Chapter 2

Materials and methods

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S. pombe strains

Most experiments were performed with the wild-type *972 h*$^-$ strain, with the exception of the experiments performed to study the translational response to the deletion of *rmt3*, where the following strains were used: FBY14 (*ade6M210 leu1-32 ura4-D18 his3-D1*) and FBY18 (*ade6M216 leu1-32 ura4-D18 his3-D1 rmt3::ura4*). Furthermore, induction and shut-off experiments of *pom1* and *rpb4* under the control of various *nmt1* promoters were performed with these strains: JB150 *(kanMX6-41nmt1-pom1*), JB151 (*kanMX6-3nmt1-pom1*), JB172 (*h*$^-$ *kanMX6-3nmt1-3HA-pom1*), JB175 (*h*$^-$ *kanMX6-41nmt1-3HA-pom1*), JB178 (*h*$^-$ *kanMX6-81nmt1-3HA-pom1*), JB394 (*h*$^-$ *kanMX6-3nmt1-rpb4*), JB395 (*h*$^-$ *kanMX6-41nmt1-rpb4*), and JB396 (*h*$^-$ *kanMX6-81nmt1-rpb4*).

S. pombe growth conditions

Cells were grown in full medium (supplemented yeast extract medium YES) or in Edinburgh minimal medium (EMM) (Moreno et al. 1991) at 32°C, except for FBY14 and FBY18, which were grown at 30°C, to a concentration of 0.3 - 0.7 OD_{600}.

For repression of genes under the control of the *nmt1* promoter, the corresponding strains were grown in EMM medium supplemented with 15 μM thiamine. To induce expression from the *nmt1* promoter, strains were washed and re-suspended in EMM medium lacking thiamine.

For the stress experiments, cells were grown in YES at 32°C. Oxidative stress was induced by the addition of H_2O_2 (SIGMA) to a final concentration of 0.5 mM, DNA-damage was induced by the addition of MMS (Fluka) to a final concentration of 0.02 % v/v, and heat stress was induced by moving the culture flask with the growing cells from 32°C to 39°C in a water bath.

Translational profiling

High-resolution translational profiling

Polysome fractionation, RNA extraction and microarray hybridizations. Cycloheximide was added to a final concentration of 100 µg/ml for 5 minutes (min) before harvesting aliquots of 50 OD_{600} of cells by centrifugation at 4ºC and washing in polysome lysis buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl, 1 mM DTT, 100 µg/ml cycloheximide, 200 µg/ml heparin). Cells were resuspended in 100 µl polysome lysis buffer with 40 U/ml RNasin (Promega) and complete protease inhibitor cocktail (Roche). Cells were lysed in 1.5 ml tubes by using a Fastprep cell disruptor with 1 g of chilled glass beads (BioSpec Products). After lysis, 400 µl of lysis buffer was added and a hole was poked in the bottom of the tube to separate the lysate by centrifugation into another 1.5 ml tube. The lysate was cleared by two centrifugations at 4ºC. Aliquots corresponding to 25 OD_{260} units in 350 µl were loaded onto an 11 ml linear 10-50% (w/v) sucrose gradient, which was prepared with the Gradient Master (Biocomp) from 10% and 50% sucrose solutions (w/v) prepared with polysome lysis buffer. The gradients were separated by centrifugation for 160 min at 35,000 rpm in a SW 40Ti rotor (Beckman). The gradients were then fractionated by upward displacement with 55% (w/v) sucrose, and fractions of ~900 µl were collected directly into tubes containing 2 ml of 8 M Guanidium-HCl using an Isco fractionation system. Corresponding fractions from 3 gradients were pooled and 5 *in vitro*-transcribed *Bacillus subtilis* mRNAs (Lyne et al. 2003) were added for normalization in the following amounts: Trp 0.09 ng/µl, Dap 0.45 ng/µl, Lys 0.9 ng/µl, Phe 4.5 ng/µl, and Thr 9 ng/µl. After addition of an equal volume of 100% ethanol, RNA from each fraction was precipitated overnight at -20ºC and centrifuged at 4ºC for 90 min. Further purification of the RNA by phenol:chloroform extraction and LiCl-precipitation was performed as described by Arava et al. (2003). RNA from each pellet was then resuspended in 20 µl of DEPC-treated H_2O. 10 µl of the RNA from each fraction was used for microarray analysis and labelled using oligo(dT)-primers and the SuperScript™ Direct cDNA Labeling System (Invitrogen) and Cy3/Cy5-dCTP (Amersham). Polysomal RNA from each fraction was hybridized against 20 µg of total RNA extracted using the hot-phenol method. Half the amount of bacterial mRNAs indicated above was added to each aliquot of total RNA before labeling. Hybridization to microarrays, slide-washing and scanning of microarrays

was performed as described (see below). Polysome profiling was performed in triplicate from three independent biological repeats, including a dye swap.

Analysis of high-resolution translational profiling data. Data from high-resolution translational profiling cannot be normalized on the assumption that the overall RNA levels from the competitive hybridization are similar between the two RNA samples. Spiked bacterial mRNAs were therefore used to normalize for different amounts of RNA in the individual fractions. Probes for the bacterial mRNAs on the microarray were first normalized locally to obtain an average signal ratio of 1. Normalization based on signal intensities was then done as described (see below). Fraction 10 in the 3rd repeat was a clear outlier showing signal intensities roughly twice as high as in the other 2 repeats and higher signals than its neighbouring fractions. Thus, all ratios for this fraction were divided by a correction factor, which was calculated based on the best correlation to the other repeats. Data and conclusions presented here did not change if the 3rd experimental repeat was omitted, but the number of mRNAs included in the data set was reduced to 3020. To be included for further analysis, mRNAs had to fulfil the following criteria: 1) there had to be microarray data for all 12 fractions from at least 2 out of the 3 repeats, excluding 1012 mRNAs, and 2) the Pearson correlation between profiles for the same gene from the different repeats had to be $\geq$0.7 (if present in all 3 repeats) or at $\geq$0.75 (if present in only 2 repeats), excluding 352 mRNAs. These criteria were fulfilled by 3598 out of the 4962 nuclear encoded protein-coding genes.

Translational properties were then determined to estimate translational efficiencies for different mRNAs. Translation profiles were calculated as the percentages of a given mRNA in each of the 12 fractions such that the total over all fractions is 100%. Ribosome occupancy for a given mRNA was calculated by adding up the percentages of this mRNA in fractions 5 to 12, which are associated with ribosomes. For the mean number of associated ribosomes, we calculated the percentages of a given mRNA for each fraction associated with ribosomes such that the total of fractions 5 to 12 was 100%. The percentage of mRNA in each fraction was then multiplied with the corresponding estimate for associated ribosomes, and these values were added up to estimate the mean number of ribosomes bound to this mRNA. (Associated ribosome numbers for each fraction were estimated by plotting the defined peaks from the polysome profile, containing 1 to 8 ribosomes, against the relative distance from the

start of the profile and fitting an exponential curve; this curve was used to determine ribosome numbers at the beginning and end of a given fraction, which were averaged to produce the mean number of ribosomes associated with this fraction.) Ribosome densities represent the mean number of ribosomes associated with each mRNA divided by its ORF length. All values were determined individually for each biological repeat and then averaged.

Medium-resolution profiling

Polysome fractionation, RNA extraction and microarray hybridizations. To study the translational response to environmental stress, medium-resolution translational profiling was performed. Preparation of cell lysates and polysome fractionation was essentially done as described for the high-resolution translational profiling, except that cycloheximide was added directly when cells were harvested. Twelve fractions were collected during polysome fractionation. Fractions 1-3, 4-6, 7-9, and 10-12 were united respectively into 4 pools. RNA from each pool was precipitated overnight at -20ºC after the addition of an equal volume of 100% ethanol. After centrifugation at 4ºC for 90 min, the pellet was air dried, and dissolved in 100 µl DEPC-treated H_2O. The RNA was then purified using RNeasy columns (Qiagen) and eluted with 30 µl DEPC-treated H_2O. 10 µl of the RNA from each of the 4 pools was used for microarray analysis and labelled using a mix of oligo(dT)-primers and random hexameres and the SuperScript™ Direct cDNA Labeling System (Invitrogen) and Cy3/Cy5-dCTP (Amersham). RNA from each pool was hybridized against labelled genomic DNA as reference (see below). Two (heat stress, DNA damage) or three (oxidative stress) complete biological repeats including a dye swap were performed.

Analysis of medium-resolution translational profiling data. Microarray-data from each of the 4 pools were normalized using our standard normalization script, which removes spots with unreliable or low signal and locally adjusts the median of ratios to 1 within a sliding window (see below). For each mRNA, where data was obtained in all 4 pools, translation profiles were calculated as the percentages of a given mRNA in each of the 4 pools such that the total over all pools is 100%. Two approaches were used to identify altered translational profiles comparing the stress condition and the corresponding control. First, the total difference between the two corresponding