ATF4 couples MYC-dependent translational activity to bioenergetic demands during tumour progression

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The c-Myc oncogene drives malignant progression and induces robust anabolic and proliferative programmes leading to intrinsic stress. The mechanisms enabling adaptation to MYC-induced stress are not fully understood. Here we reveal an essential role for activating transcription factor 4 (ATF4) in survival following MYC activation. MYC upregulates ATF4 by activating general control nonderepressible 2 (GCN2) kinase through uncharged transfer RNAs. Subsequently, ATF4 co-occupies promoter regions of over 30 MYC-target genes, primarily those regulating amino acid and protein synthesis, including eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), a negative regulator of translation. 4E-BP1 relieves MYC-induced proteotoxic stress and is essential to balance protein synthesis. 4E-BP1 activity is negatively regulated by mammalian target of rapamycin complex 1 (mTORC1)-dependent phosphorylation and inhibition of mTORC1 signalling rescues ATF4-deficient cells from MYC-induced endoplasmic reticulum stress. Acute deletion of ATF4 significantly delays MYC-driven tumour progression and increases survival in mouse models. Our results establish ATF4 as a cellular rheostat of MYC activity, which ensures that enhanced translation rates are compatible with survival and tumour progression.

Cells utilize distinct stress response pathways to overcome environmental and physiological stresses. The integrated stress response (ISR) pathway promotes cellular adaptation to various stresses such as viral infection, haeme deprivation, hypoxia, nutrient deprivation and acidosis1. The ISR kinases—double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2, encoded by Eif2ak4 in mice and EIF2AK4 in humans) and haeme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI)—sense distinct stresses and catalyse phosphorylation of the α-subunit of eIF2α. Phosphorylation of eIF2α at serine 51 attenuates general protein synthesis while enhancing the translation of select transcripts containing distinct regulatory sequences in their 5′-untranslated region14, most notably that of activating transcription factor 4 (ATF4). Once translated, ATF4 drives the transcription of genes involved in antioxidant response, autophagy, amino acid biosynthesis and transport43.

The ability of cancer cells to adapt to cell-extrinsic and -intrinsic stresses is critical for maintaining viability and growth. The ISR is essential in adaptation to extrinsic stresses present in the tumour microenvironment, such as hypoxia and nutrient deprivation49. Cancer cells also experience intrinsic stress due to activation of oncogenes that increase bioenergetic processes10-12. In this context, amplification of the MYC oncogene, a frequent event in multiple human malignancies13, causes intrinsic stress due to enhanced protein synthesis and rewiring of metabolic pathways to meet the demands of rapid cell growth and proliferation14,15. MYC upregulates protein synthesis by transactivating components of the translation machinery including initiation factors, ribosomes and tRNAs16, and targeting the translation machinery has proven to be effective in MYC-driven cancers16,17. However, protein synthesis rates need to be controlled to a critical level to sustain survival of cancer cells during tumour development. For example, increased protein synthesis has to be accompanied by a concomitant increase in the unfolding capacity and size of the endoplasmic reticulum (ER) to avoid proteotoxicity18.

We previously demonstrated that a MYC-induced increase in translation rates activates the PERK arm of ISR, which is required to support MYC-induced transformation and survival19, primarily by activating cytoprotective autophagy and attenuating Ca2+ release from the ER31. Although ATF4 has been implicated in supporting survival of cancer cells experiencing a deficit of oxygen and nutrients, its role in oncogene-induced stress is not well characterized.

Here we show that optimal ATF4 expression on MYC dysregulation requires both PERK and GCN2, the latter being activated by excess uncharged tRNAs produced by increased MYC activity.

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Induced ATF4, cooperatively with MYC, co-regulates a number of gene products, including eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), to fine-tune messenger RNA translation induced by MYC. Ablation of ATF4 results in increased ER stress and cytotoxicity, which is attenuated by mammalian target of rapamycin complex 1 (mTORC1) inhibition or PERK and eIF2α activation, residual phosphorylation of eIF2α being primarily responsible for phosphorylating eIF2α. PERK and GCN2 are required for optimal phosphorylation of eIF2α following MYC induction. Activation of MYC enhances protein synthesis, resulting in ER stress. Although PERK is primarily responsible for phosphorylating eIF2α during MYC activation, residual phosphorylation of eIF2α in PERK-knockout cells prompted us to investigate whether other ISR kinases phosphorylate eIF2α in the absence of PERK. We focused on GCN2, a kinase that we and others have shown to be activated in multiple solid tumours. Of note, we observed robust phosphorylation of GCN2 following MYC activation (Fig. 2a,b). Consistent with our

**Fig. 1 | MYC-induced ATF4 inhibits apoptosis and promotes survival.** a, Immunoblot of nuclear lysates from MEFs (left) and DLD-1 cells (right) expressing MycER and treated with 4-OHT for the indicated time to activate MYC. Thapsigargin-treated cells (TG; 0.5 µM for 4 h) were used as a positive control for ATF4 induction. b, Immunoblot of nuclear lysates from P493-6 cells treated with tetracycline (Tet; 0.1 g ml⁻¹) for the indicated time with or without washing for the indicated time. c, Immunoblot analysis of whole-cell lysates from ATF4⁺/+ and ATF4⁻/⁻ MEFs treated with 4-OHT for indicated times. d, Clonogenic survival assays were performed after activating MYC in MEFs. Representative plates from three biological replicates are shown. Colonies were counted, and the surviving fraction is shown normalized to no-treatment control. Data are represented as mean ± s.d.; two-tailed Student’s t-test. e, DLD-1 MycER cells were transfected with non-targeting (siNT) siRNA or siRNA targeting ATF4 (siATF4). Cells were treated with 4-OHT and the indicated proteins were detected by immunoblotting. Blots are representative of three biological replicates that showed similar results. Unprocessed scans of blots are shown in Supplementary Fig. 7.

**Results**

**ATF4 is induced by MYC and promotes survival.** We previously reported that activation of MYC leads to phosphorylation of PERK and eIF2α. To test whether MYC activation also induces ATF4, we analysed ATF4 expression following MYC induction in DLD-1 human colon adenocarcinoma cells and mouse embryonic fibroblasts (MEFs) stably expressing a tamoxifen-inducible MYC chimaera, MycER (hereafter, MycER cells). Treatment with 4-hydroxy-tamoxifen (4-OHT) led to accumulation of nuclear MYC and expression of ATF4 in both cell lines (Fig. 1a and Supplementary Fig. 1a). Similarly, in the human Burkitt’s lymphoma cell line P493-6, in which expression of MYC is turned off by administration of tetracycline, suppression of MYC resulted in a concomitant decrease of ATF4. When MYC levels were restored, ATF4 protein levels also recovered (Fig. 1b). These data indicate that elevated MYC induces ATF4 expression.

Notably, ablation of ATF4 in MEFs significantly enhanced MYC-induced cell death, highlighted by increased levels of apoptosis markers including cleaved (cl)-poly (ADP-ribose) polymerase (cl-PARP) and cl-caspase-3 (Fig. 1c) and reduced clonogenic survival (Fig. 1d). Similarly, knockdown of ATF4 in DLD-1 cells also markedly enhanced apoptosis following MYC activation (Fig. 1e). Collectively, these results highlight a role for ATF4 in promoting survival of transformed cells on MYC activation.
The amino acid sensor GCN2 is activated by uncharged tRNAs and is required for optimal activation of ATF4 on MYC induction. a, Representative immunoblot showing activation of GCN2 following MYC induction using cytoplasmic lysates. b, DLD-1 MycER cells were transfected with non-targeting siRNA or siRNA against PERK (siPERK), GCN2 (siGCN2) or both. ISR signalling and apoptosis were assessed by immunoblotting after MYC activation. c, Representative immunoblot analysis of cytoplasmic and nuclear lysates from GCN2−/−, GCN2−/− and ATF4−/− MycER MEFs after 16 h of MYC activation. d, DLD-1 MycER cells were pre-treated with DMSO or 50 μM ML60218 (an inhibitor of RNA polymerase III) for 2 h before MYC activation. Indicated protein levels were measured by immunoblotting from cytoplasmic lysates. e, Microarray analysis of aminoacyl-tRNAs of DLD-1 MycER cells after MYC induction at indicated times. Four independent experiments; one-way analysis of variance; *P < 0.05, **P < 0.01 and ***P < 0.001. Exact P values are listed in the source data. (−) Leu denotes Leu-deprived cells and the marked tRNAs reading Leu codons are uncharged (n = 1). IRNA probes are depicted with their cognate codon and the corresponding amino acid. Two different probes recognizing two different tRNA isoacceptors that pair to the same TTA/G Leu codon but differ in their sequence outside the anticodon were used on the arrays. Brackets show decreased charging of tRNAisoacceptors in the Leu-deprivation condition. Numbers below blots in b,d represent relative band intensities, normalized as indicated. All immunoblots are representative of three biological replicates showing similar results. Unprocessed scans of blots are shown in Supplementary Fig. 7. Meti, initiator tRNA; pol, polymerase.

Consistent with loss of ATF4, knockdown of both PERK and GCN2 enhanced apoptosis following MYC activation (Fig. 2b). Decreased expression of either PERK or GCN2 also reduced ATF4 protein levels (Fig. 2b)—this effect was more prominent in GCN2-knockdown cells. Similarly, transformed GCN2-deficient MEFs failed to induce ATF4 following MYC activation (Fig. 2c). Of note, we observed higher activation of PERK in the absence of ATF4, suggesting increased ER stress in these cells (Fig. 2c). Because GCN2-deficient cells still displayed residual eIF2α phosphorylation, yet ATF4 protein was expressed at a low level compared with GCN2-proficient cells, we hypothesized that GCN2 may regulate ATF4 at the transcriptional level. Indeed, induction of MYC resulted in a significant upregulation of ATF4 mRNA only in GCN2-proficient cells, although another well-characterized MYC-target gene—ornithine decarboxylase (ODC1)—was activated independently of GCN2 (Supplementary Fig. 1b). These results indicate that GCN2 is required for efficient ATF4 protein expression and for ATF4 mRNA transcription in response to MYC activation.

MYC increases the ratio of uncharged tRNAs leading to activation of GCN2. Amino acid deficiency leads to accumulation of uncharged tRNAs, which in turn bind to GCN2, resulting in its autophosphorylation and activation. Activated GCN2 subsequently phosphorylates eIF2α, resulting in transient inhibition of general protein synthesis in conditions of amino acid
of a gene TSS, 33 of which overlapped with ATF4-binding sites (28% of all ATF4 peaks), a significant overlap (2.1 more than expected by chance, \( P = 2 \times 10^{-3} \) by hypergeometric test) suggesting that a similar mechanism regulates a substantial subset of genes via both MYC and ATF4 (Fig. 3d). Moreover, the promoters of genes with functions in amino acid transport (SLC7A11, SLC38A1 and SLC43A1) and tRNA charging (IARS, MARS and NARS) were occupied by both ATF4 and MYC (Fig. 3e).

We validated the ChIP–seq results by ChIP with quantitative PCR (ChIP–qPCR) (Supplementary Fig. 2c). A ChIP–seq profile at a representative MYC and ATF4 target locus, \( TBC1D16 \) (Fig. 3e), shows overlapping binding of MYC and ATF4 (Fig. 3f). The ChIP–seq result suggests that MYC and ATF4 share common target genes, most of which are involved in amino acid transport and tRNA charging.

ATF4 and MYC have common overlapping DNA-binding sites.

Since ATF4 is critical for survival following MYC activation (Fig. 1c–e), we performed chromatin immunoprecipitation with sequencing (ChIP–seq) in DLD-1 MycER cells with or without MYC activation to map genes bound by ATF4 on a genome-wide scale. We identified 330 unique ATF4-binding sites that had ATF4 ChIP–seq signal significantly enriched (false discovery rate, FDR < 5%; at least fourfold enrichment) over IgG control (Fig. 3a). Levels of approximately 50% of the binding sites (165 out of 330) were significantly increased following MYC activation for 8 h (Fig. 3a). A search for de novo motifs revealed that about 90% of identified ATF4-binding sites contained a previously established mouse ATF4-binding motif \( ^{10} \) (GSE35681) (Fig. 3b). From the 165 ATF4-binding sites, we identified 79 genes (with Entrez IDs) in which ATF4 bound within 5 kb of at least one gene transcription start site (TSS). Of this subset, 16 genes are previously well-characterized ATF4 targets \( ^{12} \) (Supplementary Fig. 2a). Analysis of the 79-gene list for functional and pathway enrichment showed significant overrepresentation of 11 functional categories and 10 pathways (Supplementary Fig. 2b). As expected, one of the pathways was the unfolded protein response pathway, confirming the well-characterized role of ATF4 in this pathway. Other key functional categories were amino acid transport and biosynthesis and tRNA synthetases, underscoring the important role of ATF4 in protein synthesis (Supplementary Fig. 2b). We then analysed the list of genes for enrichment of known transcriptional regulators to identify potential ATF4 co-regulators (Fig. 3c). As expected, ATF4 itself was the top significant hit with 16 known targets and a z score indicating positive regulation (\( z = 3.88 \), based on the majority of targets being upregulated by ATF4). Intriguingly, the only other significant transcriptional co-regulator was MYC, with a z score of at least 1.5, higher than additional factors such as p53, SP1 and Pax3 that also showed significant enrichment of targets.

We then performed ChIP–seq for MYC to map its global binding following stimulation with 4-OHT in the same cells. This experiment identified 3,263 peaks at 8 h of MYC induction that were within 5 kb of a gene TSS, 33 of which overlapped with ATF4-binding sites (28% of all ATF4 peaks), a significant overlap (2.1 more than expected by chance, \( P = 2 \times 10^{-3} \) by hypergeometric test) suggesting that a similar mechanism regulates a substantial subset of genes via both MYC and ATF4 (Fig. 3d). Moreover, the promoters of genes with functions in amino acid transport (SLC7A11, SLC38A1 and SLC43A1) and tRNA charging (IARS, MARS and NARS) were occupied by both ATF4 and MYC (Fig. 3e).

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ATF4 suppresses mTORC1-dependent signalling to prevent proteotoxicity following MYC activation.

We then sought to determine the mechanism by which ATF4 exerts its pro-survival effects following MYC dysregulation. Knowing the role of ATF4 in amino acid metabolism, antioxidant response and fatty acid synthesis, we attempted to rescue ATF4-deficient cells by supplying metabolites in culture media under conditions of MYC activation \( ^{14,15} \). ATF4-deficient MEFs require the presence of non-essential amino acids and antioxidants to grow \( ^{16} \). Supplementation with additional antioxidants or long-chain fatty acids did not rescue ATF4-deficient cells (Supplementary Fig. 3a,b). Notably, supplementation with \( \alpha \)-ketoglutarate (\( \alpha \)KG), an intermediary of the tricarboxylic acid cycle, delayed apoptosis during earlier (16 h) but not later (24 h) time points of MYC activation in ATF4-deficient MEFs (Supplementary Fig. 3a,c). These results suggest that MYC activation imposes metabolic stress that can be partially relieved by \( \alpha \)KG. However, additional ATF4-dependent processes must be activated to maintain long-term cell viability.

One of the targets identified by ChIP–seq that bound both MYC and ATF4 was \( E I F 4 E B P 1 \), which encodes 4E-BP1, a negative regulator of the major cap-binding protein eIF4E and a downstream substrate of mTORC1 (Figs. 3e and 4a). mTORC1 integrates oncogenic stimuli into protein synthesis and cell growth signalling by promoting phosphorylation of p70S6 kinase and 4E-BP1 \( ^{29} \). Hyperphosphorylation of 4E-BP1 by mTORC1 leads to its dissociation from eIF4E, enhancing cap-dependent protein translation downstream of eIF4E \( ^{29} \). We confirmed that both ATF4 and MYC bind to the 4E-BP1 intron (Fig. 4a). Moreover, 4E-BP1 mRNA level was reduced in the absence of ATF4 in both DLD-1 cells and MEFs (Supplementary Fig. 4a,b). We also observed that MYC activation enhanced levels of both phosphorylated and total 4E-BP1 (Fig. 4b,d,e and Supplementary Fig. 4c) indicating that ATF4 is required for MYC induction of 4E-BP1. Of note, loss of ATF4 resulted in upregulation of PERK phosphorylation (Fig. 4c), indicating ER stress activation. These results strongly suggest that 4E-BP1, downstream of the MYC–ATF4 axis, acts as a rheostat to set a protein synthesis level compatible with MYC oncogenic activity. ATF4-deficient cells also exhibited sustained phosphorylation of p70S6 kinase (Fig. 4b and Supplementary Fig. 4c). We next examined whether mTORC1 suppression in ATF4-deficient cells had an effect on cell death during MYC activation. Indeed, inhibition of mTORC1 by rapamycin treatment led to a marked decrease in MYC-dependent apoptosis and enhanced clonogenic survival in ATF4-deficient cells (Fig. 4d and Supplementary Fig. 4c,d).

To further probe the link between ATF4 and mTORC1 effectors, we knocked down p70S6 kinase and eIF4E, two major translational regulators downstream of mTORC1 signalling in the absence of ATF4. Consistent with the results of rapamycin treatment, knockdown of p70S6 kinase reduced cell death due to ATF4
Fig. 3 | ATF4 and MYC bind to common target genes. a, ATF4 ChIP–seq identified 330 binding sites for ATF4. Blue, binding sites occupied by ATF4 in the absence of 4-OHT; purple, binding sites occupied with 4-OHT treatment; red, binding sites that are significantly increased after 8 h MYC activation. Significance of the increase in binding signal was estimated using the HOMER algorithm (FDR < 5%). b, Motifs enriched in the 330 ATF4-binding sites (human de novo) and in a mouse experiment from the GSE35681 dataset. The table shows the percentage of binding sites containing the motif in the absence of 4-OHT; purple, binding sites occupied with 4-OHT treatment; red, binding sites that are significantly increased after 8 h MYC activation. The significance of the increase in binding signal was estimated using the HOMER algorithm (FDR, Motifs enriched in the 330 ATF4-binding sites (human de novo) and in a mouse experiment from the GSE35681 dataset). The table shows the percentage of binding sites containing the motif in the absence of 4-OHT; purple, binding sites occupied with 4-OHT treatment; red, binding sites that are significantly increased after 8 h MYC activation.

deficiency when MYC was activated (Fig. 4c). Combined knockdown of p70S6 kinase and ATF4 resulted in feedback upregulation of 4E-BP1 and phosphorylated (p)-4E-BP1 levels (Fig. 4c). Additionally, knockdown of both p70S6 kinase and eIF4E further reduced cell death in the absence of ATF4 (Fig. 4c). This suggests that ATF4 is required for defences against proteotoxicity in the context of hyperactive MYC–mTORC1 signalling funneled through p70S6 kinase and eIF4E.
In support of this idea, inhibiting protein synthesis with low doses of cycloheximide led to a similar reduction in cell death in ATF4-deficient cells (Fig. 4f). However, we did not observe a general increase in rate of translation by 35S-methionine and cysteine labelling in the absence of ATF4, suggesting that synthesis of specific proteins rather than global translation is deregulated in ATF4-deficient cells and contributes to ER proteotoxicity (Supplementary Fig. 4e). Furthermore, treatment with the chemical chaperone
4-phenylbutyric acid, which can protect against proteotoxicity in other stress contexts48, also promoted survival of ATF4-deficient cells following MYC activation (Supplementary Fig. 4f). Since 4E-BP1 levels were reduced in the absence of ATF4, we overexpressed a mutant version of 4E-BP1 that cannot be phosphorylated and inactivated by mTORC110. Expression of the 4E-BP1 mutant led to decreased apoptosis in ATF4-knockdown cells (Fig. 4g), which was further reduced when combined with knockdown of p70S6 kinase (Supplementary Fig. 4g). Collectively, these data indicate that inhibiting both branches of mTORC1 signalling in ATF4-deficient cells reduces the demand of specific protein synthesis and promotes adaptation to hyperactive MYC-induced gene expression, mitigating proteotoxicity.

Inhibition of both PERK and GCN2 promotes survival of MYC-driven lymphoma-bearing mice. To test the importance of the GCN2–eIF2α–ATF4 pathway in vivo, we used a well-characterized mouse model of MYC-driven lymphoma, Eμ-Myc49, which harbours Myc coupled to the IgH enhancer and succumbs to aggressive B-cell lymphoma between 6 and 15 weeks of age50. Eμ2αk4d2 (hereafter, Gcn2−/−) mice are viable and fertile, unlike Atf4−/− mice, which display several pathological abnormalities in utero or shortly after birth10,39. We generated Eμ–Myc/+;Gcn2−/−, Eμ–Myc+/+;Gcn2−/− and Eμ–Myc+;Gcn2−/− mice (Supplementary Fig. 5a). Surprisingly, loss of GCN2 did not affect tumour initiation or progression or impact overall mouse survival (Supplementary Fig. 5b). Assessment of ISR signalling in B cells from each genotype revealed robust PERK and eIF2α phosphorylation and ATF4 upregulation in B-cell lymphoma compared with normal B cells from littermate controls, suggesting that PERK is probably compensating for loss of GCN2 (Supplementary Fig. 5c).

To test this notion, we transplanted either Eμ–Myc+/+;Gcn2−/− or Eμ–Myc+/+;Gcn2−/− lymphomas (Supplementary Fig. 5d) into C57BL/6J mice and targeted PERK by using a potent and specific PERK inhibitor, LY-494792. LY-4 treatment was well tolerated and did not affect body weight or pancreas weight51, but significantly reduced phosphorylation of PERK (Supplementary Fig. 5e,f,h). Inhibition of PERK in mice bearing Eμ–Myc+/+;Gcn2−/− lymphomas, but not those bearing Eμ–Myc+/+;Gcn2−/− lymphomas, increased overall survival (Supplementary Fig. 5g). Consistent with our in vitro data, inhibition of both PERK and GCN2 significantly reduced phosphorylation of eIF2α in the lymphoma cells (Supplementary Fig. 5h,i). Unexpectedly, inhibition of PERK and GCN2 did not reduce ATF4 protein levels, suggesting an eIF2α-independent regulation of ATF4 in the Eμ-Myc lymphoma model (Supplementary Fig. 5h). One possible explanation for the modest increase in survival of these mice is the decreased phosphorylation of eIF2α, which can exacerbate ER stress in tumour cells due to unregulated protein translation52. These results underscore a strict selective pressure for maintenance of robust ATF4 levels in MYC-driven tumours.

Acute ablation of ATF4 in lymphoma cells significantly enhances tumour-free survival and overall survival. The results with the Eμ–Myc+/+;Gcn2−/− mice indicated that maintenance of ATF4 may be important for tumour progression. Therefore, we generated conditional knockout Atf4fl/fl mice and crossed the Eμ–Myc/+ mice with Rosa26-CreER72/2 mice, which express a tamoxifen-inducible Cre recombinase enzyme. Eμ–Myc+;Rosa26-CreER72/2 mice were crossed with Atf4fl/fl mice to generate Eμ–Myc+/+;Atf4fl/fl;Rosa26-CreER72/2 and Eμ–Myc+/+;Atf4fl/fl;Rosa26-CreER72/2 genotypes (Supplementary Fig. 6a,b). Following lymphoma onset, lymphoma cells were purified and allografted by intravenous injection into C57BL/6 syngeneic mice. The recipient mice were treated either with vehicle or tamoxifen to excise Atf4 specifically in the lymphoma cells (Fig. 5b). Remarkably, excision of Atf4 significantly extended lymphoma-free and overall survival (Fig. 5c,b). This effect was not due to tamoxifen treatment because mice with Eμ–Myc/+;Atf4fl/fl;Rosa26-CreER72/2 lymphomas displayed no significant difference in lymphoma-free or overall survival (Fig. 5c and Supplementary Fig. 6c).

To further assess the requirement for ATF4 in lymphoma progression, we treated a separate cohort of mice as above after lymphoma developed. Treatment with tamoxifen resulted in efficient ablation of Atf4 mRNA (Supplementary Fig. 6d) and protein as well as reduction in expression of Atf3, an ATF4-target gene (Fig. 5d) and resulted in reduced lymph node weight (Fig. 5e). Consistent with our in vitro data (Fig. 4b and Supplementary Fig. 4c), 4E-BP1 levels were significantly reduced in lymphoma cells following ATF4 ablation (Fig. 5f). However, ATF4 expression returned to levels comparable with those of vehicle-treated groups by the endpoint of the experiment, suggesting that lymphoma cells that escaped ATF4 excision eventually formed lymphomas (Supplementary Fig. 6e).

Similar to the lymphoma model, knockdown of ATF4 in DLD-1 MycER cells expressing a doxycycline-inducible short hairpin RNA directed against ATF4 significantly delayed tumour growth (Fig. 5g). Collectively, the in vivo data support our hypothesis that ATF4 is critical for tumour growth driven by hyperactive MYC and indicate that targeting ATF4 in the context of activated MYC elicits potent anti-tumour effects.

We next assessed the levels of ISR signalling in B cells isolated from wild-type, pre-lymphoma and lymphoma-bearing Eμ–Myc mice. We noted higher expression of ATF4 in lymphoma samples compared with wild-type and pre-lymphoma samples (Fig. 6a). Interestingly, levels of p-eIF2α, p-4E-BP1, 4E-BP1 and p-p70S6 kinase were higher in both pre-lymphoma and lymphoma samples than in wild-type samples (Fig. 6a). Human Burkitt’s lymphoma cell lines also displayed robust increases in ISR signalling and 4E-BP1 levels (Fig. 6b). Similar results were seen in human breast and colon cancer cell lines compared with a human immortalized breast epithelial cell line and normal human colonocytes, respectively (Fig. 6c,d).

To investigate the relevance of our findings in human malignancy, we analysed the expression of 4E-BP1 and tested its correlation with ATF4 activity in different cohorts of cancer patients by mining The Cancer Genome Atlas (TCGA) database. Since ATF4 is primarily regulated at the translational level, we used a group of ten well-characterized ATF4 targets (including ASNS, MTHFD2 and CHOP (also known as DDIT3)) as a surrogate for ATF4 activation77. We found that 4E-BP1 levels were positively correlated with ATF4-target genes in diffuse large B-cell lymphoma (DLBCL), colorectal cancer, breast cancer and sarcoma (Fig. 6e). Notably, high expression of 4E-BP1 also correlated with poor prognosis in patients with DLBCL (Fig. 6f). These data further support the notion that ATF4-dependent modulation of MYC-driven translational alterations may play a role in progression of MYC-driven lymphomas.

Discussion

MYC activation is invariably linked with enhanced protein synthesis, which is critical for enhanced proliferation and expression of the tumorigenic properties of MYC40. However, increased translation increases demands on the ER, the site of glycosylation and folding of client proteins41. While PERK-mediated phosphorylation of eIF2α can provide initial relief from increased translation rates41, this mechanism may be insufficient for long-term survival due to a negative-feedback loop that promotes dephosphorylation of eIF2α42. This work demonstrates that ATF4 is critical for MYC-dependent tumour progression not only by supporting protein synthesis via expression of amino acid transporters and tRNA synthetases but also by preventing ‘runaway’ mRNA translation and subsequent proteotoxicity via prolonged upregulation of 4E-BP1 levels (Fig. 6g).

Since ablating ATF4 expression did not increase global translation levels following MYC dysregulation, it is likely that ATF4 negatively regulates the expression of a specific set of proteins that traffic
Fig. 5 | Acute ablation of ATF4 significantly delays MYC-driven lymphomagenesis and promotes survival of MYC-driven lymphoma bearing mice. a, Schematic showing allograft lymphoma model, in which lymphoma cells are injected via the tail vein into nine-week-old male C57BL/6J mice. Three days after lymphoma engraftment, mice are randomized to receive either vehicle or tamoxifen treatment by oral gavage for 5 d. b, Kaplan–Meier analysis of lymphoma-free survival of mice bearing Eµ-Myc;Atf4fl/fl lymphoma treated with either vehicle (veh) or tamoxifen (tam) for five days. Eµ-Myc;Atf4fl/fl lymphoma tamoxifen group, n = 9; all other groups, n = 10. Kaplan–Meier curves were analysed by two-tailed log-rank test. c, Kaplan–Meier analysis of overall survival of mice bearing Eµ-Myc;Atf4fl/fl lymphoma (left) or Eµ-Myc;Atf4+/+ lymphoma (right). Kaplan–Meier curves were analysed by two-tailed log-rank test. d, Immunoblot of B cells isolated from mice bearing Eµ-Myc;Atf4fl/fl one day after the end of tamoxifen treatment. e, Lymph node weight of mice in d. n = 3 per group; data are represented as mean ± s.d.; two-tailed Student’s t-test. f, Immunoblot of lymphoma lysates from mice in e. In d, f, lysates from three different mice per treatment were used. g, Tumour volume in 11-week-old nude mice transplanted with DLD-1 MycER cells expressing inducible short hairpin RNA against ATF4 (ishATF4). Doxycycline treatment was initiated 12 d post transplant when tumours reached around 100 cm³. One of the mice with no doxycycline treatment had to be euthanized on day 12 because the tumour reached the maximum allowable size. n = 4 no doxycycline, n = 4 + doxycycline. Two-way analysis of variance; data are represented as mean ± s.e.m. Unprocessed scans of blots are shown in Supplementary Fig. 7.
Fig. 6 | EIF4EBP1 positively correlates with ATF4-target gene expression and is associated with poor prognosis. a, Immunoblot of B cells isolated from wild-type (WT) mice (n = 3), pre-lymphoma Eμ-Myc (n = 3) or lymphoma bearing Eμ-Myc mice (n = 6). Samples from all mice in each group are shown. b, Immunoblot from two normal human primary B cells (NHBc) and Burkitt's lymphoma cell lines Raji and Ramos. c, Immunoblot from MCF10A (non-tumorigenic breast epithelial cell line) or breast cancer cell lines MCF-7 and MDA-MB-231. d, Immunoblot from normal human colonocytes (NHC) or colon cancer cell lines DLD-1 and LOVO. e, Pearson correlation between EIF4EBP1 and ATF4-target gene expression in DLBCL (n = 562), colorectal adenocarcinoma (COAD, n = 329), breast cancer (BRCA, n = 1,218) and sarcoma (SARC, n = 265). The lines show linear regression, and shaded regions are 95% confidence intervals for the regression lines. Datasets analysed are listed in the Methods. The ATF4-target genes used in this analysis are listed.

Kaplan–Meier plots of progression-free survival (left) and overall survival (right) of DLBCL patients with high or low EIF4EBP1 expression. The survival analysis using Kaplan–Meier and two-sided log-rank test between high and low EIF4EBP1-expression groups was performed on all patients with records of progression-free survival (left, n = 173) and overall survival (right, n = 171). Proposed model of ATF4 and MYC cooperation in tumour progression. All immunoblots are from n = 1 experiment; unprocessed scans of blots are shown in Supplementary Fig. 7.
4E-BP1 has been shown to inhibit cap-dependent translation and to promote survival under conditions of nutrient and oxidative stress. Interestingly, higher expression of 4E-BP1 has been noted in multiple cancers, and this high expression is associated with poor prognosis. While ATF4 has been shown to regulate 4E-BP1 in the context of extrinsic physiologic stresses, its role in regulating 4E-BP1 in the context of MYC-deregulated tumour progression was unknown. Higher expression of 4E-BP1 seems paradoxical, as a hyperactive form of 4E-BP1 blocks MYC-induced tumorigenesis. Indeed, our findings provide a context for the upregulation of 4E-BP1 expression in tumours: tumours maintain high levels of 4E-BP1 whose activity can be readily modulated by rapid phosphorylation and dephosphorylation, depending on favourable or unfavourable growth and proliferation conditions.

Our study also highlights the importance of using combined inhibition of both PERK and GCN2 in MYC-driven cancers to avoid compensation of ISR kinases in regulating p-eIF2α levels. Indeed, pharmacologic targeting of eIF2α phosphorylation in patient-derived prostate-xenograft models is effective in initiating a cytotoxic response that inhibits tumour growth. However, in the lymphoma model tested here, ATF4 levels remained high despite the near complete inhibition of upstream canonical pathways (potentially via translational regulation by mTOR), and acute ATF4 ablation significantly decreased tumour growth and prolonged survival. This strongly implies that for certain malignancies, it might be essential to directly inhibit ATF4 activity for optimal anti-tumour outcome. In sum, the ISR transcription factor ATF4 is necessary for supporting and censoring protein translation thus promoting progression of MYC-driven tumorigenesis.
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Author contributions
F.T. and C.K. conceived the experiments, analysed data and wrote the manuscript. F.T., I.I.V., N.M.L., R.O., C.S. and A.M.M. performed experiments. C.P. and Z.I. performed tRNA microarray and analysis. C.S.C., J.A.D., S.Y.F. and D.R. provided valuable reagents, important experimental suggestions and helped in manuscript editing. R.K.A. and J.Y. provided important experimental suggestions. A.K. provided bioinformatics support for ChIP–seq data analysis. W.F. and P.W. analysed patient datasets.

Competing interests
The authors declare no competing interests.

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Methods

Cell culture and reagents. Human colon adenocarcinoma cell line DLD-1, Burkitt’s lymphoma cell lines Raji and Ramos and human breast cancer cell lines MCF10A, MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection (ATCC). DLD-1 and Raji cells were maintained in DMEM (Invitrogen, 21802-029) supplemented with 10% FBS (Sigma) and 1% penicillin–streptomycin (Invitrogen). P493-6 cells were provided by D. Eick (German Research Centre for Environmental Health). P493-6, Raji and Ramos cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. MCF-7 was cultured in F-12K (Corning) supplemented with 10% FBS and 1% penicillin–streptomycin. MCF-7 cells were transfected with pEGFP-C3 plasmid (Clontech) and 35S-methionine and cysteine labelling. Cells were plated in a six-well dish. The following day, the cells were treated with 4-OHT for indicated times and labelled with 50μg/ml of [35S] Met/Cys Express Mix (PerkinElmer, N677201400) for 60 min. Cells were harvested and washed with ice-cold PBS and cells lysates were collected for protein. Equal amounts of protein was loaded and resolved by SDS–PAGE and transferred on to polyvinylidene fluoride membranes. Membranes were exposed to autoradiography films. Incorporation of 35S was quantified with Image J 1.51r software. β-Actin was used as a loading control.

Magnetic isolation of mouse B cells. Spleens and lymph nodes were excised from euthanized mice and immediately passed through a 70 μm cell strainer. Normal splenic B cells and B cells in lymphomas were isolated using mouse B-cell isolation kit (Miltenyi Biotec, 130-090-862) according to the manufacturer’s instructions.

qPCR. RNA was collected for real-time qPCR analysis using the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer’s instructions. RNA was reverse transcribed using AMV reverse transcriptase (Promega, M5108) in the presence of RNase inhibitor (Promega, N2111). qPCR was performed with Power SYBR green PCR master mix (Applied Biosystems, 4367339). QuantiSoft 6 Flex Real-Time PCR System (Applied Biosystems) was used for data analysis. Primers used for qPCR are listed in Supplementary Table 1.

Aptosis analysis. Apoptosis was detected using the annexin V apoptosis detection kit (BD Pharmingen, 556548) according to the manufacturer’s instructions. In brief, cells were collected and 1×106 cells were resuspended in 100 μl annexin-binding buffer. Five microlitres of the annexin V conjugate was added to each 100 μl of cell suspension and incubated at room temperature for 15 min. An additional 400 μl of annexin-binding buffer was added to cells. Cells were immediately analysed on a BD FACS Canto flow cytometer.

tRNA charging microarray. About 8×106 DLD-1 MycER cells were treated with 500 nM 4-OHT for 2h, 4h or 8h; ethanol was used as vehicle. To determine the fraction of charged tRNAs, total RNA was isolated in mild acidic conditions using acidic phenol (pH 4.5), whose low pH preserves the aminoacyl moiety. Each sample was split into two aliquots, one of which was oxidized with periodate which oxidizes the free non-aminoacyl groups leaving the changed tRNAs intact. Following subsequent deacylation the amino acid-protected tRNAs hybridize to Cy3-labelled RNA–DNA stem-loop oligonucleotide. The second aliquot was deacylated in 100 mM Tris (pH 9.0) at 37°C for 45 min and hybridized to Atto647-labeled RNA–DNA stem-loop oligonucleotide and designated as total tRNA. Both aliquots were loaded on RNA microarrays with RNA probes covering the full-length sequence of cytoplasmic tRNA species as described previously51. For tRNA abundance, total RNA was isolated with TriReagent (Sigma Aldrich), which was used as vehicle. The fraction of charged tRNAs, total RNA was isolated in mild acidic conditions using acidic phenol (pH 4.5), whose low pH preserves the aminoacyl moiety. Each sample was split into two aliquots, one of which was oxidized with periodate which oxidizes the free non-aminoacyl groups leaving the changed tRNAs intact. Following subsequent deacylation the amino acid-protected tRNAs hybridize to Cy3-labelled RNA–DNA stem-loop oligonucleotide. The second aliquot was deacylated in 100 mM Tris (pH 9.0) at 37°C for 45 min and hybridized to Atto647-labeled RNA–DNA stem-loop oligonucleotide and designated as total tRNA. Both aliquots were loaded on RNA microarrays with RNA probes covering the full-length sequence of cytoplasmic tRNA species as described previously51. For tRNA abundance, total RNA was isolated with TriReagent (Sigma Aldrich), which was used as a negative control. Protein G Dynabeads (10003D) was used to purify RNAs from Invitrogen. Primers used for qPCR are listed in Supplementary Table 1.

Library preparation, sequencing and analysis. CHIP DNA from two independent experiments was submitted to Wistar genomics core (Wistar Institute) for library preparation using NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645S) and NEBNext Multiplex Oligos for Illumina (E7335S) according to the manufacturer’s instructions. The library fragments were assessed on an Agilent Technologies 2100 Bioanalyzer and yielded 150–350 bp products. An Illumina NextSeq 500 instrument was used for sequencing. Select targets were validated by qPCR and primers used for qPCR are listed in Supplementary Table 1.

CHIP–seq data was aligned using bowtie2 against the hg38 version of the human genome55 and HOMER software56 was used to call significant peaks against Igs3 control or between corresponding replicate pairs of samples using the --style factor option and only uniquely aligned reads with duplicates were removed. Significant peaks that passed a FDR <5% threshold were set at least fourfold over IgG control were used to identify unique binding sites. Only results that were significant in comparison for both replicates were considered. De novo motif analysis was
performed using HOMER software among the list of sites with significant ATF4 binding in at least one condition (both replicates). Overlap of binding sites with genes was done using the Ensemble 84 transcriptome database. Genes with a binding site within 5 kb of a TSS were considered. Significance of overlap was tested with a hypergeometric test using 23,869 Ensemble genes with Entrez ID as a population size. Gene-set enrichment analysis was done using QIAGEN’s Ingenuity Pathway Analysis software (www.qiagen.com/ingenuity) using ‘canonical pathways’ and ‘upstream analysis’ options. Pathways with at least two member genes that passed FDR < 20%, with enrichment at least fivefold over the threshold and upstream regulators (transcription factors only) that passed P < 0.05 and had at least five target genes were considered. Functional and pathway enrichment analysis was done using DAVID software and FDR < 20% categories enriched at least fivefold were considered. The data were submitted to the Gene Expression Omnibus database and can be accessed using accession number GSE117240.

Animal studies. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Pennsylvania and comply with all regulations for ethical conduct of research. The Eph+/- (stock # 000278), Gen2- (stock # 008240) and Rosa26-CreERT2- (stock # 008163) transgenic mice were purchased from The Jackson Laboratory. A thymic nude mouse was purchased from Charles River Laboratory (stock no. 490). Aif4fl/fl mice on C57BL/6 background were generated by Cyagen Biosciences. In brief, LoxP sites were inserted flanking exon 2 and 3 of the Aif4 locus to create a conditional knockout when crossed with Rosa26-CreERT2-+. Eph+/-;Gen2+/+ mice were crossed with Gen2- to generate Eph+/-;Gen2-/-; Eph+/-;Gen2+/- and Eph+/-;Gen2-/- mice. Aif4fl/fl mice were crossed with Rosa26-CreERT2- to generate Aif4fl/fl;Rosa26-CreERT2-+; Eph+/-;Gen2+/+ mice. The following mice were obtained and used for transplantable lymphoma experiments: Eph+/-;Rosa26-CreERT2-+; Aif4fl/fl and Eph+/-;Rosa26-CreERT2-+; Aif4fl/fl. Primers used for genotyping are listed in Supplementary Table 1.

For transplantable lymphoma experiments, mice harbouring lymphomas were euthanized according to IACUC guidelines. Lymph nodes were collected immediately on ice and minced and passed through 70 µm cell strainer in 50% Iscove’s medium and 50% DMEM supplemented with 10% FBS, 1% penicillin–streptomycin and 4 mM glutamine. Dead cells were removed by spinning cells in Ficoll–Paque PLUS (GE healthcare, 17-1440-02) at 800 g for 10 min. Lymphoma cells were washed in PBS and 2 × 10⁶ cells were injected into nine-week-old male C57BL/6 mice. Mice were monitored for lymphoma development by palpation every other day.

For PERK inhibitor (LY-4) experiments, nine-week-old male mice were injected with 2 × 10⁶ Eph+/-;Gen2+/+ or Eph+/-;Gen2+/- lymphoma cells. After 3 d, mice were randomized to receive vehicle (Captisol, CYDEX) or 100 mg kg⁻¹ LY-4 twice a day by oral gavage 3 d after lymphoma injection for the duration of the experiment. Moribund mice were euthanized according to IACUC guidelines and lymph nodes and spleen were collected and assessed for protein levels by immunoblot.

For ATF4 excision experiments, 2 × 10⁶ Eph+/-;Rosa26-CreERT2-+; Aif4fl/fl or Eph+/-;Rosa26-CreERT2--; Aif4fl/fl lymphoma cells were injected via tail vein into nine-week-old male C57BL/6 mice. Three days after lymphoma injection, mice were randomized to receive vehicle (Captisol, CYDEX) or 100 mg kg⁻¹ every other day. Three million DLD-1 MycER ishATF4 cells were injected in the flanks of 11-week-old male nude mice and MYC was activated by treating mice with 1 mg per mouse tamoxifen 3 d after tumour injection. Once tumours reached 100 cm³, mice were treated every other day with 2 mg per mouse doxycycline for the duration of the experiment. Tumour volume was measured every two to three days and calculated as \( V = (L \times W \times H)/2 \), where \( L \) is tumour length, \( W \) is tumour width and \( H \) is height.

Statistics and reproducibility. All cell culture experiments were performed three times unless otherwise noted in the legend. GraphPad Prism 7 and Excel 2010 were used for statistical analysis. Data are presented as mean ± s.d. or mean ± s.e.m. (as indicated in figure legends) and statistical significance was determined by unpaired, two-tailed Student’s t-test. One-way analysis of variance was used to determine statistical differences in the tRNA microarray data. A P value less than 0.05 was considered statistically significant. For mouse survival analysis, Kaplan–Meier curves and log-rank tests were generated in GraphPad Prism 7 software. For xenograft experiment, two-way analysis of variance was used.

Patient data analysis. The gene expression using RNA-seq and survival information of the DLBCL dataset were obtained from The National Cancer Institute Center for Cancer Genomics website, and gene expression information of three TCGA datasets were obtained from University of California Santa Cruz Xena. For each dataset, the normalized gene expression of E1F4EBP1 and another ten ATF4-targeted genes (DDIT3, ATF3, ASNS, SLC39A1, SLC18A5, GARS, NARS, MARS, PSAT1 and MTHFD2) were standardized to Z score, then the Pearson correlation between E1F4EBP1 and average ATF4-target genes was estimated. The visualization of linear relationships in Fig. 6c was performed using seaborn software version 0.9.0 (https://zenodo.org/record/8838598). In addition, patients in DLBCL were divided into two groups according to ATF4/E1F4EBP1 gene expression: low ATF4/E1F4EBP1 expression (≤ median) and high ATF4/E1F4EBP1 expression (> median). Survival analysis using Kaplan–Meier and log-rank test between high and low ATF4/E1F4EBP1 expression groups were performed using lifelines software version 0.14.0 (https://zenodo.org/record/1523442). 

Data availability

RfNA microarray and ChIP–seq data that support the findings of this study have been deposited in the Gene Expression Omnibus under accession codes GSE116812 and GSE117240, respectively. The human COAD, BRCA and SARC datasets were derived from the TCGA Data Hub on the University of California Santa Cruz Xena platform (http://xenahubs.net/). The dataset derived from this resource that supports the findings of this study is available in the following links. COAD: https://xenahub.net/datasets?dataset=TCGA.COAD.sampleMap%2FHiSeqV2_PANCAN&host=https%3A%2F%2Fdata.xenahubs.net&removeHub=htt hp%3A%2F%2Flocal.xena.ucsc.edu%3A8223; BRCA: https://xenahub.net/datasets?dataset=TCGA.BRCA.sampleMap%2FHiSeqV2_PANCAN&host=https%3A%2F%2Fdata.xenahubs.net&removeHub=htt hp%3A%2F%2Flocal.xena.ucsc.edu%3A8223; SARC: https://xenahub.net/datasets?dataset=TCGA.SARC.sampleMap%2FHiSeqV2_PANCAN&host=https%3A%2F%2Fdata.xenahubs.net&removeHub=htt hp%3A%2F%2Flocal.xena.ucsc.edu%3A8223. The human DLBCL data were derived from the National Cancer Institute Cancer Genome project (https://gdc.cancer.gov). The dataset derived from this resource that supports the findings of this study is available at https://gdc.cancer.gov/about-data/publications/DLBCL-2018. 

Statistics source data for graphical representations and statistical analyses in Figs. 1–6 and Supplementary Figs. 1–6 are provided in Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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- BRCA: https://xenabrowser.net/datapages/?dataset=TCGA.BRCA.sampleMap%2FHiSeqV2_PANCAN&host=https%3A%2F%2Ftcga.xenahubs.net&removeHub=https%3A%2F%2Flocal.xena.ucsc.edu%3A7223
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The human DLBCL data was derived from NCI Center for Cancer Genomics (CCG) website: https://gdc.cancer.gov. The data-set derived from this resource that supports the findings of this study is available in https://gdc.cancer.gov/about-data/publications/DLBCL-2018.

Statistics Source Data giving rise to graphical representations and statistical analysis in Figures 1-6 and Supplemental Figures 1-6 have been provided as Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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**Sample size**
Statistical method was not used to predetermine sample size. Our previous experience and the standard in the field guided us in determining the sample sizes in our experiments. For most in vitro experiments, at least three independent biological replicates were used unless otherwise noted in the legend. For immunoblotting of samples from several mice, we run the samples once, but included 3+ mice per group. For overall survival curves, at least 8 mice per group were used which resulted in robust statistical significance. All sample sizes are listed in the corresponding figure legend.

**Data exclusions**
There was no exclusion of data for samples.

**Replication**
All experiments were done 3 times unless otherwise noted in the legend with similar results. All attempts for replication were successful.

**Randomization**
Mice were randomized to receive treatments as described.

**Blinding**
The cell culture experiments were not blinded because treatments and different drugs used made it difficult to blind. Mouse experiments were not blinded because the same investigator who administered the treatments (e.g., tamoxifen or LY-4) also was responsible for recording the outcomes.

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| ☒ Palaeontology                 | ☒ MRI-based neuroimaging |
| ☒ Animals and other organisms   |         |
| ☒ Human research participants   |         |
| ☒ Clinical data                 |         |

**Antibodies**

Antibodies used
ATF4 (cat# sc-200x, c-20), MYC (cat# sc-764, N-262), ATF3 (cat# sc-188, C-19) were purchased from Santa Cruz. Rabbit β-tubulin (2146), mouse cl-PARP (mouse specific, #9548, 7C9), rabbit cl-PARP (human specific, cat#9541), rabbit cl-caspase3 (cat# 9661), rabbit GCN2 (cat# 3302), rabbit PERK (cat# 3192, C33E10), rabbit p-PERK T980 (cat# 3179,16F8), rabbit p-4EBP1 T37/46 (cat# 2855, 23684), rabbit 4EBP1 (cat#9452), rabbit elf4e (cat#9742), rabbit p-p70S6K T389 (cat# 9205), rabbit p70S6K (cat#2708, 49D7), rabbit p-eIF2α SS1 (cat# 3597, 119A11) and rabbit eIF2a (cat# 9722) were all purchased from Cell Signaling Technology. Rabbit p-PERK T982 was provided by Eli Lilly, Rabbit p-GCN2 T899 (cat#ab 75836) was purchased from Abcam. Mouse β-actin (cat#A5441, AC-15) was purchased from Sigma. Mouse RNA POL II (cat# 39097) was purchased from Active Motif. All antibodies were used at 1:10000 dilution except cl-PARP, β-tubulin, PERK, p-4EBP1 T37/46, 4EBP1, p-p70S6K T389, p70S6K, eIF2α which were used at 1:2000 dilution. RNA POL II was used at 1:5000 dilution. β-actin was used at 1:50,000 dilution.

**Validation**
All commercial primary antibodies used in this manuscript were previously validated by the manufacturer for species used and western blot application. We further validated ATF4, p-PERK, PERK, p-GCN2, GCN2 p-p70S6K T389, p70S6K, p-4EBP1 T37/46,
4EBP1 and MYC primary antibodies using WT and knockout or knock down cells and different stimulations by western blotting. The bands appeared at the correct molecular weight.

**Eukaryotic cell lines**

| Policy information about cell lines | DLD-1, WT, GCN2 Knockout MEFs, RAJI, RAMOS, MCF10A, MCF7 and MDA MB231 were purchased from ATCC. ATF4 knockout MEFs were a gift from Dr. David Ron. P4936 cells were a gift from Dr. Dirk Eick. |
| --- | --- |
| Authentication | DLD-1 cells, WT, GCN2 Knockout MEFs, RAJI, RAMOS, MCF10A, MCF7 and MDA MB231 were purchased from ATCC directly. GCN2 knockout and ATF4 knock out were validated by qPCR and western blot. P4936 cells were validated by addition of tetracycline which suppresses MYC expression. |
| Mycoplasma contamination | All cell lines were tested for mycoplasma and were found negative. |
| Commonly misidentified lines (See ICLAC register) | The cell lines used are not listed in the ICLAC register. |

**Animals and other organisms**

| Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research | For E\(\mu\)-myc/+; GCN2+/+ and E\(\mu\)-myc/+; GCN2-/- survival experiment both male and female C57BL/6 strain mice were used (median age of survival was 21 weeks, 17 weeks and 24 weeks, respectively). For LY-4 and ATF4 conditional excision experiments 9-week old C57BL/6 male mice were used. Male 11-week old athymic nude mice were used for xenograft growth experiment. |
| --- | --- |
| Laboratory animals | This study did not involve wild animals. |
| Wild animals | This study did not involve field collected samples. |
| Field-collected samples | All animal experiments were approved by Institutional Animal Care and Use Committee at University of Pennsylvania. |
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**ChIP-seq**

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| Data access links | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE117240 |
| --- | --- |
| Files in database submission | File type pk_ATF4.ctr.1.txt text homer peak output pk_ATF4.ctr.2.txt text homer peak output pk_ATF4.8hr.1.txt text homer peak output pk_ATF4.8hr.2.txt text homer peak output pk_MYC.8hr.1.txt text homer peak output pk_MYC.8hr.2.txt text homer peak output ATF4.ctr.1.fastq fastq ATF4.ctr.2.fastq fastq ATF4.8hr.1.fastq fastq ATF4.8hr.2.fastq fastq MYC.8hr.1.fastq fastq MYC.8hr.1.fastq fastq IgG.fastq fastq |

| Genome browser session (e.g. UCSC) | http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&hgsid=682251473_EsSPRaBOnmIX1ooZmCAxIPKAh |

**Methodology**

**Replicates**

ChiP-seq experiment was performed in two independent experiments for each condition.

| Sequencing depth | Single end 75bp reads Sample total uniquely aligned ATF4.ctr.1 29,392,142 23,835,990 (81.10%) ATF4.ctr.2 67,579,149 54,901,709 (81.24%) ATF4.8hr.1 32,719,278 26,576,709 (81.23%) |
ATF4.8hr.2 67,658,079 55,623,324 (82.21%)
MYC.8hr.1 29,678,680 24,305,563 (81.90%)
MYC.8hr.2 67,315,122 55,446,415 (82.37%)
IgG 60,317,154 49,555,309 (82.16%)

Antibodies
- Rabbit ATF4 (cat# sc-200x, lot# G0115, c-20)
- Rabbit MYC (cat# sc-764, lot# D0413, N-262)
- Rabbit IgG (cat# sc-2027x, lot# G2516)

All antibodies were purchased from Santa Cruz.

Peak calling parameters
HOMER: peaks were called vs IgG control with parameters -style factor -fdr 0.05. Comparisons between conditions were done using -fdr 0.05 -F 1 parameters

Data quality
Only peaks present in both replicates with significant signal FDR<5% and at least 4 fold over IgG control were considered.

Number of peaks in individual experiments:

| Sample Peaks          | ATFR.ctr.1 916 |
|-----------------------|----------------|
| ATFR.ctr.2 1579       |                |
| ATFR.8hr.1 1270       |                |
| ATFR.8hr.2 2231       |                |
| MYC.8hr.1 18636       |                |
| MYC.8hr.2 22532       |                |

Software
Bowtie was used for alignment against hg38 version of the human genome. HOMER software was used to call significant peaks against IgG control.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Apoptosis was detected in DLD-1 MycER cells using the annexin V apoptosis detection kit (Invitrogen, # A23204) according to the manufacturer’s instructions. Briefly, Cells were collected and 1x10^5 cells were resuspended in 100ul annexin-binding buffer. Sul of the annexin V conjugate was added to each 100ul of cell suspension and incubated at room temperature for 15 minutes. An additional 400ul of annexin-binding buffer was added to cells. Cells were immediately analyzed on BD FACS Canto flow cytometer.

Instrument
BD FACS Canto flow cytometer

Software
FLOWJO

Cell population abundance
Purity was determined by relevant staining using flow cytometry.

Gating strategy
Cells were FSC-A and SSC-A gated, followed by FSC-A/FSC-W gating in order to select singlet cells. Untreated DLD-1, MycER cells were used as negative control.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.