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## Rapid remodeling of NTP levels enables immediate translational adaptation to energy stress in yeast

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### Summary

In *Saccharomyces cerevisiae*, glucose depletion induces metabolic reprogramming through widespread transcriptional and translational reorganization. We report that initial, very rapid translational silencing is driven by a specialized metabolic mechanism. Following glucose withdrawal, intracellular NTP levels drop drastically over 30 s before stabilizing at a regulated, post-stress set point. Programmed translational control results from the differential NTP affinities of key enzymes; ATP falls below the (high) binding constants for DEAD-box helicase initiation factors, including eIF4A, driving mRNA release and blocking 80S assembly. Contrastingly, guanosine triphosphate (GTP) levels always greatly exceed the (low) binding constants for elongation factors, allowing ribosome run-off and orderly translation shutdown. Translation initiation is immediately lost on all pre-existing mRNAs before being preferentially re-established on newly synthesized, upregulated stress-response transcripts. We conclude that enzymatic constants are tuned for metabolic remodeling. This response counters energy depletion rather than being glucose specific, allowing hierarchical inhibition of energy-consuming processes on very rapid timescales.

### Abstract

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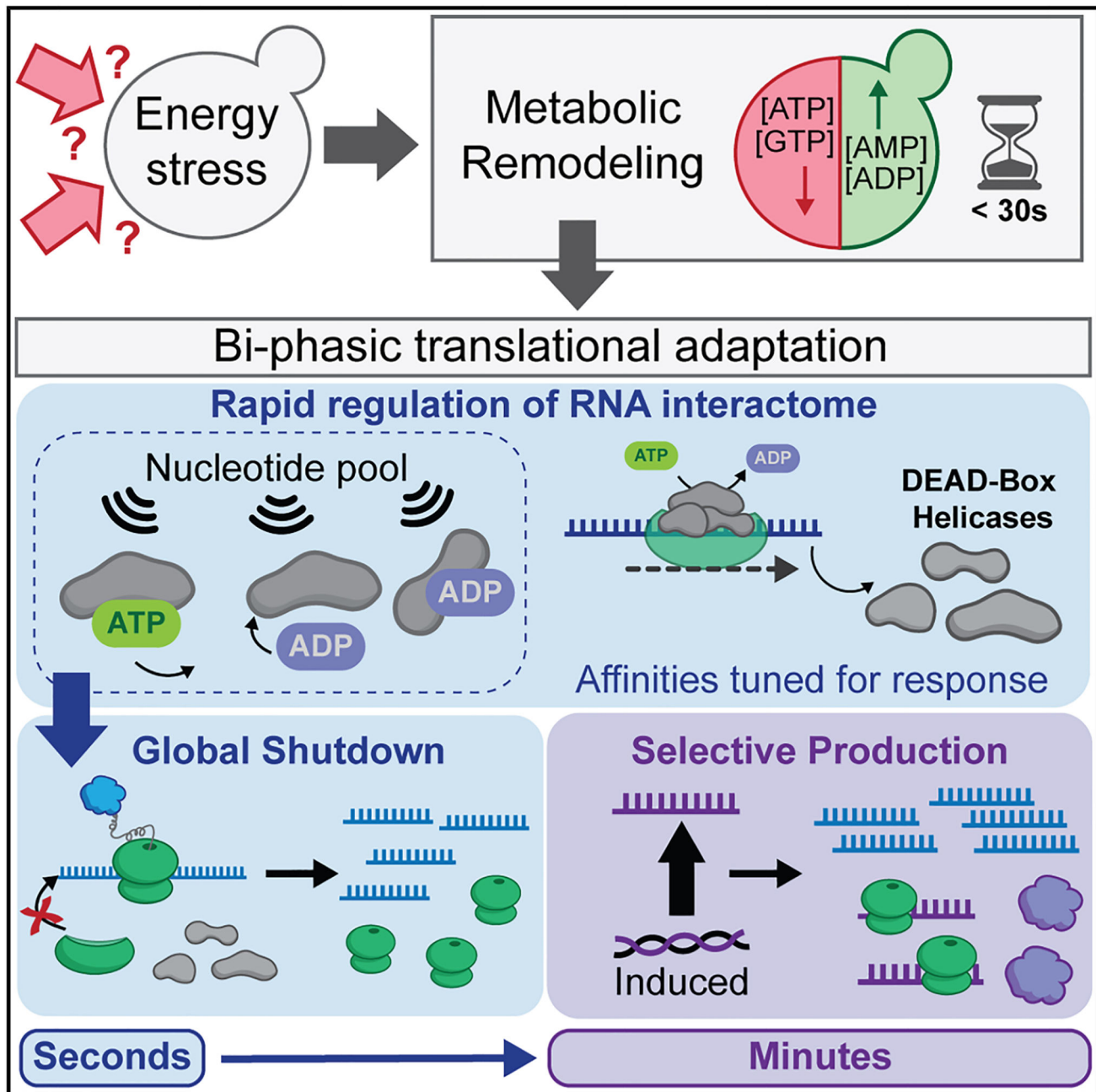
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#### Author Contributions

K.B., M.R., and D.T. conceived the project. K.B. and D.T. wrote the draft manuscript. K.B., M.R., S.S., and C.S. performed experiments. K.B., M.R., S.S., C.S., A.C., and D.T. analyzed the data. K.B. and M.R. developed techniques. All authors edited and reviewed the manuscript.

#### Declaration of Interests

D.T. is on the editorial board of *Molecular Cell*.



Graphical abstract.

## Introduction

All cells face the fundamental challenge of providing a constant supply of energy in the context of a rapidly shifting nutritional environment. The budding yeast *Saccharomyces cerevisiae* grows rapidly on glucose using “aerobic fermentation,” with ATP production almost exclusively from glycolysis (reviewed in Busti et al.<sup>1</sup>). Preferential use of aerobic

fermentation to quickly generate ATP occurs across diverse systems, including human skeletal muscles, activated T-cells, and tumors, where it is termed the “Warburg effect.”<sup>2,3</sup>

In response to glucose depletion, yeast and other organisms undergo “diauxic shift.” This entails a comprehensive transcriptional reprogramming in which hundreds of genes required for stress-responses and utilization of alternative carbon sources are upregulated.<sup>4,5</sup> Simultaneously, expression of ribosome maturation and protein synthesis factors is suppressed. However, at nutrient shift, the cellular mRNA pool is composed of pre-existing transcripts produced under glucose-replete conditions. Reprogramming is therefore complemented by more rapid post-transcriptional changes to acutely modify the functional proteome.<sup>6–8</sup> Notably, global mRNA translation is dramatically reduced within minutes.<sup>9,10</sup> This is selectively bypassed for specific stress-induced transcripts.<sup>11,12</sup> Together, this allows yeast to mount protective responses and dynamically alter metabolism immediately following glucose withdrawal.

Translation repression is a common response to numerous stresses, including temperature and osmotic shock, generally targeting initiation.<sup>13,14</sup> Notably, translational shutdown following glucose withdrawal is distinct in both speed and scale.<sup>8,15</sup> We previously reported that this results from a specific mechanism in which key initiation factors are displaced from the 5′ regions of mRNA.<sup>16</sup> Recruitment of the 40S and 60S ribosomal subunits at the start codon of a transcript is mediated by “scanning” initiation factors, which unwind secondary structures in the mRNA and promote 43S-complex loading. These are eIF4B and two DEAD-box RNA helicases, eIF4A and Ded1.<sup>17–19</sup> Following glucose withdrawal, loss of binding by these factors blocks formation of 80S ribosomes. Translation activity ceases as elongating ribosomes reach the termination codons (see Figure1A).

Remarkably, glucose-dependent initiation factor release occurred within 30 s, the earliest time point that could be tested.<sup>16</sup> These proteins are extremely abundant; eIF4A is comparable in copy number to the ribosome (~ 150,000 per cell), while eIF4B and Ded1 also exceed 25,000 copies.<sup>20</sup> The rapid regulation of such abundant targets presents a challenge: information on glucose depletion must be sensed, transmitted, and amplified to alter protein binding within seconds. Although rapid regulation of RNA-protein interactions mediates post-transcriptional responses to many cellular stresses, this is an unprecedented timescale of response.<sup>21</sup> Here, we set out to investigate the mechanisms allowing yeast cells to achieve seconds-level remodeling of RNA metabolism following glucose withdrawal.

Although implicated in the response of yeast cells to multiple stresses, depleting intracellular pH did not recapitulate the effects of glucose depletion.<sup>16</sup> Considering other metabolite regulators, we noted that the “DEAD-box” helicase initiation factors are ATP-dependent RNA-binding proteins.<sup>22,23</sup> This requirement for ATP means that their binding activity is subject to kinetic effects alongside control by protein-protein interactions.<sup>24</sup> Hypothesizing glucose-dependent changes, we discovered that rapid remodeling of NTP levels drives translation shutdown. This mechanism is distinct from the major well-characterized stress-signaling pathways and can be triggered by energy depletion without a carbon source shift. The resulting translation shutdown is global, happening prior to preferential restoration on newly synthesized mRNAs. We conclude that there is a biphasic adaptive response to energy

stress: metabolic remodeling, acting in seconds, mediates immediate protective changes in RNA metabolism, with subsequent transcriptional regulation by kinase/phosphatase cascades acting over minutes.

## Results

### Rapid NTP depletion and initiation factor displacement

The translational response to glucose withdrawal is remarkably rapid, with global initiation shutdown achieved on a timescale of seconds. This appeared to be excessively fast for regulation by conventional stress-responsive signaling cascades. Contrastingly, levels of nucleoside triphosphates (NTPs) have been reported to change rapidly following loss of glucose in *S. cerevisiae*.<sup>25,26</sup> The DEAD-box helicases are predicted to require ATP to support translation initiation, suggesting the possibility that an abrupt drop in ATP levels and/or changes in the ratio of ATP to ADP might be responsible for their displacement from mRNA 5' UTRs. In contrast, the major translation elongation factors utilize guanosine triphosphate (GTP). We hypothesized that the distinction in NTP utilization could underpin a mechanism for translation regulation acting on timescales shorter than most previous analyses.

We established rapid protocols to characterize changes in metabolites and RNA metabolism on a timescale of seconds. Nutrient shift and harvesting of exponential yeast cultures followed by metabolic quenching, was achieved within 10 s. Shift, harvesting, and UV crosslinking were achieved within 30 s (Figure S1A). NTP and nucleoside diphosphate (NDP) levels were quantified using targeted high-performance liquid chromatography (HPLC) separation coupled with UV detection.<sup>27,28</sup> Quantified NTP recoveries were used to estimate intracellular nucleoside concentrations (see STAR Methods). Rapid time courses over the first minute following glucose withdrawal were performed in biological triplicate and compared with mock shifts (Figure S1B). The results were highly reproducible.

We initially applied this approach to a switch from a medium containing 2% glucose (Glu), to a medium lacking glucose but containing 2% glycerol plus 2% ethanol (Glyc/EthOH) to mimic diauxic shift. The measured intracellular ATP level was close to 2 mM during growth on the glucose medium. Following transfer to the Glyc/EthOH medium, ATP dropped ~7-fold to below 0.3 mM by 30 s, remaining at this level through the 60-s time point (Figures 1B and S1B; Table S2). Contrastingly, the cellular ADP concentration in the glucose medium was 0.18 mM but rose to 0.45 mM by 30 s after transfer to Glyc/EthOH. As a consequence, ADP rapidly became dominant over ATP (Figures 1C and S2D; Table S2). These changes make it likely that many ATP-utilizing proteins are subject to both limiting substrate availability and product inhibition by ADP within seconds of glucose withdrawal.

This striking rate of NTP depletion is consistent with the energy requirements for translation. ATP abundance in glucose was  $1.7 \times 10^{-16}$  mol per cell, corresponding to approximately  $10^8$  ATP molecules per cell. After glucose withdrawal, ATP was depleted by  $3 \times 10^6$  molecules per second. Fast-growing yeast contain around  $1.5 \times 10^5$  ribosomes, of which around  $10^5$  are translationally engaged at any time,<sup>29</sup> while the mean rate of elongation is reported as 2.6 amino acid (aa)/s,<sup>30</sup> corresponding to total incorporation of 2.6

$\times 10^5$  aa/s. Elongation requires a minimum of three NTPs per aa incorporated, so the total cost is around  $8 \times 10^5$  NTP/s. Translation elongation alone should therefore drive ~25% of the observed ATP depletion following glucose withdrawal, with further requirements for initiation, termination, and amino acid synthesis.

Our original model for selective loss of translation initiation postulated that elongation may be favored by delayed depletion of GTP relative to ATP. However, GTP, cytidine triphosphate (CTP), and uridine triphosphate (UTP) were each depleted on a timescale comparable to ATP (Figure 1B). In particular, the starting GTP concentration of 0.46 mM fell to 89  $\mu$ M by 30 s after transfer to Glyc/EthOH (Figure S1B; Table S2). We therefore considered whether there might be differences in the NTP-binding constants for the translation initiation and elongation factors. Based on published data, eIF4A and Ded1 both show low affinity for ATP, with binding constants of 540 and 300  $\mu$ M, respectively.<sup>31,32</sup> These values are consistent with other DEAD-box ATPases reported from yeast.<sup>33,34</sup> As a consequence, ATP depletion following glucose withdrawal causes intracellular concentration to fall well below the Michaelis constant ( $K_m$ ) within 30 s. Due to DEAD-box ATPases binding ATP and RNA cooperatively, this is expected to result in release from mRNAs. Furthermore, eIF4A displays an affinity for ADP an order of magnitude greater than for ATP (Figure 1D). ADP binding promotes an “open” state with low affinity for RNA.<sup>35,36</sup> The rapid dominance of ADP following loss of glucose will therefore block further mRNA binding.

In contrast, reported binding affinities for GTPase elongation factors are very much lower: for example, 140 nM for eEF1A.<sup>37</sup> The measured GTP concentrations remain an order of magnitude above this value after transfer to Glyc/EthOH, so their continued function is expected after glucose withdrawal (Figure 1D).

To confirm the timescale of initiation factor displacement from mRNA transcripts, we developed a poly(A) interactome capture approach. RNAs were UV crosslinked to bound proteins in cells grown in glucose or at time points immediately following transfer to Glc/EthOH medium. Poly(A) + RNAs were purified from cell lysates using oligo[dT]<sub>25</sub> beads. Binding of FLAG-tagged initiation factors was quantified by western blotting and Licor imaging, using Pab1 as positive control for input and pull-down efficiency (Figures 1E and 1F). This revealed that eIF4A is lost from mRNA transcripts, reaching undetectable levels within 30 s, consistent with NTP remodeling (Figure 1E). eIF4B is the binding partner of eIF4A and showed equivalently reduced mRNA interactions over the same timescale. Recovery of eIF4B was much lower in non-crosslinked controls, and eliminated by competition with artificial poly(A) (Figure S1C), confirming binding specificity. We conclude that the entire 43S loading complex is displaced from total mRNA within 30 s.

From these results, we propose that a “differential affinity” mechanism drives translational arrest following glucose withdrawal. ATP depletion to below the binding constants for the DEAD-box helicase initiation factors (~0.35–0.54 mM) leads to reduced RNA affinity and directly blocks the translation initiation process. In contrast, GTP levels remain well above the very low (~140 nM) binding constants for elongation factors, supporting continued

function. Together, this results in orderly translation shutdown by preventing 80S ribosome assembly while allowing polysome run-off (Figure 1G).

### ATP depletion induces translational arrest independent of carbon source shift

To investigate whether changes in ATP concentrations are sufficient to arrest initiation, we characterized the translational response to intracellular ATP depletion independent of carbon source shift. For this we utilized the mitochondrial inhibitor antimycin A (AA), which blocks ATP generation through respiration. Although the BY4741 yeast strain growing in 2% sucrose shows highly similar glycolytic growth to that on glucose (see below), there is an increased metabolic contribution from respiration when utilizing the disaccharide. Under these conditions, AA strongly reduced intracellular NTP pools without an external change in carbon source availability (Figure 1H).

NTP depletion occurred on a minute timescale after AA addition, allowing comparison of the timescales of metabolic remodeling and translation arrest. Although NTP depletion after AA addition was slower than that following glucose withdrawal, by 4 min intracellular ATP levels had fallen to 0.1 mM (Figures 1I and S1D; Table S2). This is well below the binding constant of eIF4A. Polysome gradient analyses, performed after 10 min to allow time for ribosome run-off, demonstrate the loss of translation (Figure 1H). Poly(A) interactome capture at the same time point confirmed the reduction in eIF4A binding to mRNA (Figure 1J). Polysome profiling on aliquots of the same culture are consistent with reduced eIF4A binding to mRNAs driving polysome collapse (Figure S1E).

These results support ATP-dependent binding as a direct regulator of translation initiation factor function.

### NTP remodeling is actively controlled

The glycolytic enzyme hexokinase 2 (Hxk2) has been implicated in regulating transcriptional and post-transcriptional responses to glucose availability.<sup>10,38,39</sup> Consistent with previous data, a *hxx2Δ* strain showed resistance to rapid translation arrest following glucose depletion (Figure 2A). In wild-type (WT) cells, polysome collapse occurs within 10 min (Figure 1A), whereas these were maintained at 10 and 25 min in *hxx2Δ* strains, indicating ongoing translation initiation. We considered whether this might reflect reduced metabolic flux through the glycolytic pathway favoring respiratory metabolism.<sup>10,38</sup> However, *hxx2Δ* strains showed a WT growth rate on glucose (Figure S2A) and were almost insensitive to AA, indicating little dependence on mitochondrial respiration (Figure S2A). Yeast expresses two other hexose kinases (Hxk1 and Glk1), which are sufficient to maintain glycolytic flux.

Analyses of NTP levels indicate that continued translation in *hxx2Δ* mutants results instead from an altered metabolic response to glucose withdrawal (Figures 2B, 2C, and S2B–S2D). The intracellular NTP concentrations decline initially (15 s), comparable to WT cells (Figure 2B). However, ATP levels then plateau at around 1 mM, and this is maintained for at least 16 min. Concentrations of ADP and AMP also remain relatively stable in the *hxx2Δ* strain, as do the pools of CTP, UTP, and GTP (Figures 2C, S2B, and 2C). Because ATP levels



remain well above the binding constants for the DEAD-box initiation factors, these results are consistent with ongoing translation initiation.

Notably, following initial depletion, the reduced ATP concentration also remains stable in WT strains over 16 min post-stress, as do ADP and AMP pools (Figures 2D and S2D). Together, these results indicate that the depletion of NTPs and the dramatic alteration of adenylate nucleoside ratios following glucose depletion is not simply an inevitable consequence of energy deprivation. Rather, a regulated response controls remodeling to achieve a new “set point” for NTP levels.

### **Rapidly growing yeast are primed for protective post-transcriptional responses**

In previous analyses, the timescale and effects of glucose depletion were distinct from withdrawal from alternative sugars.<sup>10</sup> These less readily metabolized sugars are expected to be predominately metabolized via mitochondrial respiration. We postulated that NTP remodeling on a second time-scale occurs only in cells “primed” by fast glycolytic growth to initiate a protective adaptation of metabolism under energy stress.

We used AA to assess relative dependence on oxidative phosphorylation. Yeast growing on raffinose displayed a lag period of 14 h in response to very low doses of AA and failed to saturate (Figure 3A). Comparatively, yeast grown on sucrose demonstrated low AA sensitivity, only slightly higher than on glucose (Figures 3B and S4A). Thus, sucrose and raffinose promote primarily glycolytic or slower respiratory growth, respectively. The doubling time on sucrose medium (~97 min) is similar to that on glucose (~98 min) but substantially faster than on raffinose (148 min) (Figure S3A). Corresponding to these differences in metabolic state, we observe that transfer from the raffinose medium did not cause precipitous NTP depletion (Figures 3C and S3B), whereas shift from sucrose led to a rapid loss of ATP that was equivalent to that observed in glucose withdrawal (Figures 3D and S3C). Consistent with this, following raffinose withdrawal, eIF4A was retained on mRNA over minute-long time courses (Figure 3E). Polysomes were retained 10 min post-withdrawal, supporting ongoing translation initiation and protein synthesis. Conversely, sucrose withdrawal caused loss of eIF4A binding to mRNA in <1 min and resulted in polysome collapse (Figure 3F).

We conclude that the regulated NTP-remodeling response to carbon source withdrawal is not glucose-specific but instead a feature of fast-growing yeast. The metabolic response driving immediate, post-transcriptional changes appears specific to yeast growing rapidly by glycolysis.

### **Energy depletion drives transcriptional reprogramming**

Following glucose withdrawal, selective production of adaptive proteins depends on coordinated translational and transcriptional responses. To assess whether rapid NTP remodeling might also contribute to transcriptional adaptation, the response to sucrose withdrawal was compared with well-characterized changes following glucose derepression. Sucrose depletion and RNA sequencing (RNA-seq) were performed as described for glucose withdrawal.<sup>40</sup> Replicates showed excellent reproducibility (Figure S3D), permitting direct

comparison of the transcriptome during depletion of each carbon source (Figure S3E; Tables S3 and S4).

Yeast utilizing glucose and sucrose possessed highly similar transcriptomes, consistent with largely comparable metabolism and growth (Figures 4A, S4A, and S4B). Differences in expression were predominantly moderate and for lesser-expressed transcripts. Global gene expression patterns altered dramatically following depletion of either carbon source with striking similarity; 90% of transcripts differentially expressed following glucose depletion showed a comparable response to loss of sucrose (Figures 4B–4D). Gene Ontology (GO) over-representation analysis of the most-changed genes following either withdrawal showed enrichment for near-identical biological processes, suggesting comparable remodeling of metabolism and protein production (Figure S4C).

Yeast, and many other organisms, have glucose-specific signaling pathways (reviewed in Miles et al.<sup>41</sup>). However, the similarities in transcriptional derepression between carbon sources indicate a more general response to energy deficiency. The direct effects of ATP depletion and/or ADP accumulation on regulators may be integrated with canonical signaling pathways.

### **Immediate loss of translation initiation factors occurs globally**

Diauxic shift requires widespread reprogramming of metabolism, and protein production stops while mRNA populations are modified. NTP-depletion-driven loss of initiation factor binding is consistent with the inhibition of bulk translation following glucose withdrawal. However, translation is reported to continue or increase for important proteins that mediate stress responses and metabolic adaptation.<sup>11,12</sup> We therefore considered whether these mRNAs specifically escape initiation factor eviction following glucose withdrawal, favoring their production under stress conditions. To assess this, we compared initiation factor binding to transcript abundance before and during glucose depletion (outlined in Figure 5A).

Poly(A)<sup>+</sup> RNA-seq data were obtained during growth on Glu medium or following transfer to Glyc/EthOH for 16 min.<sup>40</sup> Abundance was quantified in sequence reads per kilobase of RNA per million mapped reads (RPKM), correcting counts for gene length and sequencing depth. Most mRNA abundances were little altered following glucose depletion, but stress-response transcripts and sugar transporters are already strongly upregulated by 16 min. Differential expression (DE) analysis identified the 100 most increased and decreased transcripts (false discovery rate [FDR] < 0.05) 16 min after glucose withdrawal (Tables S4 and S6).

As a proxy for initiation, we utilized data for RNA interactomes of eIF4A and eIF4B generated using UV crosslinking (CRAC), which identifies bound RNAs and the location of the interaction site.<sup>42</sup> Relative recoveries for each mRNA are quantified in sequence reads per million (RPM) mapped reads. Crosslinking was performed during growth on Glu and following transfer to Glyc/EthOH for 30 s or 16 min (Figure 4A).<sup>16</sup>



Both eIF4A and eIF4B are lost from almost all transcripts following 30 s in Glyc/EthOH medium, and this absence persists up to 16 min, consistent with poly(A) interactome capture (Figures S5A, S5B). Note that binding to many mRNA species falls sufficiently to be undetectable by CRAC following glucose withdrawal (boxed points in Figure 5B; blue points in Figures 5D, S5A, and S5B). To maintain these mRNAs in the dataset, they were assigned “pseudocounts” of 0.5 RPM. For eIF4A, >50% of transcripts were not detected after stress. As a consequence, we focused on eIF4B datasets for subsequent analyses.

We compared eIF4B binding with mRNAs on glucose and after transfer to Glyc/EthOH for 30 s (no glucose) (Figure 5B). Transcripts were filtered to produce a high-confidence set ( $n = 4,377$ ), with reliable quantification ( $>0.5$  RPM) in all replicates of at least one condition, thereby removing mRNAs with very low expression (Table S5). This analysis confirmed global displacement across almost all mRNAs in seconds.

At 30 s following glucose withdrawal, the 100 mRNAs that are most upregulated by 16 min showed no indication of preferential binding to eIF4B (Figure 5B, highlighted in red). Indeed, many lose binding sufficiently to fall below the detection threshold and appear in the imputed subset (lower horizontal line,  $n = 902$ ), and eIF4B binding, averaged for just this subsequently induced group, is lower than for all mRNAs (Figure 5C). Across the transcriptome, eIF4B binding immediately following glucose depletion (30 s) shows no evident preference for those mRNAs that are subsequently transcriptionally upregulated (Figure 5D).

We also compared the locations of eIF4B binding on individual mRNAs (Figures 5F and S5C), including selected abundant “housekeeping” mRNAs (shown for ACT1, RPL25, and RPB2), induced stress-responsive mRNAs (HSP12, HSP42, HSP82, HSP104, and HSP26), and upregulated glycolytic enzymes (HXK1 and GSY1). On glucose, most mRNAs show a clear peak of eIF4B binding around the AUG, as expected. At 30 s after glucose depletion, eIF4B binding was almost entirely lost on all mRNAs. At 16 min, the house-keeping mRNAs remain very abundant but show little restoration of eIF4B binding. In marked contrast, the induced genes show substantially increased eIF4B binding. However, alongside the 5' peak, there was increased binding throughout the body of the transcript. These strongly upregulated mRNA populations must largely consist of RNAs synthesized post-shift, whereas house-keeping transcripts will have been pre-dominantly produced prior to this.

We postulated that the newly synthesized pool of each mRNA species has preferential access to the translation machinery. Supporting this, we compared the re-establishment of eIF4B binding on the sets of transcriptionally induced or repressed transcripts, defined by RNA-seq (Figure 5E). 16 min after glucose depletion, strongly upregulated transcripts nearly all showed increased eIF4B binding. In contrast, downregulated transcripts predominately showed reduced eIF4B binding, with 44% lost below detectable levels.

Preferential availability of newly transcribed mRNA species might reflect exclusion from condensates that sequester pre-existing mRNAs.<sup>43</sup> As this new population is small, this

potentially favors non-specific interactions with translation initiation factors in addition to cognate 5' binding.

### Newly synthesized mRNAs are preferentially translated

In principle, binding by translation initiation factors or ribosomes might not correlate with productive translation. We therefore assessed protein synthesis by metabolic labeling of nascent polypeptides using the “clickable” methionine analog L-Azidohomoalanine (AHA). Labeling was performed for 16 min in glucose medium or for 16 min immediately following transfer from glucose to glycerol/ethanol medium (Figure 6A). Total proteins were extracted, and newly synthesized proteins were purified by copper-catalyzed covalent linkage to an alkyne-agarose resin. Following stringent washing, peptides were released by trypsin digestion and analyzed by mass spectrometry (MS). The total proteome was quantified in parallel and all replicates showed good correlation (Figure S6). Unlabeled controls were included. Total proteomes (inputs) correlated well, but this correlation is lost following purification of AHA-labeled proteins, supporting selective enrichment (Figure S7A).

During steady-state growth on glucose medium, the newly synthesized and total proteomes were in broad agreement, as expected (Figure 6B). Following transfer to Glyc/EthOH for 16 min, the control and labeled input samples were closely correlated (Figure 6C), whereas there were clear differences following purification of labeled proteins (Figure 6D). Differential proteomic expression analysis identified relatively enriched proteins following AHA labeling, whereas no proteins were significantly enriched in the unlabeled control (Figure 6E). The most highly induced proteins are stress-response factors and hexose transporters (labeled in Figure 6E). Many enriched proteins are products of the 100 most highly induced mRNAs (highlighted in red in Figure 6E). Selected corresponding mRNAs are shown (Figure 6F).

Globally, at 16 min, most proteins are largely unchanged (Figure S7B), but a set of mRNAs are induced for both transcription and nascent protein production (Figure S7C). DE analysis identified proteins with significantly different label incorporation prior to and following glucose withdrawal ( $n = 524$ ). Distinct groups of proteins showed higher label incorporation on glucose (cluster 2) or following glucose withdrawal (cluster 3), supporting preferential production under these conditions (Figure 7A).

Cluster 2, with higher translation on glucose, was enriched for housekeeping functions. These show stable mRNA levels, low protein production, and low association with eIF4B binding following glucose withdrawal (Figure 7B). In contrast, cluster 3 includes 25% of the 100 mRNAs identified by DE as most upregulated following 16 min glucose (Figure 7B, highlighted in red in scatterplots), which correlates with increased protein production and elevated binding of eIF4B.

We conclude that eIF4B binding is initially heavily reduced for nearly all mRNA and contributes to attenuated translation. Subsequently, eIF4B preferentially binds newly produced mRNA. For a select, heavily induced population produced near entirely post-stress, this results in significantly increased protein translation. This sequence-independent

mechanism promotes selective production of adaptive proteins following energy stress, dependent on temporal coordination of translational and transcriptional responses.

## Discussion

Here, we report that the immediate protective response to energy stress is driven by a specialized metabolic mechanism, distinct from the kinase networks mediating conventional glucose repression.

Translational arrest following glucose withdrawal occurs through displacement of translation initiation factors, including DEAD-box helicases eIF4A and Ded1.<sup>16</sup> Strikingly, mRNA binding was abrogated within 30 s of glucose depletion, a speed of response seemingly incompatible with conventional stress-responsive signaling cascades. DEAD-box helicases bind RNA and ATP cooperatively, whereas ADP binding promotes an open, non-RNA-bound conformation.<sup>35,36,44</sup> This suggested that altered ATP and/or ADP levels, as glycolytic flux falls, might drive translation factor release.

Analyses of nucleoside availability revealed that intracellular ATP levels drop from ~2 mM to below 0.3 mM within 30 s following glucose depletion. This concentration is below reported ATP-binding constants for DEAD-box helicases, including eIF4A (~0.35–0.54 mM).<sup>31,32,36</sup> Over the same period, ADP rises from around 0.18 to 0.45 mM. This is substantially greater than the ATP concentration or the reported eIF4A  $K_m$  for ADP (0.027 mM),<sup>35</sup> which favors the open structure with low RNA affinity. We propose that loss of ATP binding and increased ADP association directly drive release of eIF4A from all mRNA transcripts, blocking the initiation process. However, even after depletion, a minimal ATP-associated population will remain, potentially supporting the residual level of protein synthesis.

Over the initial period of glucose depletion, translation elongation is more resistant to inhibition than is translation initiation, resulting in bulk polysome run-off (Figure 1A).<sup>10,11</sup> Notably, translation elongation is more dependent on GTP than ATP. In our metabolite analyses, GTP was depleted with similar kinetics to ATP, presumably reflecting the requirement for ATP in GTP recycling. However, GTP levels remained substantially above the binding constants for elongation GTPases.<sup>37</sup> Strikingly, these show GTP-binding affinities around 3,000-fold greater than the initiation factor ATPases. We therefore proposed a mechanism based on differential affinity. NTP depletion rapidly prevents 80S ribosome assembly but allows initiated ribosomes to continue and terminate, resulting in an orderly translation shutdown.

During termination, ribosome release is mediated by eRF1-eRF3 or the homologs Dom34-Hbs1, which are GTP dependent (reviewed in Buskirk et al.<sup>45</sup>). Peptide release and 40S-60S splitting activities are stimulated by the ATPase Rli1.<sup>46</sup> However, peptide release is independent of ATP hydrolysis, whereas subunit splitting is ATP dependent,<sup>47</sup> perhaps explaining why 80S monosomes accumulate rather than free subunits. Alternatively, polysome run-off at low ATP levels may involve Rli1-independent dissociation of post-termination 80S ribosomes from mRNAs.

The metabolic response reflects energy depletion rather than being glucose specific. Depletion of ATP by inhibition of respiration without carbon source shift also resulted in the loss of initiation factor binding and ribosome run-off, supporting a direct ATP-driven mechanism. Similarly, withdrawal of sucrose caused rapid ATP depletion and translation factor loss. Sucrose supports fast growth and is largely metabolized by glycolysis. In contrast, raffinose supports slow growth and is largely metabolized by respiration. Withdrawal of raffinose did not result in substantial ATP depletion or translation factor loss. We conclude that rapid NTP remodeling is “primed” by fast glycolytic growth. Rapid ATP generation through glycolysis, with a minimal mitochondrial network, is highly efficient but results in vulnerability to energy stress.<sup>48,49</sup> NTP remodeling may “de-risk” glycolytic growth by curtailing energy-consuming processes on very rapid timescales.

The differential affinity model requires that both enzymatic constants and NTP levels are “tuned” by evolutionary selection to enable distinct responses following glucose withdrawal. We noted that, after rapid depletion during the first minute, NTP levels were approximately constant over the following 16 min. This suggested that the cells do not simply exhaust NTPs. Many cellular processes utilize NTPs, with a wide range of binding constants and turnover rates. We suggest that as the effective [NTP] drops, different NTP sinks will be progressively inhibited until residual consumption and production match and a new equilibrium is established. In practice this occurs at ~0.3 mM ATP after 30 s. Residual ATP production may reflect a low level of respiration or glycolysis from mobilization of stored glycogen.

In yeast lacking the glycolytic enzyme Hxk2, polysomes are not lost following glucose withdrawal.<sup>10,38</sup> In *hxk2Δ* yeast, ATP levels initially declined but reached equilibrium faster and at a higher set point: 1.0 mM after 15 s, which is above the binding constants of the initiation factors. We conclude that regulated metabolic remodeling controls NTP levels following glucose depletion. This higher set point is substantially above the binding constants of the initiation factors, suggesting that one or more major ATP sink is inactivated at a higher [ATP] in the mutant. A plausible candidate is the plasma membrane H<sup>+</sup>-ATPase (Pma1), which alone consumes around 30% of the ATP budget in WT cells and has been functionally linked to hexokinase in numerous studies.<sup>50–53</sup>

Notably, subsequent changes in global gene expression patterns were strikingly similar following either glucose or sucrose withdrawal. 90% of transcripts responded comparably, reflecting remodeling of central carbon metabolism and repression of protein synthesis components.<sup>4,5</sup> We postulate that a general “energy stress” program also initiates transcriptional reprogramming, independent of the canonical glucose repression network. We anticipate that subsequent metabolic remodeling allows respiratory metabolism, full restoration of ATPase function, and resumption of growth after an extensive lag.

A range of environmental insults trigger translation repression. This inactivates pre-stress mRNAs while a selective transcriptional response is mounted.<sup>54</sup> Importantly, although bulk synthesis is rapidly lost, stress-induced proteins must be selectively produced. Structural or sequence elements allowing specific evasion of translation repression have been suggested,<sup>55,56</sup> but wide-spread common features have not been found. Rather, reporter

systems indicate that timing is crucial: mRNAs transcribed after the initial stress escape repression.<sup>43,57</sup> Supporting a sequence-independent mechanism for selective translation, our analyses found no evidence that withdrawal-induced mRNAs escape initiation factor eviction following glucose withdrawal. Instead, eIF4B was globally displaced on a second timescale and then re-engaged over minutes, preferentially on transcriptionally upregulated transcripts. Concordantly, initiation factor binding patterns shifted on these transcripts to reveal non-specific interactions alongside cognate 5' binding. This indicates reduced specificity, possibly reflecting an excess of initiation factors relative to available mRNAs.

Immediately following glucose withdrawal, release of the protein-synthesis machinery leads to sequestration of free mRNAs into cytoplasmic condensates. However, newly transcribed mRNAs are excluded from condensates by an unknown mechanism.<sup>57</sup> Reduced global translation will generate free pools of translation initiation factors, but these can access only the available, newly synthesized mRNA pool. A relative excess of initiation factors may favor translation even under conditions of ATP limitation.

Ded1 and other DEAD-box helicases participate in mRNA condensate formation under stress conditions. However, condensates form via multivalent interactions and the contribution of RNA binding in the helicase active site is unclear. Ded1 is lost from the AUG-proximal regions of all mRNAs immediately after glucose withdrawal but remains associated with some coding sequences, potentially in condensates.<sup>16</sup> This indicates that alternative modes of interaction exist and can be maintained after ATP depletion, perhaps facilitating roles in condensate formation and other regulatory processes. We propose that evolutionary tuning of enzymatic constants to control NTP remodeling allows specific rapid modulation of RNA metabolism following carbon source shift.

Our findings may be relevant to other systems. For yeast, ATP abundance in glucose was approximately  $10^8$  ATP molecules per cell. After glucose withdrawal, the ATP level drops by  $\sim 8 \times 10^7$  molecules in 30 s, giving an ATP depletion rate of  $3 \times 10^6$  molecules per second. During normal metabolism in human cells, the ATP pool is reported to be around  $10^9$  molecules and is entirely turned over in around 1–2 min.<sup>58,59</sup> Therefore, a complete block in ATP production should result in an ATP depletion rate comparable to that seen in yeast. Moreover, 70%–80% ATP depletion is reported for focal ischemia following arterial occlusion (see Sims et al.<sup>60</sup> and references therein) or with combined oxygen and glucose deprivation in neuronal cultures.<sup>61,62</sup> In differentiating human cells, translation consumes around 30% of NTPs,<sup>63</sup> indicating that rapid shutdown would favor homeostasis.

NTPases function in a vast range of essential and non-essential activities. Selection for differential affinity may provide a single mechanism to coordinate comprehensive post-transcriptional adaptation to energy stress. Similar enzymatic tuning might have modulated binding constants for many metabolites, such that alterations in metabolite abundance are buffered by hierarchical loss of selected pathways.

### Limitations of the study

Loss of eIF4A and eIF4B is expected to block translation initiation, but other initiation factors may also contribute to inhibition. It remains unclear how the ATP concentration “set

point” is established and maintained as a new equilibrium following glucose withdrawal. It is unclear what features distinguish newly synthesized mRNAs from the pre-existing pool. These could be RNA structures and/or modifications, associated proteins, or localization—and these possibilities are not mutually exclusive. Finally, we do not yet know whether a similar strategy is applied in other systems as an immediate response to energy depletion or other stresses.

## Resource Availability

### Lead contact

Requests for further information and resources should be directed to, and will be fulfilled by, the lead contact, David Tollervey (d.tollervey@ed.ac.uk) Institute of Cell Biology, University of Edinburgh, Edinburgh, Scotland.

### Materials availability

All unique reagents generated in this study are available from the lead contact without restriction.

## Star★Methods

### Key Resources Table

| REAGENT or RESOURCE                                | SOURCE               | IDENTIFIER                  |
|----------------------------------------------------|----------------------|-----------------------------|
| Antibodies                                         |                      |                             |
| IRDye 680RD Goat anti-Mouse IgG Secondary Antibody | LICORbio             | #926-68070 RRID:AB_10956588 |
| IRDye 800CW Goat anti-Rat IgG Secondary Antibody   | LICORbio             | #926-32219 RRID:AB_1850025  |
| Pab1p Monoclonal Antibody                          | Invitrogen           | #MA5-47390 RRID:AB_2942372  |
| RAT Anti-DYKDDDDK Tag Antibody                     | Agilent              | #200474 RRID:AB_10597743    |
| Chemicals, peptides, and recombinant proteins      |                      |                             |
| C-18 Disks                                         | Sigma-Aldrich        | #66883-U                    |
| CSM Complete                                       | Formedium            | #DCS0011                    |
| CSM Single Drop-out -Met                           | Formedium            | #DCS0119                    |
| CSM Single Drop-out -Trp                           | Formedium            | #DCS0149                    |
| Cycloheximide (20 mg/ ml in EtOH//−20° C)          | Sigma-Aldrich        | #C7698-5G                   |
| D(+) – Glucose Anhydrous                           | Thermo Scientific    | #G-0500-53                  |
| D(+) – Raffinose Pentahydrate                      | Formedium            | #RAF04                      |
| Dynabeads™ Oligo(dT)25                             | Invitrogen           | #61002                      |
| EDTA-free cOmplete protease inhibitor cocktail     | Roche                | #11836170001                |
| GlycoBlue™ Coprecipitant                           | Invitrogen           | #AM9515                     |
| L-Azidohomoalanine (hydrochloride)                 | Cambridge Bioscience | #HY-140346A                 |
| LoBind Tube                                        | Eppendorf            | #E0030108116                |
| MOPS running buffer 20x                            | Invitrogen           | #NP0001                     |
| Nitrocellulose membrane                            | Thermo Scientific    | #88018                      |
| NuPAGE Bis-Tris 4-12% precast gradient gels        | Invitrogen           | #NP0321BOX                  |



| REAGENT or RESOURCE                                                                             | SOURCE                         | IDENTIFIER                                                                                               |
|-------------------------------------------------------------------------------------------------|--------------------------------|----------------------------------------------------------------------------------------------------------|
| NuPAGE LDS Sample Buffer (4X)                                                                   | Invitrogen                     | #NP0007                                                                                                  |
| NuPAGE™ Transfer Buffer (20X)                                                                   | Invitrogen                     | #NP00061                                                                                                 |
| Oasis WAX 3 cc Vac Cartridge                                                                    | Waters Sverige AB              | #186002492                                                                                               |
| RNase A/T1 mix                                                                                  | Thermo                         | #EN0551                                                                                                  |
| Rnasin Ribonuclease Inhibitor                                                                   | Promega                        | #N2111                                                                                                   |
| RQ1 RNase-Free DNase                                                                            | Promega                        | #M6101                                                                                                   |
| Sucrose, Extra Pure                                                                             | Thermo Scientific              | #S-8560-60                                                                                               |
| Trichloroacetic acid                                                                            | Sigma-Aldrich                  | #91228-100G                                                                                              |
| TRIzol Reagent                                                                                  | Invitrogen                     | #15596026                                                                                                |
| Trypsin Protease                                                                                | Pierce                         | #90057                                                                                                   |
| Yeast Nitrogen Base                                                                             | Formedium                      | #CYN0410                                                                                                 |
| Zirconia beads                                                                                  | Thiste Scientific              | #ZrOB05                                                                                                  |
| Critical commercial assays                                                                      |                                |                                                                                                          |
| Click-iT™ Protein Enrichment Kit                                                                | Invitrogen                     | #C10416                                                                                                  |
| RNA Clean & Concentrator-5' kit                                                                 | Zymogen                        | #R1013                                                                                                   |
| NEBNext Ultra II Directional RNA Library Prep kit                                               | NEB                            | #7760                                                                                                    |
| Poly-A mRNA magnetic isolation module                                                           | NEB                            | #E7490                                                                                                   |
| Deposited data                                                                                  |                                |                                                                                                          |
| Proteomics                                                                                      | This study                     | PRIDE: PXD064070                                                                                         |
| Raw and processed sequencing data for Sucrose and Sucrose withdrawal RNAseq experiments         | This study                     | GEO: GSE285035                                                                                           |
| Raw and processed sequencing data for Glucose and Glucose withdrawal RNAseq experiments         | Ristová et al. <sup>40</sup>   | GEO: GSE283345                                                                                           |
| CRAC sequence data                                                                              | Bresson et al. <sup>16</sup>   | GEO: GSE148166                                                                                           |
| Imaging data at Mendeley Data                                                                   | This study                     | Mendeley data: <a href="https://doi.org/10.17632/jx59t358zy.2">https://doi.org/10.17632/jx59t358zy.2</a> |
| Experimental models: Organisms/strains                                                          |                                |                                                                                                          |
| S. cerevisiae strain: BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)                                | N/A                            | N/A                                                                                                      |
| S. cerevisiae strain: FH-TIF1 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FH-TIF1)                       | Bresson et al. <sup>16</sup>   | N/A                                                                                                      |
| S. cerevisiae strain: TIF3-FH (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TIF3-HF)                       | Bresson et al. <sup>16</sup>   | N/A                                                                                                      |
| S. cerevisiae strain: hxx2Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hxx2Δ)                           | Obtained from Stefan Bresson   | N/A                                                                                                      |
| S. cerevisiae strain: MET15+ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MET15+)                         | Obtained from Aziz El Hage     | N/A                                                                                                      |
| Oligonucleotides                                                                                |                                |                                                                                                          |
| HXX2_amplification_F: GTTGTAGGAATA TAATTCTCCACACATAATAAGTACGCTAA TTAAATAAACGGATCCCCGGGTTAATTAA  | This study                     | N/A                                                                                                      |
| HXX2_amplification_R: GTAGAAAAAGGG CACCTTCTTGTTGTTCAAACCTTAATTTACA AATTAAGTGAATTCGAGCTCGTTTAAAC | This study                     | N/A                                                                                                      |
| Software and algorithms                                                                         |                                |                                                                                                          |
| Bioconductor (v3.20)                                                                            | Gentleman et al. <sup>64</sup> | <a href="https://bioconductor.org/">https://bioconductor.org/</a><br>RRID:SCR_006442                     |

| REAGENT or RESOURCE       | SOURCE                        | IDENTIFIER                                                                                                                                                                                 |
|---------------------------|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| clusterProfiler (v4.12.6) | Yu et al. <sup>65</sup>       | <a href="https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html">https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html</a><br>RRID:SCR_016884 |
| DEP (v1.28.0)             | Zhang et al. <sup>66</sup>    | <a href="https://bioconductor.org/packages/release/bioc/html/DEP.html">https://bioconductor.org/packages/release/bioc/html/DEP.html</a><br>RDID:SCR_023090_                                |
| DIA-NN (v1.9.2)           | Demichev et al. <sup>67</sup> | <a href="https://github.com/vdemichev/DiaNN">https://github.com/vdemichev/DiaNN</a> RRID:SCR_022865                                                                                        |
| edgeR (v4.2.0)            | Robinson et al. <sup>68</sup> | <a href="https://www.bioconductor.org/packages/release/bioc/html/edge.html">https://www.bioconductor.org/packages/release/bioc/html/edge.html</a> RRID:SCR_012802                          |
| Flexbar (v3.5)            | Dodt et al. <sup>69</sup>     | RRID:SCR_013001                                                                                                                                                                            |
| ggplot2 (v3.5.1)          | Wickham <sup>70</sup>         | <a href="https://ggplot2.tidyverse.org/index.html">https://ggplot2.tidyverse.org/index.html</a> RRID:SCR_014601                                                                            |
| GraphPad Prism (v10.3.1)  | N/A                           | <a href="https://www.graphpad.com/">https://www.graphpad.com/</a><br>RRID:SCR_002798                                                                                                       |
| ImageStudio (v5.2.5)      | N/A                           | <a href="https://www.licor.com/bio/image-studio/">https://www.licor.com/bio/image-studio/</a> RRID:SCR_015798q                                                                             |
| pheatmap (v1.0.12)        | N/A                           | <a href="https://rdrr.io/cran/pheatmap/">https://rdrr.io/cran/pheatmap/</a><br>RRID:SCR_016418                                                                                             |
| R Studio                  | N/A                           | <a href="http://www.rstudio.com/">http://www.rstudio.com/</a>                                                                                                                              |
| Salmon (v1.4.0)           | Patro et al. <sup>71</sup>    | RRID:SCR_017036                                                                                                                                                                            |

## Experimental Model and Study Participant Details

All *S. cerevisiae* strains used in this study were derived from the BY4741 background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The methionine prototroph (BY4741 MET15+) carrying the WT S288C MET15+ gene, was utilized for metabolic labelling (generously gifted by Aziz El Hage). The *hxx2Δ* strain was a kind gift from Stefan Bresson. Conventional homologous recombination was used for complete deletion of the *HXX2* gene, utilizing the His3MX6 cassette from the pFa6a plasmid (Addgene #41596). Oligonucleotides were designed with 50bp homology to the *HXX2* locus, detailed in key resources table. All tagged strains were constructed and utilized previously in Bresson et al.,<sup>16</sup> as described. The chromosomal copy of TIF1 (one of two genes encoding eIF4A) was N-terminally tagged with Flag-His (FH), consisting of a single Flag motif, a four-alanine spacer, and eight consecutive histidine residues (DYKDDDDKAAAAHHHHHHHH). TIF3 (eIF4B) was C-terminally tagged with the same elements in reverse (HHHHHHHHA AAADYKDDDDK), the His-Flag (HF) tag. Both strains were generated using CRISPR-Cas9.<sup>72</sup>

## Method Details

**Cell culture and medium:** Yeast strains were cultured at 30°C with constant shaking at 200 rpm in 2% synthetic complete (SC) 2% yeast nitrogen base medium supplemented with 2% carbon source, as indicated. For all experiments, saturated overnight starter cultures were inoculated into fresh media at a starting OD<sub>600</sub> of 0.05 and grown to exponential OD (0.3-0.5). Glucose, sucrose and raffinose grown cells were harvested directly from supplemented media. For treatments, cells were collected by filtration on a nitrocellulose

membrane and transferred to fresh medium. This medium contained the same carbon source (mock shift), 2% glycerol and 2% ethanol (carbon source withdrawal), or the same carbon source and either 0.2 µg/ml or 0.002 µg/ml Antimycin A (+AA), as indicated.

For poly(A)-interactome capture experiments, yeast cells were cultured in 2% synthetic dropout (SD) -TRP, supplemented with 2% yeast nitrogen base and 2% indicated carbon source. This minimizes potential interference during UV-crosslinking.

L-Azidohomoalanine (AHA) labelling experiments were performed with SD-MET media supplemented with 2% yeast nitrogen base and 2% indicated carbon source. Labelling was performed by addition of 200 µM AHA for 16 minutes, either directly to exponential glucose grown culture, or alongside carbon source withdrawal by transferred to glycerol and ethanol medium supplemented with AHA.

**Growth Curves:** For Antimycin A sensitivity assays, cultures were saturated overnight in SC medium supplemented with indicated carbon source. Subsequently, these were diluted to OD<sub>600</sub> 0.05 in 20ml fresh media and grown to an OD<sub>600</sub> of 0.3. Yeast cells were then shifted to SC medium supplemented with indicated carbon source and either 0, 0.2 or 0.02 µg/ml Antimycin A. In each case, 3 ml culture was pelleted at 4,000 rpm for 3 min, washed once and then resuspended in 3 ml shift medium. These cultures were diluted to an OD<sub>600</sub> of 0.025 in the same media, and 150 µl aliquots plated into a 96-well plate in technical triplicate. Optical density was monitored over 48 h using a Sunrise TECAN plate reader, measuring at 15 min intervals with continuous shaking at 30°C. All conditions were tested in at least biological duplicate.

**Polysome profiling:** Overnight cultures of yeast were diluted to OD<sub>600</sub> 0.05 in 100 mL SC medium supplemented with indicated carbon source, and cultured with shaking at 30°C. At OD<sub>600</sub> 0.45, cultures were either harvested directly or collected by filtration and transferred to indicated treatment medium for 10 min. For *hxx2Δ*, 25m glucose withdrawal was also performed. In all cases, cells were then pelleted by centrifugation at 4,600 rpm for 2 minutes at 4°C, washed once with 20 ml of ice-cold water, snap-frozen in a dry-ice and ethanol bath, and stored at -80°C. Biological duplicates were collected for each condition.

The technique for lysis and profiling was modified from Winz et al.<sup>73</sup> Briefly, cells were resuspended in 300 µl of buffer (20 mM HEPES-KOH, 7.4; 100 mM KOAc; 2 mM MgOAc; 0.1 mg/ml Cyclohexamide; EDTA-free protease inhibitors). The suspension was transferred to 2 mL screw-cap tubes containing 200 µL zirconia beads and lysed using a Fast Prep-24 machine (MP Biomedicals) with a pre-cooled CoolPrep™ adapter. Four cycles were performed at 6 m/s for 40 sec, with cooling on ice in between rounds. The lysate was diluted with an additional 200 µl lysis buffer, and cleared twice by centrifugation at max speed for 5 min at 4°C.

350 µg of RNA was loaded onto 10%-45% sucrose gradients in 1X gradient buffer (10 mM Tris-acetate, pH 7.4; 70 mM NH<sub>4</sub>OAc; 4 mM MgOAc) prepared using the Gradient Master (BioComp) and refrigerated prior to use. Subsequently, the gradients were centrifuged in an SW40-Ti rotor in an Optima XPN-100 Ultracentrifuge (Beckman Coulter) at 38,000 rpm

for 2.5 h at 4°C. Absorbance profiles were visualized using the Piston Gradient Fractionator (BioComp) equipped with a TRIAX flow cell (BioComp) for UV profiling. For comparative analysis, starting absorbances were zeroed and the monosome peaks then aligned.

**Metabolomics:** For each metabolomics experiment, BY4741 were grown in 150 ml SC media supplemented with indicated carbon source. At OD<sub>600</sub> 0.3, exponential cultures were harvested by filtration and either lysed immediately or subject to the indicated treatment (Figure S1A). For this, 10 ml shift media was applied directly to the filter and incubated for the time indicated. With 10 sec remaining, shift media was removed using the vacuum and the filter rapidly transferred for lysis. Where timepoints exceeded 1min, the filter was transferred to 150ml stress media and cultured with shaking at 30°C and re-filtered prior to the time-point. In all cases, cells were lysed by placing the filter in a petri dish containing 4 ml 12% Trichloroacetic acid (w/v) in 15 mM MgCl<sub>2</sub>, kept on ice. The lysis solution and filter were then transferred to a 5 ml Eppendorf tube, vortexed vigorously for 5 min and snap frozen in liquid nitrogen. These were stored at -80°C. All conditions were tested in at least biological triplicate, ensuring ~ 600 million yeast cells were collected in each experiment.

For NTP quantification, thawed samples were vortexed for 30 sec then centrifuged at 4,000 rpm for 5 min at 4°C. 1 ml of the supernatant was transferred to a 1.5 ml Eppendorf tube and neutralized twice in a 1:1 ratio with a dichloromethane (DCM)–trioctylamine (TOC) mixture (1 ml DCM and 0.28 ml TOC). Following centrifugation at 14,000 rpm for 2 min at 4°C, 900 µl of the supernatant was transferred to new Eppendorf tubes. A 100 µl aliquot of the extracted nucleoside solution was then mixed with 1.15 ml Milli-Q water, and the pH was adjusted to between 3 and 4 using 0.5 µl 6 M HCl. Subsequently, analysis was performed by HPLC UV as described in Jia et al.<sup>27</sup>

The remaining 800 µl of extracted solution was used to quantify NDPs and AMP as in Ranjbarian et al.<sup>28</sup> Briefly, samples were adjusted to pH 4.6 and cleaned by OASIS WAX-SPE. Following elution, samples were dried using a Speedvac and resuspended in 200µl water. For HPLC analysis, 20 µl was loaded and run on a 4.6 mm × 150 mm Sunshell C18-WP 2.6 µm column (ChromaNik Technologies Inc.).

This methodology produces UV chromatograms for experimental samples accompanied by NTP, NDP and AMP standards. By comparison of the peak heights or areas, the amount of NTPs, NDPs and AMPs in yeast cell samples is calculated. The result is expressed as pmol/108 cells. From these, we estimate intracellular nucleoside concentrations as in Sabouri et al.<sup>74</sup> Briefly, the volume of the soluble fraction of a haploid yeast cell was estimated as  $45 \times 10^{-12}$  g ( $\sim 45 \mu\text{m}^3$ ) by subtracting the reported dry weight from wet weight ( $15 \times 10^{-12}$  g and  $60 \times 10^{-12}$  g, respectively). Assuming a conversion of 1g/L, a volume of  $4.5 \times 10^{-14}$  L/cell was used to transform quantified NTP levels into concentrations in mM, as reported in Table S5.

**Poly(A)-Interactome Capture:** The poly(A)-interactome capture protocol is based on the experimental procedures in Castello et al.<sup>75</sup> and Matia-Gonzalez et al.,<sup>76</sup> with significant modifications.

For each experiment, Flag-tagged strains were grown in 800 ml SC -TRP media supplemented with indicated carbon source. At OD<sub>600</sub> 0.4, cultures were either utilized directly or subject to the treatment indicated using the rapid shift protocol outlined in Figure S1A. Here, the exponential culture was collected by filtration, transferred to 800 ml of relevant shift media, resuspended and incubated shaking until 20 sec prior to the time-point. All cultures were then UV-irradiated at 254 nm for 20 sec using the Vari-X-Link crosslinker.<sup>77,78</sup> Following crosslinking, cells were collected by filtration and resuspended in 50 ml ice-cold H<sub>2</sub>O. Cells were then centrifuged at 4,600xg for 2 min, and pellets immediately stored at -80°C. All conditions were tested in biological duplicate.

For lysis, cell pellets were resuspended in 800 µl lysis buffer (100 mM Tris-HCl, pH 7.5; 500 mM LiCl; 10 mM EDTA; 1% Triton X-100; 5 mM DTT; 100 U/ml RNasin; complete EDTA-free protease-inhibitor cocktail). Following addition of 1.5 ml Zirconia beads to the suspension, cells were broken mechanically using six one-minute pulses on a benchtop vortex, with cooling on ice in between. The cooled lysate was centrifuged at 4,600xg for 5 min at 4°C, and the supernatant was then transferred to Eppendorf tubes and cleared by centrifugation at 20,000xg for 20 min at 4°C. This extract was diluted to 15 mg/ml in lysis buffer. For competed controls, 500 µl aliquots of extract were supplemented with 20 µl 10 mg/ml poly(A).

To capture polyadenylated RNAs, for each extract, 200 µl (~1mg) of oligo(dT)<sub>25</sub> Dynabeads were first equilibrated by four washes in lysis buffer (as above, except 10 U/ml RNasin). These were then incubated with 500 µl (~7.5mg) extract for 40 min at room temperature, while rotating. Subsequently, using a magnet to capture the beads, the supernatant was recovered into a fresh tube from the beads for repeat incubations (described below). The beads were washed once with 500 µl wash buffer A (10 mM Tris-HCl, pH 7.5; 600 mM LiCl; 1 mM EDTA; 0.1% Triton X-100; 10 U/ml RNasin) and twice with 500 µl wash buffer B (10 mM Tris-HCl, pH 7.5; 600 mM LiCl; 1 mM EDTA; 10 U ml<sup>-1</sup> RNasin). To elute RNA-protein complexes by temperature, the beads were re-suspended in 60 µl elution buffer (10 mM Tris-HCl, pH 7.5) and incubated at 80°C for 2 min. The eluate was then collected immediately.

To fully deplete samples of poly(A) RNAs, this procedure was repeated twice further. The beads were retained, and re-equilibrated with four washes in lysis buffer before the preserved supernatant was re-applied. The three sequential eluates were then combined and treated with 4 µl RNase A/T1 mix in 20 µl RNase buffer (10 mM Tris-HCl, pH 7.5; 300 mM NaCl; 5mM EDTA, pH 7.5) for 1 h at 37°C. Recovered proteins were precipitated overnight at -20°C, using 2µl glycoblue in 1 ml acetone. These were pelleted at 20,000xg for 20 min, dried briefly and resuspended in 18 µl 1X NuPAGE LDS sample buffer supplemented with 50 mM DTT.

**Western Blot Analysis:** Western blot analysis was used to compare amounts of Flag-tagged initiation factors in inputs and eluates of poly(A) interactome capture. Poly(A)-binding protein (Pab1) acted as a control for input and pull-down efficiency.

Input samples were prepared by mixing 1  $\mu$ l 15mg/ml cell lysate with 1X NuPAGE LDS sample buffer supplemented with 50 mM DTT, and preparation of eluates is outlined above. All samples were boiled for 5 min, and resolved on a 4%–12% Bis-tris NuPAGE gel by electrophoresis at 150 V in 1X NuPAGE MOPS buffer. Proteins were transferred to nitrocellulose membrane using the Biorad Mini Trans-Blot apparatus with NuPAGE transfer buffer for 1 h at 100V. Membranes were blocked with 5% milk in PBS for 30 min, and probed overnight at 4°C with the indicated antibodies diluted in PBS with 0.1% Tween-20 (PBS-T). Following washing in PBS-T, membranes were incubated in the corresponding fluorescently labelled secondary antibody for 1 h at room temperature. Membranes were subsequently washed again, then visualized by chemiluminescent imaging using the LiCor Odyssey CLx imaging system (LICORbio). Quantitative analysis of captured images was performed with the Image Studio software (LICORbio).

The following antibodies were used: rat Anti-DYKDDDDK Tag Antibody (1:1000; Agilent, 200474), mouse Pab1p Monoclonal Antibody (1G1) (1:1000; Invitrogen, MA5-47390), IRDye 800CW Goat anti-Rat IgG Secondary Antibody (1:5000; LICORbio, 926-32219), IRDye 680RD Goat anti-Mouse IgG Secondary Antibody (1:5000; LICORbio, 926-68070).

**L-Azidohomoalanine (AHA) labelling and Click Chemistry:** To assess post-starvation protein synthesis, metabolic labelling was performed methionine analog AHA, modified with an azide moiety to allow chemoselective ligation with an alkyne group. This “click” reaction allows efficient covalent capture of labelled nascent proteins and identification by mass spectrometry.

BY4741 MET15+ were grown to OD<sub>600</sub> 0.4 in 200 ml SC -TRP media supplemented with 2% glucose. Cultures were then labelling for 16 min with AHA at final concentration 200  $\mu$ M, diluted from a 200 mM L-Azidohomoalanine (hydrochloride) (Cambridge Bioscience) stock dissolved in equimolar NaOH to neutralize pH. For glucose samples, this was added directly to the media, whereas starved samples were prepared by rapidly shifting (Figure S1A) the exponential culture to 200 mL AHA-containing SC -TRP media supplemented with 2% glycerol and 2% ethanol. Labelled cultures were incubated shaking until the timepoint, collected by filtration and resuspended in 50 mL ice-cold H<sub>2</sub>O. Cells were pelleted at 4600xg for 2 min, snap-frozen in a dry-ice and ethanol bath, and pellets immediately stored at –80°C. Unlabeled controls were prepared simultaneously, and all conditions were tested in biological triplicate.

Total protein extraction and enrichment of newly synthesized proteins was performed using the Click-iT™ Protein Enrichment Kit (Invitrogen), with minor adaptations to the protocol. For lysis, cell pellets were resuspended in 800  $\mu$ l lysis buffer (8 M urea, 200 mM Tris pH 8, 4% CHAPS, 1 M NaCl, supplemented with 2X complete EDTA-free protease-inhibitor cocktail. Cells were broken mechanically using a benchtop vortex, by addition of 1.5ml Zirconia beads to the suspension and six one-minute pulses, with cooling on ice in between. The cooled lysate was centrifuged at 4600xg for 5 min at 4°C, and the supernatant was then transferred to Eppendorf tubes and cleared by centrifugation at 20,000xg for 20 min at 4°C.



Purification of labelled proteins was then performed by copper-catalyzed coupling to an alkyl-agarose resin. For each sample, 200µL Click-iT® Enrichment Resin was washed once with RNase-free water and re-suspended in 1ml 2X Copper Catalyst Solution (freshly prepared according to manufacturer's datasheet). The lysate was then added and incubated at room temperature on a rotating wheel overnight. Subsequently, resin bound proteins were reduced and alkylated as outlined by the manufacturer. For stringent removal of non-specifically bound proteins, 10 washes were performed with each of the SDS buffer, 8 M urea and 20% acetonitrile. To release resin-bound proteins, the resin was then re-suspended in 1ml digestion buffer (100 mM Tris, 2 mM CaCl<sub>2</sub>, 10% acetonitrile), pelleted and aspirated to leave ~200ml digestion buffer and supplemented with 10 µL of 0.1 µg/µL trypsin. This was incubated at 37°C overnight, centrifuged to pellet the resin and the digest supernatant retained.

To prepare the digest for MS, the sample was acidified to pH 1-2 using 10% TFA and processed onto activated stage-tips. Briefly, three C-18 discs (Sigma-Aldrich) were placed in a 200 µL pipet tip, and washed sequentially with 50 µl methanol, 50 µl 80% acetonitrile/0.1% TFA and 70µl 0.1% TFA. The sample was then passed by centrifugation at 1300 rcf for 5 min, washed with 70 µl 0.1% TFA and stored at -20°C.

Total protein samples were prepared simultaneously from the lysate. Approximately 25µg input protein was mixed with 1x NuPAGE LDS sample buffer supplemented with 10mM DTT, loaded onto a 4%–12% NuPAGE™ Bis-Tris Mini Protein Gel and run in MOPS buffer at 190V. Subsequently, the gel was washed with water, stained with Coomassie Protein Stain for 1 h, rinsed several times with water to destain. Protein smears were excised and resulting gel fragments were destained further by two washes in 1:1 50 mM ammonium bicarbonate (ABC) and 50% acetonitrile (ACN) for 30 min at 37°C with shaking at 1200 rpm.

Proteins were then reduced and alkylated in situ. Gel fragments were covered with 10 mM DTT in 50mM ABC for 30 min at 37°C, then shrunk by 5 minutes in ACN. Following this, fragments were incubated with 55 mM iodoacetamide in ABC for 20 min at ambient temperature in the dark, washed with ABC and shrunk twice with ACN. These were then digested overnight with 13 ng/µL trypsin (Pierce) in 10mM ABC containing 10% (v/v) ACN at 37°C.

Following trypsin digestion, samples were acidified and passed on to stage-tips as outlined for eluates. To remove any remaining peptides from the gel, the fragments were incubated for 10 min in 100% ACN, which was then transferred to a 2 mL Protein LoBind tube and dried under vacuum centrifugation at 60°C. The resulting protein pellets were resuspended in 100 µL of 0.1% TFA and passed through the stage tip. These were then washed with 100 µL 0.1% TFA, and placed at -20°C prior to mass spectrometry.

**Mass Spectroscopy:** Peptides were eluted in 40 µL of 80% acetonitrile in 0.1% TFA and concentrated down to 1 µL by vacuum centrifugation (Concentrator 5301, Eppendorf, UK). The peptide sample was then prepared for LC-MS/MS analysis by diluting it to 5 µL by 0.1% TFA.

LC-MS analyses were performed on Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, UK) on a Data Independent Acquisition (DIA) mode, coupled on-line, to an Ultimate 3000 HPLC (Dionex, Thermo Fisher Scientific, UK). Peptides were separated on a 50 cm (2 µm particle size) EASY-Spray column (Thermo Scientific, UK), which was assembled on an EASY-Spray source (Thermo Scientific, UK) and operated constantly at 55°C. Mobile phase A consisted of 0.1% formic acid in LC-MS grade water and mobile phase B consisted of 80% acetonitrile and 0.1% formic acid. Peptides were loaded onto the column at a flow rate of 0.3 µL min<sup>-1</sup> and eluted at a flow rate of 0.25 µL min<sup>-1</sup> according to the following gradient: 2 to 40% mobile phase B in 150 min and then to 95% in 11 min. Mobile phase B was retained at 95% for 5 min and returned back to 2% a minute after until the end of the run (190 min).

MS1 scans were recorded at 120,000 resolution (scan range 350-1650 m/z) with an ion target of 5.0e6, and injection time of 20ms. MS2 was performed in the orbitrap at a resolution of 30,000 with a scan range of 200-2000 m/z, maximum injection time of 55ms and AGC target of 3.0E6 ions. HCD fragmentation was utilised with stepped collision energy of 25.5, 27 and 30.<sup>79</sup> Variable isolation windows were used throughout the scan range, ranging from 10.5 to 50.5 m/z. Narrower isolation windows (10.5-18.5 m/z) were applied from 400-800 m/z and then gradually increased to 50.5 m/z until the end of the scan range. The default charge state was set to 3. Data for both survey and MS/MS scans were acquired in profile mode.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD064070.

**RNAseq:** RNA sequencing for cells growing glucose and after nutrient shift was performed as described,<sup>40</sup> and datasets are deposited in GEO (detailed in Table S4). BY4741 cells were grown to OD<sub>600</sub> 0.45 in 50ml SC -TRP media supplemented with 2% sucrose. Sucrose grown samples were pelleted by centrifugation at 4,600 rpm for 2 minutes at 4°C, snap frozen in a dry-ice ethanol bath, and stored at -80°C. For depletion, cells were collected by filtration and transferred to SC -TRP media supplemented with 2% glycerol and 2% ethanol. Following 16 min, cells were collected and frozen as described for sucrose grown samples. Each condition was collected in biological triplicate.

To extract RNA, cell pellets were re-suspended in TRIzol reagent supplemented with 5 mM DTT final, transferred to 2 ml screw-cap tubes containing 200 µL of zirconia beads and lysed by three cycles (6m/s for 40 sec) in a Fast Prep-24 machine (MP Biomedicals). RNA was cleaned twice with chloroform and precipitated using 0.8 vol isopropanol. Samples were treated with DNase I and further purified using RNA Clean and Concentrator kit.

Libraries for RNAseq were prepared by the Wellcome Trust Clinical Research Facility at Western General Hospital (Edinburgh, UK) using the NEBNext Ultra II Directional RNA Library Prep kit (NEB #7760) and the Poly-A mRNA magnetic isolation module (NEB #E7490) according to the provided protocol. The libraries were sequenced using the NextSeq 2000 platform (Illumina Inc, #20038897), with paired-end, 2x 50 nt outputs.

## Quantification and Statistical Analysis

**RNAseq data analysis:** For the RNAseq experiments reported here, raw sequencing reads were first processed using Flexbar to remove Illumina adapters and low-quality bases. Reads less than 18nt or with quality threshold below 30 were discarded. These trimmed and filtered reads were aligned to the *S. cerevisiae* transcriptome using Salmon, and the reads mapping to each transcript quantified. For this, the GTF file alongside fasta files for genes, cDNAs, ncRNA were downloaded from Ensembl using genome version GCF\_000146045.2\_R64-1-1. These were input to the generateDecoyTranscriptome.sh Salmon script, producing a hybrid fasta containing the decoy sequences from the genome concatenated with the transcriptome. Indices for this decoy-aware transcriptome were then generated using the salmon indexer. The Salmon quant command was used to quantify the processed reads directly against this index.

Following processing, these new datasets were combined with our previous published RNAseq datasets generated by the same pipeline. The resulting data were analyzed in R. Quantified reads were normalized in counts per million (CPM) and filtered using the edgeR package, applying the TMM method and 'filterByExp' function (threshold per sample: CPM > 15; threshold over all samples: CPM > 20) (Table S6). Subsequently, to calculate fold changes (FC) in transcript abundance between conditions, differential expression analysis was performed in edgeR. The 'model.matrix' function was used to model experimental design, dispersion estimates were obtained using 'estimateDisp', fit to a linear model using 'glmQLFit' and expression analysis performed using 'glmQLFTest' (Table S6). The clusterProfiler package was used to analyze and visualize the overrepresentation of GO Biological Process annotations among differential expressed transcripts.

For visualization, pheatmap was used to create Spearman correlation heatmaps and cluster analysis comparing RNAseq experiments. Principle component analysis (PCA) was performed using the log transformed CPM data and plotted using biplot. Scatter graphs comparing logCPM distribution between experiments and volcano plots visualizing differentially expressed genes were created using ggplot2.

**CRAC analysis:** Our previous published CRAC datasets were re-analyzed here to address distinct questions (Figure 4A). These are for the RNA interactomes of the DEAD-box helicase eIF4A and associated factor eIF4B in glucose grown and glucose starved (16 min) *S. cerevisiae*. Utilized data is outlined in Table S4. Processing, mapping and quantification of the sequencing data was described in Bresson et al.<sup>16</sup> These repositd datasets were imported into R for analyses presented here.

Firstly, a high-confidence dataset of quantified mRNA transcripts bound under each condition was created (Table S5). Feature counts were normalized to library size in Reads per Million (RPM) and a threshold of RPM < 0.5 (~1 count) applied. Features were required to have been quantified in a minimum of two replicates, and the median of replicate values was used. Accounting for low coverage in glucose withdrawal conditions, imputation of the threshold 0.5 RPM was used to retain features present in the averaged glucose data. Retained features were then filtered by annotation as 'protein\_coding; exon' to select mRNA transcripts, and utilizing RNAseq data for corresponding conditions lowly

expressed transcripts were removed by a RPKM threshold of 10. For each protein, changes in RNA binding before and after stress were compared using scatterplots created in ggplot2, using the log transformed RPM values. For eIF4B, RPM binding was further normalized by expression using the RNAseq RPKM values at the equivalent timepoint. As mRNA populations will not alter significantly in seconds, RNAseq for glucose was used for 30 sec withdrawal. Targeted analyses of changes in eIF4B binding were performed for the top 100 most increased (induced) and decreased (repressed) transcripts following glucose withdrawal, as determined by differential expression analyses of RNAseq data (outlined above; Table S6). Bar graphs were created using GraphPad Prism 10.

In order to visualize binding across individual transcripts, the coverage at each position along the genome was calculated and normalized to the library size using genomcov from bedtools v2.27.0.<sup>80</sup> The Integrative Genomics Viewer was used to illustrate the distribution of reads across chosen transcripts.<sup>81</sup>

**Metabolic Labelling Proteomics Analysis:** The DIA-NN software platform version 1.9.2. was used to process the DIA raw files, and these were searched against the *Saccharomyces cerevisiae* complete/reference proteome in Uniprot (released in June 2019). Precursor ion generation was based on the chosen protein database (automatically generated spectral library) with deep-learning based spectra, retention time and IMs prediction. Digestion mode was set to specific with trypsin allowing maximum of two missed cleavages. Carbamidomethylation of cysteine was set as fixed modification. Oxidation of methionine, and acetylation of the N-terminus were set as variable modifications. The parameters for peptide length range, precursor charge range, precursor m/z range and fragment ion m/z range as well as other software parameters were used with their default values. The precursor FDR was set to 1%. Annotating library proteins were created with information from the FASTA database: the spectral library contained 6093 proteins and 6093 genes.

Processed protein intensity data was analyzed in R. Standard filtering steps were applied to clean the dataset, and reverse and potential contaminant were removed. The dataset contained triplicate samples of the labelled input (total) and eluate (nascent) proteins for AHA-purification (described above), alongside unlabeled controls. To reliably identify the nascent proteome, only protein groups quantified in all purification eluate samples for at least one labelled condition were retained (n=4165).

Samples were VSN normalized and missing values were imputed with the left-censored MinProb method, as proteins are not expected to be missing at random, using the proteomics specific DEP package (Table S7A). Subsequently, to quantify changes between conditions, differential expression tests were performed using test\_diff function on manually defined contrasts. Obtained *p* values were corrected to control for false discoveries using the highly stringent Bayesian/local false discoveries rate (FDR) method, and proteins with greater than 1.5-fold change ( $FDR \leq 0.05$ ) were considered differentially expressed (Table S7C). Volcano plots visualizing differentially expressed genes were created using ggplot2.

For cluster analysis of nascent protein production, proteins with significant change between +AHA purification eluates were selected using the conventional FDR in R stats. For the

resulting 524 proteins, median intensities for the input and eluate samples were z-scored and k-means clustered (k=4) (Table S7B). For visualization, pheatmap was used to create correlation heatmaps. Clusters 2 and 3 showed significantly increased label incorporation in glucose or no glucose conditions, respectively, and were analyzed further. For genes in these clusters, RNAseq and CRAC data sets were utilized to compare fold change in RNA abundance (RPKM), eIF4B binding (RPM) and protein abundance (intensity) following glucose withdrawal.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data and code availability

- All datasets will be publicly available at the time of publication. Raw and processed sequencing data for sucrose and sucrose withdrawal RNA-seq experiments are available from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) with the accession number GEO: GSE285035. Deposited datasets GEO: GSE283345 and GEO: GSE148166 were also used for analysis. Imaging data are available from Mendeley Data. (<https://doi.org/10.17632/jx59t358zy.2>). Proteomics data are available from ProteomeXchange (<https://www.proteomexchange.org/>) with the accession number PRIDE: PXD064070.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## References

1. Busti S, Coccetti P, Alberghina L, Vanoni M. Glucose Signaling-Mediated Coordination of Cell Growth and Cell Cycle in *Saccharomyces Cerevisiae*. *Sensors* (Basel, Switzerland). 2010; 10: 6195–6240. DOI: 10.3390/s100606195 [PubMed: 22219709]
2. Vander Heiden MGV, Cantley LC, Thompson CB. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*. 2009; 324: 1029–1033. DOI: 10.1126/science.1160809 [PubMed: 19460998]
3. Pearce EL. Metabolism in T cell activation and differentiation. *Curr Opin Immunol*. 2010; 22: 314–320. DOI: 10.1016/j.coi.2010.01.018 [PubMed: 20189791]

4. Broach JR. Nutritional control of growth and development in yeast. *Genetics*. 2012; 192: 73–105. DOI: 10.1534/genetics.111.135731 [PubMed: 22964838]
5. Kayikci Ö, Nielsen J. Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2015; 15 fov068 doi: 10.1093/femsyr/fov068 [PubMed: 26205245]
6. Heinrich S, Hondele M, Marchand D, Derrer CP, Zedan M, Oswald A, Malinowska L, Uliana F, Khawaja S, Mancini R, et al. Glucose stress causes mRNA retention in nuclear Nab2 condensates. *Cell Rep*. 2024; 43 113593 [PubMed: 38113140]
7. Hernández-Elvira M, Sunnerhagen P. Post-transcriptional regulation during stress. *FEMS Yeast Res*. 2022; 22 foac025 doi: 10.1093/femsyr/foac025 [PubMed: 35561747]
8. Kuhn KM, DeRisi JL, Brown PO, Sarnow P. Global and Specific Translational Regulation in the Genomic Response of *Saccharomyces cerevisiae* to a Rapid Transfer from a Fermentable to a Nonfermentable Carbon Source. *Mol Cell Biol*. 2001; 21: 916–927. DOI: 10.1128/MCB.21.3.916-927.2001 [PubMed: 11154278]
9. Janapala Y, Preiss T, Shirokikh NE. Control of Translation at the Initiation Phase During Glucose Starvation in Yeast. *Int J Mol Sci*. 2019; 20 4043 doi: 10.3390/ijms20164043 [PubMed: 31430885]
10. Ashe MP, De Long SK, Sachs AB. Glucose Depletion Rapidly Inhibits Translation Initiation in Yeast. *Mol Biol Cell*. 2000; 11: 833–848. DOI: 10.1091/mbc.11.3.833 [PubMed: 10712503]
11. Guzickowski AR, Harvey AT, Zhang J, Zhu S, Begovich K, Cohn MH, Wilhelm JE, Zid BM. Differential translation elongation directs protein synthesis in response to acute glucose deprivation in yeast. *RNA Biol*. 2022; 19: 636–649. DOI: 10.1080/15476286.2022.2065784 [PubMed: 35491906]
12. Zid BM, O'Shea EK. Promoter sequences direct cyto-plasmic localization and translation of mRNAs during starvation in yeast. *Nature*. 2014; 514: 117–121. DOI: 10.1038/nature13578 [PubMed: 25119046]
13. Crawford RA, Pavitt GD. Translational regulation in response to stress in *Saccharomyces cerevisiae*. *Yeast Chichester Engl*. 2019; 36: 5–21. DOI: 10.1002/yea.3349 [PubMed: 30019452]
14. Dever TE, Kinzy TG, Pavitt GD. Mechanism and Regulation of Protein Synthesis in *Saccharomyces cerevisiae*. *Genetics*. 2016; 203: 65–107. DOI: 10.1534/genetics.115.186221 [PubMed: 27183566]
15. Holmes LEA, Campbell SG, De Long SKD, Sachs AB, Ashe MP. Loss of Translational Control in Yeast Compromised for the Major mRNA Decay Pathway. *Mol Cell Biol*. 2004; 24: 2998–3010. DOI: 10.1128/MCB.24.7.2998-3010.2004 [PubMed: 15024087]
16. Bresson S, Shchepachev V, Spanos C, Turowski TW, Rappsilber J, Tollervey D. Stress-Induced Translation Inhibition through Rapid Displacement of Scanning Initiation Factors. *Mol Cell*. 2020; 80: 470–484. DOI: 10.1016/j.molcel.2020.09.021 [PubMed: 33053322]
17. Gao Z, Putnam AA, Bowers HA, Guenther UP, Ye X, Kindsfather A, Hilliker AK, Jankowsky E. Coupling between the DEAD-box RNA helicases Ded1p and eIF4A. *eLife*. 2016; 5 e16408 doi: 10.7554/eLife.16408 [PubMed: 27494274]
18. Sen ND, Zhou F, Harris MS, Ingolia NT, Hinnebusch AG. eIF4B stimulates translation of long mRNAs with structured 5' UTRs and low closed-loop potential but weak dependence on eIF4G. *Proc Natl Acad Sci USA*. 2016; 113: 10464–10472. DOI: 10.1073/pnas.1612398113 [PubMed: 27601676]
19. Wang J, Shin B-S, Alvarado C, Kim J-R, Bohlen J, Dever TE, Puglisi JD. Rapid 40S scanning and its regulation by mRNA structure during eukaryotic translation initiation. *Cell*. 2022; 185: 4474–4487. DOI: 10.1016/j.cell.2022.10.005 [PubMed: 36334590]
20. Ho B, Baryshnikova A, Brown GW. Unification of Protein Abundance Datasets Yields a Quantitative *Saccharomyces cerevisiae* Proteome. *Cell Syst*. 2018; 6: 192–205. [PubMed: 29361465]
21. Goswami B, Nag S, Ray PS. Fates and functions of RNA-binding proteins under stress. *Wiley Interdiscip Rev RNA*. 2023. e1825 [PubMed: 38014833]
22. Linder P, Jankowsky E. From unwinding to clamping – the DEAD box RNA helicase family. *Nat Rev Mol Cell Biol*. 2011; 12: 505–516. [PubMed: 21779027]
23. Tanner NK, Linder P. DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol Cell*. 2001; 8: 251–262. [PubMed: 11545728]



24. Ray BK, Lawson TG, Kramer JC, Cladaras MH, Grifo JA, Abramson RD, Merrick WC, Thach RE. ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. *J Biol Chem.* 1985; 260: 7651–7658. [PubMed: 3838990]
25. Weber CA, Sekar K, Tang JH, Warmer P, Sauer U, Weis K.  $\beta$ -Oxidation and autophagy are critical energy providers during acute glucose depletion in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA.* 2020; 117: 12239–12248. DOI: 10.1073/pnas.1913370117 [PubMed: 32430326]
26. Xu L, Bretscher A. Rapid Glucose Depletion Immobilizes Active Myosin V on Stabilized Actin Cables. *Curr Biol.* 2014; 24: 2471–2479. DOI: 10.1016/j.cub.2014.09.017 [PubMed: 25308080]
27. Jia S, Marjavaara L, Buckland R, Sharma S, Chabes A. Determination of deoxyribonucleoside triphosphate concentrations in yeast cells by strong anion-exchange high-performance liquid chromatography coupled with ultraviolet detection. *Methods Mol Biol.* 2015; 1300: 113–121. [PubMed: 25916709]
28. Ranjbarian F, Sharma S, Falappa G, Taruschio W, Chabes A, Hofer A. Isocratic HPLC analysis for the simultaneous determination of dNTPs, rNTPs and ADP in biological samples. *Nucleic Acids Res.* 2022; 50: e18. doi: 10.1093/nar/gkab1117 [PubMed: 34850106]
29. Warner JR. The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci.* 1999; 24: 437–440. [PubMed: 10542411]
30. Riba A, Di Nanni N, Mittal N, Arhné E, Schmidt A, Zavolan M. Protein synthesis rates and ribosome occupancies reveal determinants of translation elongation rates. *Proc Natl Acad Sci USA.* 2019; 116: 15023–15032. DOI: 10.1073/pnas.1817299116 [PubMed: 31292258]
31. Blum S, Schmid SR, Pause A, Buser P, Linder P, Sonenberg N, Trachsel H. ATP hydrolysis by initiation factor 4A is required for translation initiation in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA.* 1992; 89: 7664–7668. DOI: 10.1073/pnas.89.16.7664 [PubMed: 1502180]
32. Iost I, Dreyfus M, Linder P. Ded1p, a DEAD-box Protein Required for Translation Initiation in *Saccharomyces cerevisiae*, Is an RNA Helicase. *J Biol Chem.* 1999; 274: 17677–17683. [PubMed: 10364207]
33. Bernstein J, Patterson DN, Wilson GM, Toth EA. Characterization of the Essential Activities of *Saccharomyces cerevisiae* Mtr4p, a 3'  $\rightarrow$  5' Helicase Partner of the Nuclear Exosome \*. *J Biol Chem.* 2008; 283: 4930–4942. [PubMed: 18096702]
34. Cordin O, Hahn D, Beggs JD. Structure, function and regulation of spliceosomal RNA helicases. *Curr Opin Cell Biol.* 2012; 24: 431–438. [PubMed: 22464735]
35. Lorsch JR, Herschlag D. The DEAD Box Protein eIF4A. 1. A Minimal Kinetic and Thermodynamic Framework Reveals Coupled Binding of RNA and Nucleotide. *Biochemistry.* 1998; 37: 2180–2193. [PubMed: 9485364]
36. Yourik P, Aitken CE, Zhou F, Gupta N, Hinnebusch AG, Lorsch JR. Yeast eIF4A enhances recruitment of mRNAs regardless of their structural complexity. *eLife.* 2017; 6 e31476 doi: 10.7554/eLife.31476 [PubMed: 29192585]
37. Cavallius J, Merrick WC. Site-directed mutagenesis of yeast eEF1A. Viable mutants with altered nucleotide specificity. *J Biol Chem.* 1998; 273: 28752–28758. [PubMed: 9786872]
38. Entian KD. Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. *Mol Gen Genet.* 1980; 178: 633–637. [PubMed: 6993859]
39. Randez-Gil F, Sanz P, Entian KD, Prieto JA. Carbon Source-Dependent Phosphorylation of Hexokinase PII and Its Role in the Glucose-Signaling Response in Yeast. *Mol Cell Biol.* 1998; 18: 2940–2948. DOI: 10.1128/mcb.18.5.2940 [PubMed: 9566913]
40. Ristová M, Bexley K, Shchepachev V, Cook AG, Tollervey D. Pin4 Links Post-transcriptional and Transcriptional Responses to Glucose Starvation in Yeast. *bioRxiv.* 2024; doi: 10.1101/2024.11.13.623376
41. Miles S, Bradley GT, Breeden LL. The budding yeast transition to quiescence. *Yeast.* 2021; 38: 30–38. [PubMed: 33350501]
42. Granneman S, Kudla G, Petfalski E, Tollervey D. Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. *Proc Natl Acad Sci USA.* 2009; 106: 9613–9618. DOI: 10.1073/pnas.0901997106 [PubMed: 19482942]



43. Zedan M, Schürch AP, Heinrich S, Garciá PAG, Khawaja S, Weis K. Newly synthesized mRNA selectively escapes translational repression following acute stress. *bioRxiv*. 2024; doi: 10.1101/2024.04.14.589419
44. Yang Q, Jankowsky E. ATP- and ADP-dependent modulation of RNA unwinding and strand annealing activities by the DEAD-box protein DED1. *Biochemistry*. 2005; 44: 13591–13601. [PubMed: 16216083]
45. Buskirk AR, Green R. Ribosome pausing, arrest and rescue in bacteria and eukaryotes. *Philos Trans R Soc Lond B Biol Sci*. 2017; 372 20160183 doi: 10.1098/rstb.2016.0183 [PubMed: 28138069]
46. Young DJ, Guydosh NR, Zhang F, Hinnebusch AG, Green R. Rli1/ABCE1 recycles terminating ribosomes and controls translation reinitiation in 3' UTRs in vivo. *Cell*. 2015; 162: 872–884. DOI: 10.1016/j.cell.2015.07.041 [PubMed: 26276635]
47. Shoemaker CJ, Green R. Kinetic analysis reveals the ordered coupling of translation termination and ribosome recycling in yeast. *Proc Natl Acad Sci USA*. 2011; 108: E1392–E1398. DOI: 10.1073/pnas.1113956108 [PubMed: 22143755]
48. Bagamery LE, Justman QA, Garner EC, Murray AW. A Putative Bet-Hedging Strategy Buffers Budding Yeast against Environmental Instability. *Curr Biol*. 2020; 30: 4563–4578. DOI: 10.1016/j.cub.2020.08.092 [PubMed: 32976801]
49. Egner A, Jakobs S, Hell SW. Fast 100-nm resolution three-dimensional microscope reveals structural plasticity of mitochondria in live yeast. *Proc Natl Acad Sci USA*. 2002; 99: 3370–3375. DOI: 10.1073/pnas.052545099 [PubMed: 11904401]
50. dos Passos JB, Vanhalewyn M, Brandão RL, Castro IM, Nicoli JR, Thevelein JM. Glucose-induced activation of plasma membrane H(+)-ATPase in mutants of the yeast *Saccharomyces cerevisiae* affected in cAMP metabolism, cAMP-dependent protein phosphorylation and the initiation of glycolysis. *Biochim Biophys Acta*. 1992; 1136: 57–67. [PubMed: 1322708]
51. Belinchón MM, Gancedo JM. Glucose controls multiple processes in *Saccharomyces cerevisiae* through diverse combinations of signaling pathways. *FEMS Yeast Res*. 2007; 7: 808–818. [PubMed: 17428308]
52. Kotyk A, Lapathitis G, Horák J. Critical findings on the activation cascade of yeast plasma membrane H<sup>+</sup>-ATPase. *FEMS Microbiol Lett*. 2003; 226: 175–180. [PubMed: 13129624]
53. Mazón MJ, Eraso P, Portillo F. Specific phosphoantibodies reveal two phosphorylation sites in yeast Pma1 in response to glucose. *FEMS Yeast Res*. 2015; 15 f0v030 [PubMed: 26019146]
54. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Mol Biol Cell*. 2000; 11: 4241–4257. DOI: 10.1091/mbc.11.12.4241 [PubMed: 11102521]
55. Sen ND, Zhou F, Ingolia NT, Hinnebusch AG. Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast DEAD-box RNA helicases Ded1 and eIF4A. *Genome Res*. 2015; 25: 1196–1205. DOI: 10.1101/gr.191601.115 [PubMed: 26122911]
56. G, Wv; Z, K; B, Tk; D, Ja. Cap-independent translation is required for starvation-induced differentiation in yeast. *Science*. 2007; 317 [PubMed: 17761883]
57. Glauninger H, Bard JAM, Hickernell Wong, Airoidi EM, Li W, Singer RH, Paul S, Fei J, Sosnick TR, Wallace EWJ, et al. Transcriptome-wide mRNA condensation precedes stress granule formation and excludes stress-induced transcripts. *bioRxiv*. 2024; 2024.04.15.589678 doi: 10.1016/j.molcel.2025.11.003 [PubMed: 41289999]
58. Alberts, B, Johnson, A, Lewis, J, Raff, M, Roberts, K, Walter, P. *Molecular Biology of the Cell*. (Fourth Edition): Garland Science; 2002.
59. Skog S, Tribukait B, Sundius G. Energy metabolism and ATP turnover time during the cell cycle of Ehrlich ascites tumour cells. *Exp Cell Res*. 1982; 141: 23–29. [PubMed: 7117415]
60. Sims NR, Muyderman H. Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta*. 2010; 1802: 80–91. [PubMed: 19751827]
61. Kinoshita H, Maki T, Yasuda K, Kishida N, Sasaoka N, Takagi Y, Kakizuka A, Takahashi R. KUS121, a valosin-containing protein modulator, attenuates ischemic stroke via preventing ATP depletion. *Sci Rep*. 2019; 9 11519 doi: 10.1038/s41598-019-47993-w [PubMed: 31395936]

62. Schädlich IS, Winzer R, Stabernack J, Tolosa E, Magnus T, Rissiek B. The role of the ATP-adenosine axis in ischemic stroke. *Semin Immunopathol.* 2023; 45: 347–365. DOI: 10.1007/s00281-023-00987-3 [PubMed: 36917241]
63. Buttgerit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. *Biochem J.* 1995; 312: 163–167. DOI: 10.1042/bj3120163 [PubMed: 7492307]
64. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 2004; 5: R80. doi: 10.1186/gb-2004-5-10-r80 [PubMed: 15461798]
65. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS.* 2012; 16: 284–287. DOI: 10.1089/omi.2011.0118 [PubMed: 22455463]
66. Zhang X, Smits AH, van Tilburg GB, Ovaa H, Huber W, Vermeulen M. Proteome-wide identification of ubiquitin interactions using UbIA-MS. *Nat Protoc.* 2018; 13: 530–550. [PubMed: 29446774]
67. Demichev V, Messner CB, Vernardis SI, Lilley KS, Ralser M. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nat Methods.* 2020; 17: 41–44. DOI: 10.1038/s41592-019-0638-x [PubMed: 31768060]
68. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010; 26: 139–140. DOI: 10.1093/bioinformatics/btp616 [PubMed: 19910308]
69. Dodt M, Roehr JT, Ahmed R, Dieterich C. FLEXBAR-Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. *Biology (Basel).* 2012; 1: 895–905. DOI: 10.3390/biology1030895 [PubMed: 24832523]
70. Wickham, H. ggplot2. Springer International Publishing; 2016.
71. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods.* 2017; 14: 417–419. DOI: 10.1038/nmeth.4197 [PubMed: 28263959]
72. Laughery MF, Hunter T, Brown A, Hoopes J, Ostbye T, Shumaker T, Wyrick JJ. New vectors for simple and streamlined CRISPR–Cas9 genome editing in *Saccharomyces cerevisiae*. *Yeast.* 2015; 32: 711–720. DOI: 10.1002/yea.3098 [PubMed: 26305040]
73. Winz M-L, Peil L, Turowski TW, Rappsilber J, Tollervey D. Molecular interactions between Hel2 and RNA supporting ribosome-associated quality control. *Nat Commun.* 2019; 10: 563. doi: 10.1038/s41467-019-08382-z [PubMed: 30718516]
74. Sabouri N, Viberg J, Goyal DK, Johansson E, Chabes A. Evidence for lesion bypass by yeast replicative DNA polymerases during DNA damage. *Nucleic Acids Res.* 2008; 36: 5660–5667. DOI: 10.1093/nar/gkn555 [PubMed: 18772226]
75. Castello A, Horos R, Strein C, Fischer B, Eichelbaum K, Steinmetz LM, Krijgsvelde J, Hentze MW. System-wide identification of RNA-binding proteins by interactome capture. *Nat Protoc.* 2013; 8: 491–500. [PubMed: 23411631]
76. Matia-González AM, Laing EE, Gerber AP. Conserved mRNA-binding proteomes in eukaryotic organisms. *Nat Struct Mol Biol.* 2015; 22: 1027–1033. DOI: 10.1038/nsmb.3128 [PubMed: 26595419]
77. McKellar SW, Ivanova I, van van Nues RW, Cordiner RA, Chauhan M, Christopoulou N, Worboys W, Langford A, Jensen TH, Granneman S. Monitoring Protein-RNA Interaction Dynamics In Vivo at High Temporal Resolution Using  $\chi$ CRAC. *J Vis Exp.* 2020; 159 e61027 [PubMed: 32449723]
78. van Nues R, Schweikert G, de Leau E, Selega A, Langford A, Franklin R, Iosub I, Wadsworth P, Sanguinetti G, Granneman S. Kinetic CRAC uncovers a role for Nab3 in determining gene expression profiles during stress. *Nat Commun.* 2017; 8: 12. doi: 10.1038/s41467-017-00025-5 [PubMed: 28400552]
79. Olsen JV, Macek B, Lange O, Makarov A, Horning S, Mann M. Higher-energy C-trap dissociation for peptide modification analysis. *Nat Methods.* 2007; 4: 709–712. [PubMed: 17721543]
80. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics.* 2010; 26: 841–842. DOI: 10.1093/bioinformatics/btq033 [PubMed: 20110278]

81. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform.* 2013; 14: 178–192. DOI: 10.1093/bib/bbs017 [PubMed: 22517427]

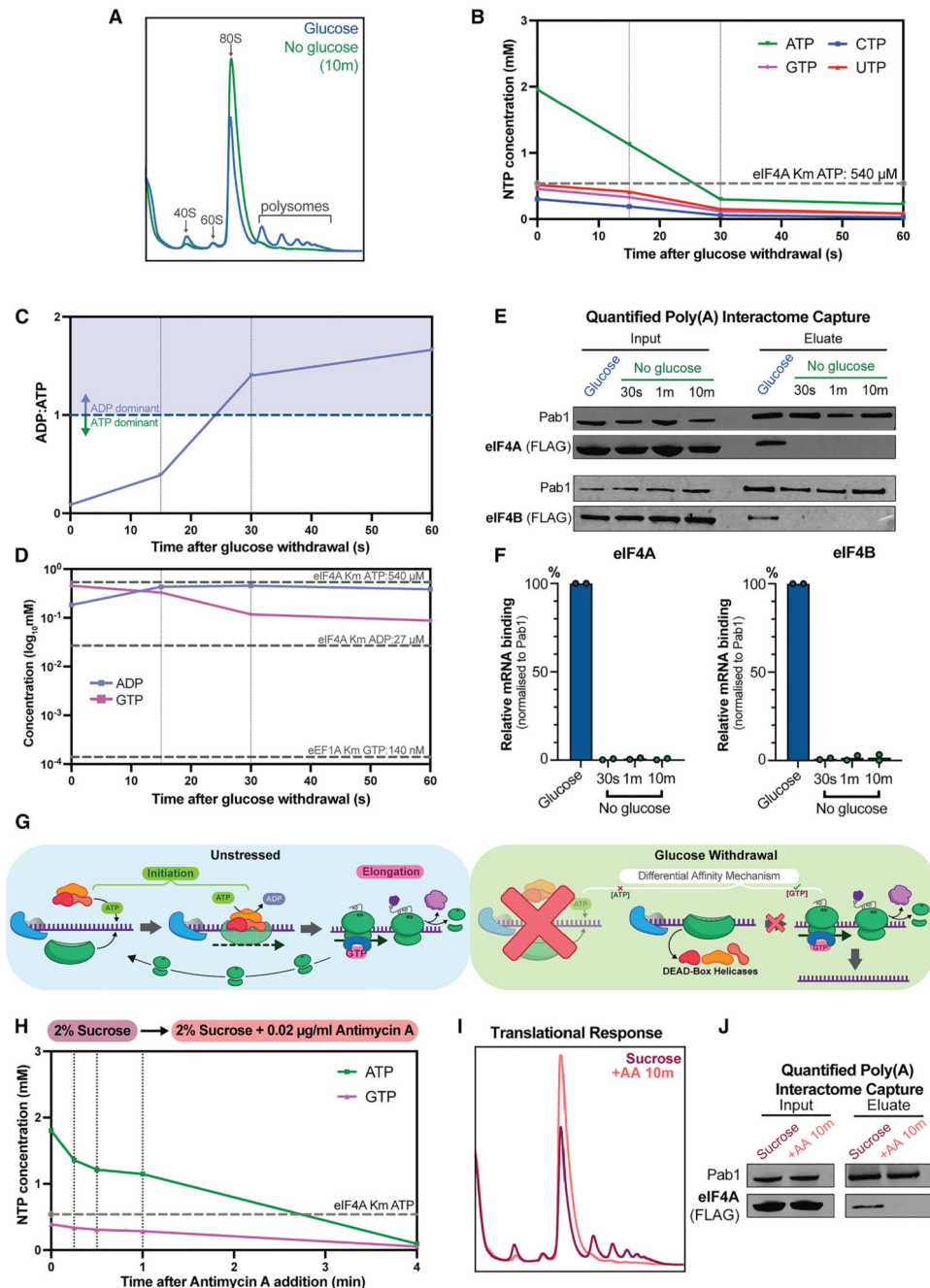


**Highlights**

- A metabolic shift drives the immediate stress response after glucose withdrawal
- Rapid stress-induced translation inhibition is triggered by NTP depletion within 30 s
- Translational adaptation to energy depletion from differential affinities of NTPases
- Programmed responses to energy depletion do not require specific signaling pathways

**In brief**

Bexley et al. report that rapid metabolic shifts mediate immediate, protective changes in RNA metabolism following energy depletion. In yeast, protein production is regulated by tuning enzymatic constants to energy charge ratios. This initiates a global shutdown within seconds in response to declining intracellular ATP, independent of the specific cause.



**Figure 1. Rapid intracellular ATP depletion directly drives translation shutdown**

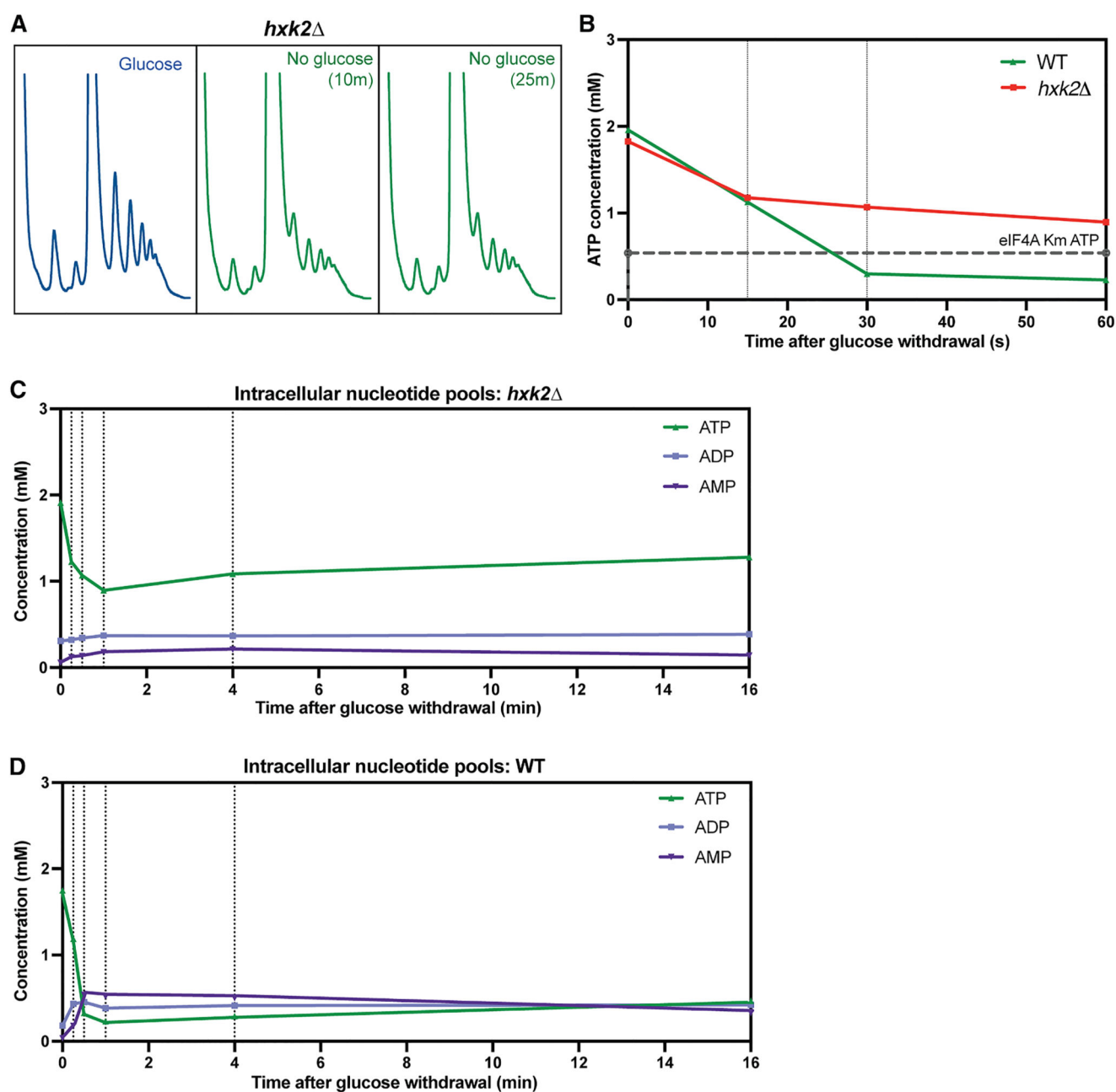
(A) Polysome gradient analyses assessing translational status on 2% glucose, and 10 min after transfer to 2% glycerol/2% ethanol (no glucose). Positions of small (40S) and large (60S) ribosomal subunits, monosomes (80S), and polysomes with multiple ribosomes are indicated.

(B) Estimated intracellular nucleoside triphosphate (NTP) pools following glucose withdrawal (mean values;  $n = 3$ ).  $K_m$  for eIF4A is indicated (dashed line).

(C) ADP:ATP ratio following glucose withdrawal (mean values;  $n = 3$ ).



- (D) GTP and ADP concentrations (log10-adjusted means;  $n = 3$ ). Binding constants for eIF4A and eEF1A are indicated (dashed lines).
- (E) Analysis of translation initiation factor recovery following *in vivo* UV crosslinking (254 nm) and poly(A) selection. Cells were irradiated during growth on 2% glucose or 30 s, 1 min, and 10 min after transfer to 2% glycerol/2% ethanol. Total proteins in cell lysates (input) and proteins recovered after mRNA binding to oligo [dT]<sub>25</sub> beads (eluate) were analyzed by western blots decorated with anti-FLAG for FLAG-eIF4A and FLAG-eIF4B.
- (F) Quantification of (E). Intensities were normalized to Pab1 as positive control for input and pull-down efficiency ( $n = 2$ ). Bar graphs represent the amount of eIF4A/4B bound to mRNA as a percentage of pre-shift level.
- (G) Proposed “differential affinity” mechanism for rapid translational arrest. In the absence of glucose, flux through the glycolytic pathway is stopped immediately and NTP production falls, while usage continues, leading to rapid depletion. ATP levels fall below the binding constants for the low-affinity DEAD-box helicases required for initiation, displacing these ATP-dependent RNA-binding protein from transcripts. Comparatively, elongation factors are high affinity and retain GTP binding, allowing translation to continue from initiated ribosomes. In the absence of recycling, polysomes run off, leaving free translation machinery and transcripts.
- (H) Intracellular ATP and GTP levels (mean values;  $n = 3$ ) during growth in 2% sucrose and following addition of mitochondrial inhibitor antimycin A (AA) to 0.02  $\mu\text{g/mL}$ .  $K_{\text{m}}[\text{ATP}]$  for eIF4A is indicated (dashed line).
- (I) Polysome gradient analyses for yeast grown in 2% sucrose and 10 min after addition of 0.02  $\mu\text{g/mL}$  AA.
- (J) Western blot analysis of translation initiation factor mRNA binding. *In vivo* UV crosslinking was performed during growth on 2% sucrose or 10 min after addition of 0.02  $\mu\text{g/mL}$  AA, followed by poly(A) selection and analysis as in (D).



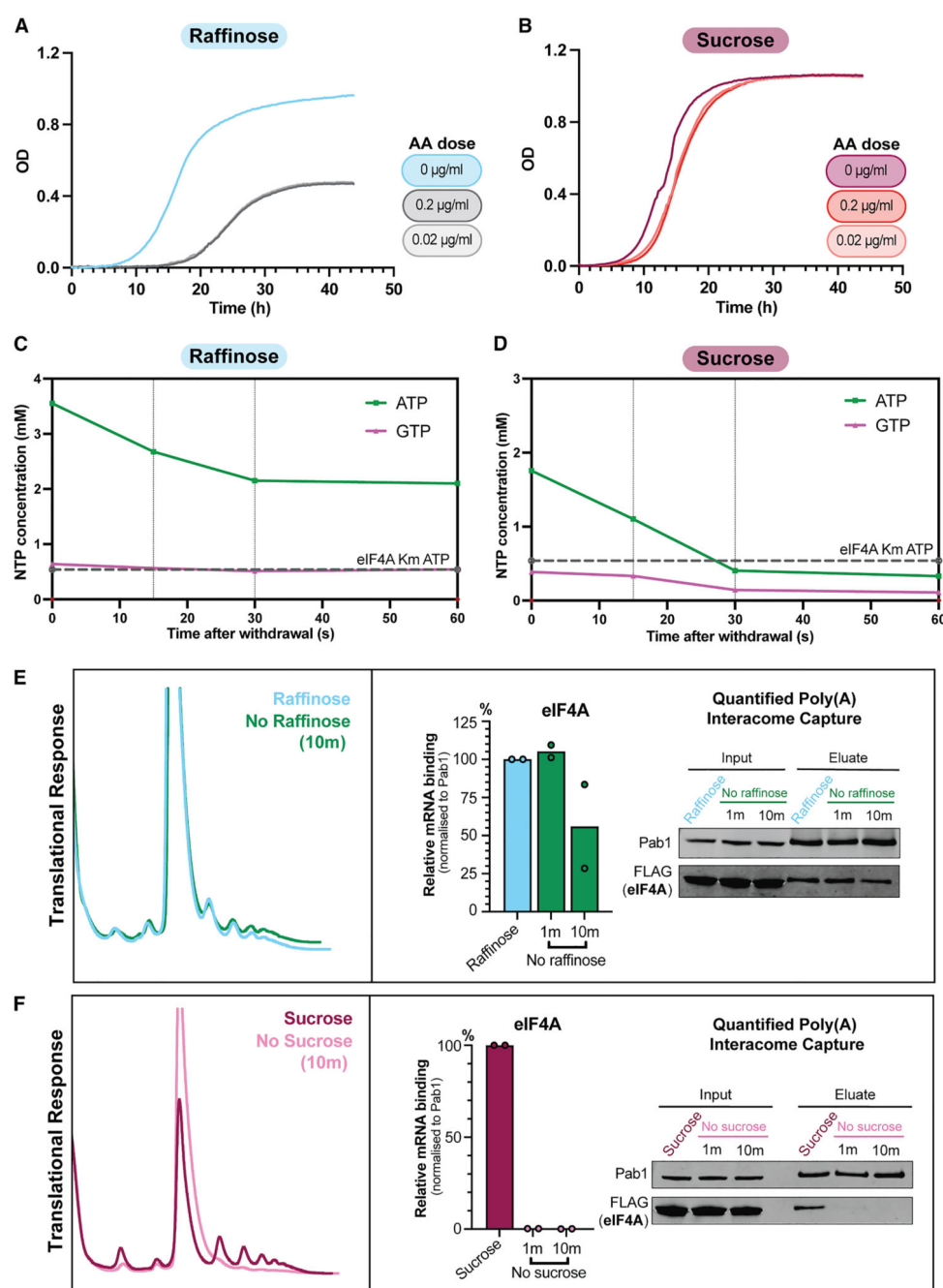
**Figure 2. *hxx2Δ* mutants reveal active control of NTP remodeling**

(A) Polysome gradient analyses in *hxx2Δ* strains assessing translational status on 2% glucose, and 10 or 25 min after transfer to 2% glycerol/2% ethanol (no glucose).

(B) Intracellular ATP concentrations (mean values;  $n = 3$ ) in wild-type and *hxx2Δ* strains following glucose withdrawal.

(C) Levels of adenosine nucleosides in *hxx2Δ* strain (mean values;  $n = 6$ , except  $n = 3$  for 15 s).

(D) Levels of adenosine nucleosides in wild type (mean values;  $n = 5$ , except  $n = 2$  for 15 s).

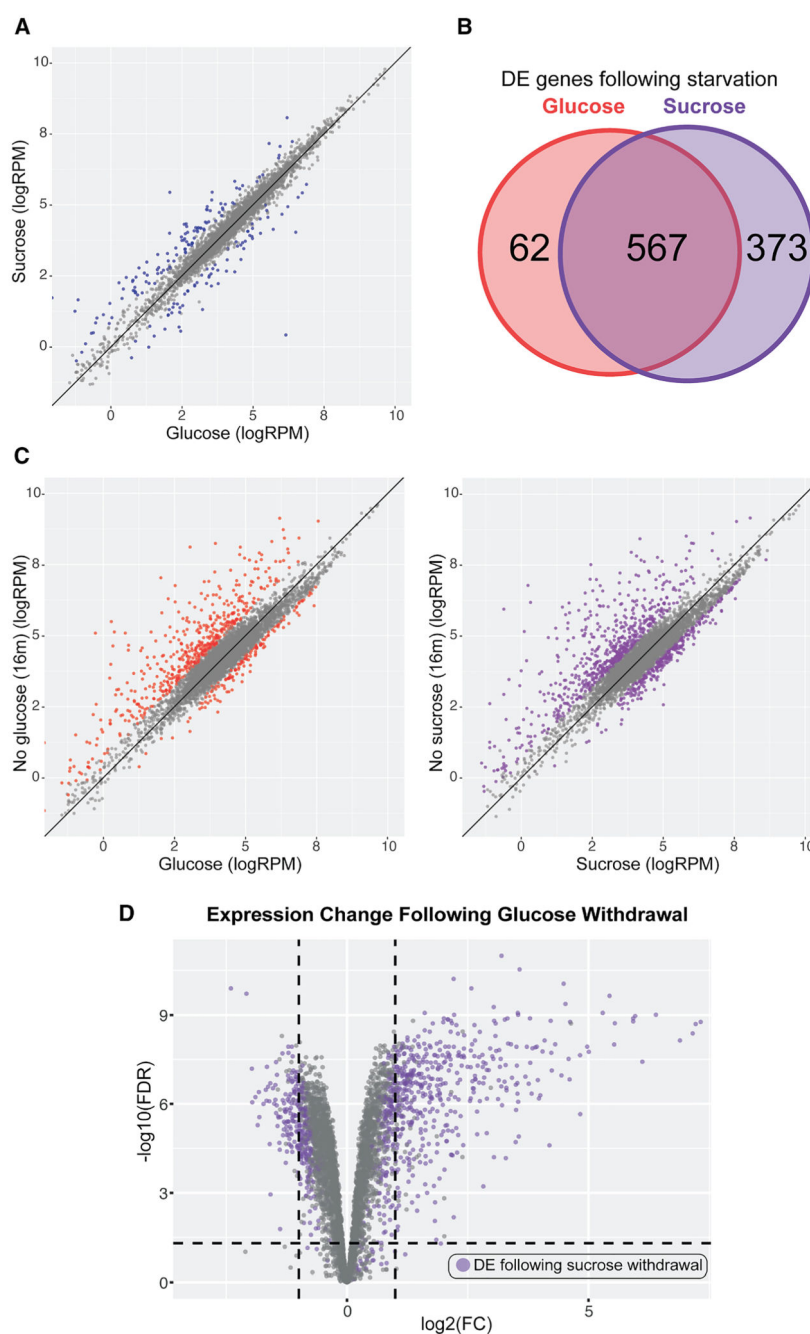


**Figure 3. Metabolic changes are dependent on the prior carbon source**

- (A) Growth curves ( $\text{OD}_{600\text{nm}}$ ) with raffinose as carbon source, in the presence of the mitochondrial inhibitor antimycin A (AA) at 0.2  $\mu\text{g/ml}$ , 0.02  $\mu\text{g/ml}$  ( $n = 2$ )
- (B) As (A) for yeast grown on sucrose.
- (C) Intracellular NTP levels (mean values;  $n = 3$ ) following shift from 2% raffinose to 2% glycerol/2% ethanol.  $K_m[\text{ATP}]$  for eIF4A is indicated (dashed line).
- (D) As (C) for carbon source shift from 2% sucrose to 2% glycerol/2% ethanol.

(E) Translational response to raffinose withdrawal. mRNA association of eIF4A was assessed using poly(A)-interactome capture as described for Figures 1D and 1E. Bar graphs show eIF4A binding relative to the pre-shift level ( $n = 2$ ). Polysome profiles 10 min after raffinose withdrawal.

(F) As in (E) for sucrose withdrawal.



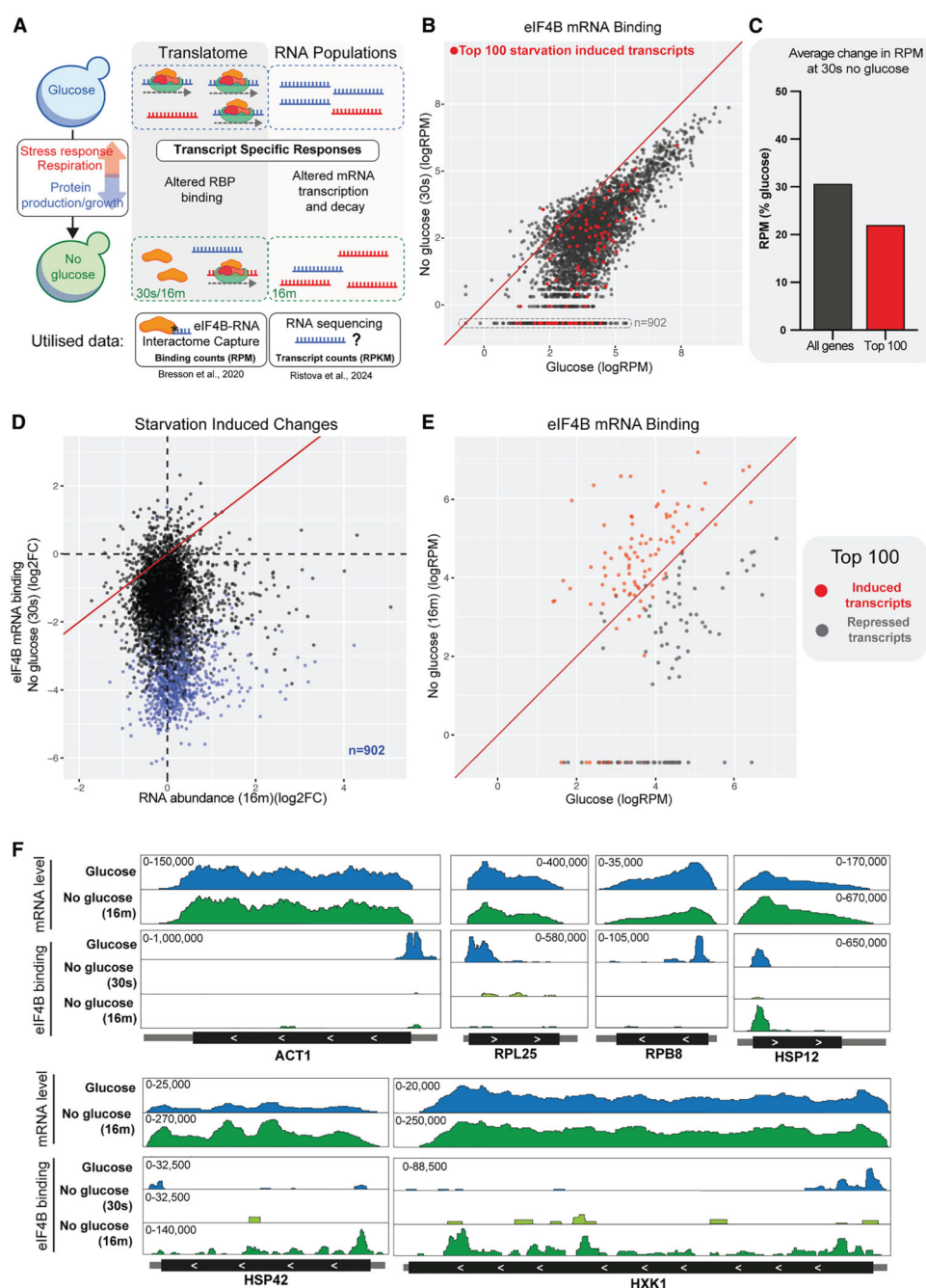
**Figure 4. Transcriptional reprogramming in response to energy depletion**

(A) Transcript abundance reads per million, RPM) in glucose versus sucrose media ( $n = 3$ ). Significantly altered mRNAs (fold change [FC]  $> 2$  and false discovery rate [FDR]  $< 0.05$ ) are colored blue.

(B) Venn diagram showing numbers of transcripts significantly altered following each carbon source withdrawal.

(C) Scatterplots comparing transcript abundance (RPM) following withdrawal of glucose (left) or sucrose (right) ( $n = 3$  in each case). Significantly altered mRNAs ( $FC > 2$ ,  $FDR < 0.05$ ) are colored.

(D) Volcano plot showing the transcript-level FC distribution after glucose withdrawal for 16 min relative to control cells. For response comparison, transcripts differentially expressed ( $FC > 2$ ,  $FDR < 0.05$ ) following withdrawal of sucrose as a carbon source are colored purple.



**Figure 5. Immediate translation arrest occurs globally**

(A) Overview of bioinformatic approach to investigate transcript-specific translational responses to glucose withdrawal. In response to glucose withdrawal, select transcripts are transcriptionally induced and translated against a background of global shutdown. To relate translation initiation factor displacement to such differential responses, transcript-level data quantifying eIF4B binding prior to or following glucose withdrawal (30 s and 16 min) was combined with time-matched RNA sequencing data.



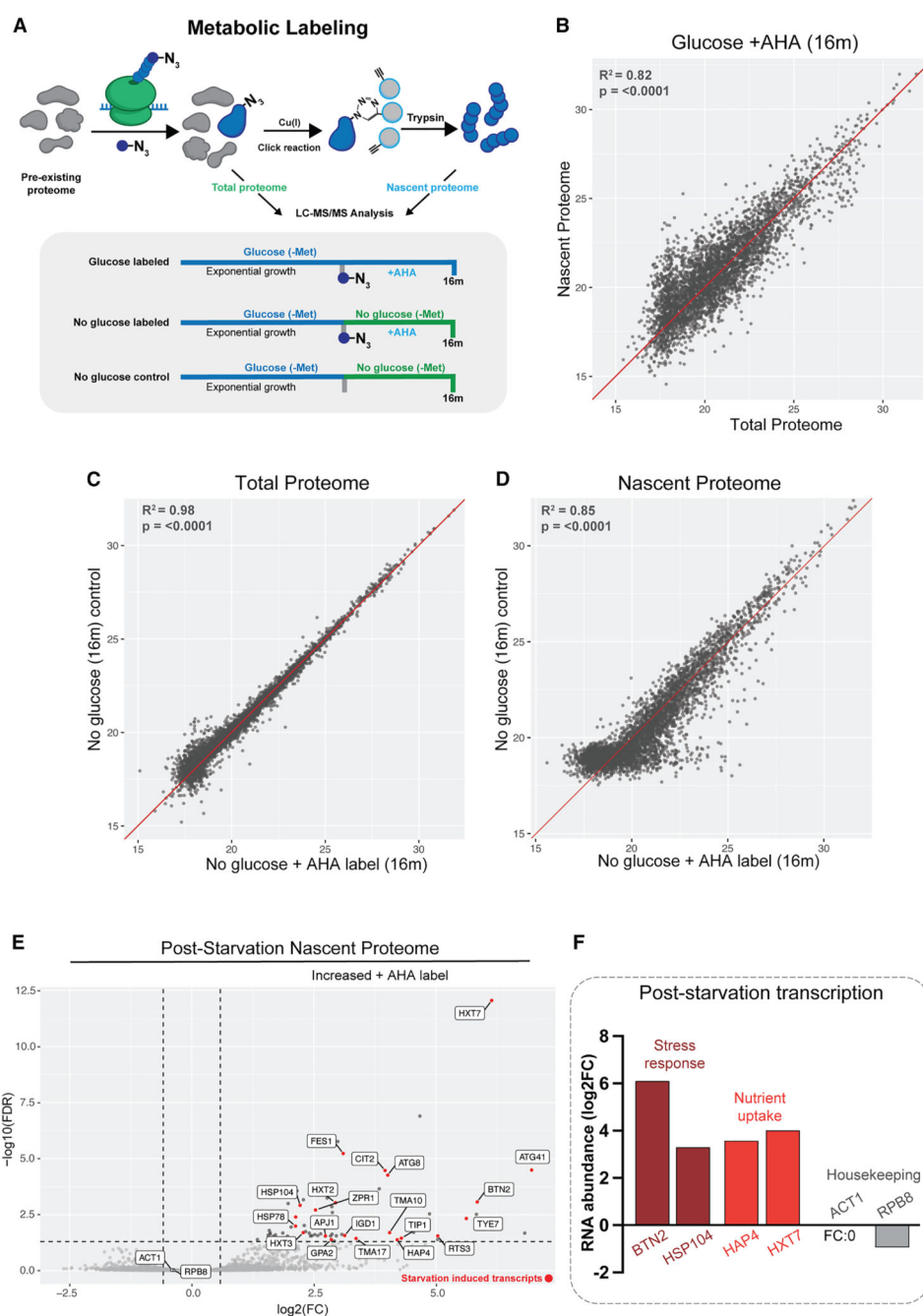
(B) Scatterplot comparing changes in mRNA binding by eIF4B prior to, or following 30 s after, glucose withdrawal. Transcript counts are normalized to library size in RPM. The 100 transcripts showing the greatest FC in abundance following 16 min glucose withdrawal (FDR < 0.05) are colored red. For transcripts not detectably bound after glucose withdrawal, pseudocounts matching the minimum threshold (RPM = 0.5) were added (lower, boxed line;  $n = 902$ ).

(C) Quantification of changes in (B) (as % of RPM in glucose) for all detected ( $n = 4,377$ ) or most induced ( $n = 100$ ) mRNAs.

(D) Scatterplot comparing changes in eIF4B binding with subsequent changes in mRNA levels following energy stress. The FC in eIF4B association 30 s after glucose withdrawal is compared with transcript abundance after 16 min. Undetected transcripts retained by pseudocount addition are indicated in blue;  $n = 902$ . The  $x = y$  line is shown in red.

(E) Scatterplot comparing changes in eIF4B binding to mRNAs of the most differentially expressed genes following 16 min glucose withdrawal. The 100 transcripts with the greatest increase (red) or decrease (black) in abundance are shown (FDR < 0.05). mRNA binding by eIF4B is presented normalized to library size, in RPM. The lower line represents mRNAs maintained by pseudocount addition.

(F) Examples of eIF4B binding to “housekeeping” mRNAs, ACT1, RPL25, and RPB8 or stress-induced mRNAs, HSP12, HSP42, and HXK1. Each set of tracks is normalized to library size (RPM), and relative scale is given in the top corner. Total mRNA abundance in RNA-seq is shown for comparison. For induced transcripts, scales differ between glucose and no glucose tracts. Open reading frames are indicated as black boxes (directionality indicated by white arrows), with UTRs as flanking gray boxes. Further examples are given in Figure S5.



**Figure 6. Metabolic labeling detects the newly synthesized translome**

(A) Scheme of metabolic labeling experiment. L-Azidohomoalanine (AHA) is incorporated into nascent polypeptides. Cu(I)-catalyzed click chemistry covalently links the azide moiety in AHA to an alkyne-agarose resin, allowing stringent washing. Purified peptides are released by trypsin digestion and analyzed by mass spectrometry (MS). Labeling was for 16 min during growth in 2% glucose or commencing immediately after shift to 2% glycerol/ethanol (no glucose). Unlabeled controls were prepared in parallel.

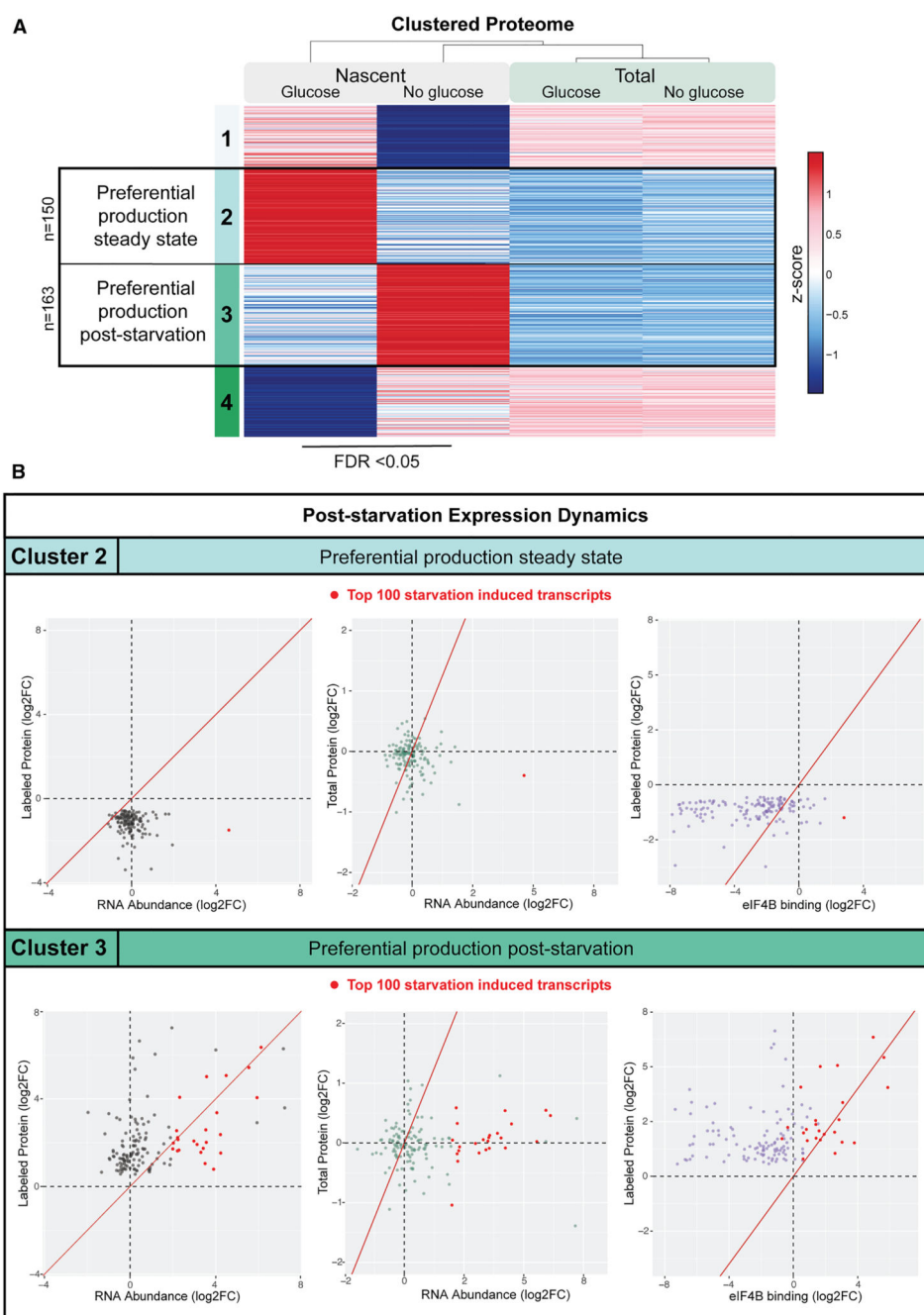
(B) Scatterplot comparing protein intensity ( $\log_2$ ) in the total and AHA-labeled proteomes during steady-state growth on glucose ( $n = 3$ ). Strength of correlation ( $R^2$ ) and significance ( $p$  value) are indicated.

(C) Scatterplot comparing total proteomes (input) for AHA-labeled and unlabeled control samples following 16 min glucose withdrawal. Median protein intensities ( $n = 3$ ) are plotted on  $\log_2$  scale.

(D) Scatterplot comparing purified proteomes (nascent) from AHA-labeled and unlabeled control samples following 16 min glucose withdrawal. Strength of correlation ( $R^2$ ) and significance ( $p$  value) are indicated.

(E) Volcano plot showing the FC in purified proteomes (nascent) between AHA-labeled and control samples prepared 16 min following glucose withdrawal. Proteins with  $FC > 1.5$  and  $FDR < 0.05$  were considered significantly differentially enriched. Enriched proteins whose mRNA levels also show significant increase ( $FDR < 0.05$ ) during glucose withdrawal are colored red and labeled. Strength of correlation ( $R^2$ ) and significance ( $p$  value) are indicated.

(F) Changes in mRNA abundances for selected proteins involved in stress response and sugar uptake, compared with mRNAs for housekeeping proteins (highlighted in E).



**Figure 7. Newly synthesized mRNAs are preferentially translated**

(A) Cluster analysis of nascent protein production. 524 proteins exhibited significantly ( $FDR < 0.05$ ) different abundance in the proteome labeled over 16 min prior to, or immediately following, glucose withdrawal. For the proteins, intensities in total and nascent proteomes were k-means clustered.

(B) Scatterplots for proteins in cluster 2 (higher label incorporation in glucose) and cluster 3 (higher label incorporation after glucose withdrawal). Graphs show FC in mRNA levels (RPKM), eIF4B binding (RPM), and protein abundance (intensity) in glucose or following

glucose withdrawal. The 100 proteins with most increased mRNA abundance following 16 min glucose withdrawal are colored red. The  $x = y$  line is shown in red; note that scales are not the same for each axis in some of the graphs.

