TRANSLATION REGULATION

A phosphorylation-regulated eIF3d translation switch mediates cellular adaptation to metabolic stress

Adam M. Lamper*†, Rebecca H. Fleming*, Kayla M. Ladd*, Amy S. Y. Lee‡§

Shutoff of global protein synthesis is a conserved response to cellular stresses. This general phenomenon is accompanied by the induction of distinct gene programs tailored to each stress. Although the mechanisms driving repression of general protein synthesis are well characterized, how cells reprogram the translation machinery for selective gene expression remains poorly understood. Here, we found that the noncanonical 5′ cap-binding protein eIF3d was activated in response to metabolic stress in human cells. Activation required reduced CK2-mediated phosphorylation near the eIF3d cap-binding pocket. eIF3d controls a gene program enriched in factors important for glucose homeostasis, including members of the mammalian target of rapamycin (mTOR) pathway. eIF3d-directed translation adaptation was essential for cell survival during chronic glucose deprivation. Thus, this mechanism of translation reprogramming regulates the cellular response to metabolic stress.

During stress, cells undergo substantial alterations to protein synthesis. Global translation is inhibited, whereas the translation of a subset of mRNAs encoding stress-responsive proteins is considerably up-regulated. Translation shutoff is mediated through inactivation of core translation initiation factors, including the 5′ cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) (*1*). How initiation factor activity is modified during stress to allow some mRNAs to escape shutoff despite carrying a conserved 5′ cap structure remains unknown. The eIF3d subunit of the 13-subunit eukaryotic initiation factor complex 3 (eIF3) is a cap-binding protein that directs noncanonical translation of the transcription factor *Jun* (*2*), raising the possibility that eIF3d could be harnessed for stress-induced translation.

To test this hypothesis, we examined changes to eIF3d-mediated translation of the *Jun* mRNA during metabolic stress in human embryonic kidney (HEK) 293T cells. Conditions of nutrient depletion caused a marked decrease in general protein synthesis (fig. S1A), yet chronic glucose depletion led to a pronounced increase in Jun protein levels (Fig. 1A). The increase in Jun expression was stress specific and did not occur with glutamine or serum starvation (Fig. 1A). Analysis of *Jun* mRNA distribution in polysome fractions revealed that elevation of Jun protein synthesis during glucose deprivation was due to increased ribosome association and translation efficiency (Fig. 1, B and C). By contrast, the eIF4E-dependent control mRNA *PSMB6* shifted to monosome and free RNA fractions under the same glucose deprivation conditions (Fig. 1, B and C). Thus, the ability to engage eIF3d is likely key to enabling the *Jun* mRNA to escape translation shutoff. We next investigated whether nutrient starvation directly changed eIF3d cap-binding activity. An autoinhibitory conformation restricts eIF3d and necessitates that the subunit must function as part of the 13-subunit eIF3 complex for 5′ cap recognition (*2*). Taking advantage of a natural HIV-1 protease (PR) cleavage site in eIF3d that occurs immediately before the cap-binding domain (fig. S1B) (*3*), we used immunoprecipitation and on-bead cleavage to isolate eIF3d cap-binding activity separately from other eIF3 complex functions (*4*–*6*) (fig. S1, C to G). eIF3d cap binding to *Jun* mRNA increased 10-fold specifically during glucose deprivation (Fig. 1D). Thus, a cellular mechanism can reprogram eIF3d cap-binding activity and translation regulation during nutrient depletion.

Protein phosphorylation is a major mechanism regulating translation factor function (*1*). Using [^{32}P]-orthophosphate labeling, we found that 48 hours after glucose starvation, the eIF3 complex underwent a phosphorylation switch, with loss of modification of an ~70-kDa protein (Fig. 1E). Protein complex digestion with HIV-1 PR confirmed that the phosphorylated protein was eIF3d (Fig. 2A). Mass spectrometry mapped the phosphorylation sites in eIF3d to positions S528 and S529 (Fig. 2B and fig. S2A).

Department of Biology, Brandeis University, Waltham, MA 02453, USA.
*These authors contributed equally to this work.
†Present address: Department of Biological Sciences, Columbia University, New York, NY 10027, USA.
‡Corresponding author. Email: amysiyinglee@gmail.com
§Present address: Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA, and Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA 02115, USA.

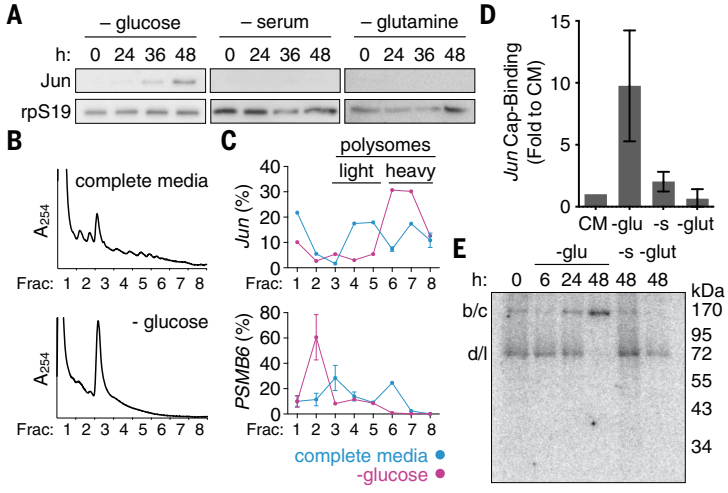

Fig. 1. eIF3d-specialized translation increases during chronic glucose deprivation. (**A**) Jun and rpS19 protein levels in HEK293T cells after nutrient deprivation. (**B** and **C**) Polysome association of *Jun* or *PSMB6* mRNA in response to glucose deprivation in HEK293T cells. *PSMB6* is a control for eIF4E-dependent translation. mRNA abundance is expressed as a percentage of total transcript recovered from all fractions. Results are plotted as the mean ± SD from a representative quantitative reverse transcription polymerase chain reaction (qRT-PCR) performed in duplicate. The results of (A) to (C) are representative of three biological replicates. (**D**) eIF3d binding to the 5′ cap of *Jun* mRNA in HEK293T cells upon nutrient deprivation. eIF3d cap binding is quantified as levels of *Jun* transcripts determined by reverse transcription and quantitative PCR, in HIV-1 PR-treated eIF3d immunoprecipitation samples compared with total input *Jun* RNA. Cap binding is normalized to samples prepared from cells grown in complete media (CM). glu, glucose; s, serum; glut, glutamine. Results are shown as the mean ± SD of three independent experiments. (**E**) Phosphorylation of eIF3 subunits after nutrient deprivation. Shown is a phosphor image of SDS–polyacrylamide gel electrophoresis (PAGE) gel resolving eIF3 subunit phosphorylation levels as detected by [^{32}P]-orthophosphate labeling of HEK293T cells and immunoprecipitation of the eIF3 complex. The results are representative of two independent experiments.

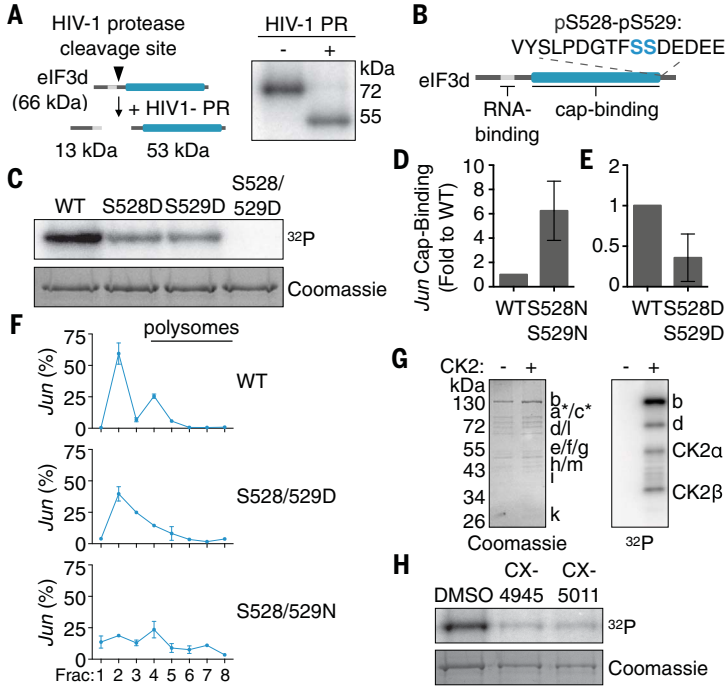

Fig. 2. CK2 phosphorylation inhibits eIF3d cap-binding activity. (**A**) Phosphorylated eIF3 subunit identification by selective cleavage with HIV-1 PR. HIV-1 PR was incubated with eIF3 complex immunoprecipitated from [^{32}P]-orthophosphate-labeled HEK293T cells. HIV-1 PR cleavage of eIF3d leads to faster gel migration. (**B**) Location of phosphorylated eIF3d residues S528 and S529 (blue) identified by mass spectrometry. (**C**) Validation of eIF3d phosphorylation sites using eIF3d-HA mutants by [^{32}P]-orthophosphate labeling in HEK293T cells. Coomassie staining is shown as a loading control. WT, wild-type. (**D** and **E**) Fold change in eIF3d binding to the 5′ cap of *Jun* mRNA by phosphoinhibitory (S528/529N) or phosphomimetic (S528/529D) mutants in HEK293T cells. Cap-binding activity was measured as in Fig. 1D and normalized to WT eIF3d. Results are shown as the mean ± SD of three independent experiments. (**F**) Association of *Jun* transcripts with ribosomal complexes in HEK293T cells in which endogenous eIF3d was replaced with eIF3d mutants through coexpression of shRNA-resistant mutants and an eIF3d-targeting shRNA. The results are representative of two independent experiments, and ribosome association was determined as in Fig. 1C. (**G**) In vitro phosphorylation of the recombinant 12-subunit eIF3 complex by incubation with purified CK2 and [γ^{32}P] ATP. Autophosphorylated CK2 subunits are indicated. (**H**) CK2 inhibition in HEK293T cells with 10 μM CX-4945 or CX-5011 decreases eIF3d phosphorylation levels as assessed by [^{32}P]-orthophosphate labeling. The results in (A), (C), (G), and (H) are representative of three independent experiments.

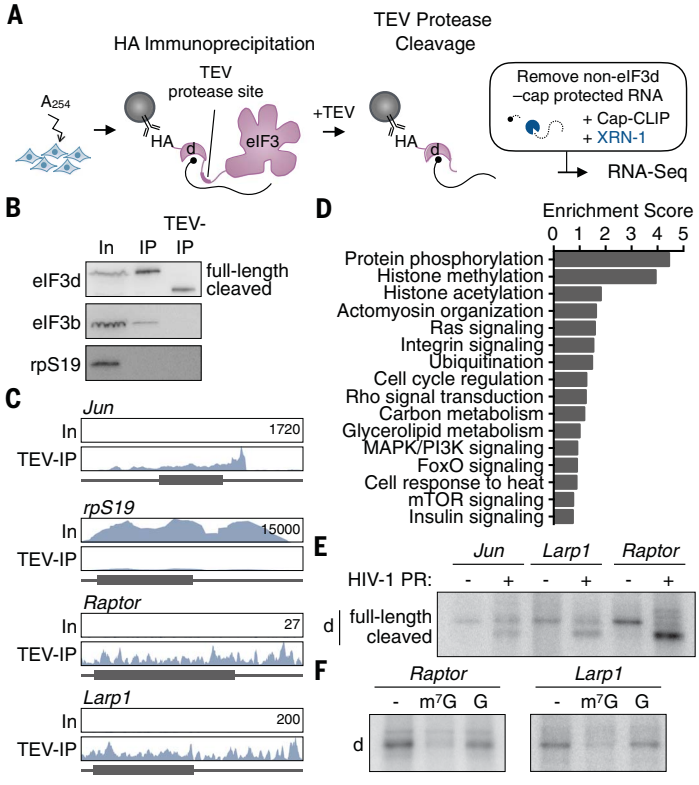

Fig. 3. eIF3d^{TEV} Subunit-Seq identifies cap-binding targets enriched in regulators of cell metabolism. (**A**) Schematic of eIF3d^{TEV} Subunit-Seq methodology. Position R131 within eIF3d was selected for TEV site insertion to minimize perturbation of eIF3d function because this linker region exhibits high phylogenetic variation and is naturally targeted by HIV-1 PR (*3*) (fig. S1B). See text for details. (**B**) Immunoblot of eIF3d^{TEV} Subunit-Seq samples. In, Input lysates; IP, anti-HA eIF3d immunoprecipitants; TEV-IP, IP samples after on-bead incubation with TEV protease. Results are representative of three independent experiments. (**C**) Read mapping to respective transcripts from eIF3d^{TEV} Subunit-Seq performed in glucose-deprived cells. The schematics represent the transcript architecture: Thin lines are the 5′ and 3′ UTRs and thick lines are the open reading frames. The annotated *y*-axis maximum is set equivalently for within each transcript for input (In) and TEV-IP samples. (**D**) Gene ontology analysis of eIF3d cap-binding targets from glucose-deprived cells. (**E**) Phosphor image of SDS-PAGE gel resolving RNase-protected ^{32}P-cap-labeled 5′ UTRs. Recombinant eIF3 complex was cross-linked to target RNAs in vitro and treated with RNase. Cap protection by eIF3d leads to the formation of a radiolabeled covalent eIF3d-cap complex. HIV-1 PR cleavage was performed to validate cross-linked subunit identity as eIF3d, as shown through the shifted migration of the cap-cross-linked complex. (**F**) In vitro eIF3d cap-cross-linking to *Raptor* and *Larp1* 5′ UTRs specifically requires a methylated cap structure. The competitor methylated cap analog m^{7}GpppG (m^{7}G) blocks eIF3d cross-linking to the 5′ UTR, unlike the unmethylated cap analog GpppG (G). The results in (E) and (F) are representative of three independent experiments.

Phosphorylation was reduced with single-site mutations and completely abolished in an eIF3d S528/529D double mutant (Fig. 2C). The eIF3d phosphorylation sites are in a location capable of influencing 5′ cap recognition, proximal to the cap-binding domain (*2*) (Fig. 2B and fig. S2B). In support of a regulatory role of phosphorylation, phosphoinhibitory (S528N/S529N) mutations increased eIF3d 5′ cap-binding activity in cells (Fig. 2D and S2C). Conversely, phosphomimetic (S528D/S529D) eIF3d mutations inhibited 5′ cap-dependent *Jun* mRNA interactions (Fig. 2E) and mirrored the reduced ability of eIF3d to interact with *Jun* when cells were in nutrient-replete conditions (Fig. 1, D and E). We next measured *Jun* translation efficiency in cells engineered to express eIF3d mutants exclusively through stable knockdown and rescue with short hairpin (shRNA)–resistant plasmids (fig. S2, D and E). *Jun* mRNA association was lost in the polysome fractions of cells expressing eIF3d S528D/S529D and gained in cells expressing eIF3d S528N/S529N

(Fig. 2F and fig. S2F). Thus, phosphorylation under nutrient-replete conditions represses eIF3d cap binding, and loss of phosphorylation upon chronic glucose deprivation relieves this inhibition.

The eIF3d phosphorylation sites are each predicted substrates of the protein kinase CK2, which recognizes the sequence motif S*/T*XXE/D with an enrichment in downstream acidic residues (fig. S3A) (*7*, *8*). CK2 is regulated by environmental stimuli (*9*), and CK2-substrate complex formation is inhibited in low-glucose conditions (*10*), in agreement with nutrient-dependent phosphorylation of eIF3d (Fig. 1E). We confirmed that CK2 was capable of modifying eIF3d in vitro in the context of the full eIF3 complex (Fig. 2G). Treatment of cells with the CK2 active-site inhibitors CX-4945 or CX-5011 (*11*) led to a loss of eIF3d phosphorylation (Fig. 2H). By contrast, eIF3d phosphorylation was unaffected by treatment with staurosporine (fig. S3B), a broadly acting kinase inhibitor for which CK2 is atypically insensitive (*12*). Additionally, CK2 inhibition increased the ability of wild-type, but not phosphomimetic, eIF3d to bind the 5′ cap (fig. S3C), further demonstrating a role for the CK2-dependent phosphorylation in regulating eIF3d.

Activation of eIF3d cap-binding activity during glucose deprivation suggests that an eIF3d-controlled gene program may be critical to supporting the cellular response to this metabolic perturbation. However, because eIF3d is an essential gene and 5′ cap recognition occurs as part of the ~800-kDa 13-subunit eIF3 complex (*2*, *5*), technical restrictions have prevented a comprehensive identification of eIF3d target mRNAs (*2*, *13*, *14*). To overcome this challenge, we developed Subunit-Seq, a method that allows for the specific release and isolation of an individual subunit domain and its associated mRNAs away from a larger multisubunit complex. We created a cell line expressing an engineered eIF3d protein with an internal tobacco etch virus (TEV) protease cleavage site proximal to the cap-binding domain and a C-terminal hemagglutinin (HA) affinity tag (Fig. 3A and fig. S4A). To perform Subunit-Seq, we isolated HA-tagged eIF3 complexes and used TEV protease to induce on-bead cleavage and specific isolation of the eIF3d cap-binding domain–RNA complexes (Fig. 3, A and B). Contaminating RNAs that did not have eIF3d-protected 5′ cap structures were removed with on-bead pyrophosphatase decapping and 5′ exoribonuclease digestion. This methodology can be readily adapted to separate the functions of other multisubunit RNA-binding protein complexes through rational insertion of TEV protease sites.

eIF3d^{TEV} Subunit-Seq revealed that eIF3d binds the 5′ cap of 668 transcripts in glucose-deprived HEK293T cells (Fig. 3C; fig. S4, B and C; and table S1). In agreement with a starvation-induced increase in cap binding, ~70% of the eIF3d targets exhibited a two-fold or higher increase in eIF3d binding upon glucose deprivation (fig. S4, D to F, and tables S2 and S3). Gene ontology analysis of the eIF3d targets identified an enrichment in cell metabolism and glucose homeostasis pathways, including mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/phosphatidylinositol 3-kinase (PI3K), and forkhead box O (FoxO) signaling (Fig. 3D). Indeed, eIF3d targets included *Regulatory-associated protein of mTOR* (*Raptor*), an essential component of the mTOR complex 1 (mTORC1) (*15*), and *La-related protein 1* (*Larp1*), a protein that blocks translation of 5′ terminal oligopyrimidine motif-containing RNAs during mTOR inhibition (Fig. 3C) (*16*, *17*). We biochemically validated targets using purified recombinant eIF3 complex and in vitro transcribed mRNAs (*2*) and observed that eIF3d bound to and protected the ^{32}P-labeled cap structure of *Raptor* and *Larp1* 5′ untranslated regions (UTRs) from RNase digestion in vitro (Fig. 3E and fig. S4G). This interaction required a 5′ methylated cap structure, with binding competed away by the competitor ligand m^{7}GpppG (Fig. 3F). Using selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) RNA structure analysis, we identified a stem loop structure in the *Raptor* 5′ UTR that is conserved with the characterized eIF3–*Jun* RNA-binding determinant (*4*), including a U-rich loop, an upper C-rich internal bulge, and a multihelical junction (fig. S5, A and B, and fig. S6). Disruption of this *Raptor* 5′ UTR stem loop by deletion or mutagenesis was sufficient to block eIF3d cap binding (fig. S5C) and translation (fig. S5, D to F), suggesting that the presence of conserved secondary structure may allow coordinated regulation of the eIF3d-directed gene program.

We next investigated whether up-regulation of the eIF3d-directed gene program through the phosphorylation switch provides an adaptive response to glucose deprivation. Lack of glucose triggered increased cell death, rounded cell morphology, and poor attachment in cells expressing a phosphomimetic eIF3d mutant (Fig. 4, A and B, and fig. S7A). By contrast, CK2 inhibition enhanced the cell survival phenotype in cells expressing wild-type, but

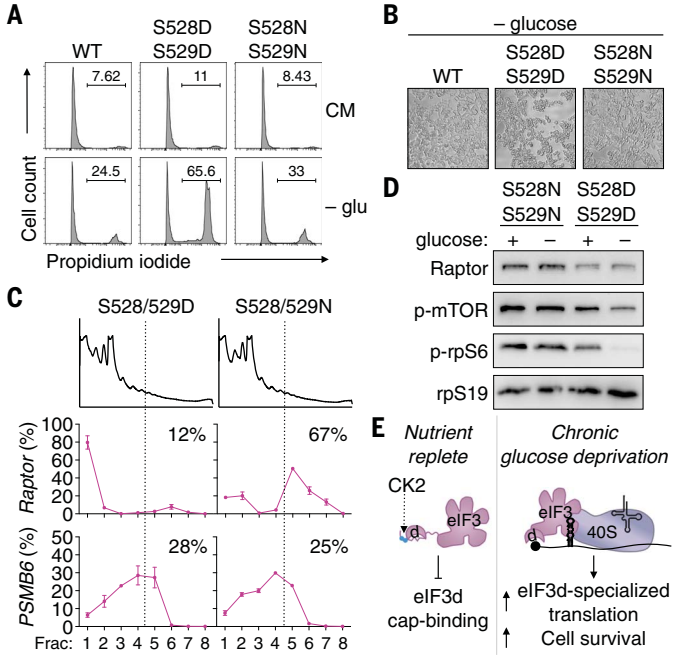

Fig. 4. Regulation of eIF3d-specialized translation through a phosphorylation switch promotes cell survival during chronic glucose deprivation. (**A**) Analysis of apoptotic cell percentage by propidium iodide staining and flow cytometry in eIF3d phosphorylation mutant cell lines. Results in (A) are representative of two independent experiments. (**B**) Representative images of the cell morphology after 48 hours of glucose deprivation in eIF3d phosphorylation mutant cell lines. Results in (B) are representative of three independent experiments. (**C**) Association of *Raptor* mRNA with ribosomal complexes in eIF3d mutant cell lines during glucose deprivation. The percentage of total RNA associated with heavy polysomes (i.e., more than three ribosomes) is indicated. Results are representative of two independent experiments, and ribosome association was determined as in Fig. 1C. (**D**) Immunoblot of mTORC1 pathway activation in eIF3d phosphorylation mutant cell lines during glucose deprivation. Results in (D) are representative of three independent experiments. (**E**) Model for phosphorylation-mediated regulation of eIF3d-specialized translation during metabolic stress.

not phosphomimetic, eIF3d (fig. S7B). Furthermore, dephosphorylated eIF3d was essential for the target mRNA *Raptor* to bypass starvation-induced global shutoff (Fig. 4C and fig. S7, C and D), whereas phosphomimetic eIF3d-expressing cells exhibited decreased Raptor protein levels (Fig. 4D). Raptor is critical for substrate recruitment and mTORC1 kinase activity (*15*). Correspondingly, during glucose deprivation, incorrect control of eIF3d phosphorylation status led to decreased levels of activated mTOR and reduced phosphorylation of rpS6, a downstream target of the mTOR pathway (*15*, *18*) (Fig. 4D). Although mTOR is rapidly inactivated within a few hours of nutrient deprivation, long-term starvation reactivates it and promotes cell survival (*19*, *20*). A protective response during chronic endoplasmic reticulum stress is also dependent on partial rescue of mTOR activity (*13*, *21*). This response is blocked by shRNA-mediated knockdown of eIF3d (*13*), which could be due to the inability of *Raptor* and the eIF3d-mediated gene program to be translated (fig. S7, E and F, and table S1).

Our results establish a model in which a phosphorylation-regulated switch in eIF3d cap-binding activity enables cellular control of eIF3d-specialized translation in response to metabolic state (Fig. 4E). These findings uncover a parallel between stress-induced regulation of eIF3d and the essential canonical translation factors eIF2 and eIF4E (*1*). During glucose deprivation, phosphorylation-regulated inhibition of eIF2 and eIF4E, through hypophosphorylation and activation of the 4E-BPs, allows cells to block general protein synthesis. The conserved but opposing regulation of eIF4E and eIF3d in response to glucose deprivation reveals that the balance of canonical versus noncanonical cap-dependent translation is a key decision point for cellular adaptation to metabolic state.

REFERENCES AND NOTES

1. B. Liu, S. B. Qian, *Wiley Interdiscip. Rev. RNA* **5**, 301–315 (2014).
2. A. S. Lee, P. J. Kranzusch, J. A. Doudna, J. H. Cate, *Nature* **536**, 96–99 (2016).
3. S. Jäger *et al.*, *Nature* **481**, 365–370 (2011).
4. A. S. Lee, P. J. Kranzusch, J. H. Cate, *Nature* **522**, 111–114 (2015).
5. K. Asano *et al.*, *J. Biol. Chem.* **272**, 27042–27052 (1997).
6. Y. Hashem *et al.*, *Nature* **503**, 539–543 (2013).
7. F. Meggio, L. A. Pinna, *FASEB J.* **17**, 349–368 (2003).
8. N. Blom, T. Sicheritz-Pontén, R. Gupta, S. Gammeltoft, S. Brunak, *Proteomics* **4**, 1633–1649 (2004).
9. O. Filhol, C. Cochet, *Cell. Mol. Life Sci.* **66**, 1830–1839 (2009).
10. S. Welker *et al.*, *Int. J. Biochem. Cell Biol.* **45**, 2786–2795 (2013).
11. R. Battistutta *et al.*, *Biochemistry* **50**, 8478–8488 (2011).
12. F. Meggio *et al.*, *Eur. J. Biochem.* **234**, 317–322 (1995).
13. B. J. Guan *et al.*, *Mol. Cell* **68**, 885–900.e6 (2017).
14. C. de la Parra *et al.*, *Nat. Commun.* **9**, 3068 (2018).
15. D. H. Kim *et al.*, *Cell* **110**, 163–175 (2002).
16. S. Hong *et al.*, *eLife* **6**, e25237 (2017).
17. R. M. Lahr *et al.*, *eLife* **6**, e24146 (2017).
18. G. G. Chiang, R. T. Abraham, *J. Biol. Chem.* **280**, 25485–25490 (2005).
19. L. Yu *et al.*, *Nature* **465**, 942–946 (2010).
20. K. J. Condon, D. M. Sabatini, *J. Cell Sci.* **132**, jcs222570 (2019).
21. B. J. Guan *et al.*, *J. Biol. Chem.* **289**, 12593–12611 (2014).

ACKNOWLEDGMENTS

We thank S. T. Aoki, K. Chat, P. Garrity, P. J. Kranzusch, J. K. Nuñez, and members of the Lee laboratory for discussions and S. R. Hildebrand, A. D. Yu, W. Xu, and R. Tomaino for experimental advice. **Funding:** This work was funded by the Charles H. Hood Foundation, the Searle Scholars Program, the Pew Biomedical Scholars Program, and a Sloan Research Fellowship (A.S.Y.L). R.H.F. was supported by NIH T32 GM007122.
Author contributions: The project was conceived by A.S.Y.L, and experiments were performed by A.M.L., R.H.F., K.M.L., and A.S.Y.L. The manuscript was written by A.S.Y.L with editing from A.M.L., R.H.F., and K.M.L., and all authors support the conclusions.
Competing interests: The authors declare no competing interests.
Data and materials availability: All sequencing data have been deposited in the Gene Expression Omnibus under accession number GSE158249.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/370/6518/853/suppl/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 to S3
References (*22*–*42*)
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

29 January 2020; resubmitted 20 August 2020
Accepted 24 September 2020
10.1126/science.abb0993

A phosphorylation-regulated eIF3d translation switch mediates cellular adaptation to metabolic stress

Adam M. LamperRebecca H. FlemingKayla M. LaddAmy S. Y. Lee

Science, 370 (6518),

Cellular adaptation during metabolic stress

Cells respond to environmental stress by down-regulating general protein synthesis and inducing selective expression of proteins required for survival. However, the mechanisms controlling this selective messenger RNA (mRNA) translation response remain poorly understood. Lamper *et al.* report that the noncanonical 5# cap-binding protein subunit eIF3d is activated upon metabolic stress in mammalian cells to reprogram cellular mRNA translation. eIF3d is activated by a switch in phosphorylation status at sites near the cap-binding pocket and enables cells to express the proteins required for the regulation of metabolism and survival during stresses, including glucose starvation. This work reveals how eIF3d-dependent, noncanonical cap-dependent translation controls the cellular adaptation to stress.

Science, this issue p. 853

Science (ISSN 1095-9203) is published by the American Association for the Advancement of Science. 1200 New York Avenue NW, Washington, DC 20005. The title *Science* is a registered trademark of AAAS.