LETTER

doi:10.1038/nature09338

Identification of a quality-control mechanism for mRNA 5′-end capping

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The 7-methylguanosine cap structure at the 5′ end of eukaryotic messenger RNAs is a critical determinant of their stability and translational efficiency[1–3]. It is generally believed that 5′-end capping is a constitutive process that occurs during mRNA maturation and lacks the need for a quality-control mechanism to ensure its fidelity. We recently reported that the yeast Rai1 protein has pyrophosphohydrolase activity towards mRNAs lacking a 5′-end cap[4]. Here we show that, *in vitro* as well as in yeast cells, Rai1 possesses a novel decapping endonuclease activity that can also remove the entire cap structure dinucleotide from an mRNA. This activity is targeted preferentially towards mRNAs with unmethylated caps in contrast to the canonical decapping enzyme, Dcp2, which targets mRNAs with a methylated cap. Capped but unmethylated mRNAs generated in yeast cells with a defect in the methyltransferase gene are more stable in a *rai1*-gene-disrupted background. Moreover, *rai1Δ* yeast cells with wild-type capping enzymes show significant accumulation of mRNAs with 5′-end capping defects under nutritional stress conditions of glucose starvation or amino acid starvation. These findings provide evidence that 5′-end capping is not a constitutive process that necessarily always proceeds to completion and demonstrates that Rai1 has an essential role in clearing mRNAs with aberrant 5′-end caps. We propose that Rai1 is involved in an as yet uncharacterized quality control process that ensures mRNA 5′-end integrity by an aberrant-cap-mediated mRNA decay mechanism.

The stability and translational efficiency of eukaryotic mRNAs are both influenced by the 5′-end cap[1–3]. The cap is co-transcriptionally added and consists of a guanine nucleoside methylated at the N7 position attached to the terminal nucleoside of the RNA by an unusual 5′–5′ pyrophosphate linkage[5,6]. Capping is carried out by a combination of three enzymatic activities[5,7], consisting of a triphosphatase, guanylyltransferase and methyltransferase[8]. Three different proteins carry out the distinct activities in yeast whereas the triphosphatase and guanylyltransferase activities are carried out by a single bifunctional protein in mammals[9]. The presence of the methyl group on the cap is essential for recognition by the cap-binding proteins CBP and eIF4E as well as the scavenger decapping enzyme DcpS[10,11]. Removal of the cap is a regulated process catalysed by the Dcp2 decapping enzyme to release m^7GDP (containing the N7 methyl moiety) and monophosphate RNA[12–14]. The exposed 5′-monophosphate RNA is subsequently subjected to degradation by the cytoplasmic 5′→3′ exoribonuclease Xrn1 to clear the mRNA body[15,16]. Interestingly, Dcp2 functions on a cap substrate with an N7 methyl moiety and does not function on an unmethylated cap[13]. This latter point raises an interesting question regarding the fate of mRNAs aberrantly lacking cap methylation.

Rai1 is a pyrophosphohydrolase that hydrolyses the 5′-end triphosphate of an uncapped RNA to release diphosphate and a monophosphorylated 5′-end RNA that could be degraded by the Rai1-interacting 5′→3′ nuclear exoribonuclease Rat1[4], indicating a possible role of Rai1 in a quality-control mechanism for 5′-end capping. To address this possibility, the activity of Rai1 on unmethylated capped mRNAs was tested. *In vitro* generated capped RNAs containing or lacking the N7 methyl moiety (m^7GpppRNA and GpppRNA, respectively) were labelled either within the 5′ cap at the γ phosphate relative to the mRNA, or uniformly labelled throughout the RNA body, and incubated with recombinant Rat1, Rai1 or both proteins simultaneously. As expected, Rat1 did not appreciably decrease the level of either full-length RNA (Fig. 1a, lanes 1–4) because they lack the required 5′-end monophosphate. Incubation of the RNAs with Rai1, however, revealed a marked decrease in the level of 5′-end-labelled unmethylated RNA (Fig. 1a, lanes 5–8, and Fig. 1b), which was further stimulated by Rat1 (Fig. 1a, lanes 9–12, and Fig. 1b). The preferential decay of unmethylated capped RNA was a function of Rai1; modest levels of reaction products were detected when this RNA was incubated with Rai1 protein (Fig. 1c, lane 4) or a single point mutant compromised in its ability to interact with Rat1 (Rai1(W159A); lane 5). As expected, background levels of activity were detected from the catalytically inactive Rai1 mutant protein containing substitutions in the cation-binding residues[4] (Rai1(E199A/D201A), lane 6). Importantly, Rai1 hydrolysis activity was stimulated by Rat1 (lane 8) and the stimulation was attenuated with a mutant Rai1 compromised in its ability to interact with Rat1 (lane 9). Consistent with the decay results above, undetectable levels of reaction products were observed on 5′-end methyl-capped RNAs (lane 14) with a relatively modest increase following the addition of Rat1 (lane 18). A direct comparison of methylated and unmethylated capped RNA decapping showed at least a tenfold greater activity on the cap lacking a methyl moiety (Fig. 1d). Moreover, the activity is restricted to capped RNA and does not function on cap structure, as hydrolysis of cap structures lacking the linked RNA was not detected (Supplementary Fig. 1). Collectively, these data demonstrate that Rai1 can preferentially remove the cap from an unmethylated 5′-end capped RNA and that this activity is enhanced by Rat1.

Surprisingly, enzymatic tests confirmed that the Rai1-decapping products corresponded to the cap analogue GpppG or m^7GpppG (Supplementary Fig. 2). Therefore, unlike the pyrophosphohydrolase activity of Dcp2 decapping, which releases m^7Gpp[12,13], or of Rai1 itself on a 5′-triphosphate RNA, which releases PPi diphosphate[4], the presence of a cap guanosine on the triphosphorylated 5′-end of an RNA converts the pyrophosphohydrolase activity of Rai1 into a phosphodiesterase-decapping endonuclease that releases the entire cap structure (GpppN) from an unmethylated capped RNA with comparable efficiencies (Supplementary Fig. 3). Nevertheless, similar to canonical decapping, the decapping endonuclease activity of Rai1 also generates a 5′-monophosphorylated RNA that is a substrate for Rat1 exoribonuclease-directed mRNA decay.

The Rai1-mediated selective hydrolysis of mRNA caps lacking the methyl residue was also observed in cells using a yeast *abd1-5* strain harbouring a temperature-sensitive cap methyltransferase. The *abd1-5* strain is unable to methylate the 5′ cap at the 37 °C non-permissive temperature and results in a reduction of *PGK1* and *ACT1* mRNA levels[17]. We reasoned that the stability of mRNAs in an *abd1-5 rai1Δ* double mutant background should increase if Rai1 was involved in hydrolysing the aberrant unmethylated capped cellular mRNAs that would be produced at the non-permissive temperature. Following a

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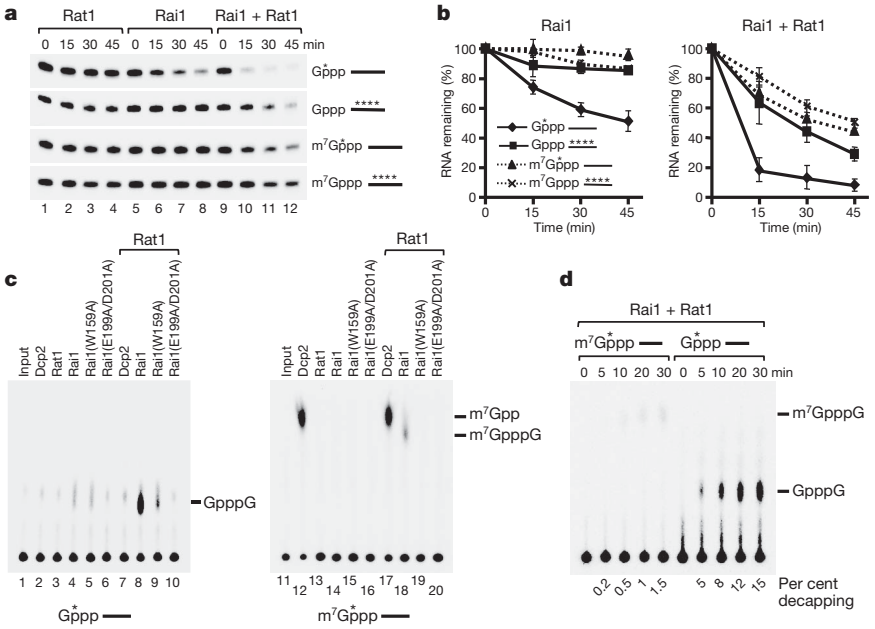
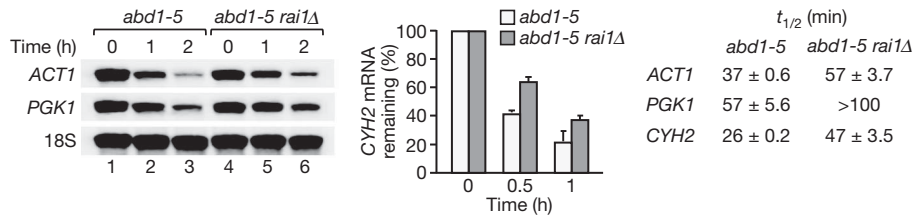


Figure 1 | Rai1 preferentially hydrolyses unmethylated capped RNA. **a**, *In vitro* transcribed ^{32}P-cap-labelled or uniform-labelled pcP RNAs with a methylated or unmethylated cap were subjected to Rat1 or Rai1 proteins and the decay of the RNAs followed at the indicated times. The RNAs used are denoted on the right and the asterisks represent the position of the ^{32}P labelling. **b**, Graph quantifying the amount of RNA remaining in the assays in **a** after Rai1 or Rai1 plus Rat1 treatment. Data are from three independent experiments normalized to a ^{32}P-labelled DNA-oligonucleotide loading control included in the stop buffer and presented relative to time zero. Error bars represent data ± 1 standard deviation (s.d.). **c**, *In vitro* decapping assays were carried out at 37 °C for 15 min as in **a** with the indicated proteins, and decapping products were resolved by polyethyleneimine-cellulose thin-layer chromatography (PEI-TLC) developed in 0.45 M $(NH_4)_2SO_4$. Migration of the cap analogue markers are shown on the right. Human Dcp2 was used as a methyl-capped-RNA-decapping positive control (panels 2, 7 and panels 12, 17). **d**, *In vitro* decapping assay using ^{32}P-cap-labelled methylated or unmethylated 5′-capped pcP RNAs were carried out with 50 nM Rat1 and Rai1 recombinant proteins for the indicated times and resolved as in **c**. Per cent decapping of three independent experiments is presented on the bottom.

45-min shift to the non-permissive temperature to enable the accumulation of unmethylated capped mRNAs, thiolutin was added to block transcription and mRNA levels were determined. Consistent with a role of Rai1 in modulating the stability of unmethylated capped mRNAs in cells, a stabilization of the *PGK1*, *ACT1* and *CYH2* (also known as *RPL28*) mRNAs was observed in the *abd1-5 rai1Δ* background (Fig. 2). These data demonstrate that the methylation state of the mRNA cap can determine its susceptibility to Rai1 and that Rai1 functions normally to clear aberrant mRNAs with an unmethylated 5′ cap.

Next we addressed the physiological significance of Rai1 decapping for mRNA stability. The stability of the *PGK1* and *ACT1* mRNAs was comparable regardless of whether Rai1 was present when cells were grown in complete media (Fig. 3a). However, considerable stabilization of the mRNAs was observed when cells were exposed to nutritional stress. Both mRNAs were substantially more stable in the *rai1Δ* strain after glucose starvation (Fig. 3b) or amino acid starvation (Fig. 3c) relative to the wild-type strain grown in the same media. These results demonstrate the importance of Rai1 in the modulation of mRNA stability after the exposure of yeast cells to stress. Although previous studies have indicated that mRNAs seem stable after nutrient stress[18], the discrepancy between our results and that of others is based on the timing of the analysis, where we set time zero at 45 min after removal of the nutrient source rather than at the time of depletion. Incorporation of the lag period provided an opportunity for a majority of the capped and methylated mRNAs that were transcribed before the onset of nutrient depletion to be cleared.

A surprising implication of these results is that mRNAs with an aberrant 5′ end are generated in yeast cells after nutrient starvation. To begin testing this hypothesis, steady-state RNA was isolated from *rai1Δ* cells grown for 45 min in either complete medium or nutrient-deprived medium. Cells were fractionated into polysome-containing P130 pellet and soluble S130 mRNP fractions. The relative ratio of the *CYH2* mRNA distribution shifted from the polysome-containing fraction to the mRNP fraction in amino-acid-starved cells, and this difference was further evident in the *rai1Δ* strain (Fig. 4a), indicating that



	$t_{1/2}$ (min)	
	abd1-5	*abd1-5 rai1Δ*
ACT1	37 ± 0.6	57 ± 3.7
PGK1	57 ± 5.6	>100
CYH2	26 ± 0.2	47 ± 3.5

Figure 2 | Rai1 preferentially hydrolyses unmethylated 5′-end-capped mRNA in yeast cells. Yeast strains harbouring the temperature-sensitive *ABD1* methyltransferase mutant allele, *abd1-5*, or the *abd1-5 rai1Δ* double mutation were grown at the 37 °C non-permissive temperature for 45 min before transcriptional block with 5 μg ml^{-1} thiolutin. RNA was isolated from cells at the indicated time points after thiolutin addition and the levels of RNA remaining were determined by northern blot analysis (*PGK1* and *ACT1*) or quantitative polymerase chain reaction with reverse transcription (RT–qPCR) (*CYH2*). Half-lives ($t_{1/2}$) of the mRNAs were determined relative to the 18S ribosomal RNA and were derived from three independent experiments. The range of half-lives obtained are consistent with previously reported thiolutin-directed transcriptional arrest measurements[30].

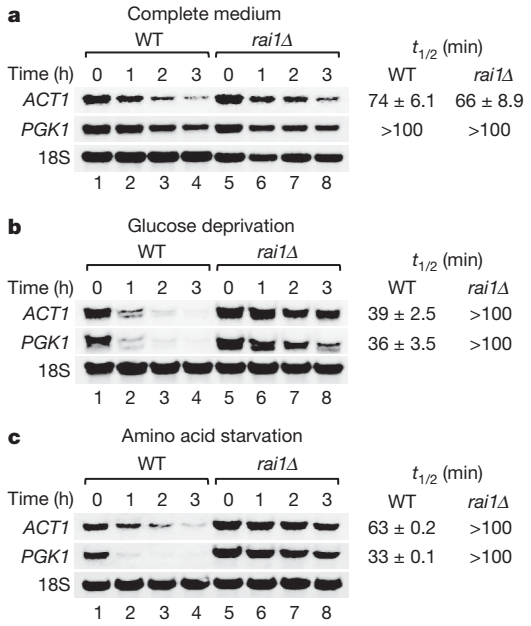


Figure 3 | Rai1 functions to clear mRNAs in cells subjected to glucose starvation or amino acid starvation. **a**–**c**,Wild-type (WT) or *rai1Δ* yeast strains were grown in complete medium at 30 °C to an $OD_{600\,nm}$ of 0.6 and subsequently cultured in complete medium (**a**), glucose-deficient medium (**b**) or amino-acid-deficient medium (**c**) for 30 min followed by addition of 5 μg ml^{-1} thiolutin. Total RNAs were isolated and detected at the indicated times after thiolutin addition by northern blot analysis, and RNA half-lives ($t_{1/2}$) quantified from three independent experiments normalized relative to the 18S rRNA are presented.

nutrient starvation promotes the generation of aberrantly capped mRNA. The presence of an aberrant 5′ cap after nutrient starvation was further substantiated in a strain disrupted for the methyl-cap-specific decapping enzyme, Dcp2. The increased mRNA stability after nutrient starvation in the *rai1Δ* background was not observed in a *dcp2Δ* background (Fig. 4b), indicating the presence of an aberrant 5′ cap resistant to Dcp2 upon nutrient starvation. Furthermore, determination of the steady-state levels of methylated capped *PGK1* and *ACT1* mRNAs by immunopurification with an anti-cap column, which specifically retains methylated but not unmethylated capped RNA (Supplementary Fig. 4), showed a 25% and 55% reduction in methylated species when *rai1Δ* cells were grown in glucose- or amino-acid-deficient medium relative to cells grown in complete medium (Fig. 4c). Therefore, despite the increase in *ACT1* and *PGK1* mRNA stabilities in the *rai1Δ* cells on nutrient starvation (Fig. 3), a decrease in the extent of methylated capped mRNA is observed under these conditions (Fig. 4c), indicating that nutrient starvation leads to the accumulation of mRNAs with aberrant 5′ ends. In addition, absence of the methyl moiety does not seem to be a consequence of demethylation after proper capping, as the ratio of spliced and unspliced *CYH2* mRNAs containing an aberrant cap remained constant under both normal conditions and nutrient starvation (Supplementary Fig. 5). Therefore, the aberrant cap does not seem to be generated from a methylated cap precursor.

Overall, we have shown that mRNA cap methylation is a regulated process that can be diminished on exposure of yeast cells to glucose or amino acid starvation. Our data, combined with reports demonstrating a stimulation in general cap methylation by Impα[19] or specific methylation by c-Myc[20] and transcriptionally linked uncapped RNAs[21], indicates that addition of the cap and methyl moiety are regulated processes that affect overall gene expression. Moreover, the accumulation of aberrantly capped mRNAs on nutrient starvation and the importance of Rai1 in clearing these mRNAs define a novel regulatory mechanism to potentially control the translation of mRNA transcribed during stress. Furthermore, Rai1 defines a new class of decapping endonuclease that specifically removes the cap structure and promotes the decay of mRNAs possessing unmethylated capped 5′ ends. The monophosphorylated 5′-end-containing mRNA products generated by Rai1 are probably degraded by Rat1, although cytoplasmic recapping[22] and recycling into the translationally competent mRNA pool

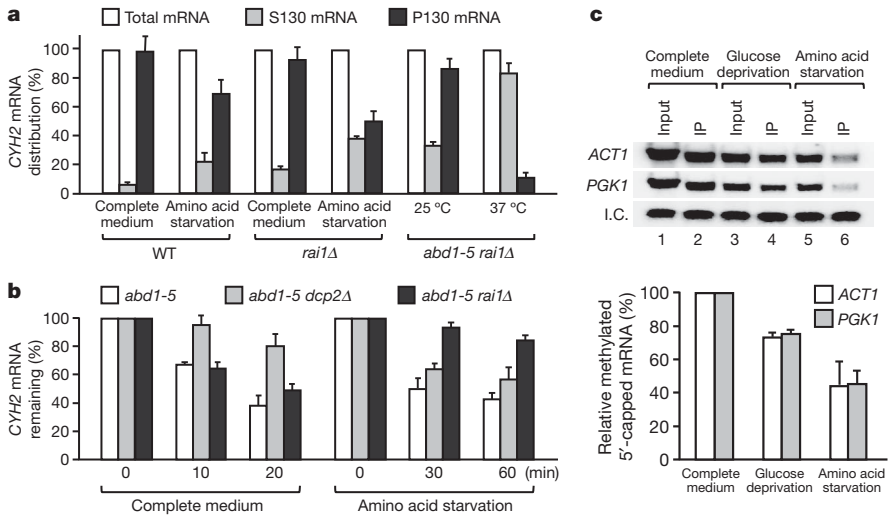


Figure 4 | Aberrantly capped mRNA levels increase in cells exposed to nutrient starvation. **a**, Amino acid starvation shifts mRNAs into the soluble mRNP fraction. The mid-log-phase yeast strains were shifted to the indicated medium and grown for 45 min before fractionation. RNA was isolated from polysome-containing fractions sedimenting at 130,000*g* (P130) and the supernatant (S130) fraction, which contained the soluble mRNP. Distribution of the *CYH2* mRNA from each fraction was determined by RT–qPCR. The *abd1-5 rai1Δ* double mutant strain grown at the permissive 25 °C (methylated capped mRNA) or non-permissive 37 °C (unmethylated capped mRNA) were used as a positive control. Results of three independent experiments are presented with error bars denoting data ± 1 s.d. **b**, Aberrantly capped mRNAs are minimally affected by the Dcp2 decapping enzyme. The indicated strains were grown in complete medium at 22 °C to an $OD_{600\,nm}$ of 0.6 and subsequently cultured in the same medium or amino acid minus medium for 45 min followed by the addition of thiolutin. The levels of *CYH2* mRNA were determined by RT–qPCR as in **a**. **c**, Methylated capped RNA was immunopurified using monoclonal anti-trimethylguanosine antibody column from cells grown at the denoted culture conditions for 45 min and were detected by northern blot analysis. Quantifications for the mRNA cap methylation were normalized to total input RNA and ^{32}P-labelled methylated capped pcP RNA internal control (I.C.) and derived from three independent experiments. IP, immunoprecipitation. The error bars represent data ± 1 s.d.

cannot be ruled out. Collectively, our data indicate that Rai1 initiates a quality-control mechanism that clears aberrant 5′-end-containing mRNAs, whether they lack a cap[4] or contain an unmethylated cap, and defines a new aberrant-cap-mediated mRNA decay surveillance mechanism that functions under normal and stress conditions to ensure the integrity of mRNA 5′ ends.

METHODS SUMMARY

RNA *in vitro* decay assay. The indicated recombinant proteins (50 nM) were incubated with ^{32}P-cap-labelled or ^{32}P-uniform-labelled pcDNA3 polylinker (pcP) generic RNA containing either m^{7}Gppp or Gppp at the 5′ end in decapping buffer as previously described[23]. The decay products were resolved by 8% urea-denaturing polyacrylamide gel electrophoresis to visualize the RNA or by polyethyleneimine-cellulose thin-layer chromatography (PEI-TLC) plates developed in 0.45 M $(NH_4)_2SO_4$ at 25 °C to detect decapping products as noted. Quantifications were carried out using a Molecular Dynamics PhosphorImager (Storm860) with ImageQuant-5 software.

Yeast growth conditions and RNA isolation. Yeast strains were grown in complete medium at 30 °C to mid-log phase and switched to medium lacking glucose or amino acids as described[24,25]. Following 30 min of growth in the new medium conditions, transcription was terminated with the addition of thiolutin (5 μg ml^{-1}) and cells were harvested at 0, 1, 2 or 3 h time points. In Fig. 2, *abd1-5* temperature-sensitive strains were grown in complete medium lacking tryptophan until mid-log phase at 25 °C and shifted to the nonpermissive 37 °C for 45 min to abolish methyltransferase activity. Cells are viable for several hours at 37 °C[17]. In Fig. 4b, strains were cultured at 22 °C to minimize further the temperature-sensitive phenotype. The cells were harvested by centrifugation and stored as pellets at −80 °C. Total RNAs were isolated with the acidic hot phenol method[26].

Cap antibody immunoprecipitations and northern blotting. Methylated capped mRNA was immunoprecipitated from 20 μg of yeast total RNA with an agarose-conjugated 2,2,7-trimethylguanosine antibody column (Calbiochem) that immunopurifies monomethyl-capped mRNA as previously described[27,28]. RNAs were detected by northern blotting carried out with [α-^{32}P]dCTP-labelled gene-specific DNA probes as described[29].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 3 February; accepted 6 July 2010.
Published online 29 August 2010.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank B. Schwer for the *abd1-5* yeast strain and A. Shatkin for discussions. This research was supported by grants from the National Institutes of Health (NIH) to L.T. (GM077175) and M.K. (GM67005).

Author Contributions X.J. and M.K. conceived the project, analysed the data and wrote the manuscript. X.J. carried out the experiments. S.X. and L.T. provided the recombinant proteins. C.O. and C.E.M. generated the yeast mutant strains. All authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.K. (kiledjian@biology.rutgers.edu).

METHODS

Yeast strains. The genotypes of all the *Saccharomyces cerevisiae* strains used in this study are listed in Supplementary Table 1. *The abd1-5* strain has been reported[17]. The *rai1* gene disruption construct was isolated from the chromosomal DNA −708 to +600 of yeast knockout strain YGL246C using PCR amplification by forward primer 5′-CCATTTCTAACAAAGTGTACCAACGAGAAACG-3′ and reverse primer 5′-CCGCAAGATGCTAGATTAGCCCGAC-3′. The *dcp2* gene disruption construct was isolated from the chromosomal DNA of a heterozygous diploid *dcp2* knockout strain YNL118C by PCR amplification with a forward primer 5′-AGCTCATAGATAATCGTCGTAAGGCTGACAC-3′ and a reverse primer 5′-TCAAGTATGGCTAAGCCGTCACAATGTC-3′. The amplified fragments were inserted into pSTBlue-1 vector (EMD) to create the plasmids pSTB-raiΔ::kanMAX4 and pSTB-dcp2Δ::kanMAX4. The *raiΔ* mutant strain and *abd1-5 raiΔ* or *abd1-5 dcp2Δ* double mutant strains were generated by transformation of *ABD1* wild-type and *abd1-5* strains respectively with either the *rai1* or *dcp2* disruption cassette DNA fragments obtained by digesting pSTB-raiΔ::kanMAX4 with HpaI or digesting pSTB-dcp2Δ::kanMAX4 with KpnI/XhoI.

Protein expression and purification. Recombinant His-tagged Rat1, Rai1 and Rai1 mutant (Rai1(W156A) and Rai1(E199A/D201A)) proteins were expressed in *Escherichia coli* BL21 (DE3) Rosetta cells with pET26b vector (EMD) and purified by Ni-NTA (Qiagen) and gel-filtration chromatography (Sephacryl S-300; GE Healthcare) as described previously[4].

RNA generation. The pcDNA3 polylinker was amplified by PCR with SP6 promoter primer and T7 promoter primer containing 16 cytosines at the 5′ end, and was used as a template to reverse transcript RNA with SP6 RNA polymerase to generate the pcP RNA as previously described[13]. The resulting RNA following transcription with SP6 polymerase will yield an RNA containing 16 guanosines at the 3′ end to minimize 3′-end exonucleolytic decay[31]. 5′-end N7-methylated or unmethylated ^{32}P-cap-labelled pcP RNAs were generated with the vaccinia virus capping enzyme by the inclusion or omission of the methyl donor *S*-adenosyl-L-methionine (SAM) in the presence of [α-^{32}P]GTP[32]. 5′-end capped N7-methylated or unmethylated ^{32}P-uniform-labelled pcP RNAs were generated by transcription with SP6 RNA polymerase under standard conditions that included [α -^{32}P]UTP and either m^{7}GpppG or GpppG-cap analogue in the respective reactions.

RNA *in vitro* decay assay. His-tagged Rat1, Rai1 or Rai1-mutant recombinant proteins (50 nM) were incubated with ^{32}P-cap-labelled or ^{32}P-uniform-labelled pcP RNAs in decapping buffer as previously described[23]. After incubation at 37 °C for the indicated times, the decay reactions were terminated with phenol:chloroform extractions and resolved by 8% denaturing polyacrylamide gel electrophoresis[32] or PEI-TLC plates developed in 0.45 M $(NH_4)_2SO_4$ at 25 °C[33]. The dried gels or TLC plates were detected by a Molecular Dynamics PhosphorImager (Storm860) and quantified using ImageQuant-5 software.

Yeast growth conditions and RNA isolation. Yeast strains were grown in standard media lacking tryptophan until mid-log phase at 25 °C. After pelleting and washing, the cells were resuspended into pre-warmed standard media lacking tryptophan, or glucose-deprivation medium, or yeast nitrogen base (YNB) amino-acid-deprivation medium (Invitrogen) for nutrient stress conditions as noted. After a 45-min incubation at 30 °C, transcription was stopped by the addition of the transcriptional inhibitor thiolutin (Sigma-Aldrich) to a final concentration of 5 μg ml^{-1}. The cells were harvested at 0, 1, 2 or 3 h post-thiolutin addition and RNAs were isolated. *abd1-5* and *abd1-5 raiΔ* strains were grown at 25 °C in standard media lacking tryptophan until mid-log phase and shifted to 37 °C for 2 h to abolish methyltransferase activity; thiolutin was added and RNA isolated at the indicated times. Yeast total RNAs were isolated with the acidic hot phenol method[26] with modifications as described[34].

Northern blotting. Twenty micrograms of total yeast RNA isolated from the indicated strains and time points were resolved on a 1% formaldehyde denaturing agarose gel and transferred to a Hybond-N membrane (GE Healthcare Life Science) for northern blot analysis as described previously[29]. Specific mRNAs were detected by [α-^{32}P]dCTP-labelled gene-specific DNA probes. Quantifications were carried out using a Molecular Dynamics PhosphorImager (Storm860) using ImageQuant-5 software.

RT–qPCR and PCR. Yeast total RNA isolated with acidic hot phenol method or immunoprecipiation-isolated RNAs were reverse transcribed into complementary DNA with M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Real-time PCR was performed with iTaq Supermix (Biorad) according to the manufacturer's instructions. Data were computed by the comparative C_T method. The *CYH2* mRNA was amplified with forward primer 5′-TAGAGGTATGGCCGGTGGTC-3′ and reverse primer 5′-ACCCAAGATC TTACCGTAACC-3′. The *CYH2* intron containing pre-mRNA was amplified with forward primer 5′-TCAAATGGTTGTAGAGAGCGC-3′ and reverse primer 5′-AGTTCAAGACTGGCTTCCAG-3′. Forward primer 5′-TTCTGGCTAACCT TGAGTCC-3′ and reverse primer 5′-AAAACGTCCTTGGCAAATGC-3′ were used to amplify the internal control 18S rRNA.

P130 and S130 fractions. Yeast cultures grown to mid-log phase were transferred to either complete medium or nutrient-deprived medium and grown for an additional 45 min before harvesting. The cells were harvested and total extracts were generated with the glass bead method[35] in the presence of 0.1 mg ml^{-1} cycloheximide, protease inhibitor (Roche) and 0.8 U μl^{-1} RNase inhibitor (Promega). The resulting extract was fractionated into a polysome-containing P130 pellet and soluble S130 mRNP fraction by centrifugation at 130,000*g* at 4 °C for 30 min. RNAs were isolated as described earlier.

Cap analogue generation, phosphatase and NDPK treatments. ^{32}P-labelled cap analogues m^{7}GpppG, GpppG and GpppGp were generated by hydrolysing ^{32}P-cap-labelled methylated or unmethylayed pcP RNA (m^{7}G*ppp RNA or G*ppp RNA) with nuclease P1 (Roche) or RNase T1 (Roche) as previously described[33]. Phosphatase and NDPK treatments were carried out with 1 U calf intestinal alkaline phosphatase (NEB) or 1 U nucleoside diphosphate kinase (Sigma) in the presence of 1 mM ATP at 37 °C for 30 min as previously described[31].

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