Saccharomyces cerevisiae metabolic labeling with 4-thiouracil and quantification of newly-synthesized mRNA as a proxy for RNA polymerase II activity
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1 **TITLE:**

2 *Saccharomyces cerevisiae* Metabolic Labeling with 4-thiouracil and the Quantification of Newly

3 Synthesized mRNA as a Proxy for RNA Polymerase II Activity

4

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16 **KEYWORDS:**

17 *S. cerevisiae*, RNA, transcription, newly synthesized RNA, 4-thiouracil, RNA polymerase II, SAGA

18 complex

19

20 **SHORT ABSTRACT:**

21 The protocol described here is based on the genome-wide quantification of newly synthesized

22 mRNA purified from yeast cells labeled with 4-thiouracil. This method allows to measure mRNA

23 synthesis uncoupled from mRNA decay and, thus, provides an accurate measurement of RNA

24 polymerase II transcription.

25

26 **LONG ABSTRACT:**

27 Global defects in RNA polymerase II transcription might be overlooked by transcriptomic studies

28 analyzing steady-state RNA. Indeed, the global decrease in mRNA synthesis has been shown to

29 be compensated by a simultaneous decrease in mRNA degradation to restore normal steady-

30 state levels. Hence, the genome-wide quantification of mRNA synthesis, independently from

31 mRNA decay, is the best direct reflection of RNA polymerase II transcriptional activity. Here, we

32 discuss a method using non-perturbing metabolic labeling of nascent RNAs in *Saccharomyces*

33 *cerevisiae* (*S. cerevisiae*). Specifically, the cells are cultured for 6 min with a uracil analog, 4-

34 thiouracil, and the labeled newly transcribed RNAs are purified and quantified to determine the

35 synthesis rates of all individual mRNA. Moreover, using labeled *Schizosaccharomyces pombe* cells

36 as internal standard allows comparing mRNA synthesis in different *S. cerevisiae* strains. Using this

37 protocol and fitting the data with a dynamic kinetic model, the corresponding mRNA decay rates

38 can be determined.

39

40 **INTRODUCTION:**

41 Cells respond to endogenous and exogenous cues, through the dynamic alteration of their gene

42 expression program. In recent years, a tremendous development of genome-wide

43 methodologies allows the precise and comprehensive description of transcriptome changes in

44 different conditions. In most transcriptomic studies, microarray hybridization or high-throughput

45 sequencing are used to quantify RNA levels from a total steady-state RNA fraction.
46 Transcriptional changes under a specific perturbation can display a wide range of possible
47 outcomes, with either specific gene expression changes or a large spectrum of genes being either
48 up- or downregulated. Gene expression is the result of a fine-tuned equilibrium—or steady-
49 state—between RNA synthesis by RNA polymerases and other processes affecting RNA levels.
50 RNA polymerase II transcription, including its three distinct phases (initiation, elongation, and
51 termination), is highly and intricately associated with mRNA processing, cytoplasmic export,
52 translation, and degradation.
53
54 Several recent studies demonstrated that mRNAs synthesis and decay are coupled mechanisms
55 and showed that transcriptional effects upon mutation or under stimuli can be overlooked when
56 quantifying total steady-state RNA. First, the detection of transcriptional changes through the
57 analyses of steady-state levels of mRNA always depends on mRNAs half-life. Once the
58 perturbation is introduced, the steady-state levels of mRNAs with long half-lives will be much less
59 affected than those of mRNAs with short half-lives. Therefore, the detectability of the changes in
60 RNA synthesis is strongly biased in favor of short-lived transcripts, while the analysis of longer-
61 lived mRNA species might fail to reveal dynamic changes in transcription rate. Second, several
62 reports have shown that, both in yeast and mammals, global changes in transcription might be
63 overlooked when analyzing the steady-state levels of mRNA. This is likely due to the mechanisms
64 that link mRNA synthesis and degradation resulting in mRNA buffering. This prompted the
65 development of new protocols to quantify mRNA synthesis uncoupled from degradation, through
66 the analysis of newly transcribed mRNA. In recent years, several alternatives have been
67 presented, including global run-on sequencing (GRO-seq)[1], and native elongation transcript
68 sequencing (NET-seq)[2,3]. Here, we are presenting a protocol initially developed in mammalian
69 cells[4-6] and then adapted to yeast[7-11], which is based on RNA labeling with a thiolated nucleoside
70 or base analog, 4-thiouridine (4sU) or 4-thiouracil (4tU), respectively.
71
72 This method specifically purifies newly transcribed RNA from the cells in which RNA are pulse-
73 labeled with 4sU with virtually no interference in the cell homeostasis. Hence, once the cells are
74 exposed to 4sU, the molecule is rapidly uptaken, phosphorylated to 4sU-triphosphate, and
75 incorporated in RNAs being transcribed. Once pulse-labeled, it is possible to extract total cellular
76 RNA (corresponding to steady-state levels of RNA), and, subsequently, the 4sU-labeled RNA
77 fraction is thiol-specifically modified, leading to the formation of a disulfide bond between biotin
78 and the newly transcribed RNA[4,5]. However, 4sU can only be uptaken by the cells expressing a
79 nucleoside transporter, like the human equilibrative nucleoside transporter (hENT1), preventing
80 its immediate use in budding or fission yeast. While one could express hENT1 in either *S. pombe*
81 or *S. cerevisiae*, an easier approach can be achieved using the modified base 4tU, since yeast cells
82 can take up 4tU, without the need of expression of a nucleoside transporter[10-13]. In fact, the
83 metabolism of 4tU requires the activity of the enzyme uracil phosphoribosyltransferase (UPRT).
84 In several organisms, including yeast but not mammals, UPRT is essential for a pyrimidine salvage
85 pathway, recycling uracil to uridine monophosphate.
86
87 An important bias in transcriptomic studies can be introduced by the normalization between
88 different samples analyzed in parallel. Indeed, many deviating factors can affect the comparative

89   analysis of the transcriptome of mutant and wild-type strains: the efficiency of cell lysis,
90   differences in the extraction and recovery of RNA, and variances in scanner calibration for
91   microarray analyses, among others. As discussed above, such variations can be particularly
92   misleading when global effects on RNA polymerase II transcription are expected. An elegant
93   mean to accurately compare mRNA synthesis rates between different samples was designed by
94   using the distantly related fission yeast *Schizosaccharomyces pombe* as an internal standard. For
95   that, a fixed number of labeled *S. pombe* cells is added to the *S. cerevisiae* samples, either wild-
96   type or mutant cells, prior to cell lysis and RNA extraction[10]. Subsequently, both steady-state and
97   newly synthesized RNAs from *S. pombe* and *S. cerevisiae* are quantified either by RT-qPCR or *via*
98   the use of microarray chips or high-throughput sequencing[10]. Combining these data with kinetic
99   modeling, absolute rates of mRNA synthesis and decay in budding yeast can be measured.
100
101 In the framework of this manuscript, we will show how the analysis of newly transcribed RNA
102 allowed to reveal a global role for the coactivator complexes SAGA and TFIID in RNA polymerase
103 II transcription in budding yeast[14-16]. Importantly, past studies quantified steady-state mRNA
104 levels in *S. cerevisiae* and suggested that SAGA plays a predominant function on a limited set of
105 yeast genes which are strongly affected by mutations in SAGA but relatively resistant to TFIID
106 mutations[17-19]. Surprisingly, the SAGA enzymatic activities were shown to act on the whole
107 transcribed genome, suggesting a broader role for this co-activator in RNA polymerase II
108 transcription. Decreased RNA polymerase II recruitment at most expressed genes was observed
109 upon the inactivation of SAGA or TFIID, suggesting that these coactivators work together at most
110 genes. Hence, the quantification of newly transcribed mRNA revealed that SAGA and TFIID are
111 required for the transcription of nearly all genes by RNA polymerase II[14-16]. The implementation
112 of compensatory mechanisms emerges as a way for the cells to cope with a global decrease in
113 mRNA synthesis which is buffered by a simultaneous global decrease in mRNA degradation. SAGA
114 adds to the list of factors having a global effect on RNA polymerase II transcription, such as RNA
115 Pol II subunits[10], the Mediator coactivator complex[20], the general transcription factor TFIIH[21,22],
116 and indirectly, elements of the mRNA degradation machinery[9,10,23]. Such compensatory events
117 were universally observed in SAGA mutants, accounting for the modest and limited changes in
118 steady-state mRNA levels despite a global and severe decrease in mRNA synthesis[14]. Similar
119 analyses were also performed in a *BRE1* deletion strain, resulting in a complete loss of histone
120 H2B ubiquitination. Interestingly, a much milder but consistent global effect on RNA polymerase
121 II transcription could be detected in the absence of Bre1, indicating that metabolic labeling of
122 newly transcribed RNA in yeast can detect and quantify a wide range of changes in mRNA
123 synthesis rates.
124
125 **PROTOCOL:**
126
127 **1. Cell Culturing and Rapamycin Depletion of a SAGA Subunit**
128
129 1.1. For each *S. cerevisiae* strain and replicate, including wild-type or control strains, inoculate a
130 single colony from a fresh plate onto 5 mL of YPD medium (2% Peptone, 1% yeast extract, and
131 2% glucose).
132

1.2. Grow *S. cerevisiae* cells overnight at 30 °C with constant agitation (150 rpm).

134

135 1.3. Measure the optical density at 600 nm (OD_{600}) and dilute the culture to an OD_{600} of
136 approximately 0.1 in 100 mL of YPD medium and let it grow up until the OD_{600} is around 0.8.

137

138 1.4. In parallel, inoculate a single colony of *S. pombe* cells from a fresh plate onto 50 mL of YES
139 medium (0.5% yeast extract; 250 mg/L adenine, histidine, uracil, leucine, and lysine; 3% glucose)
140 and grow the cells overnight at 32 °C with constant agitation (150 rpm).

141

142 1.5. Measure the OD_{600} of the *S. pombe* overnight culture and dilute the culture to an OD_{600} of
143 approximately 0.1 in 500 mL of YES medium and let it grow up until the OD_{600} is approximately
144 0.8.

145

146 1.6. For each *S. cerevisiae* anchor-away strain and replicate, including wild-type or control strains,
147 inoculate a single colony from a fresh plate onto 5 mL of YPD medium (2% Peptone, 1% yeast
148 extract, and 2% glucose).

149

150 1.7. Grow the cells overnight at 30 °C with constant agitation (150 rpm).

151

152 1.8. The next morning, measure the OD_{600}, dilute the culture to an OD_{600} of approximately 0.1 in
153 100 mL of YPD medium and let it grow until the $OD_{600} \approx 0.8$.

154

155 1.9. Add 100 µL of rapamycin to the culture from a stock solution of 1 mg/mL (final rapamycin
156 concentration of 1 µg/mL) and let the cells incubate at 30 °C with constant agitation for the time
157 necessary for the protein of interest to be conditionally depleted from the nucleus (usually, 30
158 min is adequate). For the control, use a similar yeast culture, but instead of adding rapamycin,
159 add the equivalent volume of dimethyl sulfoxide (DMSO).

160

161 **2. 4tU Labeling with *S. pombe* as a Spike-in (Counting)**

162

163 2.1. Prepare a fresh solution of 2 M 4-thiouracil. Once prepared, keep it at room temperature
164 and away from light.

165

166 2.1.1. Accurately weigh 64.1 mg of 4-thiouracil for each *S. cerevisiae* culture and dissolve it in 250
167 µL of dimethylformamide (DMF) or in 250 µL of DMSO.

168

169 2.1.2. For the *S. pombe* culture to be used as spike-in, weigh 320.5 mg of 4-thiouracil and dissolve
170 it in 1,250 µL of DMSO.

171

172 2.2. Add the 4-thiouracil solution to *S. cerevisiae* and *S. pombe* cultures for a final concentration
173 of 5 mM and incubate them for 6 min with constant agitation at 30 °C and 32 °C, respectively.

174

175 2.3. After 6 min, remove a small aliquot of each culture for cell counting. Count the cells using an
176 automatic cell counter or a Neubauer chamber.

177

2.4. Collect the cells through centrifugation (2,500 x g) for 5 min at 4 °C.

2.5. Discard the supernatant, wash the cells with ice-cold 1x PBS, and centrifuge again (2,500 x g, 4 °C, 5 min).

2.6. Calculate the total number of cells in each of the *S. cerevisiae* and *S. pombe* samples.

2.7. Resuspend the cells in 5 mL of ice-cold 1x PBS and mix *S. cerevisiae* with *S. pombe* cells with a 3:1 ratio.

2.8. Centrifuge the cells (2,500 x g, 4 °C, 5 min), remove the PBS, flash-freeze the cells in liquid N_2, and store the sample at -80 °C until further use.

3. RNA Extraction and DNase Treatment

3.1. Thaw the cells on ice for approximately 20 - 30 min.

3.2. Proceed with the RNA extraction using a yeast-RNA extraction kit (**Table of Materials**) with a few adaptations.

3.3. Per each sample, pour 750 µL of ice-cold Zirconia beads into a 1.5-mL screw cap tube supplied with the kit. Keep in mind that per each tube, RNA from up to 10^9 cells can be efficiently extracted. Hence, prepare the number of tubes necessary for each sample. For example, an *S. cerevisiae* culture of 100 mL ($OD_{600} \approx 0.8$) can render around 2×10^9 to 3×10^9 cells, leading up to a total of 2.7×10^9 to 4×10^9 cells in total (after the spike-in with a third of the *S. pombe* cells). In this case, up to 3 - 4 reaction tubes per each sample/condition/mutant/replicate would be required.

3.4. Per each 1×10^9 cells, add 480 µL of the lysis buffer provided with the kit, 48 µL of 10% SDS, and 480 µL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v).

3.5. Mix the cells using a vortex mixer and transfer them to the tubes containing the ice-cold Zirconia beads.

3.6. Accommodate the tubes on a vortex mixer adaptor, turn the vortex at maximum speed, and beat for 10 min to lyse the yeast cells (in a room at 4 °C). Alternatively, perform lysis of the cells in an automatic bead-beater.

3.7. Centrifuge tubes at 16,000 x g for 5 min at room temperature and carefully collect the upper phase (RNA-containing phase) to a fresh 15-mL falcon tube. Typically, the volume recovered per each tube is around 500 - 600 µL.

3.8. To the 15-mL tubes containing the partially purified RNA, add the binding buffer provided

with the kit and mix thoroughly. Per each 100 μL of RNA solution, add 350 μL of binding buffer (*i.e.*, when the aqueous-phase solution volume is 600 μL, 2.1 mL of the binding buffer should be added).

3.9. To the previous mixture, add the 100% ethanol and mix thoroughly. Per each 100 μL of RNA solution, add 235 μL of 100% ethanol (*i.e.*, when the aqueous-phase solution volume is 600 μL, add 1.41 mL of ethanol).

3.10. Apply up to 700 μL of the mixture from step 3.9 to a filter cartridge assembled in a collection tube, both provided with the kit.

3.11. Centrifuge for 1 min at 16,000 x *g*. If the centrifugation duration was not enough for the total volume to pass through the filter, repeat the centrifugation for 30 s.

3.12. Discard the flow-through and reuse the same collection tube. Add another 700 μL of RNA-binding buffer-ethanol solution to the filter and centrifuge again at 16,000 x *g* for 1 min.

3.13. Discard the flow-through and repeat steps 3.11 and 3.12 until the RNA solution is finished.

3.14. Wash the filter 2x with 700 μL of washing solution 1. Collect the washing solution *via* centrifugation at 16,000 x *g* for 1 min and always keep the collection tube.

3.15. Wash the filter 2x with 500 μL of washing solution 2. Collect the washing solution *via* centrifugation at 16,000 x *g* for 1 min and always keep the collection tube.

3.16. Centrifuge the tubes once again at 16,000 x *g* for 1 min to completely dry the filter.

3.17. Transfer the filter cartridge to the final collection tube (RNA-appropriate tube) and elute RNA with 50 μL of DEPC-treated, RNase-free H_2O (preheated to 100 °C).

3.18. Centrifuge for 1 min at 16,000 x *g*.

3.19. Elute RNA again (to the same tube) with 50 μL of preheated DEPC-treated, RNase-free H_2O. Make sure that all the volume has passed through the filter; otherwise, centrifuge for longer periods.

3.20. If multiple tubes for one single sample are used, pool them all in one tube.

3.21. Quantify and check the purity of the sample using the appropriate equipment.

Note: While 4tU is only incorporated within newly synthesized RNA, there is a chance of minor contamination with DNA. For that reason, it is always advisable to treat the samples with DNase I. For that, use the reagents provided with the RNA-extraction kit (**Table of Materials**) following the manufacturer's recommendations.

4. Thiol-specific Biotinylation of Newly Synthesized RNA

4.1. Adjust the concentration of the RNA obtained with section 3 of the protocol to 2 mg/mL. Aliquot 200 µg of total RNA, heat it for 10 min at 60 °C, and immediately chill it on ice for 2 min.

4.2. To the RNA aliquot, add the reagents mentioned below in the following order: 600 µL of DEPC-treated, RNase-free H$_2$O, 100 µL of biotinylation buffer (100 mM Tris-HCl [pH 7.5] and 10 mM EDTA, in DEPC-treated, RNase-free H$_2$O), and 200 µL of biotin-HPDP from a stock of 1 mg/mL biotin-HPDP in DMSO or DMF.

4.2.1. In some situations, the biotin-HPDP solution tends to precipitate, likely due to its low solubility in water. In this situation, increase the volume of DMSO/DMF up to 40% of the reaction volume (to the RNA sample, add 400 µL of DEPC-treated H$_2$O, 100 µL of biotinylation buffer, and 400 µL of biotin-HPDP from a 0.5 mg/mL stock).

4.3. Incubate the sample at room temperature and protected from light for 3 h, with gentle agitation.

4.4. After incubation, add an approximately equal volume of chloroform to the tubes and mix vigorously.

4.5. Spin the sample at 13,000 x g for 5 min, at 4 °C. This step allows the removal of excess biotin that did not biotinylate the RNA. Alternatively, perform this step using phase-lock tubes (heavy). For that, spin down the phase-lock tubes for 1 min at 13,000 x g, add the RNA mixture and an equal amount of chloroform, mix them vigorously, and centrifuge them at 13,000 x g for 5 min, at 4 °C.

4.6. Carefully transfer the upper phase into new 2-mL tubes.

4.7. Add one-tenth of the volume of 5 M NaCl and mix the sample.

4.8. Add an equal volume of isopropanol, mix the sample thoroughly, and spin it at 13,000 x g for at least 30 min, at 4 °C.

4.9. Cautiously remove the supernatant and add 1 mL of ice-cold 75% ethanol.

4.10. Spin at 13,000 x g for 10 min, at 4 °C.

4.11. Carefully remove supernatant, quick-spin the tube, and remove the remaining ethanol solution. Make sure that the RNA pellet does not dry.

4.12. Suspend the RNA in 100 µL of DEPC-treated, RNase-free H$_2$O.

5. Purification of Newly Synthesized Fraction from Total and Unlabeled RNA Using Streptavidin-coated Magnetic Beads

5.1. Heat the biotinylated RNA for 10 min at 65 °C and then chill the samples on ice for 5 min.

5.2. Add 100 µL of streptavidin-coated magnetic beads to the biotinylated RNA (at a final volume of 200 µL). Specifically, it is recommended to use the beads indicated in the **Table of Materials**, since, after conversations with other laboratories, these seemed to be the more consistent and reliable.

5.3. Incubate the sample with slight shaking for 90 min, at room temperature.

5.4. Place the columns provided with the kit (**Table of Materials**) in the magnetic stand.

5.5. Add 900 µL of room-temperature washing buffer (100 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1 M NaCl, and 0.1% Tween 20, in DEPC-treated, RNase-free H_2O) to the columns (pre-run and equilibrate).

5.6. Apply beads/RNA mixture (200 µL) to the columns.

5.7. Collect the flow-through in 1.5-mL tubes and apply it again to the same magnetic column. If necessary, keep this flow-through as it represents the unlabeled RNA fraction.

5.8. Wash the columns 5x with increasing volumes of washing buffer (600, 700, 800, 900, and 1,000 µL).

5.9. Elute the newly synthesized RNA with 200 µL of 0.1 M DTT.

5.10. Perform a second elution, 3 min later, with an equal volume of 0.1 M DTT.

5.11. After eluting the RNA, add 0.1 volumes of 3 M NaOAc (pH 5.2), 3 volumes of ice-cold 100% ethanol, and 2 µL of 20 mg/mL glycogen (RNA-grade) and let the RNA precipitate overnight, at -20 °C.

5.12. Recover the RNA by centrifugation (13,000 x g for 10 min, at 4 °C) and resuspend it in 15 µL of DEPC-treated, RNase-free H_2O. The proportion of labeled-to-total RNA is expected to be around 2% - 4% (usually more toward the lower end), rendering approximately 2.0 µg of newly synthesized RNA. This quantity is enough to do several qPCR experiments, as well for microarray/sequencing analyses.

6. RT-qPCR Validation of the Different Fractions

6.1. Synthesize cDNA from 2 µg of total RNA or 10 µL of labeled RNA using random hexamers and the reverse transcriptase of choice, according to the manufacturer's instructions (**Table of**

6.2. Amplify the cDNA by real-time qPCR using a standard protocol (**Table of Materials**).

Note: All samples should be run in triplicate from a minimum of two biological replicates. Correct all raw values for the expression of *S. pombe* tubulin.

7. Microarray Hybridization

7.1. Hybridize RNA samples onto preferred microarray chips according to the manufacturer's instructions (for this specific protocol, see **Table of Materials**). Briefly, prepare biotinylated cRNA targets from 150 ng of RNA using the Premier RNA Amplification Kit (**Table of Materials**), according to the manufacturer's instructions. Hybridize 4 mg of fragmented cRNAs for 16 h at 45 °C and 60 rpm on microarray chips.

7.2. Wash, stain, and scan the chips using the indicated station and scanner (**Table of Materials**). Extract the raw data (CEL Intensity files) from the scanned images using the command console (AGCC, version 4.1.2).

7.3. Further process the CEL files with Expression Console software version 1.4.1 to calculate probe set signal intensities, using the statistics-based algorithms MAS 5.0 with default settings and global scaling as normalization method.

Note: The trimmed mean target intensity of each chip was arbitrarily set to 100. Perform all experiments using at least two independent biological replicates. Normalize raw data to the *S. pombe* signal and calculate fold changes in total and newly synthesized RNA levels.

8. Data Analysis using an Existing R Pipeline

8.1. Calculate synthesis and decay rates using a pipeline and R/Bioconductor package publicly available, as previously described[8,10].

REPRESENTATIVE RESULTS:
When performing metabolic labeling of newly transcribed RNA, several aspects need to be controlled: the time and efficiency of the labeling, the spike-in proportion, the extraction protocol, and the biotinylation efficacy (including signal-to-noise ratio), among others. These conditions have been extensively and methodically shown by others[7,10,11]. Here we mainly focus on the interpretation and immediate analyses that can be performed once the samples have been processed, either by RT-qPCR, microarray, or sequencing. The analyses of different mutant strains demonstrate the power of the method to detect not only a dramatic global decrease in mRNA synthesis, as in the case of SAGA mutant *S. cerevisiae* strains, but also a very mild reduction of RNA polymerase II activity upon suppression of histone H2B monoubiquitination.

Our analyses of SAGA enzymatic activities suggested a broad recruitment to chromatin[15], which

397 was not revealed by the analysis of steady-state mRNA levels in SAGA mutant strains. As RNA
398 polymerase II recruitment was impaired upon SAGA inactivation, we decided to analyze whether
399 mRNA synthesis rates would be globally affected. Hence, wild-type or mutant *S. cerevisiae* strains
400 were exposed to 4tU for a 6-min period, to label newly transcribed RNAs. After mixing with the
401 spike-in labeled cells (*S. pombe*) in a proportion of 3:1, total RNA was extracted, and newly
402 synthesized RNA was biotinylated and purified according to the protocol presented here, as in
403 the chronogram shown in **Figure 1**. Labeled RNAs were purified from a total of 200 µg of RNA,
404 ensuring that the amount of purified product would be sufficient for any downstream
405 application. As an initial and systematic step before any genome-wide analysis, newly synthesized
406 RNA purification was validated by RT-qPCR. Genes were selected according to different
407 parameters, including a level of expression, regulatory pathways, and a dependence on different
408 RNA polymerases.
409
410 To confirm that this protocol specifically purifies labeled RNA, we quantified the levels of
411 transcripts in fractions purified from wild-type cells that were cultured with or without 4tU.
412 Negligible background levels of the analyzed RNAs were detected from cells that were not
413 exposed to 4tU (**Figure 2A**). Newly transcribed RNA purification was further validated by the
414 observed enrichment of the intron-containing *ACT1* pre-mRNA (data not shown). After validation
415 of the quality of the samples, we tested whether mRNA synthesis would be affected upon
416 deletion of *SPT20*, which is known to disrupt the SAGA complex assembly. As reported by others,
417 mRNA quantification performed on total (steady-state) RNA from the *spt20Δ* strain revealed
418 mostly unchanged or mildly reduced levels for the tested genes (**Figure 2B**). Similar results were
419 obtained for strains deleted for *BRE1* (**Figure 2B**). In contrast, the analysis of newly transcribed
420 RNA from the *spt20Δ* strain revealed a dramatic decrease in mRNA synthesis by three- to fivefold
421 for all tested genes (**Figure 2C**). The loss of Bre1 led to a more discreet but still visible decrease
422 in newly synthesized mRNA levels for the studied genes (**Figure 2C**). In good agreement with a
423 role of SAGA and Bre1 in RNA polymerase II transcription, the loss of either Spt20 or Bre1 did not
424 affect the expression of *RDN25* or *snR6* genes, transcribed by RNA polymerase I and RNA
425 polymerase III, respectively (**Figure 2C**).
426
427 However, strains deleted for structural subunits of the SAGA complex, like *SPT7* or *SPT20*, display
428 severe slow-growth phenotypes which may account for the observed transcriptional alterations.
429 To rule out undesired secondary effects, we conditionally depleted Spt7 from the nucleus, using
430 anchor-away *S. cerevisiae* strains[14,24]. Upon 60 min of rapamycin treatment, prior to the pulse-
431 labeling with 4tU (see **Figure 1** for a schematic representation), newly transcribed mRNA levels
432 were reduced to a similar extent as that observed in the deletion strain (**Figures 2D** and **2E**). This
433 analysis, thus, confirmed our former results and validated the protocol for this inducible
434 depletion system. In a time-course analysis, where cells were exposed to rapamycin for a time
435 spanning from 0 to 240 min, reduced expression was evidenced immediately after 15 min of
436 exposure to the drug. More interestingly, steady-state mRNA levels tended to initially diminish
437 but returned to normal levels after 60 min, an indication that a compensatory mechanism takes
438 place in the meantime (**Figure 2E**).
439
440 Altogether, the labeling and quantification of newly synthesized RNA allowed revealing new

441 regulatory roles for the SAGA complex. The described protocol could also reveal moderate effects
442 on RNA polymerase II activity and was successfully applied to conditional depletion yeast strains.
443
444 One of the downstream applications for the purified newly synthesized RNA is a genome-wide
445 quantification of transcripts using microarray hybridization or sequencing (4tU-seq). While high-
446 throughput sequencing is more quantitative, sensitive and informative, microarray hybridization
447 can be very helpful to determine whether the global mRNA levels are altered. In this context
448 where normalization is pivotal, we added a spike-in organism to the sample that we aimed to
449 analyze. Specifically, we mixed *S. cerevisiae* cells to *S. pombe* cells in a ratio of 3:1, both being
450 previously exposed to 4tU. When the purified, labeled RNAs are subjected to high-throughput
451 sequencing, any standard library preparation protocol can be used, most often following
452 ribosomal RNA depletion. Normalization between 4tU-seq data from different samples has been
453 performed by adding either labeled RNA from a different species (*i.e.,* mixing *S. cerevisiae* and *S.*
454 *pombe* cells as above)[22] or *in vitro*-transcribed, thiolated spike-in RNA[12,25-27]. Commercially
455 available microarray chips contain probes for the whole transcriptome of both budding and
456 fission yeast, allowing the quantification of mRNA from both organisms in one single experiment.
457 Microarray hybridizations were performed with total and newly synthesized RNA validated by
458 RT-qPCR as indicated above (wild-type, *spt20Δ*, and *bre1Δ*). In addition, we included a strain that
459 does not support monoubiquitination of histone H2B, through point mutation of the
460 ubiquitinable residue (*K123R*). Because the exact same proportion of *S. pombe* cells were used
461 in the different samples and replicates, it is possible to linearly rescale the arrays' intensities so
462 that the total and labeled *S. pombe* probes have the same median intensity. This normalization,
463 or rescaling, can be performed using a *Bioconductor* package developed by the laboratory of
464 Patrick Cramer and is used in parallel in all analyzed samples, total and labeled fractions and wild-
465 type and mutants strains[8]. Briefly, the input of this pipeline is an Excel file containing the probes
466 and their intensity (MAS5 or RMA) for all the samples and fractions. After excluding probes that
467 are outside the range of detection, the signal intensity is rescaled, taking into account the
468 intensity values of the *S. pombe* probes. Finally, you can map the probes to the budding and
469 fission yeast genomes, ending with a matrix containing the expression values normalized for the
470 spike-in. These values can be further processed (see next section) or used as is. In the following
471 example, we determined the fold change of every transcript between the mutant and the wild-
472 type strain.
473
474 The analyses were performed for both steady-state or newly synthesized levels of RNA and
475 plotted against their statistical significance (*p*-value) (**Figure 3**). In agreement with other studies,
476 when total RNA levels were analyzed, only a few genes had their expression altered, either up-
477 or downregulated (**Figures 3A** - **3C**). However, the analysis of newly transcribed RNA led to
478 strikingly different conclusions. The newly transcribed mRNA levels of more than 4,000 genes
479 were significantly reduced by at least twofold upon deletion of *SPT20*, suggesting a global positive
480 effect of SAGA on RNA polymerase II transcription in budding yeasts (**Figure 3D**). Additionally, in
481 the *bre1Δ* and *K123R* mutants, the results were more discreet: most genes appeared to have
482 their expression reduced, but the extent of downregulation and the number of significantly
483 affected genes (≈ 300 - 500) was indeed more limited (**Figures 3E** and **3F**).
484

485 As previously mentioned, steady-state or total levels of mRNA are dictated by the tight
486 equilibrium between synthesis and decay (**Figure 4A**). When RNA polymerase II transcription is
487 globally impaired, two scenarios can be pictured: (i) either the total mRNA levels decrease
488 globally, as a response to reduced synthesis but constant decay, or (ii) mRNA degradation is
489 decreased to the same extent, resulting in mostly unchanged steady-state mRNA levels. The
490 second scenario has been reported for several conditions, including in the context of SAGA or
491 TFIID disruption[9,10,14,16,22]. A procedure called comparative dynamic transcriptome analysis
492 (cDTA) based on 4tU labeling and dynamic kinetic modeling allows us to determine mRNA
493 synthesis and infer the decay rates for every transcript[8,10]. Once again, we took advantage of the
494 data collected for the previously mentioned strains (wild-type, *spt20Δ*, *bre1Δ*, and *K123R*). As
495 expected, upon deletion of *SPT20*, we observed a simultaneous decrease in mRNA synthesis and
496 degradation rates when compared to the wild-type strain. In this mutant strain, the
497 compensation was almost optimal, with an average decrease in synthesis of 3.8-fold and an
498 average decrease in decay of 4.1-fold (**Figure 4B**), corroborating why only limited transcriptional
499 changes could be detected on total mRNA levels (**Figure 3A**). In the two other mutant strains
500 (*bre1Δ* and *K123R*), concomitant changes in mRNA synthesis and decay were also observed, but
501 the changes were on a much smaller scale and more dispersed (**Figures 4C** and **4D**).
502
503 **FIGURE AND TABLE LEGENDS:**
504 **Figure 1: Schematic representation of the metabolic labeling of RNA using 4tU**. Freshly prepared
505 4tU is added to the culture medium and cells are labeled for 6 min. Labeled *S. cerevisiae* and *S.*
506 *pombe* cells are mixed in a ratio of 3:1 and total RNA is extracted. Afterward, newly synthesized
507 RNA is biotinylated and can be purified using streptavidin-coated magnetic beads. Finally, total
508 (steady-state) RNA and labeled newly synthesized RNA can be used in a variety of downstream
509 applications, including RT-qPCR and microarray hybridization or sequencing.
510
511 **Figure 2: Analysis depicting transcriptional changes in both steady-state and newly transcribed**
512 **RNA determined by RT-qPCR.** (**A**) RNA levels of five different genes were quantified from the
513 labeled RNA fraction purified from wild-type cells that were either exposed or not to 4tU. The
514 next two panels show (**B**) total and (**C**) newly synthesized RNA quantification by RT-qPCR for wild-
515 type (WT), *spt20Δ*, and *bre1Δ* yeast cells. Spt7 anchor-away strains, untreated or treated with
516 rapamycin for 60 min, were labeled with 4tU, and (**D**) total or (**E**) newly transcribed RNA was
517 quantified by RT-qPCR. (**F**) This panel shows the time-course analysis of changes in steady-state
518 and newly synthesized mRNA upon Spt7 nuclear depletion. For all samples, mRNA levels for five
519 RNA polymerase II genes were quantified by RT-qPCR. RNA polymerase I and RNA polymerase III
520 genes (*RDN25* and *snR6*, respectively) were used as control. Expression values (mean ± SD of
521 three independent experiments) were normalized to the spiked-in *S. pombe* signal and set to 1
522 in the control sample. Panels **D** - **F** have been modified from Baptista *et al.*[14].
523
524 **Figure 3: Genome-wide analyses of mRNA levels using total or labeled RNA fractions**. ZTHese
525 panels show volcano plots showing fold changes in (**A** - **C**) steady-state mRNA levels or (**D** - **F**)
526 newly synthesized mRNA levels relative to their significance (*p*-value). The fold changes (FC) were
527 calculated as the log2 of the ratio of the expression value of each gene after normalization to the
528 *S. pombe* signal in the (**A** and **D**) *spt20Δ*, (**B** and **E**) *bre1Δ*, or (**C** and **F**) K123R strain *versus* the

529   expression value of the same gene in wild-type *S. cerevisiae*. A total of 5,385 genes were
530   analyzed, and thresholds of twofold change (blue dot: more than a twofold decrease; yellow dots:
531   more than a twofold increase) and 0.05 *p*-values were considered. Panels **A** and **D** have been
532   modified from Baptista *et al.*[14].

534   **Figure 4: Parallel changes in mRNA synthesis and mRNA decay results in mRNA buffering**. (**A**)
535   This panel shows a schematic representation of the outcome of RNA synthesis perturbation on
536   steady-state RNA levels. (**B** - **D**) These panels show the calculation of mRNA synthesis and the
537   decay rates from the analyses of total and newly synthesized RNA. The synthesis and decay rates
538   were determined for each *S. cerevisiae* transcript in (**B**) *spt20Δ*, (**C**) *bre1Δ*, and (**D**) K123R. Changes
539   (calculated as the log2 of the ratio between mutant and wild-type) in synthesis rates were plotted
540   against changes in decay rates.

542   **DISCUSSION:**
543   While genome-wide tools to analyze changes in transcription are still improving, the sole analysis
544   of the transcriptome through the quantification of steady-state levels of RNA might not
545   accurately reflect changes in RNA polymerase II activity. Indeed, mRNA levels are regulated not
546   only by RNA synthesis but also by their maturation and degradation. To measure mRNA synthesis
547   uncoupled from mRNA degradation, distinct protocols have been developed in recent years for
548   the analysis of nascent transcription in both yeast and mammals.

550   One of the most widely used protocols for the quantification of nascent transcription is GRO-
551   seq[1]. While GRO-seq's main advantage is its capacity to unveil transcriptionally engaged and
552   active polymerase at high resolution and low background, it has two distinct points that decrease
553   its attractiveness: (i) it is technically challenging and requires the isolation of nuclei and, (ii) nuclei
554   manipulations introduce some perturbations to the system[28]. Another alternative is NET-seq,
555   which relies on the sequencing of the 3' ends of transcripts obtained upon immunoprecipitation
556   of the extremely stable ternary complex formed between nascent RNA, template DNA, and RNA
557   polymerase II. This approach allows the characterization of nascent RNAs at a base pair resolution
558   and can explore mRNA processing events through immunoprecipitation of modified RNA
559   polymerase II (serine-5 phosphorylation, for instance)[2,3,29]. Nevertheless, it presents some
560   obstacles, from which we highlight three. First, while antibodies targeting RNA polymerase II are
561   usually highly specific, it depends on antibody efficiency. Second, RNA might be prone to
562   degradation during incubation periods, thus leading us back to the previous point (less efficient
563   antibodies might require longer incubation times, leaving the RNA more susceptible to
564   degradation)[29]. Third, and especially in mammals, the MNase digestion and size selection might
565   exclude unique sequence alignment[29,30].

567   Here we presented a detailed protocol for the metabolic labeling of newly synthesized RNA using
568   4tU in *S. cerevisiae* that has different advantages in comparison to other available approaches.
569   Because transcription is highly sensitive to perturbations, cells should be maintained in the most
570   physiological conditions. For instance, GRO-seq implies the arrest of transcriptionally engaged
571   RNA polymerase II through exposure of the cells/nuclei to sarkosyl. However, sarkosyl treatment
572   has been described as inhibitory of several cellular processes[11]. In this case, metabolic labeling

573 with 4tU or 4sU at the described concentration is non-perturbing and does not visibly affect the
574 cell homeostasis, especially for short periods of exposure. In contrast to other methods using
575 transcription inhibition to measure mRNA half-lives, cDTA or 4tU-seq and fitting with dynamic
576 modeling determines degradation rates of every single mRNA in unperturbed cells. Hence, one
577 single method can simultaneously address both the synthesis and the decay rates of the whole
578 transcriptome on a specific cell type. Since cDTA or 4tU-seq takes advantage of highly reliable
579 normalization methods, namely through the usage of spike-in, different datasets can be analyzed
580 together and directly compared. Finally, metabolic labeling and quantification of newly
581 synthesized RNA is a technique that can be easily implemented in any molecular biology
582 laboratory as it does not require any specific equipment. This likely explains the rather large
583 dissemination of this technique, which tends to be used more and more systematically to explore
584 RNA production and degradation in yeasts, as well as in higher eukaryotes.

586 RNA can be labeled in living cells using other nucleoside analogs, namely 5-bromouridine
587 (BrU)[31,32] or 5-ethynyluridine (EU)[33,34]. The isolation of BrU pulse-labeled RNA relies on anti-BrdU
588 antibody purification which may have different efficiencies between experiments. EU-labeled
589 RNA can be covalently conjugated to biotin using click chemistry leading to a non-reversible
590 conjugation in contrast with thiol modification, which can be reversed with reducing agents. BrU
591 and EU labeling have been used in mammalian cells to determine genome-wide RNA decay
592 rates[31,32] or to assess nascent RNA synthesis[34,35]. However, BrU or EU labeling has not been
593 described in *S. cerevisiae*. Indeed, uptake of nucleoside analogs requires the expression of
594 nucleoside transporter and, thus, these methods appear less flexible in budding yeast than
595 labeling with 4tU.

597 The duration of 4tU labeling can be adapted and varies according to the question. When assessing
598 mRNA synthesis in *S. cerevisiae*, a short labeling pulse of 6 min ensures that the newly transcribed
599 mRNA levels are minimally affected by RNA degradation. Considering that the lag time before
600 the modified nucleotide can be incorporated into the nascent RNA is less than 1 min, this 6-min
601 labeling duration warrants that a reasonable amount of newly synthesized RNA can be purified.
602 Such protocol, as defined by Miller *et al.*[11], has been used in different *S. cerevisiae* mutants to
603 reveal global changes in mRNA synthesis and RNA decay[9,10,22,26,27,36]. A longer labeling duration
604 (3 h) was used in a metabolic pulse-chase labeling with 4tU to determine decay rates of all mRNAs
605 in *S. cerevisiae*[12]. Finally, extremely short (from 1.5 min) 4tU labeling has been used to examine
606 RNA processing kinetics in budding and fission yeast[13,25,37].

608 Nevertheless, this method has some limitations, such as the relatively low yield of labeled RNA
609 recovered at the end of the whole protocol. While in yeast, there is no limitation in the starting
610 material, and this can be an issue when analyzing metazoan cells, specifically if this protocol is
611 being utilized *in vivo*. One of the limiting steps of the protocol is biotinylation of the labeled RNA
612 pool, whose efficiency is far from complete and was estimated to modify one in three 4sU
613 residues in labeled RNA[5]. It recently showed that the use of methanethiosulfonate (MTS)-biotin,
614 instead of biotin-HPDP, increases the yield of recovered RNA[38]. However, according to
615 unpublished results from this research group and results from Rutkowski and Dölken, MTS-biotin
616 is not fully thiol-specific, leading to the purification of unlabeled RNA, which is particularly

617   problematic when low amounts of labeled RNA are purified[39]. Another limitation of the metabolic
618   labeling using 4tU/4sU is the inherent speed at which RNA polymerase elongates. The average
619   speed of RNA polymerase II was estimated to be approximately 3.5 kb/min[40-42]. Hence, in a 6-min
620   labeling, the polymerase will have the capacity to transcribe 15 - 20 kb. Thus, in an experiment
621   with a short pulse-labeling with 4tU/4sU, only the 3' end part of newly transcribed RNA is labeled,
622   while the 5' end regions were pre-existing before the addition of 4tU/4sU. Thus, the purification
623   of labeled RNAs also enriches pre-existing 5' ends of transcripts, particularly for long transcripts
624   as in mammalian cells. In order to overcome this bias, in a refined protocol called TT-seq, the
625   extracted labeled RNA is fragmented by sonication prior to the purification of newly synthesized
626   species[43].
627
628   Altogether, 4tU-seq is an excellent way to address transcriptional changes in a given context.
629   Nevertheless, and while the protocol is fairly simple, it consists of several different steps, being
630   ultimately relatively extensive. First and foremost, since this protocol handles RNA from start to
631   finish, it is mandatory that all the requirements necessary for a clean and degradation-free
632   sample are met. Hence, all reagents should be RNase-free, and all the materials should be
633   dedicated to manipulating RNA, cleaning everything thoroughly and regularly with RNase
634   decontamination solution. Second, while biotin reaction is highly specific, the excess of biotin-
635   HPDP that did not react should be removed from the sample (to avoid the saturation of the
636   streptavidin beads with biotin that is not covalently bound to RNA, for example). Third, as
637   described in the protocol, the use of the indicated streptavidin-coated magnetic beads is highly
638   recommended, since the several research groups we have talked to have all indicated that these
639   beads led to lower background levels. Fourth, appropriate quality and experimental controls
640   should be used. Include samples derived from cells that were not exposed to 4tU/4sU. These
641   samples will be of great value to address background levels and to make sure that, during the
642   experiment, a contamination with non-labeled RNA does not occur. Also, another excellent
643   alternative to test whether the sample is enriched for newly synthesized RNA is to perform an
644   RT-qPCR using primers against an intro-containing gene. The primers should be complementary
645   to the 3' end and 5' end of two consecutive exon and intron, or *vice versa*. Finally, prior to the
646   sequencing of microarray hybridization, validate the samples by RT-qPCR. For this, different
647   genes with different levels of expression and regulation should be selected and analyzed.
648   Optimally, this should be performed for the two different fractions of RNA (steady-state and
649   newly synthesized RNA).
650

658

659   **DISCLOSURES:**
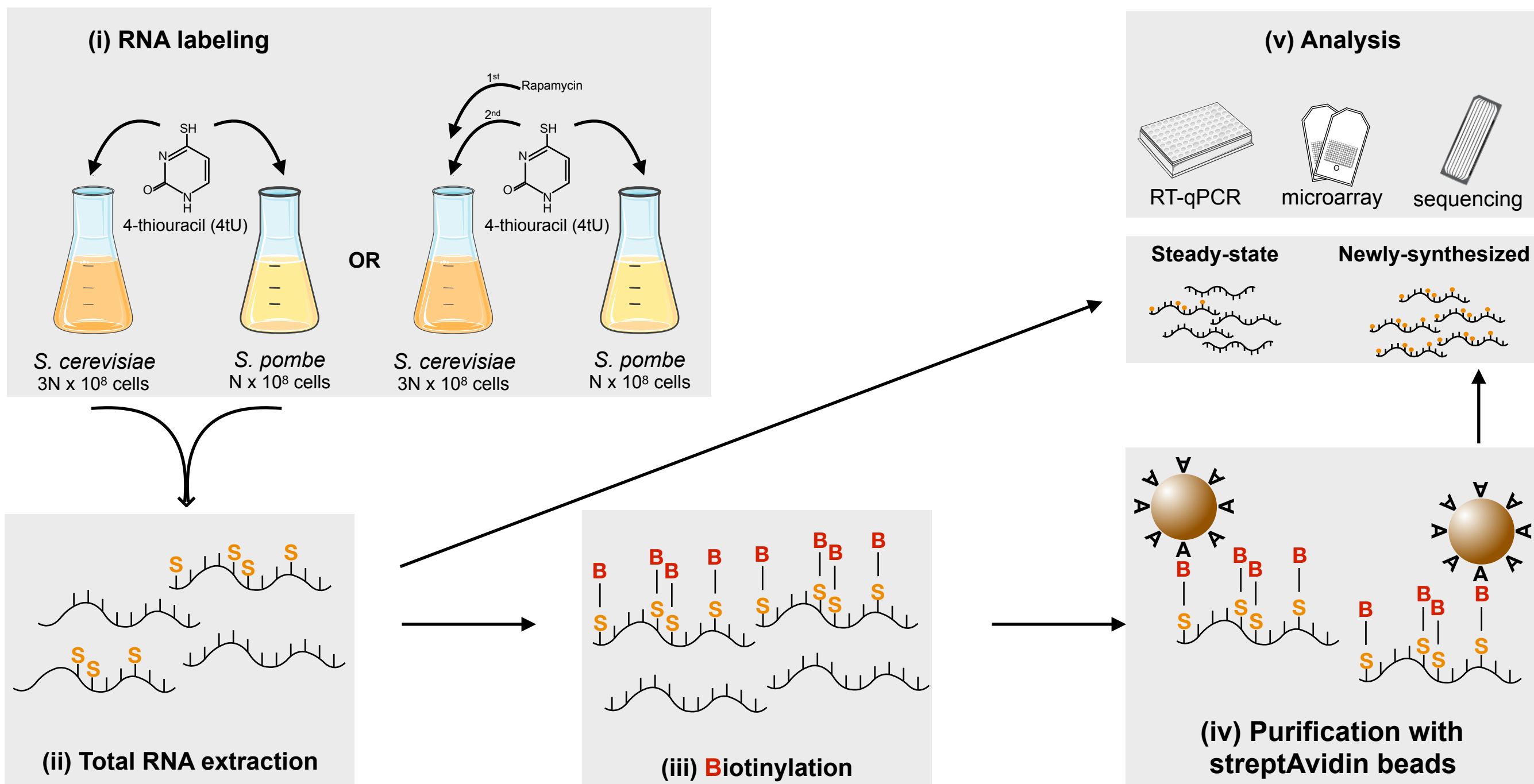660   The authors have nothing to disclose.

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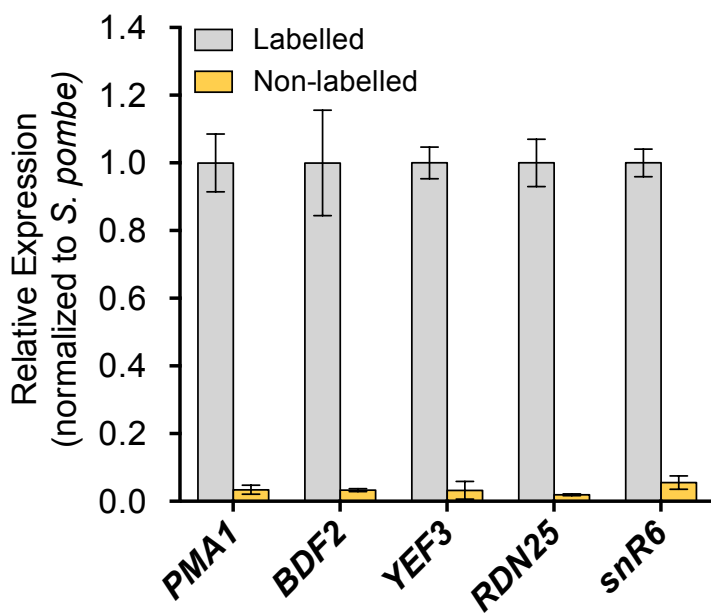791 *Molecular Biology.* **14** (9), 796-806 (2007).

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802

Figure 1   [Click here to download Figure Baptista Fig1.pdf ±](#)

Figure 1

Figure 2

Click here to download Figure Baptista Fig2.pdf ⬇

Wild-type cells

Unlabelled cells

Deletion strains

Total RNA

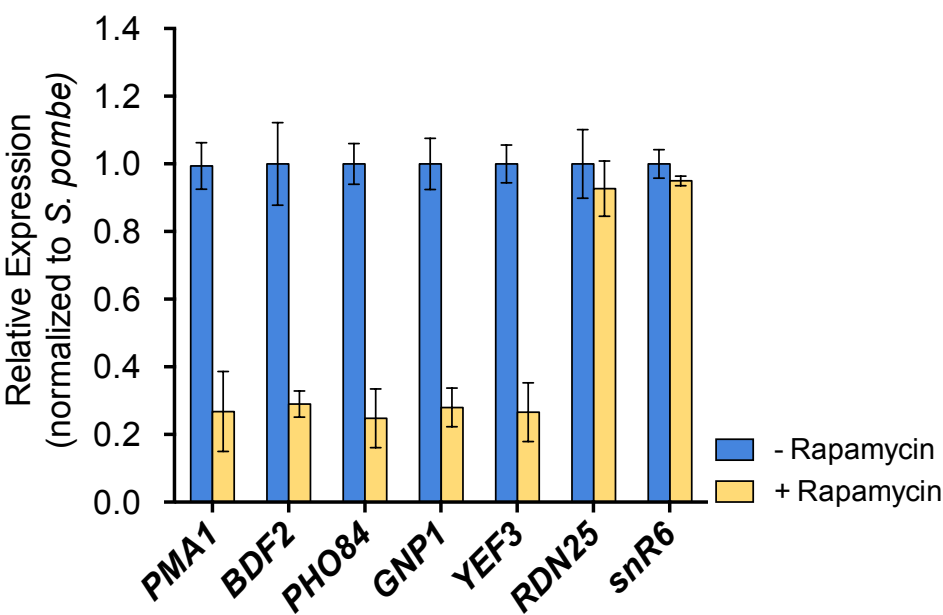Newly-synthesized RNA
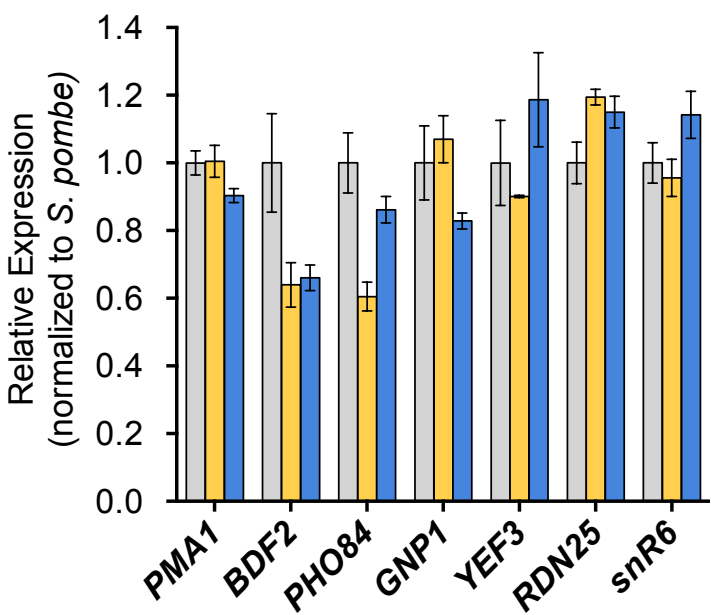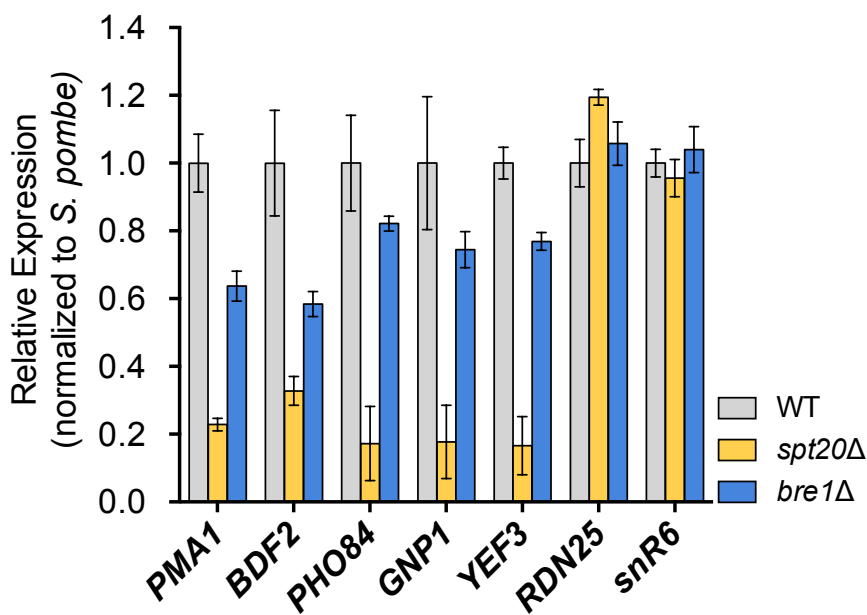
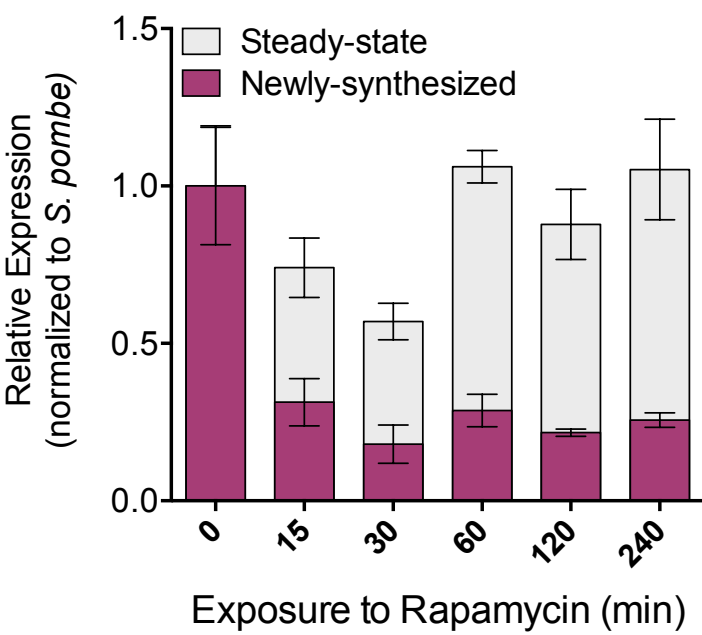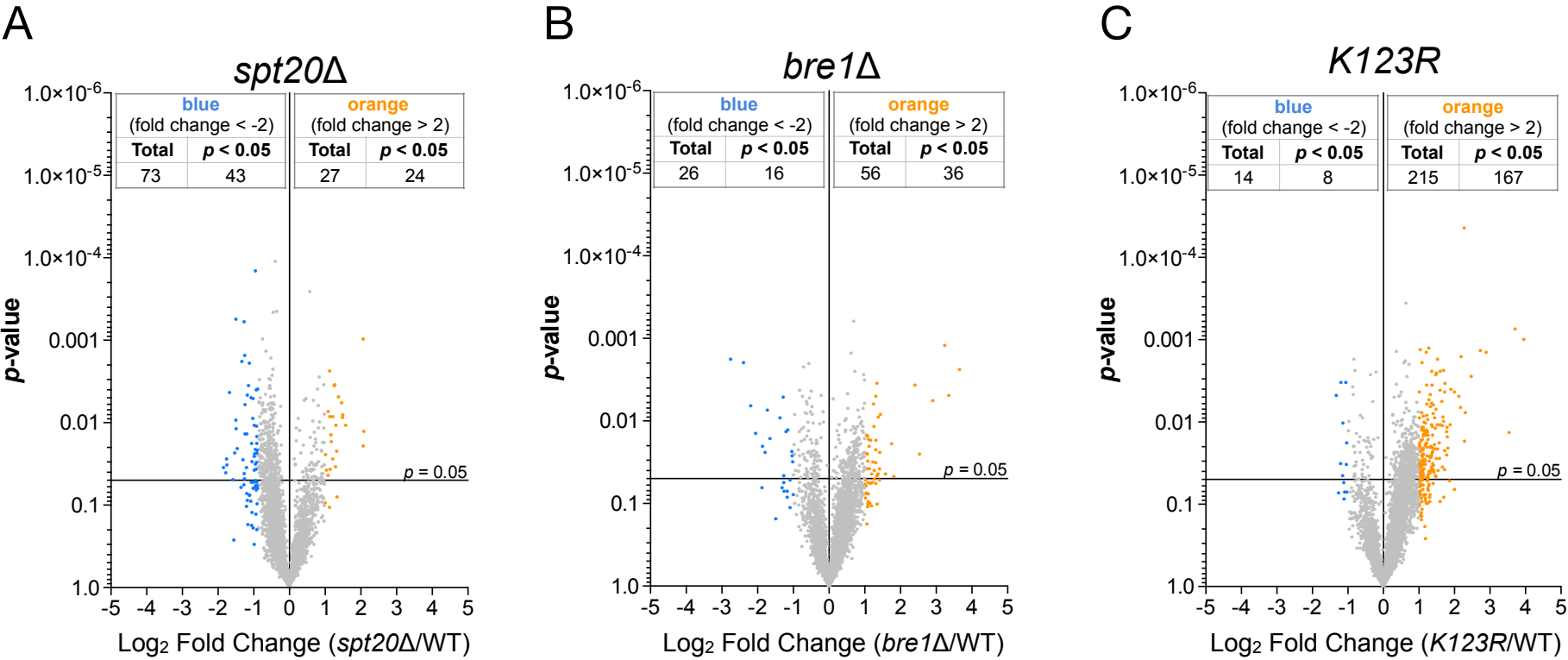**Spt7 anchor-away strain**

Total RNA

Newly-synthesized RNA

Figure 3
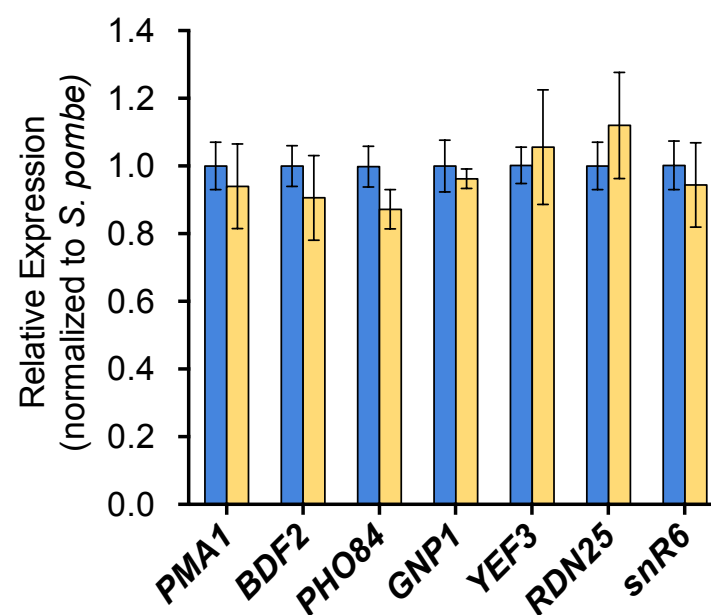
Total mRNA

Newly-synthesized mRNA

Figure 4
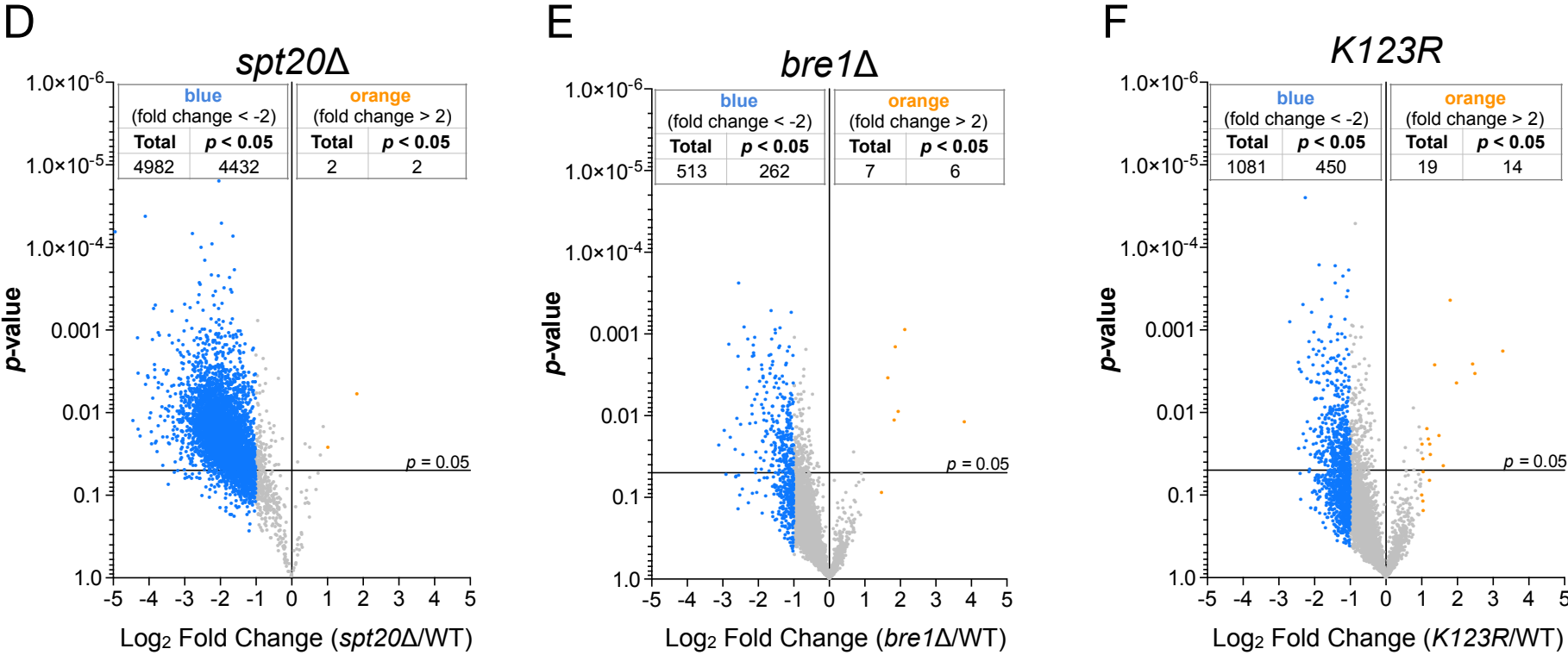
A

Global inhibition of RNA transcription

steady-state

without RNA buffering

with RNA buffering
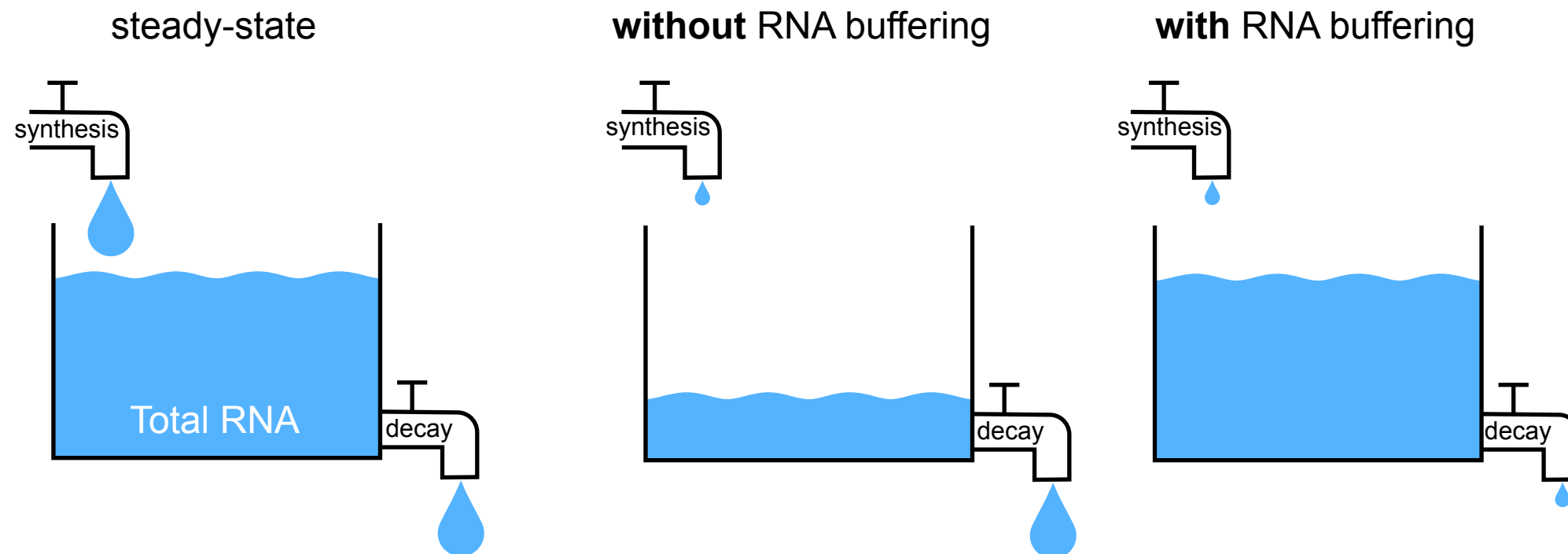
B

spt20Δ
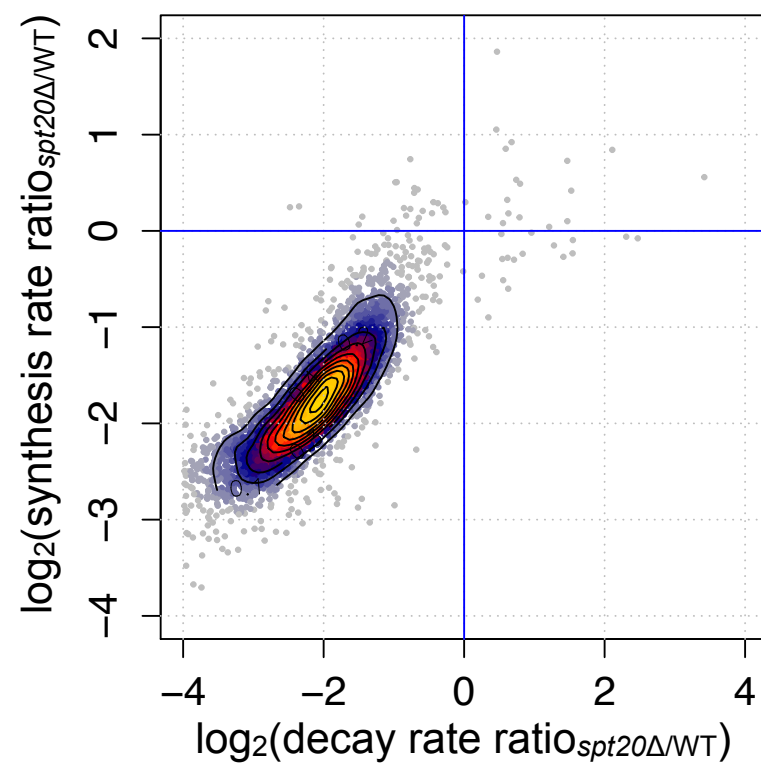
C

bre1Δ
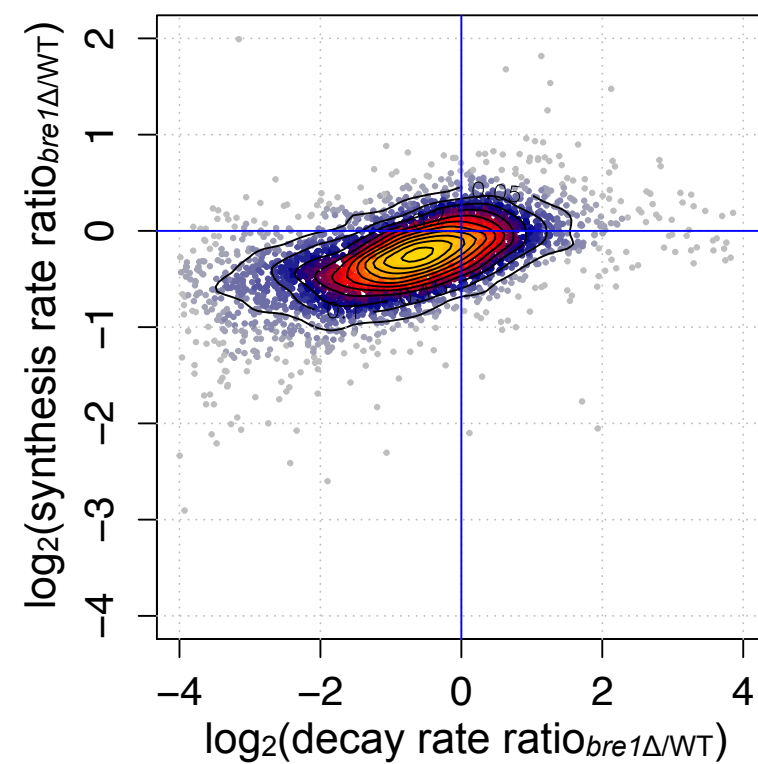
D

K123R
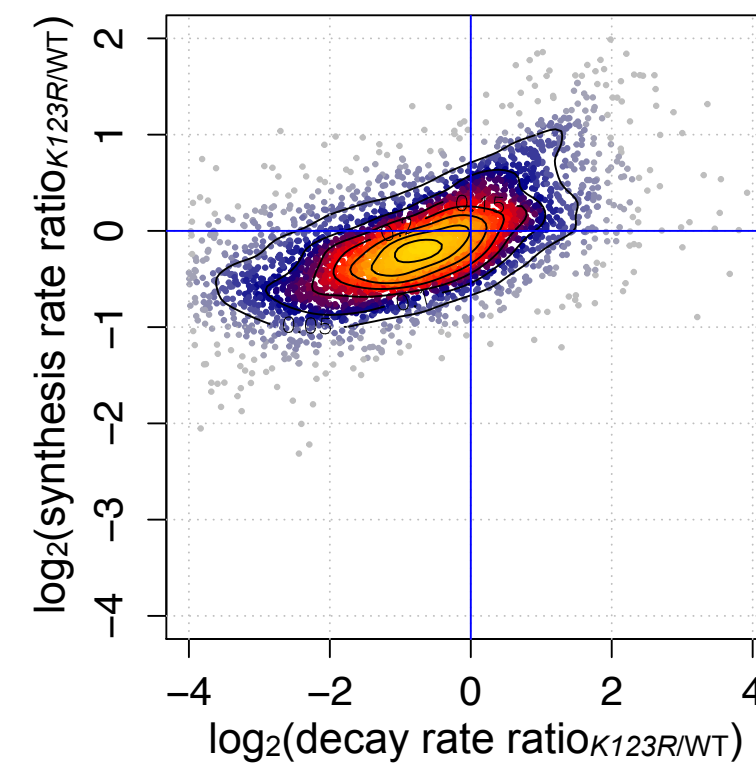
Name of Material/ Equipment	Company	Catalog Number	Comments/Description
4-Thiouracil	Sigma-Aldrich	Cat# 440736	
Rapamycin	Euromedex	Cat# SYN-1185	
Countess II FL Automated Cell Counter	ThermoFisher	N/A	
RiboPure RNA Purification kit, yeast	ThermoFisher	Cat# AM1926	
NanoDrop 2000 Spectrophotometer	ThermoFisher	ND-2000	
TURBO DNA-free Kit	ThermoFisher	AM1907	
EZ-Link HPDP Biotin	ThermoFisher	Cat# 21341	
Thiolutin	Abcam	ab143556	
µMACS Streptavidin kit	Miltenyi Biotec	Cat# 130-074-101	
Transcriptor Reverse Transcriptase	Roche	03 531 295 001	
SYBR Green I Master	Roche	4707516001	
GeneChip Yeast Genome 2.0	ThermoFisher	900555	
GeneChip Fluidics Station 450	ThermoFisher	00-0079	
GeneChip Scanner 3000 7G	ThermoFisher	00-0210	

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Strasbourg, 19th of June, 2018

Dear Editor,

You will find enclosed a second revision of our manuscript entitled "*Saccharomyces cerevisiae* metabolic labeling with 4-thiouracil and quantification of newly-synthesized mRNA as a proxy for RNA polymerase II activity" by Tiago Baptista and Didier Devys that we wish to submit for publication to JoVE. I apologize for the delay in providing our revised manuscript.

In this revised version, we made changes to address Editorial comments. We hope that you will find these changes adequate.

You will find below a point by point answer to the editor's comments.

Thank you for considering our revised manuscript.

Best regards,
Didier Devys

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We carefully proofread the manuscript and we hope that there no remaining spelling mistakes.

2. There are still a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.
We did our best to avoid overlap with previously published work. The only significant overlap is with our earlier Molecular Cell paper (Baptista et al., 2017) which can be expected. Moreover, it is not so easy for non-native English speaker to use always original language which I could do more easily in French.

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