Gcn4 (ATF4) drives a methionine-dependent anabolic program by enabling metabolic precursor supply.

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Abstract:

How cells manage biomolecule supply to enable constructive metabolism for growth is a fundamental question. Methionine directly activates genomic programs where translation and metabolism are induced. In otherwise amino acid limitations, methionine induced ribosomal biogenesis, carbon metabolism, amino acid and nucleotide biosynthesis. Here, we find that the methionine-induced anabolic program is carbon-source independent, and requires the 'starvation-responsive' transcription factor Gcn4/ATF4. Integrating transcriptome and ChIP-Seq analysis, we decipher direct and indirect, methionine-dependent roles for Gcn4. Methionine-induced genes enabling metabolic precursor biosynthesis critically require Gcn4; contrastingly ribosomal genes are partly repressed by Gcn4. Gcn4 binds promoter-regions and transcribes a subset of metabolic genes, driving amino acid (particularly lysine and arginine) biosynthesis. This sustained lys/arg supply maintains high translation capacity, by allowing the translation of lys/arg enriched transcripts, notably the translation machinery itself. Thus, we discover how Gcn4 enabled metabolic-precursor supply bolsters protein synthesis demand, and drive a methionine-mediated anabolic program.

Introduction

In order for cells to sustain growth, and thereby proliferation, a controlled supply of biosynthetic precursors is essential. These precursors include amino acids for not just protein translation, but also metabolite synthesis, nucleotides (to make RNA and DNA), lipids, ATP and several co-factors. Such a balanced cellular economy therefore entails multiple regulatory systems that sense nutrients, and control global responses in order to manage metabolic resources and ensure coordinated growth outputs. Here, the model eukaryote, *Saccharomyces cerevisiae*, has been instrumental in building our general understanding of global nutrient-dependent responses, for identifying what are 'limiting metabolites' for a cell, addressing resource allocations within cells, as well as to uncover conserved mechanisms of nutrient-sensing (Boer et al., 2010; Brauer et al., 2006; Dikicioglu et al., 2011; Gresham et al., 2011; Gurvich et al., 2017; Gutteridge et al., 2010; Metzl-Raz et al., 2017; Saldanha et al., 2004; Zaman et al., 2008).

Interestingly, several studies from yeast and other systems show that the presence of some metabolites, even in nutrient-limited conditions, directly induces cell growth programs. A now well-studied example is that of acetyl-CoA. At sufficient concentrations of acetyl-CoA, cells exit quiescence, complete the cell-division cycle, and activate global gene expression programs enabling increased cell growth and division (Cai and Tu, 2011; Cai et al., 2011; Krishna and Laxman, 2018; Kuang et al., 2014; Pedro et al., 2015; Rowicka et al., 2007; Shi and Tu, 2013; Wellen and Thompson, 2010). In this context, the availability of methionine (and its metabolite S-adenosyl methionine) strongly correlates with cell 'growth states' (Lees et al., 2017; Sutter et al., 2013; Walvekar et al., 2018b). In mammals, strong associations exist between methionine availability and tumor growth (Halpern et al., 1974; Sugimura et al., 1959), and methionine availability influences cancer therapy, by controlling one-carbon and nucleotide metabolism (Gao et al., 2019b, 2019a). Most pertinently, studies in yeast directly show that methionine controls cell growth. In cells growing in otherwise amino acid limited medium and a poor carbon source (lactate), supplementing methionine inhibits autophagy (Wu and Tu, 2011), and increases cell growth and proliferation (Laxman et al., 2014; Sutter et al., 2013), with effects stronger than the addition of all other non-sulfur amino acids provided together. For sustaining such growth, cells must manage a steady supply of metabolic precursors as well as high rates of translation. We recently discovered that methionine triggers a hierarchically organized anabolic program, where cells strongly induce ribosomal genes, and key metabolic nodes including the pentose phosphate pathway, as well as glutamate, all amino acid, and nucleotide biosynthesis (Walvekar et al., 2018b). This directly suggests that methionine acts as a growth signal, and enables an entire anabolic transformation, where the biosynthetic precursors that sustain growth are sufficiently produced. However, the mechanisms by which methionine induces the synthesis of these biosynthetic precursors are unclear. Unexpectedly, this previous study suggested that the transcription factor Gcn4 might support this anabolic program (Walvekar et al., 2018b). But the actual role played by Gcn4 in such an anabolic program was both unclear and unforeseen.

Gcn4 (called ATF4 in mammals) is a transcriptional master-regulator, conventionally studied for its role during starvation and stress (Hinnebusch, 2005; Mascarenhas et al., 2008; Natarajan et al., 2001; Yang et al., 2000). During severe amino acid starvation, the translation of Gcn4 mRNA increases, through the activation of the Gcn2 kinase, and subsequent eIF2-alpha phosphorylation (Hinnebusch, 1994, 2005; Hinnebusch et al., 2004). This resultant increase in Gcn4 protein allows it to function as a transcriptional activator, where it induces transcripts primarily involved in amino acid biosynthesis, thereby allowing cells to restore amino acid levels and survive severe starvation (Hinnebusch, 2005; Mittal et al., 2017; Natarajan et al., 2001; Rawal et al., 2018). Almost our entire

current knowledge of Gcn4 function comes from studying its roles during starvation. In contrast, this recent study surprisingly found that when methionine is abundant, cells induce Gcn4, in a context of high cell proliferation (Walvekar et al., 2018b). How might Gcn4 sustain this methionine-dependent anabolic program?

In this study, we show that Gcn4 is induced by methionine in a carbon source independent manner, and controls essential components of an anabolic program. Notably, methionine-induced Gcn4 directly transcribes genes required for amino acids and transamination reactions, and indirectly regulates other essential 'nitrogen' metabolic processes. This enables cells to rapidly assimilate nitrogen into amino acids, as well as nucleotides, when methionine is abundant. We elucidate the direct and indirect, methionine-dependent roles of Gcn4, to separate the metabolic component from the induction of translation-related genes. Finally, we discover the critical importance of Gcn4-enabled biosynthetic precursor supply in sustaining an anabolic program, and particularly for maintaining a high translation capacity. Through this, we show how a transcriptional master-regulator, conventionally viewed as a 'survival-factor', can drive resource production to sustain an anabolic program induced by a sentinel metabolite, methionine.

Results

Methionine induces an anabolic response independent of carbon sources

Earlier studies using prototrophic yeast growing with lactate as a sole carbon source observed that adding methionine, in otherwise amino acid limited conditions, inhibits autophagy and promotes growth (Sutter et al., 2013; Wu and Tu, 2011). In these conditions, methionine activates a hierarchically organized anabolic program at the level of gene expression as well as metabolic state (Walvekar et al., 2018b). Here, multiple nodes that lead to anabolism are induced. This includes increased expression of ribosomal transcripts, and induced expression and metabolic flux through the pentose phosphate pathway and glutamate biosynthesis, which provide co-factors and precursors supporting high amino acid and nucleotide production (Walvekar et al., 2018b). In summary, methionine acts as a potent anabolic signal for cells. However, given that lactate (the carbon source used in these prior studies) is a non-preferred carbon source (especially for yeast), we first asked if such a methionine-dependent genome-wide response is universal, and independent of carbon source. To address this, we studied the effect of methionine supplementation in high glucose medium, as glucose is the preferred carbon source for yeast.

We estimated the global transcriptional outputs of wild-type yeast cells in response to methionine using mRNA sequencing. The experimental design was as follows: prototrophic yeast cells growing in complex, glucose-rich medium (YPD) were shifted to either synthetic minimal medium with glucose (**MM_{gluc}**), or this same medium supplemented with 2 mM methionine (**MM_{gluc}+Met**). No other amino acids were supplemented. Cells were collected after 1 hour of the shift, and RNA extracted for further processing (Figure 1A). The mRNAs were enriched using polyA enrichment, and sequenced using Illumina 2500 for 50 cycles (see material and methods). For every experimental condition, data were obtained from two biological replicates. Normalized read counts between the biological replicates are well correlated (Figure S1). For each strain we obtained ~30-35 million uniquely mapped reads. From these data, we found a methionine-dependent, differential expression of 969 genes (fold change > 1.5 fold) in MM_{gluc}+Met, relative to MM_{gluc}. Of these differentially expressed genes, 574 genes were induced by methionine, and 395 genes were downregulated (Figure 1A & Supplementary WS1). A Gene-ontology (GO) based analysis was carried out to understand the functional categories of the differentially expressed genes using g:Profiler - a GO-based analysis tool (Raudvere et al., 2019). A GO-based analysis of the upregulated genes shows a significant enrichment of amino acid biosynthesis, cytoplasmic translation, mitochondrial translation, and nucleotide biosynthesis (hypergeometric test, p-value <= 0.05) (Figure 1B, and

Supplementary WS3). In contrast, the downregulated genes are involved in sulfur compound metabolic process (which is expected, since methionine is supplemented), ion transport, carbohydrate metabolism, hexose transport (Figure 1C, Supplementary WS3). These results show that supplementing only methionine, even in standard glucose medium, strongly induces amino acid biosynthesis, nucleotide biosynthesis and translation related genes– all of which are hallmark signatures of an anabolic program (Airoldi et al., 2009; Brauer et al., 2008; Xiao and Grove, 2009).

These groupings obtained for cells growing with glucose as the carbon source (MM_{gluc}) resembled our prior studies carried out in cells growing with lactate as a sole carbon source (MM_{lac}) (Walvekar et al., 2018b). Therefore, to better-compare the methionine dependent response across carbon sources, we first classified genes involved in amino acid biosynthesis and nucleotide biosynthesis genes as an '*anabolic gene group*', constituting 158 genes. Out of these 158 anabolic genes, ~40% of the genes are upregulated in MM_{gluc}+Met (63 out of 158), with a fold increase of > 1.5 fold. 67% of these genes induced in MM_{gluc}+Met overlap with genes induced in MM_{lac}+Met (Fisher exact test p-value 1.4×10^{-13}). These data indicate that the induction of anabolic genes by methionine is universal, regardless of the carbon source used (Figure 1D & Supplementary WS2). Similarly, we next grouped ~390 genes that encode for proteins involved in various steps of translation as '*translation related genes*'. Out of 390 translation related genes, ~170 genes were upregulated in MM_{gluc}+Met. As with the anabolic genes considered earlier, a significant proportion of these translation genes overlapped with genes induced upon adding methionine to cells growing in lactate (Fisher exact test p-value $<10^{-10}$) (Figure 1D & Supplementary WS2). Thus, we find that the methionine-dependent induction of an anabolic program is carbon source independent.

As a final, functional output of this anabolic program, we had observed that supplementing methionine to cells growing in lactate strongly increased *de novo* amino acid and nucleotide synthesis (Walvekar et al., 2018b). We tested the conservation of this output in minimal glucose medium, using a quantitative, targeted LC/MS/MS based flux approach (Walvekar et al., 2018a). We pulsed N^{15}-labelled ammonium sulfate, or C^{13-}labelled glucose to cells in MM_{gluc} or MM_{gluc}+Met, and measured label incorporation into newly synthesized amino acids, or nucleotides. We observed a strong increase in *de novo* amino acid and nucleotide biosynthesis upon methionine supplementation (Figure 1E & 1F). Collectively, these results reiterate that the increased anabolic response due to methionine is not only at the level of transcription, but is also reflected by the metabolic state of the cell, independent of the carbon source available.

GCN4 regulates the anabolic component of the methionine-dependent transcriptional program

Gcn4 is a transcriptional master-regulator, conventionally studied in the context of starvation and other stress conditions, as part of the integrated stress response (Hinnebusch, 2005; Hinnebusch and Natarajan, 2002; Mascarenhas et al., 2008; Natarajan et al., 2001; Pakos-Zebrucka et al., 2016). Therefore, nearly all existing studies of Gcn4 have used pharmacological inhibitors of amino acid biosynthesis, such as 3-amino triazole (3-AT) or sulfo meturon (SM) to induce Gcn4 (Akhter and Rosonina, 2016; Albrecht et al., 1998; Mittal et al., 2017; Natarajan et al., 2001; Rawal et al., 2018). Indeed, our current understanding of Gcn4 function comes primarily from contexts of nutrient-stress and starvation conditions. In contrast, our observations show a Gcn4 induction by methionine (Walvekar et al., 2018b), coincident with increased cell growth and proliferation. This is very distinct from conditions of starvation and low growth, and hence, we wanted to better understand this possible novel role of Gcn4, in an anabolic program.

First, we examined if the methionine-dependent increase of Gcn4 protein is also observed in cells using glucose as a carbon source. Gcn4 protein levels strongly increase when methionine is supplemented to MM_{gluc} (Figure 2A and Figure S2A). This observation reiterates that methionine

induces Gcn4 protein levels in cells in minimal medium (irrespective of the carbon sources). We next dissected how much of the methionine dependent anabolic program is mediated by Gcn4. To address this, we compared transcriptomes of wild type and *Δgcn4* cells in MM_{gluc}+Met. For every sample we generated about 30-35 million reads of 50bp length. Transcriptome data obtained from two biological replicates were analyzed and the normalized read counts between the replicates were very well correlated (Figure S1). By comparing the transcriptome of WT and *Δgcn4* cells in MM_{gluc}+Met condition, we found a very strong, Gcn4-dependent response in the presence of methionine. ~900 genes were differentially expressed in Δ*gcn4*, compared to wild type cells in MM_{gluc}+Met. Here, 514 genes were upregulated, and 398 genes were downregulated in *Δgcn4* cells in the presence of methionine (fold change cut-off of >= 2 fold) (Figure 2B & Supplementary WS1). Contrastingly, in MM_{gluc} medium (without supplemented methionine), far fewer genes (~160) showed any differential expression at all in *Δgcn4* relative to WT (Figure 2C). These data strongly suggest that the effect of the loss of Gcn4 on transcription is methionine-specific, and support a critical role for Gcn4 in a methionine-dependent context.

To better understand the global consequences of the loss of Gcn4 in methionine-rich conditions, we functionally categorized these differentially expressed genes using g:Profiler (Raudvere et al., 2019). A GO-based analysis of upregulated genes in *Δgcn4* shows a strong enrichment of the categories of 'cytoplasmic translation', as well as 'ncRNA processing', 'RNA maturation' and 'RNA methylation' (Figure 2E). This surprisingly revealed that the transcripts associated with protein translation, which were already induced by methionine, further increase in the absence of Gcn4. Also interestingly, among the translation related genes, a subset of genes associated with 'mitochondrial translation' are downregulated in *Δgcn4,* while the 'cytoplasmic translation' related genes are induced in *Δgcn4* (and are even higher than in wild-type cells in methionine). In contrast, the genes that are strongly downregulated in *Δgcn4* cells are primarily involved in amino acid biosynthetic processes, nucleotide biosynthetic processes, mitochondrial translation, NADP metabolic processes and pyruvate metabolism (Figure 2D & Supplementary WS3). This reveals that Gcn4 is essential for the induction of genes involved in these metabolic processes, which is a majority of the methionine induced anabolic program.

From these data, we understand that Gcn4 regulates the cytoplasmic and mitochondrial translation related genes differently. Here, there is a somewhat contradictory observation, as follows. Several earlier studies have reported, repression of ribosomal gene by the elevated Gcn4 level in cells (Bose et al., 2012; Joo et al., 2010; Mittal et al., 2017). In contrast, here, despite high Gcn4 levels in cells, methionine strongly induces ribosomal and translation related genes. This suggests additional regulation of ribosomal transcripts by methionine, while the induced Gcn4 keeps this ribosomal gene expression under check. Collectively, we find that the metabolic component of the methionine dependent transcriptional program requires Gcn4. Gcn4 activates the transcription of genes involved in amino acid biosynthesis, as well as nucleotide biosynthesis. Gcn4 does not induce other processes, and represses cytoplasmic translation related genes, under this condition.

Gcn4 strongly binds to its target promoters in the presence of methionine

The above results suggest that the metabolic component of the methionine-dependent transcriptional reprogramming is mediated by Gcn4. Given this, the obvious question to ask was, how much of this transcriptional output is directly (vs. indirectly) regulated by Gcn4? To address this, we performed chromatin immunoprecipitation (ChIP)- sequencing of Gcn4 in MM_{gluc} and MM_{gluc}+Met conditions. Notably, while prior studies of Gcn4 ChIP have been carried out by using amino acid synthesis inhibitors to induce Gcn4 (chemical induction) (Mittal et al., 2017; Rawal et al., 2018), to our knowledge this is the first study of Gcn4 ChIP under conditions of high anabolism and proliferation, where a natural amino acid (methionine) is abundant, and where Gcn4 is induced.

First, we asked what is the Gcn4 DNA binding activity when induced by methionine. We performed ChIP of Gcn4 (with a FLAG-epitope incorporated into the C-terminus in the endogenous *GCN4* locus), using cells grown in MM_{gluc} and MM_{gluc}+Met, with MM_{gluc} essentially acting as a control. We considered the peaks that are represented in both the biological replicates for further analysis. Notably, normalized read counts of biological replicates are very well correlated (Figure S2B). Here, we identified 320 Gcn4 binding peaks in the cells grown in MM_{gluc}+Met, whereas, there were no consensus peaks observed in replicate samples of cells grown in MM_{gluc} (Figure 3A & Supplementary WS4). Enhanced Gcn4 occupancy on the target gene promoter in MM_{gluc}+Met condition was further validated using ChIP-qPCR analysis (Figure S5). This shows that the GCN4 occupancy on DNA increases in the presence of methionine, and confirms direct binding of Gcn4 to DNA in methionine-rich conditions.

Next, we analyzed the Gcn4 binding signals around the transcription and translation start site of the genes found within 750bp around the identified peaks. Transcription start site data available for cells in log phase grown in YPD (rich, complex medium with glucose) - the nearest possible condition to that used in this study - was obtained from the YeasTSS database (McMillan et al., 2019). The TSS identified using the CAGE method reported in this database was used for our analysis. Interestingly, a majority of the Gcn4 binding peaks in MM_{gluc}+Met are found upstream of these annotated transcription start sites (Figure 3B). Further, a similar analysis with the translation start sites of the target genes shows higher read coverage upstream of the translation start site (Figure S3).

We also analyzed the genomic features of the identified peaks using the HOMER program package (Heinz et al., 2010). Notably, we observed a very strong enrichment of Gcn4 binding to the promoter region of the targets. 263 out of 320 peaks bind within the promoter region of target genes (-1kb to +100bp around the TSS), while the remaining peaks bind at intergenic regions (11), exons (17) or close to transcription termination sites (29) (Figure 3C & Supplementary WS4). This is notably different from recent studies of Gcn4 binding to DNA, in contexts where Gcn4 is induced during severe amino acid biosynthesis inhibition (Mittal et al., 2017; Rawal et al., 2018). In these starvation contexts, most of the Gcn4 binding peaks are found in the coding region of genes. To further address this, for the non-coding and the ORF peaks regions reported in the previous study (Rawal et al., 2018), we calculated the Gcn4 binding signal in our Gcn4 ChIP seq data (from the MM_{gluc}+Met condition). Strikingly, we find that the signal in ORF peaks is significantly lower than the non-coding peak under MM_{gluc}+Met condition (p-value < 10e-8) (Figure S4). Whereas, a similar analysis performed using the Gcn4 ChIP-seq data from (Rawal et al., 2018) show little differences in the signal intensity between ORF and Non-coding peaks (p-value of 0.002). These analysis further confirms that, in this context of methionine dependent induction of Gcn4, its binding is highly specific and restricted to the promoter regions of genes. The genes at which promoter peaks are found are very similar between the Rawal study and our study.

Two possibilities explain this data, based entirely on the contexts in which the role of Gcn4 is being studied. First, the amounts of Gcn4 protein (as induced by methionine) will be different from the other conditions tested, as the mode of induction of Gcn4 is entirely different in these studies. Therefore, since any protein's affinity to its target depends on its dosage in the cell as well as the presence of other competing factors, there will be differential binding affinity to the targets, as is well known for most transcription factors (Aow et al., 2013; Kribelbauer et al., 2019). Second, the context of Gcn4 induction is entirely distinct. In this context Gcn4 is supporting a strong anabolic program, while in all other contexts studied, Gcn4 is part of a nutrient stress/survival response. Hence this response in cells is distinct from other Gcn4 responses during extreme amino acid starvation.

Next, we searched for the enrichment of any sequence motifs in the peaks identified in the MM_{gluc}+Met condition using The MEME-suite (Bailey et al., 2009). We found that these peaks were enriched for the conserved Gcn4 binding motifs (Arndt and Fink, 1986; Mittal et al., 2017; Oakley and Dervan, 1990). Strikingly, 81% (260 out of 320) of the peaks that we identify have at least one of

the variants of the Gcn4 binding motif 'TGANTCA' (Figure 3D), showing that Gcn4 in this context still primarily recognizes its high-affinity DNA binding motif. Collectively, our data shows that in the presence of methionine, increased Gcn4-protein occupancy on the target-sites increases. These data further suggest that regardless of the mode of Gcn4 induction, it binds specifically to a conserved motif.

Direct and Indirect targets of Gcn4 in methionine dependent induction of Gcn4

We therefore asked how much of the Gcn4-dependent transcriptional response (in the presence of methionine) is directly regulated by Gcn4? To identify direct targets of Gcn4 in methionine-dependent gene regulation, we overlaid the transcriptome data (*Δgcn4 vs WT* in MM_{gluc}+Met) with the ChIP-seq data (from MM_{gluc}+Met). Out of the 398 genes that are downregulated in Δ*gcn4,* 133 are direct targets of Gcn4 (Figure 4A & Supplementary WS4). Contrastingly, Gcn4 directly regulates only 24 out of 514 upregulated genes (Figure 4C & Supplementary WS4). These results strengthen the role of Gcn4 as a transcriptional activator. GO-based analysis of the genes directly transcribed by Gcn4 reveals an enrichment primarily of amino acid biosynthetic genes. Notably, indirectly activated targets are enriched for nucleotide biosynthesis, the pentose phosphate pathway, and mitochondrial translation (Figure 4B, Supplementary WS3). In addition to the amino acid biosynthetic genes, Gcn4 directly activates genes involved in other critical functions, particularly the Sno1 and Snz1 genes – which are components of pyridoxal synthase, required for transamination reactions that lead to amino acid synthesis, and Nde1- the NADH dehydrogenase (Figure 4E). Some genes, notably Sno1 and Snz1 are present adjacent to each other on the genome, and are regulated bidirectionally by Gcn4 (Figure 4E). This is not exclusive to the conditions used in this study, but also other conditions that induce Gcn4 (Natarajan et al., 2001) and these genes have Gcn4 binding sites in its promoter (Nishizawa et al., 2008). In contrast to the Snz1 and Sno1 pair that is bidirectionally activated by Gcn4, the Trm1 and Mdh2 pair of genes are bidirectionally repressed by Gcn4 (Figure 4F). These data show that in the presence of methionine, Gcn4 directly increases the expression of primarily the amino acid biosynthetic arm, whereas the methionine-dependent activation of nucleotide biosynthetic genes, pentose phosphate pathway, mitochondrial translation related genes are indirectly regulated by Gcn4. Collectively, a majority of the metabolic component of the methionine-dependent anabolic program is regulated by Gcn4.

As discussed, in the presence of methionine Gcn4 directly upregulates the genes of various amino acid biosynthetic pathways (Figure 5A). In this context, subsets of amino acid biosynthetic genes are very strongly induced. Notably, every single gene of arginine biosynthetic pathway, and nearly every gene of lysine, histidine and branched chain amino acid biosynthetic pathways are directly, and strongly activated by Gcn4 (Figure 5A, 5B, 5C). This hints that Gcn4 might be critical for the supply of particularly arginine and lysine, during the methionine mediated anabolic program.

Finally, Gcn4 can negatively regulate (repress) ribosomal and translation related genes (Joo et al., 2010; Mittal et al., 2017; Natarajan et al., 2001; Rawal et al., 2018). In agreement with earlier ChIP-seq studies (Mittal et al., 2017; Rawal et al., 2018), we also find that Gcn4 indirectly represses translation related genes, except for the following- RPL14B, RPS9A,RPL36A and RRP5, these are directly repressed by Gcn4 under this condition (Figure 4D). Through this repressive activity, Gcn4 likely enables cells to manage the extent of ribosomal gene induction due to methionine.

Summarizing, the anabolic program mediated by Gcn4 (in the presence of methionine) can be broken into two parts. First, Gcn4 directly induces amino acid biosynthesis genes, as well as transamination reactions. As part of a feed-forward program, the nucleotide biosynthesis genes and

the PPP (which complete the methionine-mediated anabolic program (Walvekar et al., 2018b) are also indirectly induced. Further, the ribosomal/translation related genes that are induced by methionine in wild-type cells are further induced upon the loss of Gcn4 in this condition, suggesting that Gcn4 manages the extent of ribosomal gene induction due to methionine.

Gcn4 controls metabolic flux towards increased amino acid biosynthesis

To directly estimate the contribution of methionine-induced Gcn4 towards individual amino acid biosynthesis, we used a targeted LC/MS/MS based approach (Walvekar et al., 2018a) to measure amino acid synthesis flux, based on stable-isotope incorporation. Here, we pulsed N^{15} labelled ammonium sulfate into MM_{gluc} and MM_{gluc}+Met medium, where cells were already growing, rapidly isolated cells and extracted metabolites, and measured label-incorporation into newly synthesized amino acids. Consistent with the transcriptome data, we found strongly increased amino acid biosynthesis in MM_{gluc}+Met. Expectedly, the loss of *GCN4* severely decreased the flux towards amino acid biosynthesis, indicating that amino acid biosynthesis is severely arrested in *Δgcn4* (Figure 6A & Figure S6). Here, most strikingly, there is a near-complete loss of arginine and lysine biosynthesis, and a strong reduction in histidine biosynthesis in *Δgcn4* cells, which is greater than the reduction in other amino acids.

These data show that methionine-induced Gcn4 controls the supply amino acids, particularly of arginine, lysine and histidine.

Gcn4 dependent lysine and arginine supply is essential to maintain high translational capacity induced by methionine.

From our data thus far, it is clear that Gcn4 helps supply cells with several metabolites, particularly amino acids, when methionine is abundant. However, it is not yet fully apparent how important this Gcn4 induction is. Separately, methionine also strongly induces translation/ribosomal genes, and this is even further enhanced in the absence of Gcn4. We predicted that the supply of precursor metabolites by Gcn4 might be critically limiting for the high rates of macromolecular biosynthesis that sustain growth. Specifically, we hypothesized that this Gcn4-dependent availability of arginine and lysine could constrain the cell from fueling high rates of translation.

Although the amino acid compositions of proteins are evolutionarily optimized, our understanding of amino acid supply vs demand remains woefully inadequate (Hofmeyr, 2008; Hofmeyr and Cornish-Bowden, 2000). As amino acids are the building blocks of proteins, translation naturally depends on available amino acid pools in the cell. We therefore wondered if there were categories of proteins that were particularly enriched for arginine and lysine, within the genome, and if this had any correlation with Gcn4 function. For this, we divided the total number of proteins in the *S. cerevisiae* genome into three bins based on the percentage of arginine and lysine content of the protein (%R+K). The bin1 comprises of 1491 proteins with the lowest percentage of R+K (bin1; < 10% R+K), bin2 has 3033 proteins with moderate %R+K content (bin2; 10-13% R+K), and bin3 comprises of the 1501 proteins, with very high %R+K (bin3; >13% R+K) (Figure 7A). We next asked if these bins were enriched for any groups of functional pathways (based on Gene Ontology). Bin1 and bin2 have very large, disparate groups of GO terms, with no unique enrichment. However, bin3 was strongly enriched for ribosomal and translation related genes (Figure 7B). This arginine and lysine distribution in translation related genes are significantly higher compared to genome wide distributions (Wilcox test, p-value < 10^{-10}) (Figure 7C). Thus, translation related proteins are highly enriched for arginine and lysine amino acids.

Next, we asked if there is any correlation between the genes regulated by Gcn4 (in MM_{gluc}+Met), and the %R+K composition of those encoded proteins. Strikingly, we noticed that a significant

proportion of the genes that are repressed by Gcn4 fall in bin3 (~40%, Fisher's exact test P-value < 10^{-10}) (Figure 7D & Supplementary WS5). Therefore, a significant proportion of the genes induced by methionine, and further induced in Δ*gcn4* are arginine and lysine rich. This suggests an even deeper management of overall, methionine-induced anabolism by Gcn4, where the translation of these arginine and lysine enriched proteins, which will be required for high translation, require Gcn4-dependent precursors.

Given this startling observation, we asked if cells still sustain the synthesis of R+K rich genes when Gcn4 is absent. To evaluate this unambiguously, we designed inducible, luciferase-based reporters to estimate the translation of a series of R+K enriched genes, which are induced in cells in MM_{gluc}+Met (and further increased upon the loss of Gcn4). We designed a plasmid, in which the gene of interest (GOI; amplified from the genomic DNA of *S. cerevisiae*) was cloned in frame with the luciferase coding sequence, in such a way that the entire fragment (GOI+Luciferase) will be under the control of an inducible promoter (Figure S7). Using this system, measuring luciferase activity after induction will estimate the specific translation of this gene. This therefore accounts for only newly synthesized protein, and avoids mis-interpretations coming from already existing protein in the cells before methionine addition. We picked 4 candidate genes (RPL32, STM1, NHP2, RPS20), which are all transcriptionally strongly upregulated in MM_{gluc}+Met (Supplementary WS1), and further induced in Δ*gcn4* in the same conditions (Figure 7E), and cloned them in the plasmid in frame with luciferase. We subsequently measured luciferase activity in cells after 30 minutes of induction with β-estradiol, in both wild-type and Δ*gcn4* cells. Strikingly, all the candidate reporter genes showed a 3-5 fold reduction in translation in *GCN4* deficient cells, compared to WT cells (Figure 7F). These data reveal that Gcn4 is critically required to maintain the translation capacity of the cell. Finally, to determine if this reduced translation capacity in Δ*gcn4* is due to the reduced supply of arginine and lysine (in MM_{gluc}), we supplied both amino acids (2mM each) to wild-type and Δ*gcn4* cells growing in the presence of methionine, in a similar experiment as above, and measured luciferase activity after induction. Notably, the supply of arginine and lysine substantially rescued the expression of these reporter proteins, by increasing their translation ~2-3 fold (Figure 4G).

Collectively, we find that Gcn4 is central to the methionine dependent anabolic response. Gcn4 enables the sufficient supply of amino acids, particularly arginine and lysine, for the translation of ribosomal proteins. This in turn maintains the high translational capacity needed by the cell, to sustain the anabolic program and drive growth.

Discussion

A central theme emphasized in this study is mechanisms by which cells manage resource allocation, supply and demand during cell growth. Recent studies in model organisms like yeast and *E. coli* focus on protein translation, and the need to 'buffer' translation capacity during cell growth (Hui et al., 2015; Metzl-Raz et al., 2017). These studies alter our perception of how translation is regulated during high cell growth. However, the process of cell growth requires not just translation reserves (in the form of ribosomes), but also a constant supply of biosynthetic precursors to meet high demand. This includes: amino acids to sustain translation as well as drive metabolic functions, nucleotide synthesis (for DNA replication, transcription and ribosome biogenesis), and sufficient reductive capacity (for reductive biosynthesis). Even the production of ribosomes is an extremely resource-intensive process (Warner et al., 2001). While our understanding of translation-regulation in these contexts is constantly improving, how metabolic and biosynthetic components are managed, and couple with translation, remain poorly understood.

Here, using yeast as a model, we obtain striking, mechanistic insight into how cells sustain the supply of biosynthetic precursors, during a methionine-induced anabolic program. Earlier studies in yeast had observed that in otherwise amino acid limited conditions; just the addition of methionine

strongly induces an anabolic program (Walvekar et al., 2018b). This expectedly includes an induction of genes involved in ribosomal biogenesis and translation. In addition, cells strongly increase metabolic processes that sustain anabolism; primarily the pentose phosphate pathway (which enables nucleotide synthesis, as well as supplies molecules that allow reductive processes), trans-amination reactions, a strong induction in amino acid biosynthesis, and nucleotide synthesis (Walvekar et al., 2018b). Relatedly, cells utilize a sulfur-modified tRNA residue to sense methionine, to partly control carbon flow (through the pentose phosphate pathway) (Gupta et al., 2019). In this study, we now show how the presence of methionine controls this anabolic program, including maintaining high translation capacity, by co-opting (and strongly inducing) the Gcn4 transcription factor, as summarized in a schematic model (Figure 7F).

Gcn4 critically, directly sustains the increased biosynthesis, and therefore supply, of amino acids and (indirectly) nucleotides, and this sustains this methionine-induced anabolic program. These anabolic precursors have multiple metabolic functions, but also directly enable the cell to maintain a high translation capacity. Notably, the methionine-dependent induction of genes required for ribosome biogenesis and translation has a nuanced regulation by Gcn4. Gcn4 represses ribosomal genes (consistent with earlier reports), and in this context thereby appears to balance or moderate the overall induction of ribosomal genes by methionine. The methionine-dependent induction of ribosomal genes is likely controlled by directly activating the TOR pathway, a known regulator of ribosomal biogenesis, via S-adenosylmethionine (Gu et al., 2017; Martin et al., 2004; Mayer and Grummt, 2006; Sutter et al., 2013). However, despite the high expression of ribosomal gene mRNAs in *Δgcn4* cells (in the presence of methionine), and the indication of an apparent 'growth signature' transcriptional profile with high ribosomal transcripts (Bosdriesz et al., 2015; Brauer et al., 2008; Klumpp et al., 2013; Tu et al., 2005; Warner et al., 2001), cells cannot sustain the required rates of protein synthesis, or maintain the high translation capacity required for growth. This is because the translation machinery itself is highly enriched for arginine and lysine amino acids, and so cannot be maintained at high levels without a constant supply of lysine and arginine. In the presence of methionine, the increased synthesis (and therefore supply) of these two amino acids depends almost entirely on induced Gcn4. After all, to sustain high growth, and anabolic programs, cells need to maintain the required high rates of translation, and ribosomal capacity. Thus, through Gcn4, cells can deeply couple translation with metabolism, and manage sufficient resource allocations to sustain increased anabolism.

Notably, this phenomenon contrasts from the traditional, well known roles of Gcn4 during amino acid starvation or an 'integrated stress response' (Dey et al., 2010; Mascarenhas et al., 2008). In any context of rapid growth and proliferation, cells must increase the availability of biosynthetic molecules. For example, such situations where cells divert metabolic programs to support macromolecular biosynthesis and sustain growth, is a classic hallmark of cancer (Vander Heiden et al., 2009; Keibler et al., 2016; Mayers and Vander Heiden, 2015). Cells must also coordinate responses between protein synthesis and energy/biosynthetic metabolism, hence managing biosynthetic supply becomes of central importance. In this methionine-dependent context, cells co-opt Gcn4, which is conventionally viewed as a 'survival/stress' transcription factor, to provide this supply of metabolic precursors. Relevantly, recent studies of the mammalian ortholog of Gcn4 (ATF4) now report high ATF4 activity in several cancers (Pällmann et al., 2019; Singleton and Harris, 2012; Tameire et al., 2019; Ye et al., 2010). These studies suggest that ATF4 induction is critical for tumor progression during nutrient limitation, possibly by providing otherwise limiting metabolites (Tameire et al., 2019; Ye et al., 2010). Separately, observations over decades note that many tumors depend on sulfur amino acids, especially methionine (Breillout et al., 1990; Poirson-Bichat et al., 2000), and methionine restriction critically determines tumor progression (Gao et al., 2019b, 2019a). Our study directly demonstrates how Gcn4 can provide biosynthetic precursor supply to sustain anabolism, in an otherwise limiting environment. Speculatively, could the ability of methionine to

induce proliferation in cancers rest upon the induction of ATF4, which controls the supply of amino acids and other biosynthetic precursors?

Summarizing, here we discover how Gcn4 fuels an anabolic program in cells due to the presence of methionine. This expands the roles of what is conventionally viewed as a 'starvation' factor, during a contrasting, high anabolism state. Our study provides an illustrative perspective of how cells can manage the supply of important biosynthetic precursors, when a specific growth cues (methionine) induces high biosynthetic demands that need to be coordinately sustained, in order to maintain anabolism and cell growth.

Materials and Methods

Strains and growth conditions

A fully prototropic yeast strain *Saccharomyces cerevisiae* strain from a CEN.PK background (van Dijken JP et al., 2000) was used in all experiments. For all the medium-shift experiments, overnight grown cultures were sub-cultured in fresh YPD (1% Yeast extract and 2% Peptone, 2% Glucose) medium with an initial OD_{600} of ~0.2. Once the OD_{600} of the secondary culture reached 0.8 – 0.9, cells were pelleted down and washed and shifted to minimal media MM_{gluc} (MM- Yeast Nitrogen Base with glucose as a carbon source) and MM_{gluc} +Met (MM_{gluc} with 2mM methionine). For the luciferase assay (described later), overnight grown cultures were prepared by growing the cells in YPD with the antibiotic 1mM Nourseothricin (NAT). The secondary culture was started with an initial OD_{600} of ~0.5 in YPD + NAT and incubated at 30°C and 250 rpm for 4 hours. After 4 hours of incubation, cells were washed once in MM_{gluc}, and shifted to the MM_{gluc} +Met or MM_{gluc} met+arg+lys. 2mM concentration of each amino acid was used wherever required, unless mentioned otherwise. All the wash steps before shifting to minimal medium were done by centrifuging the cells at 3500 rpm for 90 seconds.

Western blot analysis

Yeast cells with a 3x-FLAG epitope sequence chromosomally tagged at the carboxy-terminus of Gcn4 (endogenous locus) were used to quantify Gcn4 protein levels using western blotting (Supplementary table 1). Overnight grown cells were sub-cultured into fresh YPD medium, with an initial OD of 0.2 and grown to an OD_{600} of 0.8. Cells were pelleted down at 3500 rpm for 1.5 minutes, cell pellets were washed once in MM_{gluc}, re-harvested and shifted to MM_{gluc} and MM_{gluc} + Met after 1 hour of the shift. ~5 OD_{600} of cells were harvested by centrifugation, and proteins were precipitated in 400 µl of 10% trichloro acetic acid (TCA), and extracted by bead beating with glass beads. Lysates were centrifuged to precipitate all proteins, and total protein pellets were resuspended in 400 µl of SDS-Glycerol sample buffer. Protein concentrations were quantified using Bicinconinic assay kit (G-Biosciences, 786-570) and equal concentrations of proteins were loaded into the 4-12% Bis-tris polyacrylamide gel (Thermo Fisher,NP0322BOX) and resolved using electrophoresis. Resolved proteins were transferred to nitrocellulose membranes and detected by standard Western blotting using monoclonal anti-FLAG M2- mouse primary antibody (Sigma Aldrich, F1804) and HRP labelled anti-mouse IgG secondary antibody (Cell Signalling technology, 7076S). Blots were developed using enhanced chemiluminescence reagents (Advansta, K-12045) imaged using Image quant. A different part of each gel (cut out) was Coomassie stained in order to compare total protein loading amounts.

mRNA sequencing and data analysis

Overnight grown cells of WT and *Δgcn4* strains were sub-cultured in YPD, with a starting OD_{600} of 0.2 and grown till they reached an OD_{600} of 0.8-0.9. YPD grown cells were pelleted down at 3500 rpm for 90 seconds and washed once with MM_{gluc}. Washed cells were shifted to MM_{gluc} and MM_{gluc}+Met, and cells remained in this fresh medium for ~1 hr. The cells were collected an hour after this shift and RNA was isolated by a hot acid phenol method as described (Collart and Oliviero, 2001). mRNA libraries were prepared using TruSeq RNA library preparation kit V2 (Illumina) and quality of the libraries were analyzed using bioanalyser (Agilent 2100) and libraries were sequenced for 51 cycles using Illumina HiSeq 2500 platform. The raw data are available in NCBI-SRA under the accession PRJNA599001. The transcriptome data were aligned and mapped to the *S. cerevisiae S288C* genome downloaded from the saccharomyces genome database (SGD), using the Burrows-Wheeler Aligner (Li and Durbin, 2009) and the mapped reads with mapping quality of ≥ 20 were used for further analysis . Number of reads mapped to each gene was calculated and read count matrix was generated. The EdgeR package was used for normalization and differential gene expression analysis (Robinson et al., 2010). Differentially expressed genes with a fold change above 1.5 or 2 fold, with a stringent p-value cutoff of <= 0.0001 were considered for further analysis. Normalized read counts was calculated for every sample as described earlier (Walvekar et al., 2018b). Normalized read counts between the replicates are well correlated with the Pearson correlation coefficient (R) is more than 0.9 (Figure S1). GO analysis of the differentially expressed genes were carried out using g:Profiler (Raudvere et al., 2019).

Chromatin Immunoprecipitation sequencing and data analysis

a. Cell growth conditions and sample collection

For ChIP sequencing, overnight grown cells were re-inoculated in fresh YPD medium (RM), with the initial OD_{600} of 0.2 and incubated at 30°C until the OD_{600} reached 0.8-0.9. Subsequently, 100 mL of culture was pelleted down, washed and shifted to MM_{gluc} and MM_{gluc}+Met. After 1 hour of the shift, cells were fixed using 1% formaldehyde, after which the fixing was quenched with 2.5M glycine.

b. Spheroplasting of fixed cells

Fixed cells were treated with 2-mercapto ethanol, and resuspended in 5 ml of spheroplasting buffer containing 1M sorbitol, 0.1M sodium citrate, 10mM EDTA, and distilled water, with 4mg/ml of lysing enzyme from *Trichoderma harzianum* (Sigma L1412-5G). This suspension was incubated at 37°C for 5 hours.

c. Lysis and ChIP

Spheroplasts were pelleted down at 1000 rpm, washed twice with Buffer 1 (0.25% Triton X100,10mM EDTA,0.5mM EGTA, 10mM sodium HEPES pH 6.5) and twice with Buffer 2 (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM Sodium HEPES pH 6.5), washed spheroplasts were resuspended in lysis buffer (50mM sodium HEPES pH 7.4, 1% Triton X, 140mM NaCl,0.1% Sodium deoxy cholate,10mM EDTA) and lysis and DNA fragmentation were carried out using a bioruptor (Diagenode, Nextgen) for 30 cycles (30 sec on and off cycles). Lysates were centrifuged to remove the debris and clear supernatant was used for chromatin immunoprecipitation (ChIP). Immunoprecipitation was carried out by incubating the lysate with the monoclonal anti-FLAG M2-mouse primary antibody (Sigma Aldrich, F1804) and protein G Dynabead (Invitrogen, 10004D). Beads were washed sequentially in low salt, high salt and LiCl buffers, TE buffer and protein-DNA complex were eluted using elution buffer as reported earlier (Lelandais et al., 2016). Decrosslinking of the immuno-precipitated proteins were carried out by using a high concentration of NaCl and incubation at 65°C for 5 hours followed by proteinase-K treatment and DNA purification. Mock samples were also prepared in parallel, except the antibody treatment. Libraries were prepared for the purified IP DNA and mock samples (NEBNext Ultra II DNA library preparation kit, Catalog no-E7103L) and sequenced using Illumina platform HiSeq 2500. Two biological replicates were maintained for all the samples. The raw data are available in NCBI-SRA under the accession ID PRJNA599001.

ChIP sequencing reads were mapped to the *S. cerevisiae S288C* genome downloaded from SGD. The reads with mapping quality < 20 were discarded, and the remaining reads were used for further analysis. The number of reads mapped to every 100bp non-overlapping bins were calculated using 'exomedepth' function of R-package GenomicRanges (Lawrence et al., 2013). Read counts were normalized by dividing the number of reads falling within each bin by the total number of reads fall within the range of μ±x, where, μ=mode of the distribution of read counts of each bin, x = median absolute deviation of all the bins that has a number of reads that are less than the mean of the distribution. Subsequently, the regions that have normalized read counts of above 2 were considered for further analysis. The binding regions which are separated by < 200bp were merged to give a single peak. The peaks which are conserved in both the replicates with the overlap of at least 50bp were considered as *bona fide* binding regions of Gcn4. Genes which are encoded around 750 bp on both sides of the peaks were listed in the Supplementary WS4

Peak feature annotation and motif analysis

Genomic features of the peaks were identified using the annotatePeak.pl function of the HOMER tool (Heinz et al., 2010). For motif analysis, nucleotide sequences corresponding to the peak intervals were extracted from the genome and motif identification was performed using 'meme' function of MEME-suite (Bailey et al., 2009).

Direct and Indirect target analysis

To annotate the genes corresponding to the peaks identified, the open reading frames that are encoded within 750 bp on both sides of the peak regions were considered as 'possible Gcn4 binding targets'. Gene sets which are differentially expressed in *Δgcn4* relative to WT under MM_{gluc}+Met condition, with a fold change of > 2 (~900 genes) were termed 'Gcn4 regulatory targets'. While comparing these gene lists, the genes which intersect between these two gene sets are considered as 'direct Gcn4 binding targets' and the rest of the genes of 'Gcn4 regulatory targets' are 'indirect targets of Gcn4'.

Metabolic flux analysis using LC/MS/MS

To determine if the rates of biosynthesis of amino acids are altered in MM_{gluc}+Met and Gcn4 dependent manner, we measured ^{15}N-label incorporation in amino acids. We used ^{15}N-ammonium sulfate with all nitrogen atoms labelled. Cells grown in YPD were shifted to fresh minimal medium (with the appropriate carbon source as indicated), containing 0.5 X of unlabelled ammonium sulfate (0.25%) and MM_{gluc}+Met containing 0.5X of unlabelled ammonium sulfate (0.25%). After 1 hour of shift to minimal media, cells were pulsed with 0.25 % of ^{15}N labelled ammonium sulfate and incubated for 5 minutes or 15 minutes as indicated. After the ^{15}N pulse, metabolites were extracted and label incorporation into amino acids was analyzed using targeted LC/MS/MS protocols as described earlier (Walvekar et al., 2018a, 2018b).

Luciferase based translation reporters for lysine and arginine enriched genes

To measure the translation of specific transcripts that encode arginine and lysine enriched proteins, the ORF of the following proteins, RPL32, NHP2, STM1, RPS20 were amplified from the genomic DNA isolated from WT CEN.PK strain of *S. cerevis*iae. The amplified ORFs (without the stop codon) were ligated to the luciferase cDNA amplified from pGL3 (Supplementary table 2). The resulting fragment with '$ORF_{RK\ rich\ genes}$ *+ luciferase*' were cloned in a centromeric (CEN.ARS) plasmid pSL207, a modified version of the plasmid used in the earlier study (McIsaac et al., 2011). Luciferase expression in this construct is under the control of inducible promoter, which can be induced by ß-estradiol (McIsaac et al., 2011). The resulting plasmids with the following genes RPL32, NHP2, STM1, RPS20 cloned in frame with luciferase and under the inducible GEV promoter were named pSL218, pSL221, pSL224,

pSL234 respectively (Figure S7 and Supplementary table 2). SL217 is a plasmid where only the luciferase cDNA amplified from pGL3 plasmid was cloned under the inducible promoter, serves as a control. All these plasmids generated have ampicillin selection (for amplification) and Nourseothricin resistant cassettes (NAT^r) for selection in yeast. The generated plasmids were transformed to the WT and *Δgcn4* strains. To measure the translation of the genes cloned upstream of luciferase, these strains having plasmid were grown in YPD for overnight with an antibiotic NAT. Overnight grown cultures were shifted to fresh YPD+NAT with an initial OD_{600} of 0.4, and grown for 4 hours at 30ºC. After 4 hrs of incubation in YPD, cells were washed and shifted to the MM_{gluc} and MM_{gluc}+Met. After 1 hour of the shift, cultures were split into two equal parts, one part of the culture was induced with 200nM ß-estradiol (Sigma Aldrich-E8875) and the other half was left as a mock-induced control. After 30 minutes of induction cells were harvested by centrifugation at 4ºC at 3000 rpm and washed with lysis buffer (1X-PBS containing 1mM PMSF), after 3 washes, were resuspended in 200µl of lysis buffer. Lysed the cells by bead beating at 4º C (1min ON and 1 min OFF for 10min). After lysis, the protein concentrations were measured by BCA assay kit. Equal concentrations of protein were used for measuring the luciferase activity. Luciferase activity was measured using luciferase assay kit (Promega, E1500) and the activity was measured using a luminometer (Sirius, Titertek Berthold Detection systems). Luciferase activity (measured as Relative Light Units per Sec (RLU/sec)) were normalized with its respective uninduced control. Similar experiments were carried out under different media conditions supplemented with different amino acids, where the conditions are mentioned in the respective sections. The relative difference in luciferase activities between the strain types and media conditions were used to estimate changes in the active translation of these proteins.

Statistical tests

R-Packages and Graph pad prism 7 were used for visualizing data and performing statistical tests. The respective statistical tests used, and the statistical significance was mentioned wherever required.

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Author contributions

RS and SL conceived the study, RS, ASW, ASN and SL designed experiments, RS and AW performed experiments, RS, ASW, AS and SL analyzed data, RS, AS and SL wrote the manuscript.

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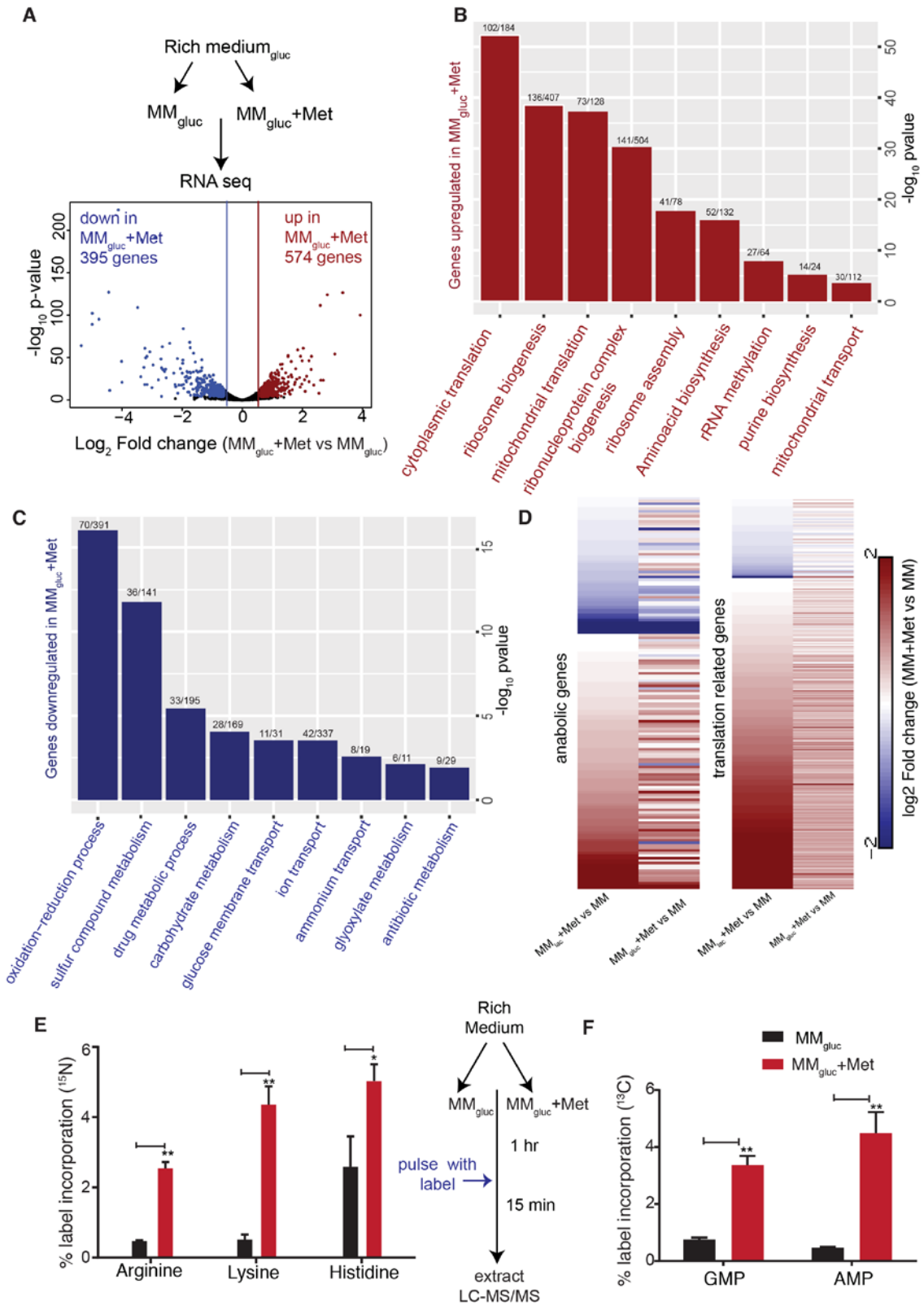

Figure 1: ***Methionine induces an anabolic response independent of carbon sources***

A. A volcano plot representing differentially expressed genes in MM_{gluc}+Met, relative to the MM_{gluc}. Genes that are upregulated and downregulated in MM_{gluc}+Met (fold change of ≥ 1.5, p-value cut off of 10^{-4}) are highlighted in red and blue respectively.
B. A bar plot showing the most significantly enriched GO categories of the genes induced by methionine (upregulated in MM_{gluc}+Met). The GO terms shown here are significantly enriched terms with the corrected p-value < 0.05 (hypergeometric test, Bonferroni Correction) (also see Supplementary WS3 for complete GO analysis results). For this, the numbers of genes induced (to the number of genes in that category) are also indicated within each bar.
C. A bar plot illustrating the most significantly enriched GO categories of genes downregulated by methionine (downregulated in MM_{gluc}+Met). The GO terms shown here are significantly enriched terms with the corrected p-value < 0.05 (hypergeometric test, Bonferroni Correction) (also see Supplementary WS3 for the more detailed GO analysis results). For this, the numbers of genes induced (to the number of genes in that category) are also indicated within each bar.
D. Heat maps showing the transcriptional induction of anabolic and translation related genes altered by methionine, i.e. in MM+Met relative to MM for cells grown in different carbon sources such as glucose (MM_{gluc}) and lactate (MM_{lac}) .
E. Quantitative LC/MS/MS based targeted metabolic flux measurements, using N^{15} ammonium sulfate labeling, to estimate nitrogen flux towards new amino acid synthesis in MM_{gluc}+Met compared to MM_{gluc}. The experiment was performed as illustrated in the schematic flow diagram. Data shown are the average of two biological replicates (with technical duplicates), with standard deviation.
F. Quantitative LC/MS/MS based targeted metabolic flux measurements, using C^{13} glucose labeling, to estimate carbon flux towards new nucleotide synthesis in MM_{gluc}+Met compared to MM_{gluc}. Data shown are the average of two biological replicates (with technical duplicates), with standard deviation. * p-value < 0.05, ** p-value <0.01.

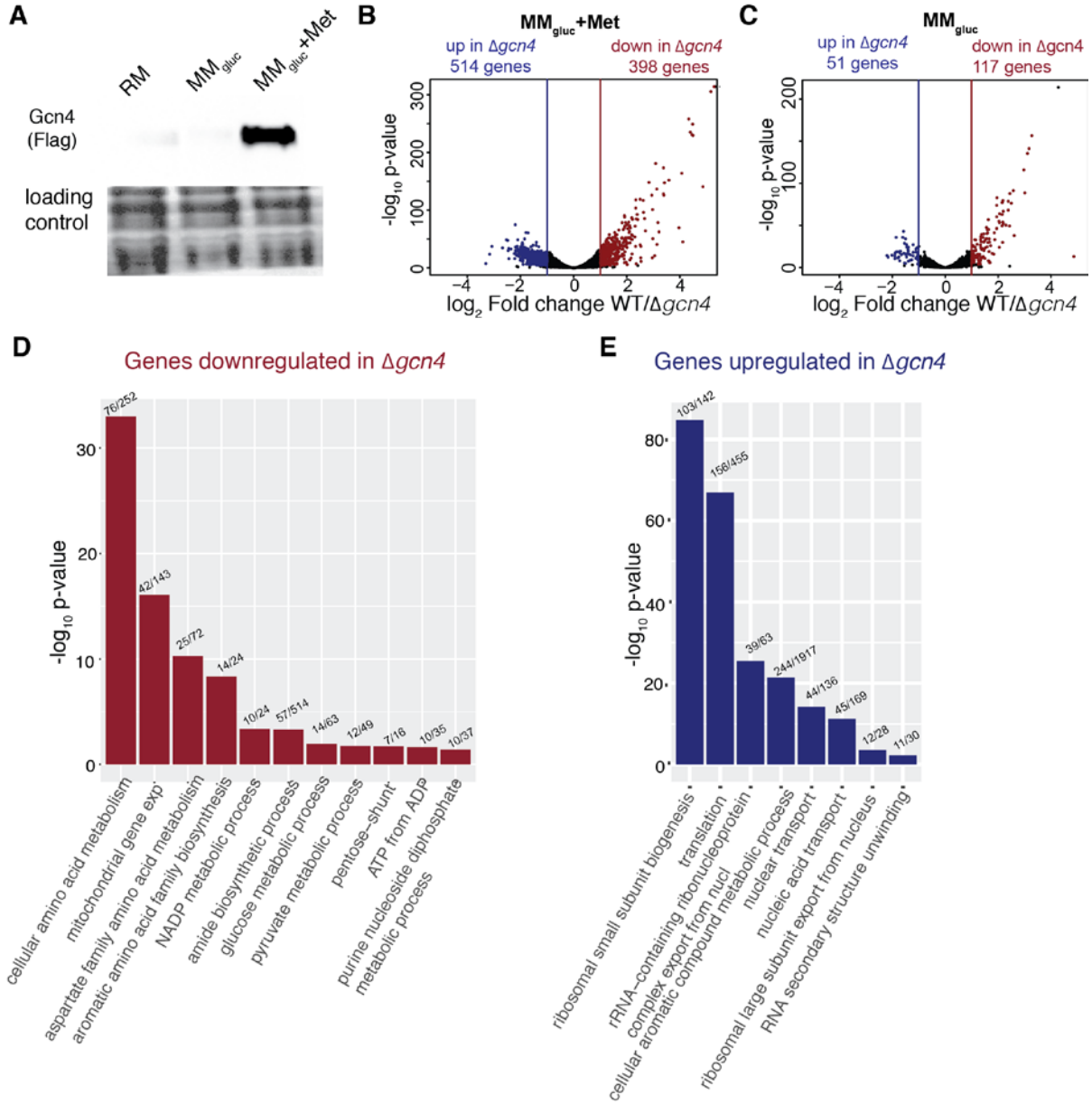

Figure 2: ***GCN4 regulates the anabolic component of the methionine-dependent transcriptional program***

A. Gcn4 is strongly induced by methionine addition to minimal medium. A representative western blot shows high Gcn4 protein levels in MM_{gluc}+Met (Gcn4 tagged with the Flag epitope at the endogenous locus). RM - rich medium, MM_{gluc} - minimal medium without amino acids and with glucose as a carbon source, and MM_{gluc}+Met - minimal medium without amino acids and with glucose as a carbon source supplemented with 2mM methionine. Also see Supplementary Figure S2.

B. A volcano plot comparing transcript expression in WT with *Δgcn4* cells (grown in MM_{gluc}+Met). Genes induced with a fold change cut-off of ≥ 2, and p-value ≤ 10^{-4} are shown in red, and the downregulated transcripts (similar cut-off parameters) are shown in blue. The numbers of differentially expressed transcripts are also indicated.

C. A volcano plot comparing transcript expression in WT with *Δgcn4* cells (grown in $MM_{gluc.}$ Genes induced with a fold change cut-off of ≥ 2, and p-value ≤ 10^{-4} are shown in red, and the downregulated transcripts (similar cut-off parameters) are shown in blue. The numbers of differentially expressed transcripts are also shown.

D. GO based analysis and grouping of transcripts down regulated in *Δgcn4.* All the terms shown here are significantly enriched terms with the corrected p-value < 0.05 (hypergeometric test, Bonferroni Correction). Also see Supplementary WS3.
E. GO based analysis and grouping of the transcripts up-regulated in *Δgcn4.* All the terms shown here are significantly enriched terms with the corrected p-value < 0.05 (hypergeometric test, Bonferroni Correction). Also see Supplementary WS3.

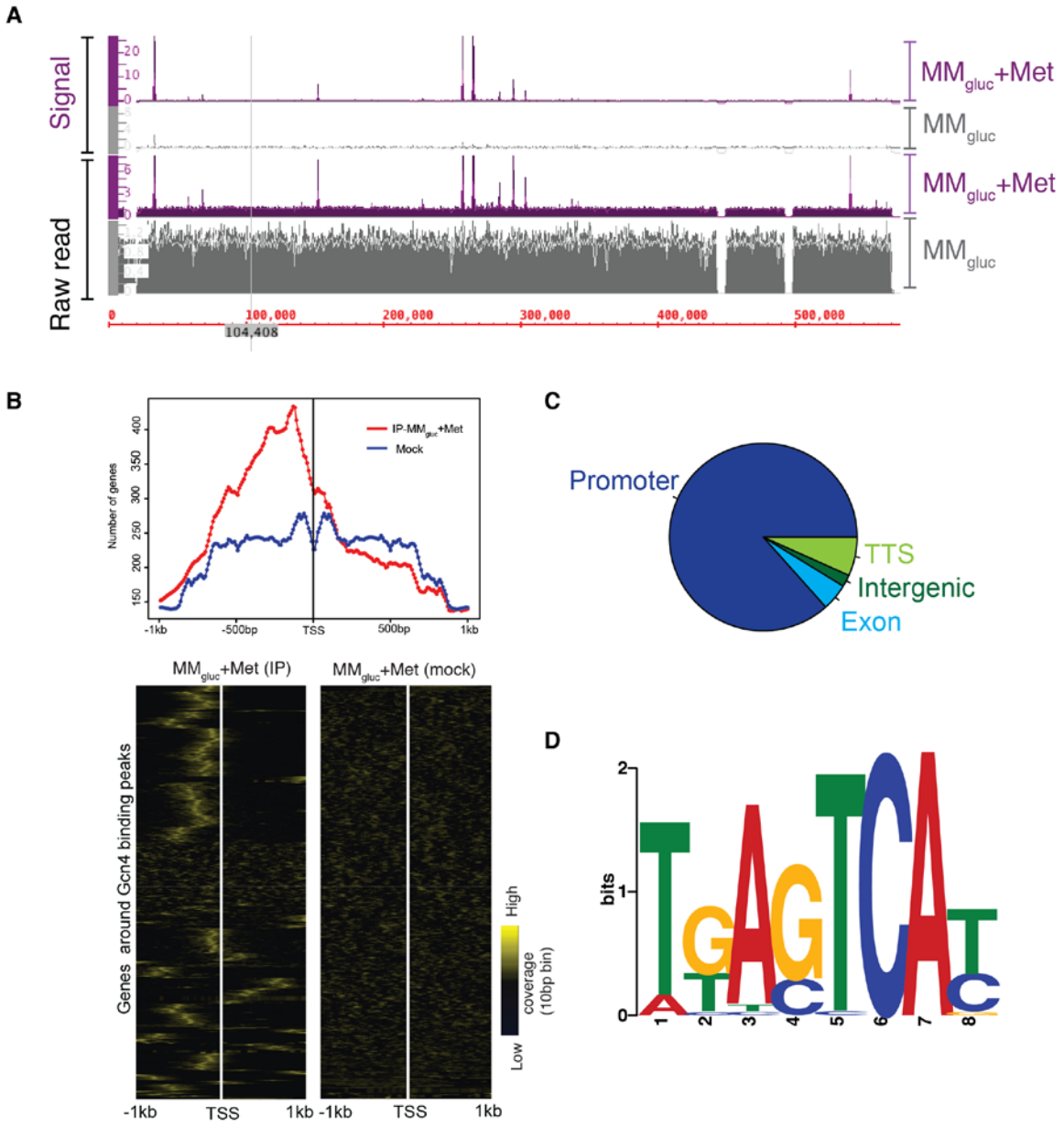

Figure 3: ***Gcn4 strongly binds to its target promoters in the presence of methionine***

A. Genomic tracts showing Gcn4 binding to DNA regions in MM_{gluc}, MM_{gluc}+Met. Raw read counts and signal around the binding region of Gcn4 are shown. Also see Supplementary Figure S2.

B. (Top) Density plots showing that most target genes have Gcn4 binding peaks upstream of the Transcription Start Site (TSS) in the ChIP samples (red), whereas no such enrichment of genes is observed in mock samples (blue). (Bottom) A heat map showing read coverage for Gcn4 binding, including 1kb upstream and downstream of predicted/known transcription start sites (TSS) of target genes. All the genes that fall in the vicinity of 750bp around the identified Gcn4 binding peaks are considered to be target genes. The heat map on the left shows read coverage in IP samples, and on the right shows coverage in mock-IP (control) under in MM_{gluc+} Met condition. Also see Supplemental Figure S3 which shows read coverage for Gcn4, in the context of the translation start site (ATG) of each gene.

C. A pie chart showing the genomic features of the identified peaks annotated using 'annotatepeak' function of Homer tool (Heinz et al., 2010). Also see Supplemental Figure S4, which compares Gcn4 genome occupancy in this methionine-induced anabolic condition, with Gcn4 genome occupancy during extreme amino acid starvation (from (Rawal et al., 2018)).
D. Consensus binding motifs identified in Gcn4 binding peaks from MM_{gluc}+Met conditions.

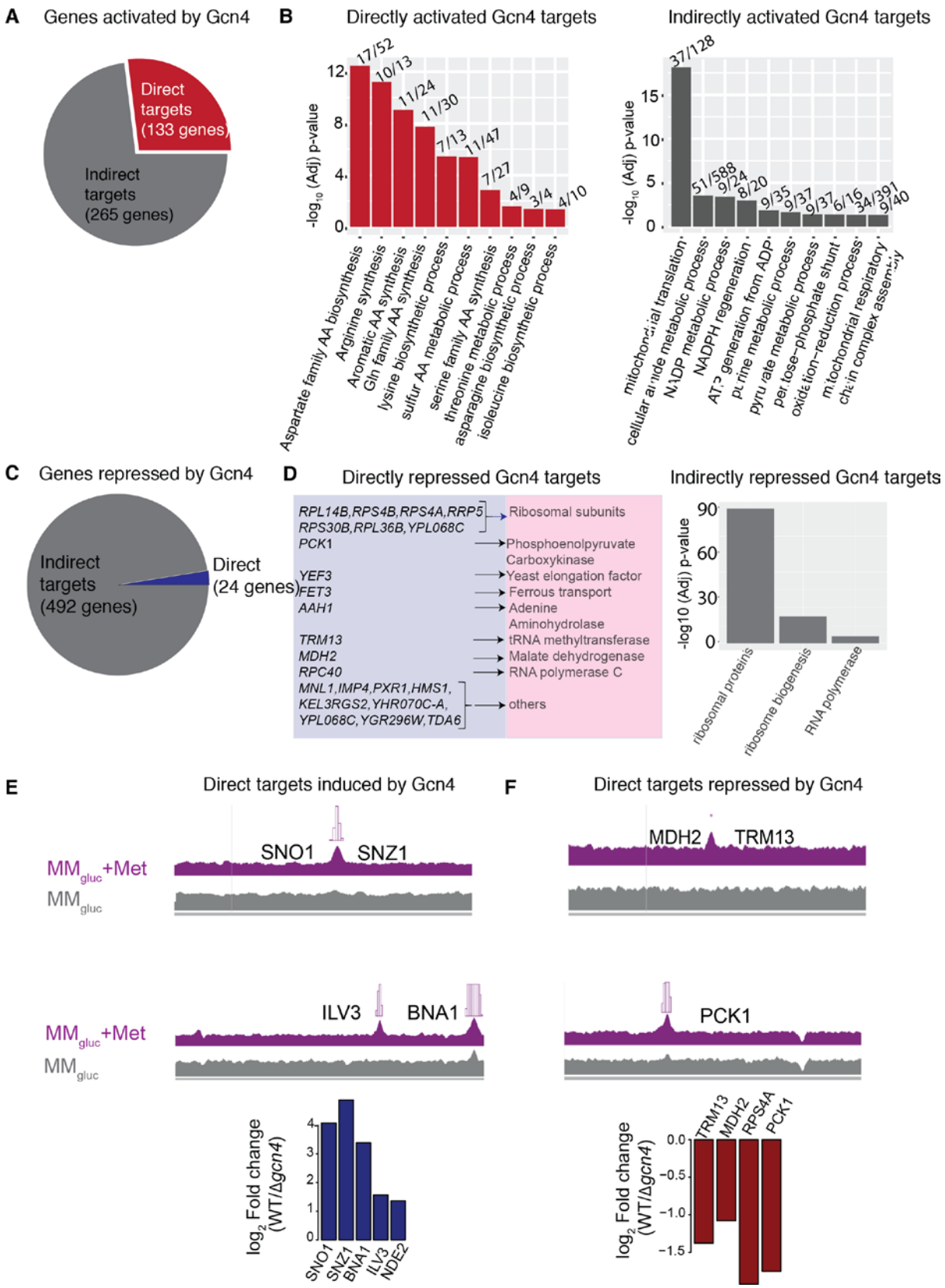

Figure 4: ***Direct and indirect targets of Gcn4 in methionine dependent induction of Gcn4***

A. Pie chart showing number of Gcn4 targets directly/indirectly activated by Gcn4.
B. Bar plots shows enriched GO term and the corresponding -log10(p-value) for the genes which are directly or indirectly activated by Gcn4 in the MM_{gluc}+ Met condition.
C. Pie chart showing number of Gcn4 targets directly/indirectly repressed by Gcn4.

D. List of genes repressed by Gcn4 and the bar plots shows enriched GO term and the corresponding -log10(p-value) of the genes which are indirectly repressed by Gcn4 in MM_{gluc}+ Met condition.
E. Genomic tract showing the Gcn4 peaks for the selected genes that are activated directly by Gcn4 (top panel) and their gene expression values as measured using RNA seq (bottom panel)
F. Genomic tract showing the Gcn4 peaks for the selected genes which are directly repressed by Gcn4 (top panel) and their gene expression values as measured using RNA seq (bottom panel)

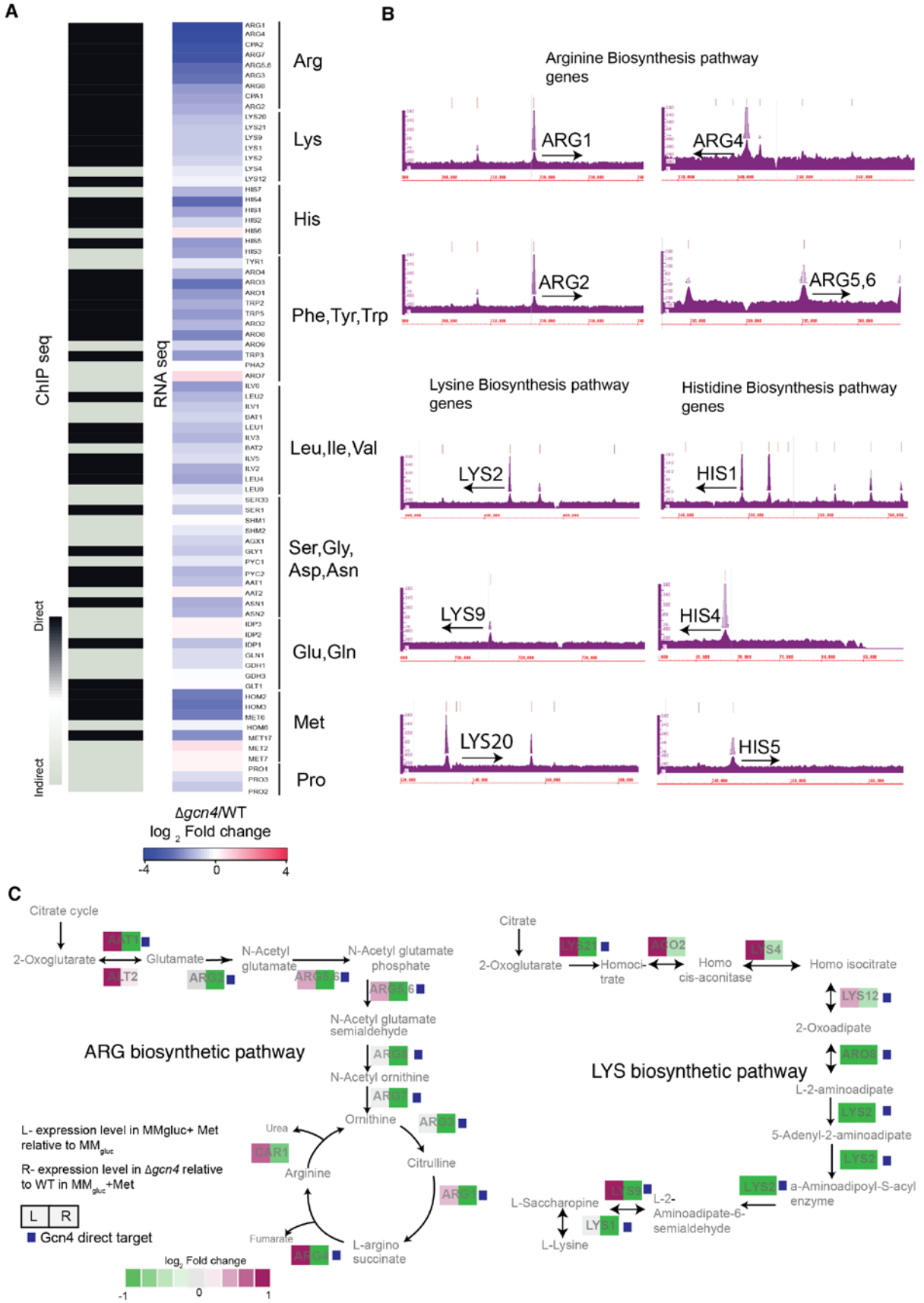

Figure 5: ***Gcn4 tightly regulates the expression of specific amino acids***

A. Comparing direct targets of Gcn4 transcription, and gene expression profiles of WT and *Δgcn4* cells (Gcn4 dependence). The heat map on the left shows whether the indicated gene (involved in amino acid biosynthesis) is directly or indirectly regulated by Gcn4 based on ChIP-Seq data

from cells in MM_{gluc}+Met medium. The black color indicates a direct target of Gcn4 and grey indicates an indirect target (Gcn4 does not bind the promoter of this gene). The heatmap on the right shows the gene expression fold changes in *Δgcn4* relative to WT cells, grown in MM_{gluc}+Met medium.

B. Integrated Genome Browser (IGB) tracts, showing Gcn4 binding peaks on the promoters of the genes for arginine, lysine and histidine biosynthetic pathways.
C. Representative pathway maps of the arginine biosynthetic pathway. This map shows the fold change in gene expression due to methionine (MM_{gluc}+Met compared to MM_{gluc}) in WT cells (**left box**), the change in gene expression due to loss of Gcn4 (WT compared to *Δgcn4*) in the presence of methionine (MM_{gluc}+Met) (**right box**). Genes that are direct targets of Gcn4 are also indicated with a small purple box next to the gene name.
D. A representative pathway map of the lysine biosynthetic pathway, represented similar to that of arginine biosynthesis, shown in panel C.

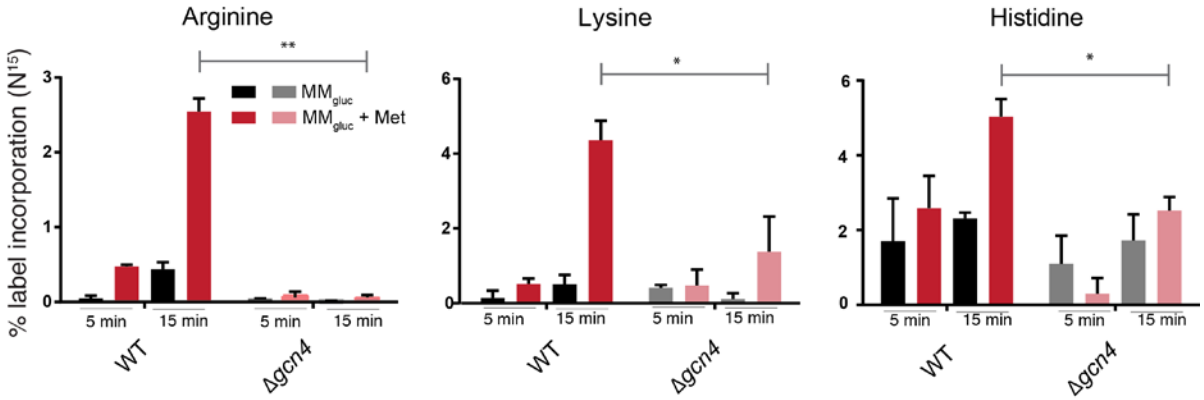

Figure 6: ***Gcn4 controls metabolic flux towards increased amino acid biosynthesis***

A. Methionine dependent, increased arginine and lysine biosynthesis depend entirely on Gcn4. Data from quantitative LC/MS/MS based metabolic flux analysis experiments, using N^{15} ammonium sulfate labeling to estimate new amino acid synthesis in a methionine and Gcn4 dependent manner, are shown. The comparisons are between WT and *Δgcn4* cells treated identically (as shown earlier in Figure 1F), in MM_{gluc}+Met medium. The data are presented for arginine, lysine and histidine synthesis. Also see Figure S6. *p<0.05,**<0.01 (t-test).

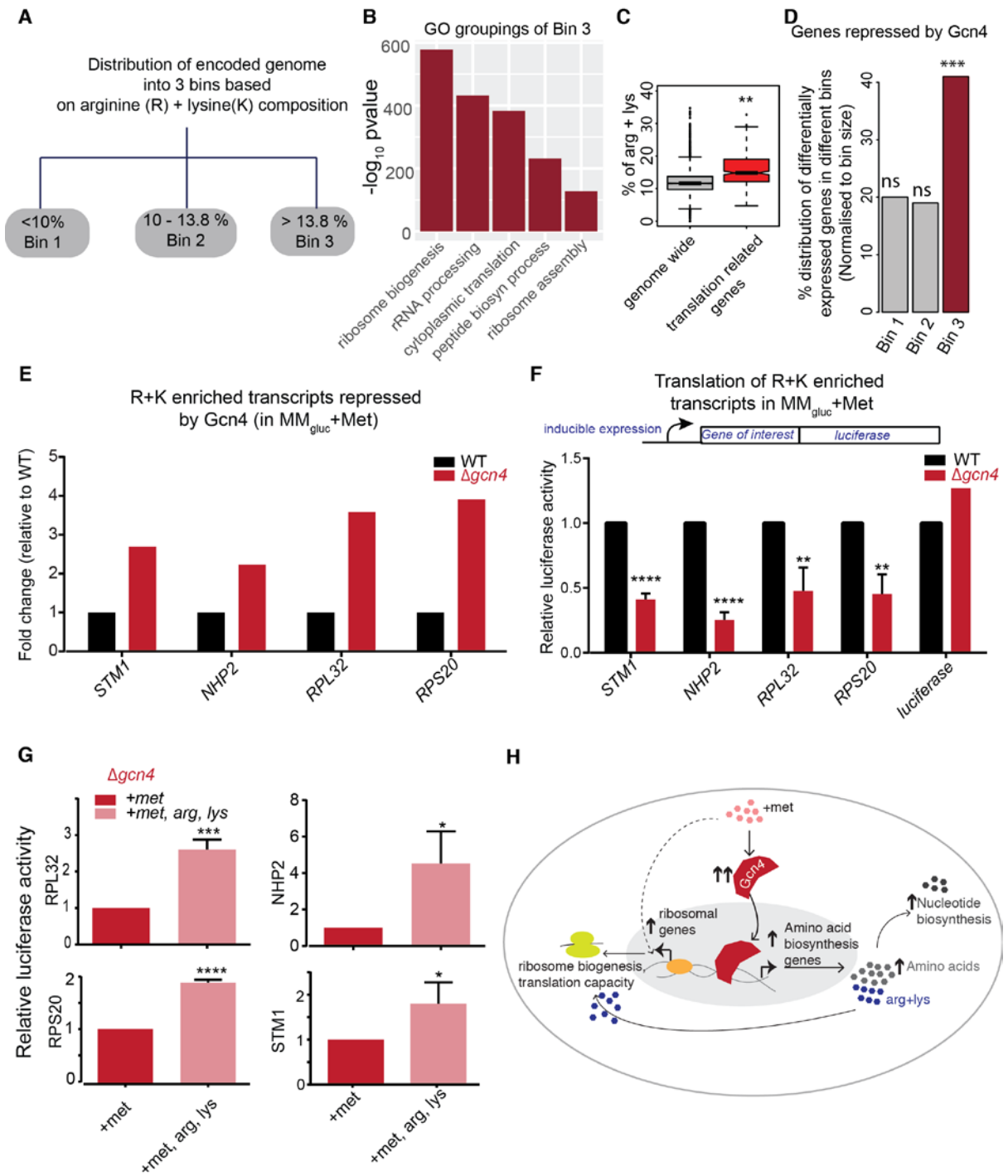

Figure 7: ***Gcn4 dependent lys and arg supply is essential to maintain high translational capacity due to methionine.***

A. "Binning" of the yeast proteome into three equal parts, based on the percentage of arginine and lysine in these proteins. The percentages of arg+lys in these bins are indicated.
B. GO based analysis reveals that bin3, which has the high percentage of arginine and lysine, is significantly enriched for ribosomal and translation related genes. The graph plots the most enriched GO term against -log10(P value).
C. Boxplot, comparing the arginine and lysine composition of the entire proteome (excluding translation related genes), and the translation related genes. The translation related genes have a significantly higher than genome wide composition of arginine and lysine.

D. Barplots, indicating in which bin (Figure 1A) the genes repressed by Gcn4 (i.e. induced in *Δgcn4*) fall under. A significant majority of the genes repressed by Gcn4 are enriched for arginine and lysine rich (Fisher exact test, p<10e-10).
E. Arg/lys enriched genes are induced in *Δgcn4* cells in methionine supplemented medium. Barplots comparing relative transcript amounts for select, highly induced, arginine and lysine enriched genes, between WT and *Δgcn4* cells. Data shown are taken from the RNA seq data.
F. Arg/lys enriched gene transcripts cannot be translated in *Δgcn4* cells in methionine supplemented medium. Barplots, comparing the relative amount of proteins translated (in a methionine-dependent manner), for the genes shown in Figure 7E. These selected genes were cloned in frame with luciferase in an inducible system, to create a translation-reporter, and translation of these were induced in WT or *Δgcn4* cells in methionine supplemented medium (MM_{gluc}+Met). Data shown are mean+/- SD from ≥ 3 biological replicates. *p<0.05,**p<0.01,****<0.0001 (t-test).
G. Supplementing arginine and lysine restores translational capacity in *Δgcn4* cells. Barplots, comparing the relative amount of proteins translated (in a methionine-dependent manner), for the genes shown in Figure 7F, in methionine-supplemented medium (MM_{gluc}+Met) or in MM_{gluc}+Met+Arg+lys . Data shown are mean+/- SD from ≥ 3 biological replicates. *p<0.05,***p<0.001,****<0.0001 (t-test).
H. A mechanistic model illustrating how high Gcn4 enables a methionine dependent anabolic response, by supplying amino acids, and maintaining translation capacity.