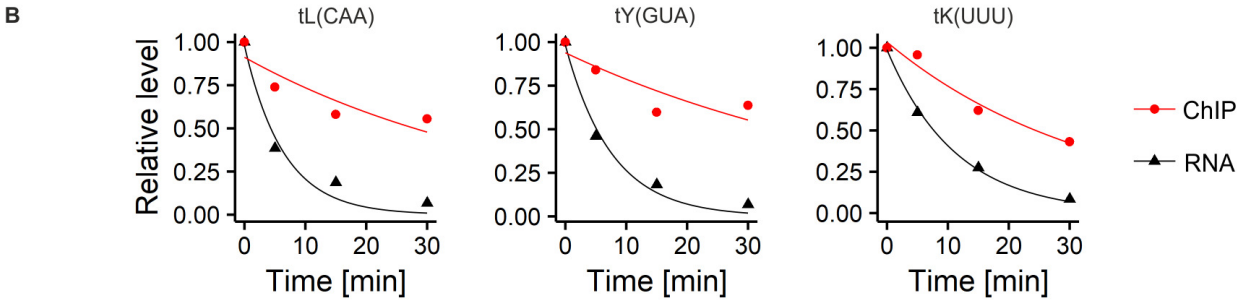








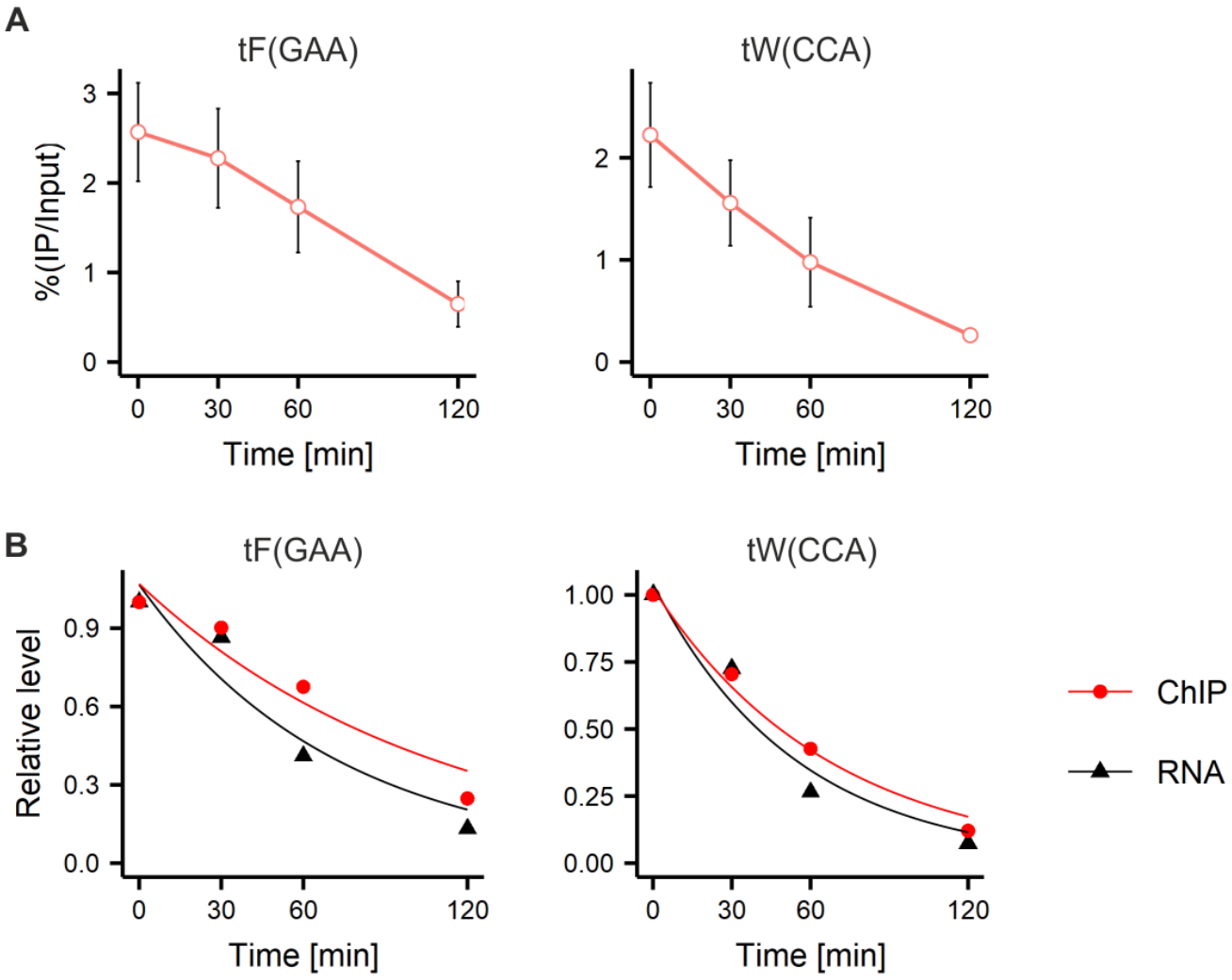




Model formula

$y = ae^{-\lambda x}$

	RNA		ChIP		
tRNA	a	λ	a	λ	P-Value
tL(CAA)	0.979	0.156	0.912	0.022	**2.817e-05**
tY(GUA)	0.982	0.132	0.939	0.018	**2.194e-06**
tK(UUU)	0.988	0.089	1.034	0.030	**1.388e-04**



Model formula

$y = ae^{-\lambda x}$

	RNA		ChIP		
tRNA	**a**	**λ**	**a**	**λ**	**P-Value**
tF(GAA)	1.067	0.014	1.069	0.009	**0.090**
tW(CCA)	1.041	0.018	1.024	0.015	**0.322**

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