Crtc modulates fasting programs associated with 1-C metabolism and inhibition of insulin signaling

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Contributed by Marc Montminy, February 4, 2021 (sent for review December 2, 2020; reviewed by John B. Hogenesch and Jerry C. Yin)

Fasting in mammals promotes increases in circulating glucagon and decreases in circulating insulin that stimulate catabolic programs and facilitate a transition from glucose to lipid burning. The second messenger cAMP mediates effects of glucagon on fasting metabolism, in part by promoting the phosphorylation of CREB and the dephosphorylation of the cAMP-regulated transcriptional coactivators (CRTCs) in hepatocytes. In *Drosophila*, fasting also triggers activation of the single Crtc homolog in neurons, via the PKA-mediated phosphorylation and inhibition of salt-inducible kinases. *Crtc* mutant flies are more sensitive to starvation and oxidative stress, although the underlying mechanism remains unclear. Here we use RNA sequencing to identify Crtc target genes that are up-regulated in response to starvation. We found that Crtc stimulates a subset of fasting-inducible genes that have conserved CREB binding sites. In keeping with its role in the starvation response, Crtc was found to induce the expression of genes that inhibit insulin secretion (*Lst*) and insulin signaling (*Impl2*). In parallel, Crtc also promoted the expression of genes involved in one-carbon (1-C) metabolism. Within the 1-C pathway, Crtc stimulated the expression of enzymes that encode modulators of *S*-adenosyl-methionine metabolism (*Gnmt* and *Sardh)* and purine synthesis (*ade2* and *AdSl)*. Collectively, our results point to an important role for the CREB/CRTC pathway in promoting energy balance in the context of nutrient stress.

CRTC | CREB | fasting metabolism

In *Drosophila*, adipokinetic hormone (AKH) and insulin-like peptides (dilps) function as orthologs of glucagon and insulin, respectively (1–6). AKH promotes fasting metabolism by binding to the AKH receptor, which is primarily expressed in the fat body, the fly equivalent of the adipose and liver (7). Ligand binding stimulates cellular cAMP accumulation and PKA activation.

Decreases in insulin signaling during fasting stimulate the dephosphorylation and nuclear translocation of the forkhead protein Foxo (8–12), which promotes the expression of gluconeogenic genes. In parallel, increases in cAMP/PKA signaling stimulate Crtc activity by phosphorylating and inhibiting the salt-inducible kinases (SIKs), which otherwise phosphorylate and sequester cAMP-regulated transcriptional coactivators (CRTCs) in the cytoplasm (13–16). Mammalian studies have revealed an important role for CREB and CRTC2 in promoting gluconeogenic gene expression in liver by binding to relevant promoters (13) in parallel with FOXO.

Crtc is not detectably expressed in *Drosophila* fat body, where fasting gluconeogenesis and lipolysis occur, however. Rather, Crtc is primarily produced in neurons, where it appears to promote energy balance and resistance to starvation, in part through induction of the neuropeptide Y homolog sNPF (17). In turn, sNPF appears to reduce energy expenditure by promoting gut epithelial integrity. Whether Crtc stimulates other metabolic programs that support starvation resistance is unclear, however.

In *Drosophila*, fasting up-regulates about 100 genes, many of which are essential for nutrient sensing and for glucose and lipid metabolism (18, 19). Here, we identify Crtc-regulated genes within the core set of fasting-inducible genes. Using flies with a knockout (KO) of Crtc, CrebB, or Sik2, we found a subset of fasting-inducible genes in the fly head that are down-regulated in both CrebB and Crtc KO flies and up-regulated in SIK2 mutants. Most of these genes are likely direct CrebB targets because they have promoter-proximal CrebB binding sites that are conserved among different *Drosophila* species. These fasting-inducible CrebB/Crtc target genes encode inhibitors of insulin signaling and secretion as well as components of one-carbon (1-C) metabolism that are also up-regulated in response to mitochondrial stress. These results point to a broader involvement of the CREB:CRTC pathway in coordinating the transcriptional response to starvation.

Results

CRTC Is Required for Starvation Resistance. The timely induction of catabolic genes during fasting is critical in maintaining energy balance. Neuronal Crtc is necessary for resistance to starvation and oxidative stress in flies (15). *Crtc* mutant flies are sensitive to starvation and have shorter lifespans relative to wild type (Fig. 1 *A*–*D*). *Crtc* mutants exhibit normal body size, and they express dilp genes at levels comparable to wild type (*SI Appendix*, Fig. S1 *A* and *B*). Arguing against a sickly phenotype, *Crtc* mutants display comparable climbing behavior to wild-type flies (Fig. 1*E*) and they have increased resistance to thermal stress (Fig. 1*F* and *SI Appendix*, Fig. S1 *C* and *D*).

Based on the starvation sensitivity of *Crtc* mutant flies, we performed RNA sequencing (RNA-seq) studies to identify Crtc-regulated genes that mediate these effects in the head. This analysis revealed 91 genes that were up-regulated twofold or

Significance

Fasting in humans stimulates a transition from glucose to lipid metabolism via the induction of catabolic programs in response to increases in circulating glucagon and decreases in insulin. The transcription factor CREB and its coactivators, the CRTCs, stimulate the expression of gluconeogenic genes in liver. CREB and CRTC are conserved in *Drosophila*; they act primarily in neurons to promote energy balance. Mutant flies lacking the Crtc gene are more sensitive to starvation due to reductions in fat and glycogen stores. Using RNA sequencing, we identified CREB/CRTC regulated genes in flies that promote survival during starvation by inhibiting insulin signaling and promoting one-carbon metabolism. Our results provide insight into the mechanisms by which organisms maintain energy balance in response to fasting.

Author contributions: J.B.T. and M.M. designed research T.W. and X.Z. performed research; T.W., E.W., J.B.T., and M.M. analyzed data; M.M. edited versions of the manuscript; and T.W. wrote the paper.

Reviewers: J.B.H., Cincinnati Children's Hospital Medical Center; and J.C.Y., University of Wisconsin.

The authors declare no competing interest.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2024865118/-/DCSupplemental.

Published March 15, 2021.

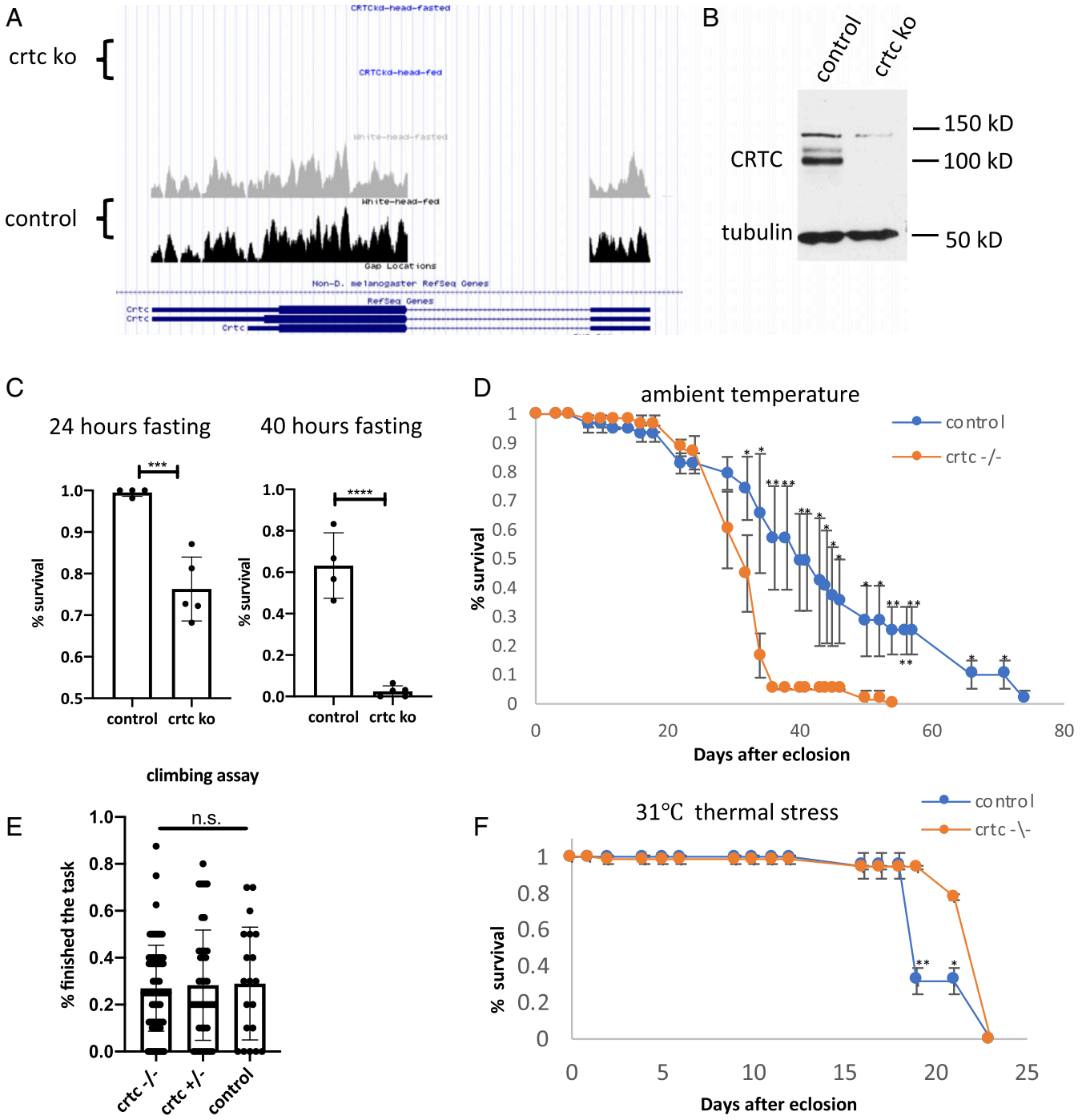

Fig. 1. CRTC is required for starvation resistance. (*A*) UCSC genome browser view showing loss of *Crtc* transcripts in *Crtc* KO flies. (*B*) Western blot showing Crtc protein amounts in wild-type and *Crtc* KO flies. (*C*) Survival rates of w1118 control and *Crtc* KO flies after 24-h and 40-h fasting. (*D*) Comparison of lifespan at ambient temperature. Blue curve, wild-type flies. Yellow curve, *Crtc* KO flies. (*E*) Climbing assay showing mobility of *Crtc* KO flies relative to *Crtc* heterozygous flies and wild-type control flies. (*F*) Effect of 31 °C thermal stress on survival of *Crtc* KO compared to wild-type flies. ***$P < 0.001$; ****$P < 0.0001$; n.s., not statistically significant.

better (fragments per kilobase million [FPKM] ≥10) and 92 that were down-regulated after 16-h fasting relative to ad libitum feeding (Fig. 2*A* and *SI Appendix*, Tables S1 and S2).

We also identified genes that are modulated during fasting with an adjusted *P* value of less than 0.05 (fold change ≥2; three replicates for feeding conditions and two replicates for fasting conditions). This analysis yielded 85 fasting-up-regulated genes and 60 fasting-down-regulated genes (Fig. 2*B* and *SI Appendix*, Tables S3 and S4). Genes identified by both strategies were assembled into a core list containing 70 fasting-inducible and 51 fasting-inhibited genes (Tables 1 and 2).

Gene Ontology (GO) analysis of these 70 fasting-up-regulated genes revealed an enrichment for metabolic pathways, including amino acid and purine metabolism, 1-C metabolism, redox regulation,

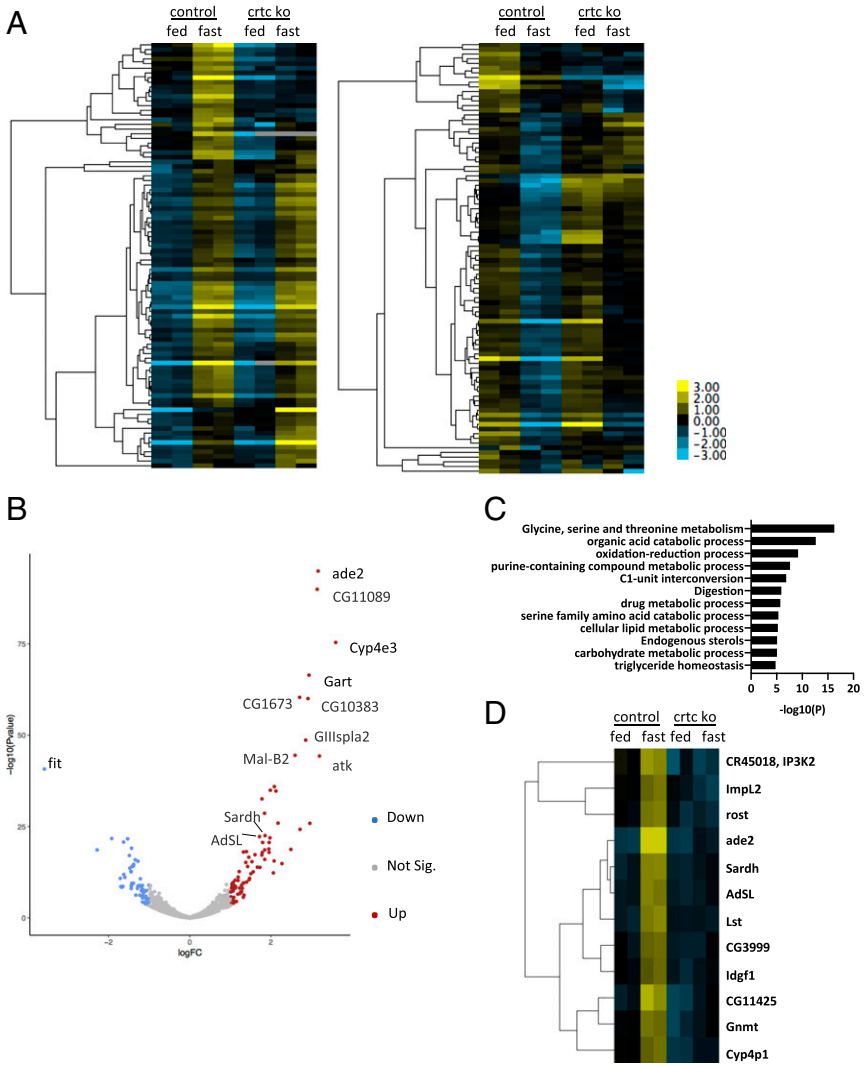

Fig. 2. CRTC is required for induction of fasting-inducible genes. (*A*) Heat map showing fasting-responsive genes in wild-type and *Crtc* KO flies. Conditions are indicated. The cutoff fold change >2 between feeding and fasting in wild-type flies; FPKM read >10 in at least one (fed/fasted) condition. Ninety-one fasting-inducible genes (*Left*) and 92 fasting-down-regulated genes (*Right*) in the control fly head identified under these criteria. Two biological replicates for control and *Crtc* KO for both feeding and fasting conditions. (*B*) Volcano plot showing fasting-responsive genes in wild-type fly heads. Blue and red dots in the volcano plot indicate genes with fold change >2 by fasting treatment and adjusted *P* value <0.05. Blue dots are fasting-down-regulated gene and red dots are fasting-up-regulated genes. (*C*) GO enrichment analysis with the core list of fasting-inducible genes. Multiple metabolic processes, including amino acid, lipid, and carbohydrate metabolic processes, are enriched by GO analysis. (*D*) Heat map showing relative expression of 12 Crtc-modulated fasting-inducible genes.

and lipid and carbohydrate metabolism (Fig. 2*C*). Indeed, over 40% of fasting-inducible genes (29 of 70) are associated with metabolic pathways by Kyoto Encyclopedia of Genes and Genomes analysis (*SI Appendix*, Table S5).

CRTC Is Required for Induction of Fasting-Inducible Genes. To identify metabolic programs that are modulated by Crtc, we selected for genes that are up-regulated by fasting in wild-type and that are down-regulated in *Crtc* mutant heads relative to control. This analysis yielded 12 putative CREB/CRTC target genes (Fig. 2*D* and *SI Appendix*, Table S6). In keeping with the proposed role of CREB, 11 of the 12 target genes have multiple conserved CREB binding sites within 2 kb upstream of the transcription start site (TSS) (*SI Appendix*, Table S7). A number of these genes, including *Gnmt*, *Sardh*, *Ade2*, and *Adsl*, encode enzymatic components of the 1-C metabolic pathway. Other genes within this pathway are also induced during fasting, but they appear to be CRTC-independent; these include *ade3*, *pug*, *Nmdmc*, *CG11089*, and *Shmt* (*CG3011*).

We sought to distinguish between genes that are dependent on Crtc uniquely during fasting from genes that are dependent on Crtc more generally under both feeding and fasting conditions. In the fed state, 123 genes are down-regulated in *Crtc* mutant relative to wild type (*SI Appendix*, Fig. S1*E*); in the fasted state, 117 genes are down-regulated in *Crtc* mutant heads (*SI Appendix*, Fig. S2*A*). Of these 117 genes, 70 are expressed at lower levels in *Crtc* mutants under both feeding and fasting conditions relative to wild type (*SI Appendix*, Table S8), while 46 are down-regulated in *Crtc* KO only during fasting (*SI Appendix*, Table S9). Although some changes in target gene expression are head-specific, others are also present in the body (*SI Appendix*, Fig. S2*B*). Nevertheless, fasting-inducible genes in the head appear to be functionally similar to those in the body by GO analysis (*SI Appendix*, Fig. S2*C*).

Based on the importance of CREB for recruitment of CRTCs to relevant targets, we evaluated whether the induction of Crtc-inducible genes during fasting is dependent on the *Drosophila* CREB homolog *CrebB*. For these experiments we used flies

Table 1. Core list of fasting-inducible genes in the fly head

Gene name	Gene function
Aay	Phosphoserine phosphatase
ade2	Phosphoribosylformylglycinamidine synthase
Adgf-D	Adenosine deaminase-related growth factor D
AdSL	Adenylosuccinate Lyase
Amy-d	Alpha-amylase
Amy-p	Alpha-amylase
Atk	Artichoke (atk) encodes a leucine-rich-repeat extracellular matrix protein
Bmm	Triacylglycerol lipase
Cyp4ac3	Cytochrome P450-related
Cyp4e2	Cytochrome P450-related
Cyp4e3	Cytochrome P450-related
Cyp4p1	Cytochrome P450-related
Cyp6a2	Cytochrome P450-related
Cyp6a8	Cytochrome P450-related
Cyp6w1	Cytochrome P450-related
Etf-QO	Electron-transferring-flavoprotein dehydrogenase
Fbp	Fructose-bisphosphatase
GIIIspla2	Phospholipase A2
GLS	Glutaminase
Gnmt	Glycine *N*-methyltransferase
Idgf1	Imaginal disk growth factor 1
ImpL2	Negative regulator of insulin signaling
Lip4	Lipase
Lst	Negative regulator of insulin signaling
mag	Lipase
Mal-B2	Alpha-glucosidase
Mcad	Medium-chain acyl-CoA dehydrogenase
Nmdmc	NAD-dependent methylenetetrahydrofolate dehydrogenase
Pepck2	Phosphoenolpyruvate carboxykinase
pug	Formate–tetrahydrofolate ligase
Reg-2	—
Sardh	Sarcosine dehydrogenase
Sclp	—
Shmt	Serine hydroxymethyl transferase
Sirup	Starvation-up-regulated protein
Spat	Serine pyruvate aminotransferase
Spn88Eb	—
Srr	Serine racemase
Thor	Translation regulation
Tsp42Ed	—
yip2	Acetyl-CoA C-acyltransferase
CG10383	Hydrolase
CG11089	Phosphoribosylaminoimidazolecarboxamide formyltransferase
CG11425	Phosphatidate phosphatase
CG11899	Phosphoserine transaminase
CG14022	Acylphosphatase
CG1441	—
CG1673	Branched-chain amino acid transaminase
CG16758	Purine-nucleoside phosphorylase
CG3036	—
CG32687	—
CG34136	—
CG3999	Glycine dehydrogenase
CG42751	—
CG42806	—
CG42876	—
CG5321	Gamma-butyrobetaine dioxygenase
CG5953	—
CG5955	L-threonine 3-dehydrogenase
CG5966	Carboxylesterase
CG6287	Phosphoglycerate dehydrogenase
CG6767	Phosphoribosyl pyrophosphate synthetase
CG7059	—
CG7530	—
CG7763	—
CG8249	—
CG8468	—
CG8654	—
CG9547	Glutaryl-CoA dehydrogenase
CG9757	—

mutant for $CrebB^{\Delta 400}$, a deletion in the transactivation and bZIP domains that disrupts the activity of this transcription factor (17). RNA-seq studies of fly head samples revealed six genes that are down-regulated in both *Crtc* and *CrebB* mutant flies during fasting (*SI Appendix*, Table S10). They are *Adsl*, *Sardh*, *Lst*, *CR45018*, *ade2*, and *ImpL2*.

Modulation of Fasting-Inducible Genes by Sik2. The genes identified above using *Crtc* and *CrebB* mutants are likely to include direct targets of CrebB/Crtc as well as genes involved in potential downstream secondary effects. To help distinguish between these, we carried out a genomic analysis of the regulatory regions of the genes. Since CREB/CRTC promote target gene expression by binding to cAMP response elements (CREs), we performed a whole-genome analysis of palindromic CRE sites (TGACGTCA) as well as half-CRE sites (TGACG or CGTCA).

The *Drosophila* genome contains 2,062 palindromic sites and 141,688 half-CRE sites. To identify binding sites that are more likely to be functional, we examined their conservation across *Drosophila* species. For each CRE site, a conservation score was assigned according to the mean PhasCons score from 142 insects (20) (*SI Appendix*, Fig. S3*A*).

Using all RefSeq genes in the *Drosophila* DM6 database, we calculated CRE scores for promoters containing CRE sites within 2 kb upstream or 500 bp downstream of the TSS. The CRE scores were determined based on the presence of a full-site or half-site, the distance of the CRE site from the TSS, the conservation of the CRE site among different *Drosophila* species (20), and the number of sites on the promoter. CRE scores for the fly genes ranged from 0 to 4.43 (*SI Appendix*, Fig. S3*B* and Table S11); 85% of fly genes have either no consensus CRE or a poorly conserved CRE site (less than 1.20); 15% have multiple conserved CRE sites (CRE scores greater than or equal to 1.20) (*SI Appendix*, Fig. S3*C*).

Within the set of 70 core fasting-inducible genes, 16 have CRE scores greater than or equal to 1.20 (Table 3) and are therefore more likely to function as targets for CREB/CRTC. We found an enrichment of genes with higher CRE scores among fasting-inducible genes compared to genes in the whole genome (hypergeometric distribution *P* value 0.0241078; *SI Appendix*, Fig. S3*D*); 50% of Crtc-dependent fasting-inducible genes have CRE scores

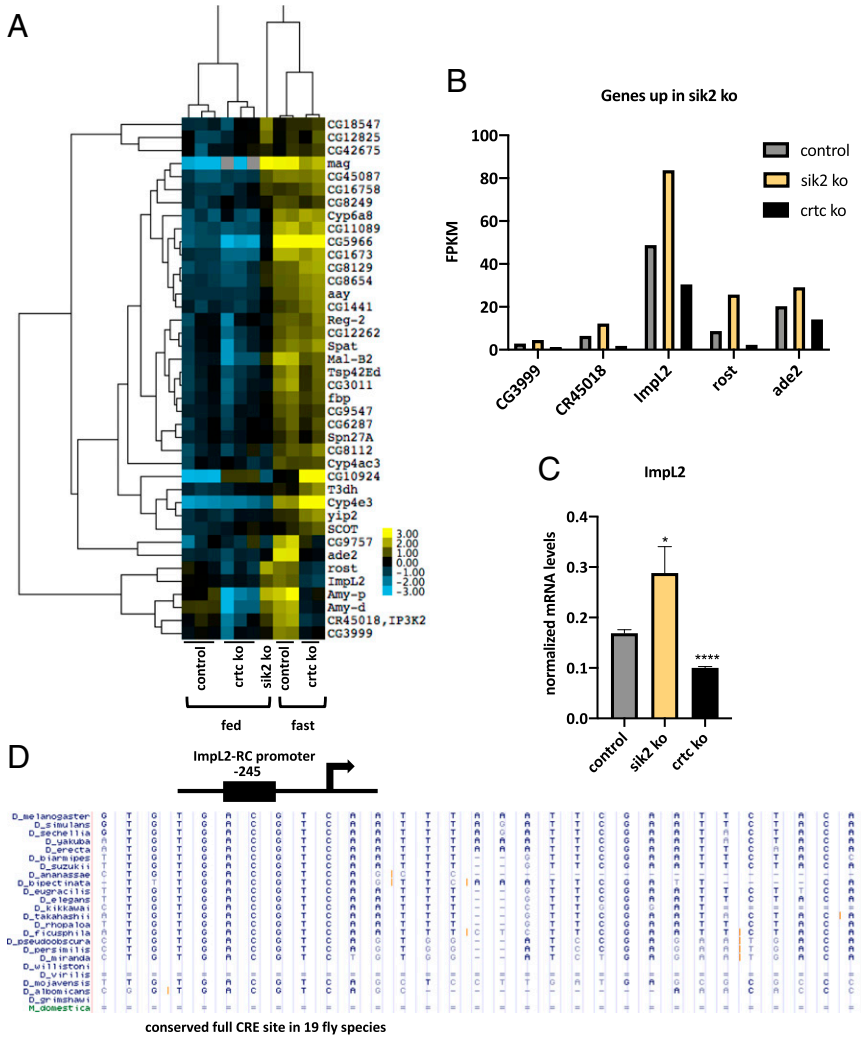

Fig. 3. Modulation of fasting-inducible genes by Sik2. (*A*) Heat map of fasting-inducible genes that are up-regulated in heads from *Sik2* KO flies under feeding conditions. Three biological replicates of head mRNA from control and *Crtc* KO in the fed state, head sample. Two biological replicates of control and *Crtc* KO in the fasted state. (*B*) Examples of CRTC-dependent fasting-inducible genes up-regulated in *Sik2* mutant flies in the fed state. Bar graph shows data from RNA-seq studies. (*C*) RT-qPCR result showing up-regulation of CRTC-dependent fasting-inducible gene, ImpL2, in *Sik2* mutant under feeding conditions. $*P < 0.05$; $****P < 0.0001$. (*D*) Conserved CRE site in the ImpL2-RC promoter of 19 *Drosophila* species.

Table 2. Core list of fasting-down-regulated genes in the fly head

Gene name	Gene function
Cbt	Transcription factor
CCHa2	Regulator of Dilps
Clect27	Chitin Binding Protein
Cyp313a1	—
ELOVL	Fatty acid elongase
fit	Satiety hormone
Gbs-70E	Glycogen binding subunit
GNBP-like3	—
IM14	Immune induced molecule
Listericin	Antibacterial protein
Lsp1beta	Larval serum protein
Lsp2	Larval serum protein
MFS9	Major Facilitator Superfamily Transporter 9
Nplp2	Neuropeptide-like precursor
Nplp3	Neuropeptide-like precursor
Obp99b	Odorant-binding protein 99b
Ork1	Open rectifier potassium channel
PGRP-SB1	*N*-acetylmuramoyl-L-alanine amidase
phu	Alkaline phosphatase
Pisd	Phosphatidylserine decarboxylase
sug	Transcription factor
sxe2	Carboxylesterase
Tig	Extracellular matrix protein
tobi	Target of brain insulin
TotX	Secreted peptides
vkg	A subunit of Collagen IV
CG10026	—
CG10516	—
CG11741	—
CG13607	—
CG14298	—
CG14439	—
CG14567	—
CG14688	—
CG15096	—
CG15282	—
CG16772	—
CG18302	—
CG3348	—
CG3630	—
CG4000	—
CG42821	—
CG4462	—
CG4797	—
CG5162	Carboxylesterase
CG5773	—
CG6126	—
CG7203	—
CG9416	—
CG9436	Alcohol dehydrogenase
CG9498	—

greater than 1.20 (*SI Appendix*, Table S7), suggesting that they are likely direct targets of CREB/Crtc.

We wondered whether activation of Crtc itself would be sufficient to stimulate expression of fasting-inducible genes. CRTC activity is primarily modulated by SIKs, which phosphorylate and sequester CRTC in the cytoplasm under basal conditions through interaction with 14-3-3 proteins. In mammals, deletion of *SIK* genes leads to CRTC dephosphorylation, nuclear entry, and constitutive activation of CREB target genes. The fly has two SIK genes (*Sik2* and *Sik3*), and Sik2 has been shown to promote the fasting response by modulating Crtc activity (16). We examined Crtc target gene expression in *Sik2* mutant flies, in which the genomic region encoding the catalytic domain is deleted (*SI Appendix*, Fig. S3*E*). *Sik2* mutants show increased starvation resistance, while *Sik2* and *Crtc* double-mutant flies show starvation sensitivity similar to *Crtc* mutant flies (16), indicating that Sik2 regulates the fasting response at least in part via a Crtc-dependent mechanism.

Within the list of 91 fasting-inducible genes, 44% are up-regulated in *Sik2* mutants under ad libitum feeding conditions (Fig. 3 *A*–*C*). Indeed, five of the Crtc-dependent genes are also more highly expressed in *Sik2* mutants relative to wild type. Notably, *ImpL2* and *ade2* genes were identified on this list, both of which contain highly conserved CREs (Fig. 3*D*). These results indicate that loss of Sik2 in neurons is sufficient to induce expression of a subset of fasting-inducible genes that are targets of Crtc.

Crtc Stimulates Expression of Genes Involved in 1-C Metabolism and Inhibition of Insulin Signaling. We explored the mechanism of Crtc-dependent induction of target genes. *Drosophila* Crtc is most similar to mammalian CRTC1 in that both are predominantly expressed in neurons. In mammalian cell lines, increases in intracellular cAMP or cytosolic calcium promote CRTC1 dephosphorylation and nuclear translocation (21). Crtc1 is activated in part by the Ser/Thr phosphatase calcineurin in response to calcium signaling. Exposure to the calcium ionophore, A23187, triggers CRTC1 activation (22).

We compared effects of cAMP and calcium agonists on Crtc activity. Although treatment with dibutyryl cAMP promoted dephosphorylation of CRTC, exposure to calcium ionophore (A23187) appeared more effective in *Drosophila* Kc167 cells (Fig. 4*A*). Moreover, Crtc dephosphorylation in response to A23187 was blocked by cotreatment with the calcineurin inhibitor cyclosporin A (CsA), indicating that, in addition to cAMP, increases in Ca^{2+} signaling also trigger the activation of *Drosophila* Crtc.

The subset of fasting-inducible genes that are CRTC-dependent includes *Gnmt* and *Sardh*, which encode glycine *N*-methyltransferase (*Gnmt)* and sarcosine dehydrogenase (*Sardh*), two key enzymes in the 1-C pathway that regulate *S*-adenosyl-methionine (SAM) metabolism. SAM serves as a methyl donor for a variety of methyltransferases, including enzymes involved in histone methylation. SAM levels are associated with lifespan and aging (23); overexpression of Gnmt, which catalyzes the conversion of SAM to sarcosine, promotes longevity, while decreases in Gnmt, as we observed in *Crtc* mutants, lead to shorter lifespans. Messenger RNA (mRNA) amounts for both *Gnmt* and *Sardh* are significantly down-regulated in *Crtc* KO flies during fasting relative to wild type (Fig. 4 *B* and *C*). Gnmt protein levels are also reduced in *Crtc* KO flies (Fig. 4 *D* and *E*). The dysregulation of *Gnmt* and *Sardh* expression in *Crtc* mutant flies may contribute to their shorter lifespan (Fig. 1*D*).

Also prominent within the subset of fasting-inducible CRTC targets are *ade2* and *AdSL*, which encode 1-C pathway enzymes for de novo purine synthesis (24, 25). Remarkably, five of the seven genes encoding enzymes for IMP synthesis in *Drosophila* (*Prat*, *Prat2*, *ade2*, *ade3*, *ade5*, *AdSL*, and *CG11089*) are induced more than threefold by fasting in the fly head, and at least two of these, *ade2* and *AdSL*, are putative CRTC targets (*SI Appendix*, Fig. S4*A*). Taken together, these studies suggest that CRTC modulates enzymes in the 1-C pathway that are required for protein methylation and purine synthesis (Fig. 4*F*).

In addition to the 1-C enzymes, CRTC targets also include *Lst* and *Impl2* (*SI Appendix*, Fig. S4 *A* and *B*), which function as negative regulators of insulin secretion and signaling (26–32). Both genes have conserved CREB binding sites; they are induced in heads but not bodies of fasted flies (*SI Appendix*, Fig. S4*B*). The Crtc-dependent induction of these modulators of

Table 3. Fasting-inducible genes with highly conserved CRE sites

Gene	CRE score	Encoding
GLS	2.73	Glutaminase
Lst	2.49	Limostatin
ade2	1.99	Phosphoribosylformylglycinamidine synthase
Pepck2	1.96	Phosphoenolpyruvate carboxykinase 2
Nmdmc	1.85	NAD-dependent methylenetetrahydrofolate dehydrogenase
Srr	1.66	Serine racemase
pug	1.5	Methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formate-tetrahydrofolate ligase
CG5966	1.45	Carboxylesterase
CG6767	1.42	Ribose-phosphate diphosphokinase
ImpL2	1.4	Imaginal morphogenesis protein-Late 2
Adgf-D	1.34	Adenosine deaminase
CG11425	1.31	Phosphatidate phosphatase
Gnmt	1.26	Glycine *N*-methyltransferase
Thor	1.25	A eukaryotic translation initiation factor 4E binding protein
CG1673	1.24	Branched-chain amino acid transaminase
CG9547	1.2	N.A.

N.A., not assessed.

insulin action in the head during fasting is consistent with the inhibition of insulin signaling under starvation conditions.

Discussion

Starvation triggers the expression of catabolic programs that maintain energy balance in part by increasing lipid burning. The CREB coactivator Crtc has been shown to function importantly in starvation resistance (15); *Crtc* mutant flies have decreases in glycogen and triglyceride stores that are partially corrected upon reexpression of Crtc in neurons. To identify metabolic programs that are regulated by neuronal Crtc, we compared the transcriptomes of wild-type and *Crtc* mutant flies under feeding and fasting conditions. Nearly 20% of fasting-inducible genes in the head are modulated by Crtc. These are likely to include direct targets of CrebB/Crtc as well as genes involved in potential downstream secondary effects. Fifty percent of Crtc-dependent fasting-inducible genes have high CRE scores, suggesting that they are likely direct targets of CREB/Crtc. Future studies in which genes are acutely disrupted could additionally help resolve issues of primary vs. secondary effects.

Superimposed on its effects on sNPF expression (17), neuronal Crtc also appears to stimulate the expression of genes involved in 1-C metabolism (*Gnmt*, *Sardh*, *ade2*, and *AdSL*) and in inhibition of insulin secretion and signaling (*ImpL2* and *Lst*). Within the set of 1-C enzymes, glycine *N*-methyltransferase (GNMT) has been found to regulate cellular concentrations of SAM, a methyl donor for many methyltransferase enzymes (33). GNMT reduces cellular SAM levels by converting it to sarcosine. In addition to its induction by Crtc during starvation, Gnmt is also up-regulated by FOXO in the fly fat body (34). Notably, Gnmt overexpression in fat body does not appear to increase starvation resistance, pointing to a potential role for Gnmt expression in other tissues. Reducing SAM levels extends lifespan in worms (35). SAM levels are increased with aging in flies (23). The down-regulation of Gnmt in *Crtc* mutant flies may contribute to their shorter lifespan. Future studies should reveal the extent to which changes in SAM alter neuronal function in response to fasting.

In addition to its effects on *Gnmt* and *Sardh*, Crtc was also found to regulate the expression of 1-C enzymes involved in purine biosynthesis. Mitochondrial impairment due to mutation of the kinase PINK1 promotes mitochondrial biogenesis via the induction of genes in the 1-C pathway (36). Indeed, a number of the same 1-C genes that are induced by fasting are also up-regulated in PINK1 flies. Similarly, knockdown of mitofusin expression causes an impairment in mitochondrial function that triggers up-regulation of the 1-C pathway in *Drosophila* (37). Changes in mitochondrial activity are thought to trigger a retrograde calcium signaling pathway that promotes expression of nuclear encoded genes for mitochondrial repair. We found that Crtc is efficiently dephosphorylated in response to calcium signals. Future studies should reveal whether induction of the 1-C pathway during fasting is important for mitochondrial biogenesis.

Crtc was also found to promote the expression of two inhibitors of insulin secretion and signaling. *ImpL2* was identified as a *Drosophila* homolog of the mammalian insulin-like growth factor 7 (IGFBP7) that binds to dilp2 and antagonizes insulin action (27). In mammals, circulating IGF-binding proteins (IGFBPs) bind to IGFs, and this binding not only prolongs the half-lives of IGFs (38) but also modulates their activity. Seven IGFBPs (IGFBP1–7) have been identified in mammals; they compete with IGF receptors for binding to IGFs (39). The up-regulation of ImpL2 protein during fasting is detectable in the fly larval fat body; its up-regulation contributes to inhibition of IIS signaling. Our results indicate that the induction of *ImpL2* transcription in the head during fasting is dependent on Crtc. Future studies should reveal the extent to which loss of Impl2 in neurons impairs energy balance via an effect on IIS signaling in the brain versus other peripheral tissues.

In addition to its effect on Impl2, Crtc was also found to inhibit insulin signaling via induction of limostatin (Lst), a decretin hormone that appears to block insulin secretion during fasting (32). Although Lst is produced in endocrine cells of the gut, our data indicate that Lst expression in the brain also contributes to effects of this hormone on energy balance.

Collectively, our results demonstrate that, in addition to its effects on sNPF expression and gut barrier integrity, the CREB/CRTC pathway promotes starvation resistance by modulating genes involved in insulin signaling and 1-C function. The identification of relevant Crtc positive neurons in the brain that express these target genes during fasting should provide further insight into this process.

Materials and Methods

Fly Stocks, Starvation Assay, and Climbing Assay. Fly lines were maintained at 25 °C on standard food medium, except for starvation and thermal stress assays. *Crtc* and *CrebB* mutant flies were generated as described previously (15, 17). The control flies are w1118. Fasting treatment was conducted as described previously (15). Male flies 3 to 5 d old were transferred to vials

BIOCHEMISTRY

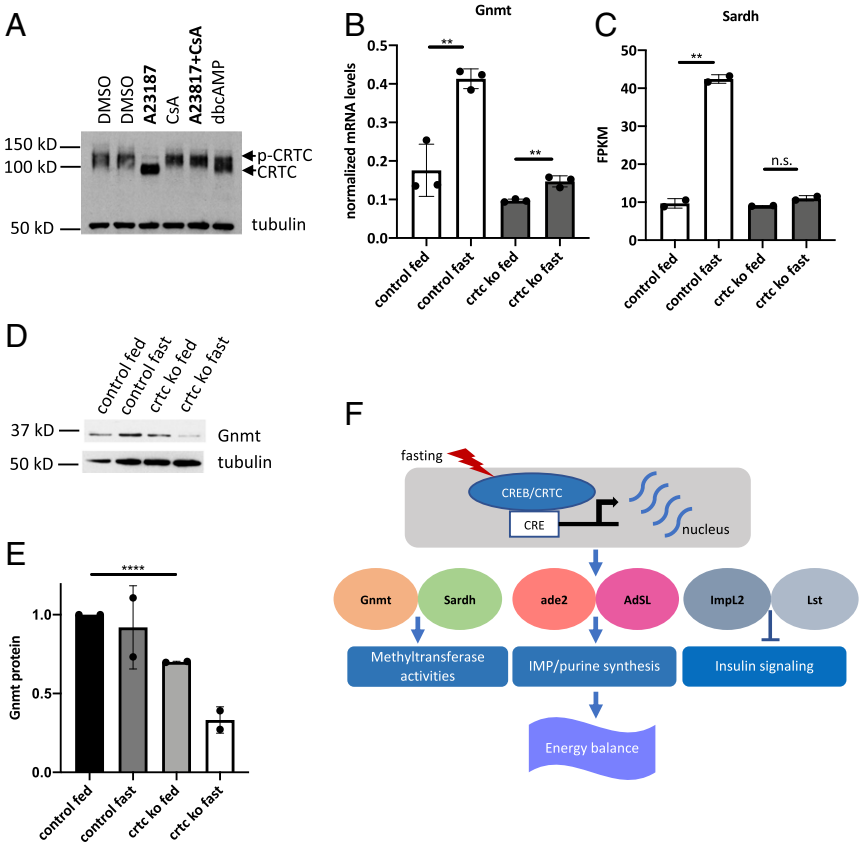

Fig. 4. Crtc stimulates expression of genes involved in 1-C metabolism and inhibition of insulin signaling. (*A*) Western blot of Crtc following 0.5-h treatment with db/cAMP, A23187, CsA, or vehicle in Kc167 cells. (B) RT-qPCR assay showing Gnmt mRNA levels in adult fly head. (*C*) Sardh mRNA levels in adult fly head; genotypes and conditions shown. (*D*) Immunoblot showing GNMT protein levels in adult fly head. (*E*) Quantification of GNMT protein levels in adult fly head, normalized to tubulin. (*F*) Model of neuronal CRTC function in *Drosophila*. CRTC is activated in response to fasting signals, where it promotes energy balance by mediating induction of CREB target genes that function in 1-C metabolism and inhibition of insulin signaling. $^{**}P < 0.01$; $^{****}P < 0.0001$; n.s., not statistically significant.

containing 1% agar in phosphate-buffered saline, with filter papers soaked with water. For starvation assay, dead flies were scored every 4 to 8 h.

Male flies 3 to 5 d old were used in the climbing assay. The percentage of flies that finished the task, climbing 5 cm within 10 s, was recorded and compared among different genotypes.

Longevity Assay and Thermal Stress Condition. Longevity assay was conducted as described previously (40), with some modifications. Newly eclosed adults, 20 to 25 flies per vial, were collected and maintained on standard food medium and transferred to new food twice weekly. For mortality analysis, deaths were scored five or six times every 7 d. For the thermal stress condition flies were maintained at 31 °C.

RNA Extraction and Purification. Flies used for RNA extraction were collected and frozen in liquid nitrogen. Typically, each sample contains 100 flies. Separation of fly head and body was achieved by forcing the frozen sample through a mesh. One milliliter of TRIzol reagent was then added to each sample and samples were pestled quickly. The mixture was then incubated at room temperature for 5 min, followed by 12,000 rcf centrifugation for 10 min at 4 °C. The supernatant was then transferred to a new tube and 200 μL of chloroform was added. The sample was shaken vigorously by hand, followed by incubation at room temperature for 3 min. The mixture was then centrifuged at 10,000 rcf for 15 min at 4 °C. The upper aqueous phase was transferred to a new RNase-free tube and 0.5 mL of isopropanol was added. The mixture was incubated at room temperature for 10 min, followed by 12,000 rcf centrifugation for 10 min at 4 °C. The supernatant was then discarded and the pellet was washed with 1 mL of 75% ethanol. The mixture was then centrifuged at 7,500 rcf for 5 min at 4 °C. The supernatant was discarded and the sample was centrifuged again briefly to remove the last of liquid. The sample was air-dried for 10 min and the pellet was resuspended in 50 μL RNase-free water.

The extracted RNA was further purified using RNease kit (Qiagen); 350 μL buffer RLT and 250 μL 70% ethanol were mixed with the 50-μL RNA sample. The mixture was loaded on to an RNeasy column in a 2-mL collection tube and centrifuged for 15 s at 8,000 rcf. The column was transferred to a new collection tube and 500 μL buffer RPE was added. The mixture was centrifuged at 8,000 rcf for 15 s and 2 min, and flow-through was discarded after each centrifugation. The column was transferred to a new tube and centrifuged at full speed for 1 min to remove all remaining liquid. Fifty microliters of RNase-free water was added to each sample and the sample was incubated at room temperature for 2 min, followed by 1-min full-speed centrifugation. The RNA concentration was measured using Nanodrop.

RNA-Seq. RNA-seq libraries were constructed using NEBNext-Ultra kits (New England Biolab) according to the manufacturer's instructions. Quantitation of libraries was done by Qubit (Invitrogen) and run on a MiSeq instrument with paired-end 75-bp reads using v3 chemistry (Illumina). Data were analyzed by tophat2 and cuffdiff against the *Drosophila* DM3 genome build for heat maps and STAR and DESeq2 against the *Drosophila* DM6 genome build for volcano plots. The GEO accession number is GSE156577. GO enrichment analysis was performed on differentially expressed genes using Metascape or WebGestalt.

RT-PCR Analysis. Total RNA was isolated using TRIzol reagent (Invitrogen), and 1 μg of RNA was used to make complementary DNA (cDNA) with a Transcriptor first-strand cDNA synthesis kit according to the manufacturer's instructions (Roche). Relative mRNA expression was determined with SYBR green master mix (Roche) by Light-Cycler 480 qPCR machine (Roche). Relative mRNA expression was calculated by $2-\Delta\Delta CT$ methods. Rp49 mRNA was used as housekeeping gene for mRNA.

The following qPCR primers were used in this study:

rp49-forward (F)-GCTAAGCTGTCGCACAAATG;

rp49-reverse (R)-GTTCGATCCGTAACCGATGT;

Gnmt-(F)-GCCTTCGATAAGTGGGTCAT, Gnmt-(R)-TGCTCCTGAATGTCGTCGTA;

ImpL2-(F)-GGCTCCAAGACCATCTATGC, ImpL2-(R)-GGCGTCAGACGAGAGGAG;

Lst-(F)-AACGGACAGCGTACTCTGATTT, Lst-(R)- TTGATTCGATAATGCGTTCG.

Cell Culture and Treatment. Kc167 cells were cultured in CCM3 medium (HyClone). Cells were treated with A23187 (5 μM), db-cAMP (100 μM), and CsA (5 μM) as described in the figure legends. For Western blot analysis, cells were treated for 30 min; for RT-qPCR analysis, cells were treated for 2 h.

Western Blot. Sodium dodecyl sulfate (SDS) protein loading buffer (2×) was added to cells or fly samples. The mixture was then sonicated, followed by 5-min denaturation at 95 °C. After 5-min centrifugation at 13,000 rpm, the supernatant was kept for SDS polyacrylamide gel electrophoresis analysis. The antibodies used were against CRTC, Gnmt, and tubulin (05-829; Millipore Sigma). CRTC antibody was generated as described previously (15). Gnmt antibody was kindly provided by Masayuki Miura, The University of Tokyo, Tokyo, Japan.

Statistical Analysis. Data are presented as means ± SD. Statistical differences between two groups were determined by unpaired Student's *t* test. *P* values less than 0.05 are considered statistically significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Data Availability. RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (accession no. GSE156577) (41).

ACKNOWLEDGMENTS. This work was supported by NIH grant R01 DK083834, the Leona M. and Harry B. Helmsley Charitable Trust, the Clayton Foundation for Medical Research, and the Kieckhefer Foundation.

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