High-resolution dynamics of the transcriptional response to nutrition in 

*Drosophila*: a key role for dFOXO

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ADULT ORGANISMS REGULATE their nutrient intake to provide sufficient energy to support locomotion, biosynthetic processes, and reproduction while limiting oxidative damage due to excess metabolic activity. This complex balance is achieved via multiple nutrient sensing systems that control food intake, storage, and utilization. Two major nutrient sensing systems, the insulin and target of rapamycin (TOR) pathways, are highly conserved between *Drosophila* and mammals (16, 43). In addition, as reported in studies carried out 30 yr ago, *Drosophila* produce both hyperglycemic and hypoglycemic hormones that respectively regulate mobilization and storage of carbohydrate (41, 59). The source of the hypoglycemic hormone has recently been identified as a set of neurosecretory cells that produce insulin-like peptides (dILPs) (57). Ablation of the dILP-secreting cells raised circulating sugar levels and caused growth inhibition (57) similar to that induced by mutations of the insulin-like receptor gene, *inr*, and of the gene encoding the insulin receptor substrate homolog, *chico* (3, 5, 7). The TOR pathway also regulates growth in *Drosophila melanogaster*, as it does in mammals (37, 72). Furthermore, mutations that lead to decreased function of either the insulin or dTOR nutrient-sensing pathways in *Drosophila* extend lifespan (9, 25, 60). Mutations that decrease activity of the nutrient sensing system may mimic, at least in part, the effects of decreased nutrient uptake or dietary restriction (DR), which extends lifespan in species as diverse as worms, flies, and mammals (4). Overall, the insulin and TOR nutrient-sensing systems are likely to play a fundamental and conserved role in coordinating complex aspects of the adult phenotype - growth, reproduction, and lifespan - in response to nutrients.

Regulation of these complex responses will require harmonized changes in the expression of many genes. How this control is affected is largely unclear. Unanswered questions include: what transcriptional changes are under the control of the nutrient-sensing systems, what is the temporal and hierarchichal relationship of these changes, what magnitude of change is biologically relevant, and are genes within a functional metabolic network coregulated at the transcriptional level? Recent work with *D. melanogaster* has begun to examine these questions in the context of transcriptional profiles (4, 48, 73). Zinke et al. (73) identified numerous genes whose expression was altered when rapidly growing second instars were starved or fed only sugar. Striking changes in genes involved in fat metabolism, protein translation, and several putative transcription factors were detected. These initial data, however, were limited to a few time points and did not resolve differences with less than fourfold change. Pletcher et al. (48) examined global changes in transcript abundance with aging and as a function of nutrient concentration in the adult food medium. They identified 2,079 genes whose transcript abundance significantly varied across diet, most of which changed by <1.5-fold. Although this study made repeated measures throughout the adult lifespan, the first was made 3 days after flies were transferred to restricted diet. Therefore, the design would not detect transcriptional changes immediately associated with the induction of the nutrient response. Remarkably, the physiology of *D. melanogaster* is extremely responsive to diet, as evidenced by the full adjustment of age-specific mortality and reproduction within 2–3 days of shifting adults between relatively rich and diluted diets (8, 17, 34). Thus, the transcriptional response to changes in nutrients might occur over a short time scale.

More detailed knowledge of the transcriptional response to nutrients is critical to our understanding of metabolic disease...
pathogenesis, especially in light of recent evidence that decreased expression of genes of mitochondrial oxidative metabolism is associated with insulin resistance and diabetes (38, 45). Furthermore, the links between nutrient-sensing systems and regulation of oxidative phosphorylation genes are unclear, and disease-associated decreases are small, in the range of 20%, requiring sensitive methods of detecting global transcriptional changes.

To understand how genes and gene networks respond to nutrition requires analysis of transcript dynamics across the time scale of the physiological response. To date, time series analysis of transcription dynamics has been limited to manipulations with yeast or cells in culture. These experimental designs synchronize a population of cells, impose an acute manipulation (e.g., drug or gene-induction treatment), and sample at short intervals across the phase of the biological change while the cells remain synchronized (e.g., 53). Here we apply key features of this design to study the transcript dynamics in whole adult D. melanogaster as they undergo an acute physiological response to refeeding: synchronize the physiological state of all animals, impose an abrupt manipulation, sample from controls and treated groups at each time point, and sample at short intervals relative to the time frame of the physiological response. Finally, we apply a computational, nonparametric change-point analysis to detect when genes undergo meaningful change and to estimate the magnitude and likelihood of these changes.

We characterized the transcriptional response in adult females to refeeding on dietary protein. To synchronize their physiological state at eclosion, we removed yeast from medium of third instar larvae. This produces an eclosion cohort where insulin-like peptide staining in the brain insulin-producing cells (IPC) is uniformly compressed in all individuals and oogenesis is fully repressed in all females (67). When synchronized yeast-deprived females are fed yeast, within 12 h the IPC expand and secrete insulin-containing vesicles, and vitellogenesis is activated. Accordingly, our analysis describes the transcriptional dynamics of metabolic and physiological networks across the initial 12 h of this physiological response. Furthermore, we compare sets of transcripts modulated in response to refeeding to candidate targets of dFOXO, the D. melanogaster ortholog of Caenorhabditis elegans daf-16 and mammalian FOXO1 and FOXO3a (22, 26, 49). dFOXO activity is modulated in D. melanogaster by insulin signaling through an insulin-like receptor that controls phosphatidylinositol-3 kinase/dAkt (22, 26, 49). Since yeast restriction impedes secretion of insulin-like peptides in adult flies (67), we expect dFOXO to be activated in yeast-deprived females and to be relatively inactivated when adults are re-fed. To identify potential nutrient-mediated dFOXO transcription targets in the adult fly we compared the transcriptional responses upon yeast refeeding to the message profiles from Drosophila S2 cells that expressed a constitutively active dFOXO-A3 construct (49).

Remarkably, dFOXO target genes represent 28% of the total nutrient-responsive genes, implicating dFOXO as a major factor in organizing the transcriptional response to nutrients. Our data suggest that dFOXO plays a central role in coordinating changes in nutrient sensing, protein synthesis and growth, fatty acid oxidation and storage, and genes of mitochondrial biogenesis. In addition, we have identified a putative Drosophila homolog of the mammalian transcriptional coactivator, peroxisome proliferator-γ coactivator-1 (PGC-1), as a dFOXO target gene, suggesting that mitochondria biogenesis is linked to insulin signaling via dFOXO-mediated repression of a PGC-1 homolog.

**METHODS**

*Drosophila culture*. Following Tu and Tatar (67), D. melanogaster (yw) were reared synchronously to third instars on standard cornmeal-sugar-yeast medium at 25°C and transferred at 76 h old to sugar-agar-cornmeal medium without yeast. Virgin females were collected within 8 h of eclosion and placed on sugar-agar-cornmeal medium without dietary yeast. At day 4, females were redistributed in groups of 30 into vials with yeast-free medium or with standard medium supplemented with live-yeast. Two females were collected at each of 12 sample times from 15 vials within each treatment group (NY, control; Y, re-fed); samples were collected every 60 min. To provide replicated estimates of expression profiles prior to the study (baseline samples), four samples of 30 females were collected just before we initiated the time series collections.

Message isolation and hybridization. Samples were frozen in liquid nitrogen at each collection time point and stored at −80°C. RNA was extracted from all samples simultaneously by lysis in a FastPrep FP120 multitube homogenizer (Q BioGen). Flies were added to 1 ml of Trizol (Invitrogen) and 10 μg of glycogen in iced FastRNA tubes (cat. no. 6040-600) and spun twice for 8 s at speed setting “6;” tubes were iced between spins. RNA was extracted by phase separation and precipitation following Trizol protocols. At the Brown University Genomics Core Facility, RNA quality was verified on agarose gels and reverse transcribed. The cDNA was amplified by in vitro transcription, producing 5 μg of total product. Products were hybridized to Affymetrix GeneChip Drosophila_1 Genome Arrays in sets of four, each included a pair of time matched Y and NY treatments sampled from one early and one late hour. The raw scan data from each chip were exported to DNA-Chip Analyzer (http://www.dchip.org) and normalized across chips by the invariant difference selection algorithm with gene expression given by the model-based expression index. Two samples from the control group produced insufficient RNA (4 and 8 h); values to calculate expression ratios in the controls of these time points were interpolated from data of control samples at adjacent hours.

Change-point statistics. The cumulative sum statistic (CUSUM) estimates the time of a change point in sequentially ordered data (6). The CUSUM function is the cumulative difference between the average among all values and each value taken in time order. A change point is estimated at the time with the largest absolute change of this cumulative function that includes a sign change. A complementary statistic for the change point is based on the mean square error (MSE) statistic, which places the change point as the time that minimizes the sum of the squares of the errors about the means on both sides of the point. Following the bootstrap methods proposed by Taylor (62), GeneTrace uses CUSUM to calculate the change point, and for each gene it estimates the probability the change point estimated both by CUSUM and by MSE would be drawn from a sample distribution of change points derived from random permutations of the observed time series. Because CUSUM and MSE statistics are insensitive to the order of time points on either side of a mean shift, the bootstrapped data represent a homogeneous sample of all potential permuted time series from the observed data. For each gene time series, GeneTrace returns an estimated change point (CUSUM estimator) and the two-tailed probability of this statistic from a sample of 10,000 random permutations across the 16 ordered expression ratios. The GeneTrace computational platform is available at http://www.brown.edu/Research/Tatar_Lab/GeneTrace; information on its operation is in Supplemental Methods. (The online version of this article contains supplemental material; there are eight supplemental tables, one supplemental figure, and a supplemental methods section.
associated with this paper.) Microarray datasets have been deposited in GEO with accession number GSE562.

RESULTS AND DISCUSSION

High-resolution time series of transcript abundance. Variance among individuals within treatment groups can reduce the power to detect change in transcript abundance. To minimize this source of noise, we imposed diet restriction in third instars (Fig. 1A). This procedure synchronizes the physiological state among emerging adults and induces a uniform pattern of suppressed insulin secretion (67). Newly eclosed virgin females were maintained on yeast-free medium until 4 days old and then transferred either to medium with yeast (Y-treatment) or to control medium without yeast (NY-control). Baseline samples were collected prior to refeeding (arbitrarily designated as hours −3, −2, −1, and 0) and every 60 min for the next 12 h after the transfer (hours 1–12). RNA was isolated from both Y-treatment and NY-control as described in METHODS and hybridized to Affymetrix GeneChips. The relative message abundance at each hour was determined as the ratio of the Y-treatment relative to its time-matched NY-control. Message abundance in the baseline samples was calculated relative to the mean among baseline samples.

These data produce a time-ordered sequence of expression ratios. To characterize the response at each gene we determined if and when its expression ratio in the time series presented a change point, a nonrandom change in the arithmetic mean of the series. In the analytical platform GeneTrace, change points were estimated by the nonparametric CUSUM, and the two-tailed probability of each was numerically calculated from permuted data. See METHODS and supplemental online material.

GeneTrace identified 3,519 genes with significant change point when the significance threshold for each gene was $\alpha = 0.005$ (Table S1). Based on 14,000 tests at this threshold we expect 70 false-positive inferences. Overall, the distribution of observed change points (Fig. 1B) differed significantly from expectation if observed change points were sampled uniformly from among the 12 h ($K$-spectrum $D$-test, $D_{obs} = 0.46$, $P < 0.001$). Likewise, the observed mean among change points ($\bar{x} = 5.09$ h) occurred earlier than expected from either a uniform or normal distribution ($P < 0.01$). In the first hour following refeeding, transcript ratios increased for 40 genes and decreased for 18 genes. Additional first change points accumulated until 7 h postrefeeding, after which time relatively few changes were detected (Fig. 1B). The decrease in observed change points occurs while individuals remained physiologically synchronous since the coefficient of variation among expression ratios did not increase in late relative to early hours (data not shown). While the rarity of change points at later times is not likely to arise from loss of synchrony in the sample, we cannot rule out that fewer significant change points at hours 8–12 may be accounted for by reduced power at these time points. At the boundaries it is more difficult to detect change points because on one side of a putative change point there are a smaller number of observations available to precisely estimate the mean expression ratio. Our detection of change points in the early hours of the time series is less likely to be an underestimate because we have buffered this boundary with four samples prior to refeeding. Decay in power to detect change points is more likely in the late hour time samples. This loss of power at the boundaries, however, does not invalidate any changes that were found. Importantly, of the changes we do detect at any hour, most were very small (Fig. 1C); only 19% of the 3,519 ratios were >1.5-fold. These data suggest that the physiological response to nutrient uptake involves many subtle changes in message abundance that alter systems of transcripts on a global scale.

Categorical response: gene ontology. Many of the genes with significant change points are annotated as gene ontology biological processes involving cell growth and maintenance, macromolecular and nucleic acid-related metabolism, signal transduction, and organogenesis (Table 1). Among 1,148 biological process terms annotated for this array, 128 categories were significantly overrepresented in the response to nutrient supply (Table S2). These include many aspects of carbohydrate, fatty acid, and RNA metabolism, DNA replication, RNA processing, translation, and protein catabolism. Terms associ-
bolic enzymes may serve to support the biosynthesis of nucleo-
tides required to provision embryos with maternal mRNA.

Transcriptional response in nutrient sensing and metabo-
lism. Overall these data reveal a global response to nutrition
that involves rapid change in functional sets of transcripts
whose products mediate metabolic physiology. Below, we
elaborate a few of the many responsive metabolic networks:
nutrient sensing via insulin and TOR, fuel metabolism,
mitochondria biogenesis, and the control of histone deacetylases.
Each illustrates the potential for discovery with these data and
reinforces how metabolic physiology is globally adjusted
through small yet coordinated change at the level of mRNA.

Nutrient sensing via insulin and TOR. Insulin/IGF signaling
in Drosophila regulates a variety of biological processes,
including growth, reproduction, and aging (16, 43, 60). It
functions as a systemic nutrient signaling system through
circulating diLPs (encoded by dilp) (5, 21). TOR signaling
also provides nutrient sensing (19) but in an exclusively cell
autonomous manner. Gain-of-function mutations in either
the insulin or the TOR pathway stimulate cell growth, whereas
hypomorphic mutations decrease growth (16) and, in some
cases, extend lifespan (9, 25, 60). While these regulatory
actions of insulin/IGF and TOR are understood in terms of
signal transduction cascades, our data indicate an additional
level of control: nutrients coordinate across the expression
of components of the nutrient sensing systems themselves.

Transcripts encoding proteins of the insulin/IGF and TOR
pathways decreased within 3 h of refeeding (Fig. 2, A and C),
suggesting they were upregulated in the absence of yeast.
InRNAs encoding the InR, the insulin receptor substrate-1
homolog chico, and phosphatidylinositol-dependent kinase-1,
PK61C, synchronously declined by 30–40% (Fig. 2A). These
dynamics contrast with the stasis of the dilp loci; there was no
change observed in transcripts for insulin ligands over this time
course (Fig. 2B). It is possible that changes in mRNAs encoding
the diLPS, produced in a small set of neurosecretory cells,
would be obscured in the whole organism RNA samples
analyzed here. However, these patterns are consistent with
observations in yeast-deprived adults; diLP peptides are
present but compact within the pars intercerebralis neurons of
the brain (67). These peptides are rapidly processed and
secreted along the medial secretory neuron when adults are refed.
Thus, the response to nutrition in D. melanogaster involves
immediate secretion of stored diLPS possibly with delayed
transcription of the ligand.

The InR controls the activity of the transcription factor
FOXO through its phosphorylation of FOXO by the down-
stream kinase Akt (1). While dfoxo and akt1 expression were
unchanged in refed flies (Fig. 2D), mRNAs for InR and the
translational repressor, 4eBP, were reduced (Fig. 2, A and C).
InR and 4eBP, a component of the dTOR pathway, were
previously defined as dFOXO-induced genes in S2 cells ex-
pressing a constitutively nuclear dFOXO mutant, dFOXO-A3
(49). Here we integrate into our analysis additional, previously
unpublished data from these dFOXO-A3 cells. There is exten-
sive agreement between these independent data sets (Table
S4), indicating that many of the genes responsive to refeeding
in D. melanogaster are potential transcriptional targets of
dFOXO. We found that 995 genes that significantly changed
upon refeeding in adults were inversely regulated following
induction of dFOXO-A3. These include several components of

Table 1. Distribution of nutrient responding messages
categorized across Gene Ontology biological
processes (level 4)

<table>
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<th>Ontology ID</th>
<th>Ontology Term</th>
<th>Percent</th>
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<td>GO:0043170</td>
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<td>Nucleobase-sial-oxide &amp; nucleic acid metabolism</td>
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<td>regulation of metabolism</td>
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<td>lipid metabolism</td>
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<td>dorsal/ventral pattern formation</td>
<td>1.04</td>
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Percent is relative to genes with level 4 Gene Ontology (GO) annotation; only the categories representing >1.0% are shown.
the insulin signal transduction cascade (e.g., INR, PK61C), which reinforces the hypothesis that cells can autonomously regulate their own insulin sensitivity in response to the systemic level of insulin peptides (49). Naturally, detailed molecular analyses are needed to verify inferences for specific genes and to discriminate direct from indirect action of dFOXO. An important problem relevant to metabolic diseases is to understand the specific way this within-cell feedback network moves from the repressed state back to insulin sensitivity.

Key components of the TOR pathway also respond transcriptionally to nutrients (Fig. 2C). Amino acid uptake stimulates TOR to phosphorylate 4e-BP, p70 S6 kinase (S6K), and other targets (19). In refeed flies, 4eBP mRNA was dramatically downregulated (Fig. 2C). Thus, 4e-BP activity is rapidly regulated at both the posttranslational and transcriptional levels. The early and dramatic downregulation of 4eBP transcripts (Fig. 2C) suggests this gene functions as a key gatekeeper in the activation of translation in response to nutrient resupply (63, 64). Similar regulation was observed in protein-starved larvae (73). While refeeding may have stimulated translation by repression of 4eBP, it appeared to simultaneously reduce the signaling capacity of the TOR pathway. Transcripts encoding the G protein Rheb, an upstream activator of dTOR, decreased by 36%, and mRNA for S6K decreased by 16% (Fig. 2C). In addition, a homolog of the binding protein required for repression of TOR by rapamycin, encoded by fkbp12, was upregulated (Fig. 2E). Additional members of the FK506 binding protein family, including fkbp39, fkbp59, and fkbp13, increased in parallel with fkbp12 (Fig. 2E). Depending on the interactions with endogenous substrates, which are unknown, the transcription changes in the FK506 proteins along with those in Rheb and S6K may desensitize the dTOR pathway to nutrient stimuli upon refeeding, much as we propose for insulin signaling within cells. Notably, some pathway components were not regulated acutely at the transcriptional level by nutrients, including dTOR itself and Tsc1 (Fig. 2D).

In contrast to the decline of 4e-BP mRNA, components required to initiate translation of 5′-cap mRNA are coordinately induced, including the initiation factors eIF4E and eIF4B, the release factors eRF1 and eRF3, and the scaffolding protein eIF4G (Fig. 2F). Notably, eIF4G competes with 4eBP for binding sites on eIF4E (19). Transcripts of eIF4E and eIF4G increase with strikingly similar kinetics, suggesting they share regulatory control. Of 35 general translation factors...
identified in the Drosophila genome (30), 25 were upregulated in refed flies (Table S5). Since the loss of 4e-BP message was rapid relative to the increase in these initiation factors, we suggest that translational inhibition is immediately released upon refeeding but the addition of capacity to initiate translation occurs only gradually.

Fuel metabolism and biosynthesis. When nutrients are re-supplied after a fast, mammals shift fuel metabolism from fatty acid oxidation to fatty acid biosynthesis and glucose oxidation (12, 52). The mechanisms of this transition are typically understood in terms of allosteric control by metabolic substrates and through large transcriptional changes of key enzymes. Here we find that the metabolic response to nutrient resupply in D. melanogaster involves small changes in many genes that function together in metabolic pathways. Message for proteins involved in fatty acid oxidation was reduced 35–41% within 4 h of refeeding (Fig. 3A). These include mitochondrial carnitine palmitoyltransferase-1 and long chain acyl-CoA synthetase, which were inversely regulated in starved larvae (73). Concurrently, message declined for the subunits of AMP-activated kinase and pyruvate dehydrogenase kinase (PDHK), enzymes that are typically active during a low energy state (Fig. 3A). Expression of glycolytic enzymes 6-phosphofructokinase and hexokinase was downregulated (~20 and ~34%, respectively), while mRNA level increased for enzymes of the TCA cycle such as isocitrate dehydrogenase and citrate synthase (Fig. 3B), consistent with a shift from glycolytic to oxidative glucose metabolism. Three subunits of phosphorylase kinase were downregulated following refeeding (Fig. 3B), suggesting decreased sensitivity to glycogenolytic signals. Transcripts of fatty acid synthetic enzymes were elevated (Fig. 3A), including citrate lyase, glucose 6-phosphate dehydrogenase, acetyl-CoA carboxylase, and the subunits of fatty acid synthase, β-ketoacyl-ACP synthase, and ACP-S-malonyltransferase. Expression of serine-palmitoyl transferase, which catalyzes the first step in ceramide and sphingolipid biosynthesis, was also elevated (Fig. 3A). Together, these changes would decrease fatty acid oxidation, increase fatty acid biosynthesis, and promote glucose oxidation by the TCA cycle (Fig. 3C).

The TCA cycle also serves a biosynthetic function in a process described as cataplerosis where four- and five-chain carbon skeletons are removed from the TCA cycle to be converted to glucose, fatty acids, or nonessential amino acids (44). Cataplerosis makes use of many enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), glutamate dehydrogenase, aspartate aminotransferase, and citrate lyase (44). The D. melanogaster homolog (CG17725) of human PEPCK encoded by PCK-1 did not vary with nutrition. However, mRNA encoding CG10924, the ortholog of human PCK-2 increased. PCK-2, unlike the gluconeogenic enzyme PCK-1, functions in both mitochondria and cytosol (36) and participates in TCA-mediated biosynthesis (44). Remarkably, mRNAs for each of the cataplerotic enzymes mentioned above increased along with CG10924 (Table S6). Cataplerosis is balanced by anaplerosis, the addition of intermediates to the TCA cycle. The major anaplerotic enzyme is pyruvate carboxylase, and mRNA encoding this enzyme was upregulated 52% upon refeeding (Table S6). Overall, resupply of nutrients elevated coordinated sets of transcripts that support multiple functions of TCA-associated metabolism.
The temporal coordination of such messages suggests they share some transcriptional control. Indeed, data from dFOXO-A3 cells suggest that dFOXO coordinates the balance between oxidation of glucose and fatty acids and between fatty acid oxidation and biosynthesis. mRNAs encoding enzymes of fatty acid oxidation and fatty acid biosynthesis were inversely regulated by refeeding and dFOXO (Table S4). In addition, PDHK was reduced in refed flies, and this gene is regulated by FOXO1 in mammalian tissue (15, 27). How dFOXO might influence PECK in D. melanogaster is ambiguous. Consistent with the inhibition of Pck1 by insulin in mammals, the message for D. melanogaster CG17725 increased in dFOXO-A3 cells and in starved larvae (73) and decreased in KC167 cells treated with insulin (22). In refed adults, however, message for CG17725 remained static while transcripts for CG10924 (Pck2) increased. Furthermore, there was no change in mRNA for CG10924 or other cataplerosis-associated genes or TCA cycle enzymes in dFOXO-A3 cells. This suggests that TCA-associated carbon metabolism, at least in D. melanogaster, is nutrient responsive but may not be regulated tightly by insulin-IGF via dFOXO.

Mitochondria metabolism and biogenesis. Given the change in mRNA encoding proteins involved in intermediary metabolism upon refeeding, we expected to find transcriptional changes in genes underlying mitochondrial function. Indeed, many genes whose products directly participate in mitochondrial substrate transport and ATP synthesis were coordinately upregulated (Table S7). These included at least 13 genes encoding proteins with functions in electron transport and thirteen substrate carriers (Table S7). Indeed, of 232 genes assigned Gene Ontology (GO) functions associated with mitochondria, 54% changed with refeeding, and 91% of these were increased.

Most strikingly, there was a marked and rapid increase in transcripts encoding genes with roles in mitochondrial biogenesis, such as membrane preprotein translocases, chaperones, mitochondrial DNA binding proteins, and nuclear encoded mitochondrial ribosome proteins (Tables S7, S8). Of 60 nuclear encoded mitochondrial ribosomal genes on the array, expression of 54 was increased an average of 44% at 5–7 h after refeeding (Table S8). The induction of mitochondrial ribosomal proteins contrasts with the relative stasis of transcripts encoding proteins of the cytoplasmic ribosome. Of the 39 cytoplasmic ribosome genes represented on the array, mRNA for seven were moderately increased and two were reduced. An expansion of mitochondria within cells may reflect de novo adipogenesis in adult fat body when females first feed on yeast, similar to what is observed in mammalian 3T3-L1 cells that increase mitochondrial density as they differentiate into adipocytes (69). The observed increase in mitochondrial gene expression may also reflect activity in skeletal muscle cells, given that nutrients have been shown to regulate expression of mitochondrial oxidative phosphorylation genes in mammalian muscle (42).

How nutrients regulate mitochondrial capacity is poorly understood. Here we see that many nutrient-induced transcripts encoding proteins of mitochondria biogenesis were broadly repressed in cells expressing dFOXO-A3 (Tables S7, S8). Of the 54 mRNAs for mitochondrial ribosome proteins that increased upon refeeding, 42 were downregulated by dFOXO-A3 (Table S8). Likewise, of the 25 upregulated genes with functions in mitochondrial biogenesis, 14 were repressed in dFOXO-A3 cells (Table S7). In contrast, only six of the 53 nutrient-regulated, nuclear-encoded genes for mitochondrial function were inversely regulated by dFOXO-A3. Therefore, insulin, presumably via dFOXO, has a strong and selective impact on genes associated with mitochondrial biogenesis.

One candidate to mediate mitochondrial biogenesis is PGC-1. PGC-1α of mammals stimulates mitochondrial biogenesis in muscle, brown adipose tissue, and adipogenic 3T3-L1 cells (50, 33, 70). Interestingly, we find a single ortholog of a PGC-like peptide in D. melanogaster (CG9809) whose mRNA increases upon refeeding (+49% at 7 h, Fig. 4) and declines in dFOXO-A3 cells (down 1.4-fold, Table S4). CG9809 encodes a predicted protein of 1,088 amino acids that exhibits 68 or 52% homology with mammalian PGC-1α or PGC-1β, respectively, in their COOH-terminal RNA-binding motif (Fig. 5). Other domains in common with its mammalian homologs include an arginine-serine-rich domain located NH2-terminal to the RNA-binding motif, an acidic NH2-terminal domain and leucine-rich motifs, although the canonical LXXLL nuclear receptor binding motif is not present. However, the LXXLL motif is not absolutely required for coactivator binding (51), and CG9809 does contain a variant of this motif near its COOH terminus, FXDL, which has been reported to function in nuclear receptor binding (20). Notably, this sequence is conserved in all mammalian PGC-1 family members (Fig. S1).

Fig. 5. Domains of CG9809 in common with human peroxisome proliferator-γ coactivator (hPGC)-1α and PGC-1β, and percent homology of the human sequences with that of Drosophila CG9809. The acidic amino-terminal, Arg/Ser-Rich (RS) and RNA-recognition motif (RRM) domains are indicated. Also noted is the sequence F(D/E)XXL common to the carboxy-terminal domains of all PGC-1 family members.

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Beside a parallel based on sequence, orthologous targets of PGC-1 were elevated in refeed flies and were reduced by dFOXO-A3 in S2 cells, including mitochondrial transcription factor A (Table S7; Ref. 70). Consistent with data presented here, insulin was reported to upregulate PGC-1α and PGC-1β mRNA levels in human muscle (33), although the mechanism was unclear. Our data suggest that insulin signaling may be a conserved regulator of normal mitochondrial biogenesis via FOXO-mediated control of PGC-1-like cofactors. Importantly, this suggests a mechanistic basis for the prevalent mitochondrial dysfunction in humans with insulin resistance and diabetes (38, 45, 46).

Histone deacetylases and cofactors of nuclear hormone complexes. Histone deacetylases mediate the dietary response to stress and of aging. Overexpression of the NAD-dependent deacetylase sir2 extends lifespan in C. elegans and D. melanogaster (54, 65). Mutation of the zinc-dependent histone deacetylase rpd3 extends D. melanogaster lifespan as does DR, and both conditions increase sir2 message (55). Here we find that refeeding simultaneously reduced transcripts of sir2, increased those of rpd3, and coordinately modulated mRNAs for other potential acetylation factors of nuclear hormone receptors (diskette, HDAC6, HDAC3) (Fig. 4). Furthermore, message for several of these genes, including sir2, rpd3, and HDAC3, are inversely regulated in dFOXO-A3 cells (Table S4). This is consistent with the reported regulation of SirT1 expression by insulin (10) and Foxo3a (40) in mammalian cells. In light of the recent reports that mammalian SIRT enhances the transcriptional activity of FOXO (11, 14, 68), upregulation of Sir2 transcription by dFOXO suggests a conserved feed-forward mechanism.

The control of acetylation factor expression provides an additional way for nutrients to regulate metabolism at the level of transcription. Nuclear hormone receptors (NHRs) interact with complex sets of transcription factors, cofactors and partner NHRs. The activity of each complex is influenced by ligand binding, but also by modifications including protein acetylation (35, 56). For example, SIRT, the mammalian homolog of sir2, represses adipocyte differentiation via an interaction with the adipogenic NHR PPARγ that appears to be dependent on the NCOR/SMRT corepressor (47, 71). Conversely, lipolysis is promoted by SIRT activity (47). Thus, activation of SIRT in adipocytes under low nutrient conditions would repress storage and promote release of energy stores. Our data now suggest that Sir2 modulation of energy storage is directly linked to the nutrient-sensing pathway via insulin-regulated dFOXO activity. The histone deacetylase rpd3 also appears to be a dFOXO target, but its message increases dramatically in refeed D. melanogaster, while message for sir2 declines (Fig. 4), consistent with repression in the low nutrient condition when dFOXO is activated. Rpd3 may also interact with the NCOR/SMRT corepressor, via Sin3A, a scaffold protein of deacetylation complexes in yeast and mammalian cells (18, 28). mSin3A and dSin3A have been shown to associate with the fly functional homolog of the mammalian NCOR/SMRT corepressor called SMTNER (SMRT-related protein) (66). Interestingly, mRNA encoding the fly HDAC3 homolog increases somewhat later (Fig. 4), and it is also a component of the NCOR/SMRT corepressor complex (31). Thus, nutrient-mediated regulation of HDACs via dFOXO may modulate gene expression by altering the components of NCOR/SMRT corepressor complexes. The expression of two additional messages are strongly correlated to rpd3 (Fig. 4), and, like rpd3, both are repressed by FOXO-A3 (Table S4): CG9809, the candidate ortholog of PGC-1, and CG3994, the ortholog of the yeast zinc transporter ZRT1 that is required for Rpd3p to suppress transcription of SIR2 (2). The temporal concordance among rpd-3, CG9809, and CG3994 is consistent with coregulation by dFOXO. Furthermore, it suggests a coordinated alteration in the composition of NHR coactivators and SMTTER corepressors in D. melanogaster in response to nutrition.

Finally, as with each of the metabolic networks featured in this discussion, the components of the acetylation system respond to nutrients through a coordinated control of transcripts where many changes are as small as 25%, subtle differences, we assert, that are statistically reliable and biologically important.

Conclusion. Control of the metabolic response to nutrition is complex and multifaceted. Although a role for transcription is well recognized, it is thought to primarily involve regulation of key, rate-limiting steps. Our fine-grained, genome-wide analysis reveals a different perspective: in response to nutrient intake extensive networks of metabolic function are coordinated through transcription. These changes, moreover, are extremely subtle yet biologically profound. While a large change in the activity of one element within a metabolic pathway can have little effect on flux (23), small coordinated changes in transcript levels throughout the network can alter the global metabolic state (13, 58). Coordination of messages on this scale requires control through common signals. We find that many of the genes that vary in response to refeeding are also influenced by dFOXO expressed within S2 cells. This suggests that upon feeding, release of insulin ligands induces an acute yet widespread response via modulation of dFOXO activity. It remains to be determined whether dFOXO affects the >900 candidate target genes (Table S4) directly or indirectly and to understand the relative contribution of induction vs. message stability and degradation in determining how transcript abundance changes with nutrient uptake (e.g., Ref. 32). To assess the potential for dFOXO to act as the direct

![Fig. 6. Proposed role of dFOXO in integrating nutrient levels, via insulin signaling, with expression of genes involved in nutrient sensing, energy utilization and storage, mitochondrial biogenesis, growth, and lifespan. Arrows symbolize activation, bars symbolize inhibition.](http://physiolgenomics.physiology.org/)
transcription factor of these candidate genes we searched for sequence patterns enriched in their promoter regions. Two clusters of genes can be defined in Table S4: those whose expression increases in S2 cells with activated dFOXO and decreases upon yeast refeeding (cluster 1) and those that decrease in dFOXO-A3 cells and increase with refeeding (cluster 2). The 500-bp upstream flanking sequences of genes from these clusters were analyzed using the POCO and POXO platforms to identify nucleotide patterns that are overrepresented in contrasting sets of sequences and relative to the background sequence (24). Patterns with significant enrichment were then screened with the JASPAR database to identify recognized transcription factor binding sites (similarity index >0.70).

Several patterns were enriched in cluster 1 and simultaneously deficient in cluster 2 and showed similarity within tolerance to forkhead family transcription factor binding sites. Four sequences (TANANAAT, AAATNTTA, TAAANATT, TGCTTANT) were similar to the motif annotated as FOXQ1, A(A/T)(A/T)GTTA(T/G)(A/T). Two patterns (TAAACNT, GTTAANAA) were similar to the motif GTAAACA(A/T) (annotated as FOXD1), which is often taken as the consensus FOXO response element of invertebrates. Among genes of cluster 1, 81% of the tested promoters contained at least one of the six patterns, suggesting that FOXO functions as a direct transcriptional activator of many targets induced under low nutrient conditions. Of special interest is the sir2 gene that, based on analysis with the FOXD1 and FOXQ1 models, contains nine putative sites within the first upstream 500 bp (Fig. S2).

In contrast to these cases, screening for FOXD1 and FOXQ1 motifs in the upstream 500 bp of several cluster 2 genes with strong induction upon refeeding revealed putative FRE sites to be relatively rare. However, the potential for dFOXO to act as an inhibitory transcription factor is suggested in some instances. CG9809, the PGCl homolog described above, and rpd3 both contained putative dFOXO binding sites (Fig. S2; 3 instances in dPGCl and 10 instances in rpd3), although two of the sites in the PGCl-1 promoter fully overlapped.

Overall, these screens suggest dFOXO acts directly to induce the cluster 1 genes of Table S4 but primarily indirectly to repress the complementary set. The analysis identifies two potential response element motifs through which dFOXO may act. Notably, the consensus and an alternative motif (CTTATCA) were found enriched among genes of C. elegans responding to genetic manipulations of the daf-16 transcription factor (39). In both organisms, it will be important to verify function of these putative sites through explicit molecular dissection.

In conclusion, the impact of FOXO upon nutrient metabolism is not simply through the linear transmission of insulin signals into the cell. By regulating the transcription of multiple systems controlling nutrient sensing, utilization, and storage, FOXO integrates the cell’s sensitivity to systemic nutrient signals (insulin) with the appropriate cellular response to local nutrient conditions (Fig. 6). Clearly, FOXO transcription factors play a central, evolutionarily conserved role in energy sensing and metabolism within the cell. This pivotal position suggests that modulation of FOXO activity would have broad therapeutic potential for the treatment of metabolic diseases.

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