Carbon Catabolite Repression Regulates Amino Acid Permeases in *Saccharomyces cerevisiae* via the TOR Signaling Pathway*

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We have identified carbon catabolite repression (CCR) as a regulator of amino acid permeases in *Saccharomyces cerevisiae*, elucidated the permeases regulated by CCR, and identified the mechanisms involved in amino acid permease regulation by CCR. Transport of L-arginine and L-leucine was increased by ~10–25-fold in yeast grown in carbon sources alternate to glucose, indicating regulation by CCR. In wild type yeast the uptake (pmol/10⁶ cells/h), in glucose versus galactose medium, of L-[^14]C-arginine was (0.24 ± 0.04 versus 6.11 ± 0.42) and L-[^14]C-leucine was (0.30 ± 0.02 versus 3.60 ± 0.50). The increase in amino acid uptake was maintained when galactose was replaced with glycerol. Deletion of gap1Δ and agp1Δ from the wild type strain did not alter CCR induced increase in L-leucine uptake; however, deletion of further amino acid permeases reduced the increase in L-leucine uptake in the following manner: 36% (gnp1Δ), 62% (bap2Δ), 83% (Δ[bap2-tat1]). Direct immunofluorescence showed large increases in the expression of Gnp1 and Bap2 proteins when grown in galactose compared with glucose medium. By extending the functional genomic approach to include major nutritional transducers of CCR in yeast, we concluded that SNF/MIG, GCN, or PSK pathways were not involved in the regulation of amino acid permeases by CCR. Strikingly, the deletion of TOR1, which regulates cellular response to changes in nitrogen availability, from the wild type strain abolished the CCR-induced amino acid uptake. Our results provide novel insights into the regulation of yeast amino acid permeases and signaling mechanisms involved in this regulation.

The preferred mode of metabolism in *Saccharomyces cerevisiae* is fermentative, and the preferred carbon source is glucose; other carbohydrates such as galactose or maltose and non-fermentable carbon sources such as ethanol and glycerol could also be utilized by yeast (1). Substitution, reduction, or removal of glucose from the laboratory growth medium, however, is known to have important physiological and genomic consequences in yeast (1-3).

The presence of a high concentration of glucose in the growth medium represses transcription of multiple genes including those involved in alternative carbohydrate and mitochondrial metabolism (1, 3). This phenomenon is known as carbon catabolite repression (CCR),

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2 The abbreviations used are: CCR, carbon catabolite repression; WT, wild type; GFP, green fluorescent protein; NCR, nitrogen catabolite repression.

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buffer. Radiolabeled amino acid uptake was initiated by adding an equal volume (100 μL) of transport buffer containing 5 μM (2.8kBq) L-[14C]-labeled amino acid to the yeast cell suspension and incubating at room temperature for timed periods. After a specified period, the uptake reaction was stopped by a rapid filtration technique (16). Briefly 100-μl aliquots of the radioactive cell suspension were filtered through a nitrocellulose filter (0.45-μm pore size) and washed immediately four times with 4 mL of ice-cold transport buffer. Filters were placed in liquid scintillation vials and counted on a Beckman LS6000 liquid scintillation counter (Beckman-Coulter). To determine the background activity cells were filtered and washed at time 0, and the resultant value was subtracted from that obtained after timed incubations. In certain experiments, non-radiolabeled amino acids were added to the uptake buffer at the specified concentrations. All uptake measurements were made in duplicate in at least three independent experiments.

RNA Isolation and Semiquantitative PCR—Total RNA was isolated from wild type yeast cells following 24 h growth in glucose or galactose YNB using RNeasy isolation kit (Qiagen) following the manufacturer’s protocol. Isolated total RNA was used as a template for reverse transcription (using an Omniscript reverse transcriptase kit from Qiagen) prior to semiquantitative PCR. The following pairs of oligonucleotide primers (5′–3′) are the sense strand, forward primer; A5 is the antisense strand, reverse primer) were used in the PCR reaction to detect the indicated amino acid permease genes: AGP1, ATGTCGTTGCTGCAA-GTCTCT(S), GGTCGATCTGTCTGCTGCTGCTGCT(S); BP2, TAGAAATGCTATCTTTCAGAAA(S), ACACCGAAATGATAAGCTT(AS); GNP1, ACATTATGACGCTTGGTAAT(S), ACACCAGAAATCAGACTC(S); TAT1, TAAAAATGGACCATGATGCT(S), GCACCCAGAATGCTGATCC(S); CAN1, TAGCAATTGACAAATCAGAA(S), TGCTACACACTTTCACATT(AS). Following amplification using Qiagen Taq polymerase with the following cycling parameters: 94 °C for 3 min; 30 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 1 min and 30 s, followed by 72 °C for 7 min, PCR products were run on a 1.4% agarose gel and visualized using standard molecular biological techniques. An internal control reaction using oligonucleotide primers to detect the actin gene was run in parallel.

Immunofluorescence of Green Fluorescent Protein (GFP)-tagged Yeast Strains—Yeast with GFP-tagged amino acid permeases were grown under conditions described above in glucose- or galactose-containing media. Immunofluorescence was performed using a Bio-Rad Radiance 2100 confocal system (Bio-Rad) coupled to a Nikon TE300 inverted microscope equipped with a Nikon 60× oil-immersion lens (numerical aperture = 1.4) with an argon laser line (488 nm) and a transmitted light detector for bright field images. Fluorescence and bright field images were acquired sequentially and analyzed using NIH Image] software.

RESULTS

We measured the uptake of neutral, cationic, and anionic amino acids in WT (M3750) strain grown in glucose or galactose media. The transport of all L-14C-amino-acids tested was linear for at least 40 min (Fig. 1) in both glucose or galactose media; subsequent uptake experiments were performed at 15 or 20min. l-α-Leucine, a neutral amino acid, is transported by all major neutral amino acid permeases (BAP2, BAP3, GNP1, TAT1, AGP1, and GAP1) in S. cerevisiae (17). The uptake of l-[14C]Leucine in WT (M3750) yeast grown in galactose medium was ~10-fold greater compared with those grown in glucose medium (Figs. 1 and 2). The addition of 5 μM unlabeled l-leucine almost completely abolished (90 ± 3%, n = 3) the l-[14C]leucine uptake measured in cells grown in galactose medium, indicating saturability. l-α-[14C]Alanine, another neutral amino acid, whose main carriers in S. cerevisiae are

| TABLE 1

| Genotype of amino acid permease deleted yeast strains  
| All strains detailed were MATα- and ura3-deleted. Details of the construction of the amino acid deleted strains can be found elsewhere (13). |
| Wild type  
| gap1Δ agp1Δ  
| gap1Δ agp1Δ gnp1Δ  
| gap1Δ bap2Δ  
| gap1Δ gnp1Δ (bap2-tat1)  
| gap1Δ (bap2-tat1) bap3Δ tat2Δ  
| gnp1Δ gnp1Δ Δ (bap2-tat1) bap3Δ tat2Δ  
| gnp1Δ (bap2-tat1) bap3Δ tat2Δ  
| l-arginine deleted strain, reverse primer) were used in the PCR reaction to detect the indicated amino acid permease genes: AGP1, ATGTCGTTGCTGCAA-GTCTCT(S), GGTCGATCTGTCTGCTGCTGCTGCT(S); BP2, TAGAAATGCTATCTTTCAGAAA(S), ACACCGAAATGATAAGCTT(AS); GNP1, ACATTATGACGCTTGGTAAT(S), ACACCAGAAATCAGACTC(S); TAT1, TAAAAATGGACCATGATGCT(S), GCACCCAGAATGCTGATCC(S); CAN1, TAGCAATTGACAAATCAGAA(S), TGCTACACACTTTCACATT(AS). Following amplification using Qiagen Taq polymerase with the following cycling parameters: 94 °C for 3 min; 30 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 1 min and 30 s, followed by 72 °C for 7 min, PCR products were run on a 1.4% agarose gel and visualized using standard molecular biological techniques. An internal control reaction using oligonucleotide primers to detect the actin gene was run in parallel. |
likely to be AGP1, PUT4, and DIP5 (17), showed a much smaller increase in uptake (~3-fold) in WT M3750 grown in galactose compared with those grown in glucose (Fig. 2).

The uptake of cationic amino acid L-[14C]arginine (5 μM) was also time-dependent (Fig. 1); a 25-fold increase in l-arginine uptake was observed (Figs. 1 and 2) in WT strain grown in galactose medium compared with glucose, which was also saturable, as addition of 5 mM L-arginine inhibited this uptake by 93 ± 2% (n = 3). Transport of L-[14C]lysine, another cationic amino acid, increased ~10-fold in the M3750 yeast grown in galactose medium compared with those in glucose medium (Fig. 2). The level of uptake of L-[14C]glutamate (5 μM) was relatively lower than that for l-lysine or l-arginine transport in M3750 grown in glucose (0.008 ± 0.002 pmol/10⁶/h cells, Fig. 2). However, there was a significant (5-fold) increase in L-[14C]glutamate uptake in M3750 grown in galactose medium (Fig. 2). Concurrent with the increase in uptake of amino acids, there was a 15 ± 1-fold increase in the oxygen consumption in yeast grown in 2% galactose compared with those grown in 2% glucose (n = 3).

Is the increase in amino acid transport in S. cerevisiae grown in galactose medium compared with those in glucose because of CCR or galactose induction? To answer this question we measured L-[14C]leucine and arginine uptake in M3750 grown in a non-fermentable carbon source, namely glycerol. There was an 8 ± 0.5 and 15.2 ± 0.3-fold (n = 3) increase in the uptake of L-[14C]leucine and arginine, respectively, in M3750 grown in glycerol compared with glucose medium. A 5.3 ± 0.5-fold increase (n = 3) was also observed for L-[14C]alanine uptake in galactol compared with glucose medium. These results indicate that the increase in amino acid transport is because of carbon catabolite repression.

We then tested other strains to determine the universality of CCR of neutral and cationic amino acid permeases in yeast. In another MATA strain BY4741, there was an 8- and 10-fold increase in leucine and arginine uptake, respectively, in yeast grown in galactose compared with glucose medium (Fig. 3); there was also an increase of 6 ± 0.4-fold in L-[14C]lysine and 3.2 ± 0.2-fold in L-[14C]glutamate uptake in yeast grown in galactose compared with glucose-containing medium in the BY4741 strain (similar results were obtained for the WT M3750 strain, Fig. 2). In a MATA type (Bio 101 strain) the uptake of both l-leucine and l-arginine showed increases of between 5–10-fold (Fig. 3) in yeast grown in galactose compared with glucose medium. In the diploid, INVSc1 strain there was a 5-fold increase in the uptake of both l-leucine and l-arginine (Fig. 3). These results indicate that there is an increase in the uptake of l-leucine and l-arginine when yeast are grown in galactose compared with glucose containing medium for MATA and MATA and haploid and diploid strains of S. cerevisiae.

Yeast amino acid permease expression and activity are thought to be regulated via nitrogen catabolite repression (NCR) or nitrogen catabolite inactivation (12, 18–20). A survey of the literature suggested that virtually no information exists on regulation of yeast amino acid permeases through CCR. After establishing that amino acid transport in S. cerevisiae is activated by CCR, we performed experiments to identify the permeases regulated by CCR and the mechanisms by which CCR transduces this activation.

To identify which neutral amino acid permeases might be functionally activated by CCR, we used the progeny of the WT M3750 strain sequentially deleted of all major neutral amino acid permeases (agp1, gap2, gap3, gnp1, gnp1, tat1, and tat2). Subsequent to growth of these strains in glucose or galactose medium and measurement of L-[14C]leucine transport (as it is a common substrate for these permeases (17)), we have been able to ascribe the neutral amino acid per-
meases activated because of CCR. Fig. 4 shows L-[14C]leucine (5 μCi) uptake measured in wild type and progeny strains deleted of the following seven major yeast amino acid transporters: AGP1 and GAP1 (transport all common 20 L-amino acids), GNP1, BAP2, and BAP3 (also transport leucine but display a narrower substrate specificity to that observed for AGP1 or GAP1), and TAT1 and TAT2 (transport aromatic amino acids in addition to leucine) (17). L-[14C]Leucine uptake in the gap1Δ agp1Δ strain showed no difference compared with the wild type indicating that these permeases did not contribute to the observed increase in amino acid transport. However additional gene deletions reduced galactose-induced leucine transport in comparison to the wild type by 36% (gnp1Δ), 62% (bap2Δ), and 83% (Δ(bap2-tat1)). Deletion of bap3 from the gap1Δ Δ(bap2-tat1) background did not cause any further decrease of L-[14C]leucine uptake, indicating that these two permeases are not regulated by CCR. Considered in their entirety, these results indicate that the bulk of the increase in L-leucine uptake by CCR occurs via BAP2, GNP1, and TAT1, whereas AGP1, GAP1, or BAP3 are not regulated by CCR.

The CCR-regulated L-[14C]leucine uptake was also abolished in the ssy1Δ yeast (Fig. 4). SSY1 is an amino acid permease homologue that mediates amino acid-induced transcription of leucine transporters (e.g. BAP2 and TAT1) by "sensing" amino acids (particularly leucine) in the yeast growth medium (21). Abolition of the CCR-regulated L-[14C]leucine uptake in ssy1Δ yeast indicates that the mechanism of CCR of amino acid transporter activity is downstream of the Ssy1p regulation of the transcription of leucine permeases in yeast. The increase in the transport of cationic amino acid L-14C]arginine in the galactose medium was largely maintained in the ssy1Δ yeast (8.5 ± 1.3-fold increase in galactose compared with glucose medium, n = 3). These results are consistent with the idea that Ssy1p is not implicated in the regulation of the major L-arginine transporter CAN1 (21).

To elucidate the molecular mechanism by which the increase in amino acid permease activity occurs under CCR, we first tested the hypothesis that this may be because of increase in gene transcription for the amino acid permeases. Semiquantitative PCR (Fig. 5) showed there was a small (~1.5-3-fold) increase in the RNA transcripts of BAP2, GNP1, TAT1, and CAN1, whereas no differences were observed for AGP1 or the actin control genes. However, this small increase in transcription is not likely to explain the large increases observed in the activity of amino acid permeases. We therefore used the BAP2 and GNP1 strains (the two major permeases responsible for CCR-induced increase in amino acid uptake) from the yeast-GFP clone collection (15) to assess whether their protein expression was increased under CCR. The yeast-GFP clone collection is designed to express full-length proteins, tagged at the carboxyl-terminal end with GFP, from their endogenous promoters. A very faint GFP signal was observed for Bap2-GFP and Gnp1-GFP yeast grown in glucose YNB medium, although this is not visible under optimal imaging conditions (Fig. 6, A and E). However, following growth in galactose YNB medium there was a large increase in the expression of Bap2-GFP (Fig. 6B) and Gnp1-GFP (Fig. 6F). These results indicate that translation of BAP2 and GNP1 messages is greatly enhanced under CCR. It is also apparent from a comparison of Fig. 6, B and F (Bap2-GFP and Gnp1-GFP, respectively, both grown in galactose-containing medium) that the GFP signal is greater for Bap2. This increase (2.5 ± 0.3-fold) in the GFP signal for Bap2 compared with
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FIGURE 6. Direct immunofluorescence was measured in yeast strains with GFP-tagged BAP2 and GNP1 using a Bio-Rad Radiance 2100 confocal system. Bap2-GFP fluorescence in yeast grown in 2% glucose YNB (A) compared with 2% galactose YNB medium (B) is shown. Micrographs C and D are corresponding bright field images of A and B. Gnp1-GFP fluorescence of yeast grown in 2% glucose YNB (E) compared with yeast grown in 2% galactose YNB medium (F) is shown. G and H are corresponding bright field images of E and F. Representative pictures of experiments performed in triplicate are presented. Identical image acquisition and analysis parameters were applied when acquiring and analyzing images from yeasts grown in glucose- or galactose-containing medium.

Gnp1 largely reflects their respective functional contribution to the CCR-induced increase in \( ^{14} \text{C} \)-leucine uptake.

Distinct signaling pathways have been delineated in response to CCR in \( S. \text{ cerevisiae} \) (4, 5). We next considered the signaling mechanisms that may be involved in the functional activation of amino acid permeases in response to CCR. We tested the hypothesis that functional activation of amino acid permeases by CCR was controlled via the SNF1/MIG1 signaling pathway by measuring \( ^{14} \text{C} \)-leucine and arginine uptake in the mig1Δ (BY4741 background) strain. As shown earlier (Fig. 3), neutral and cationic amino acid uptake increased by 10–15-fold when the parent BY4741 was grown in galactose compared with glucose. However, CCR-induced amino acid uptake was similar in both mig1Δ and the parent (BY4741) strains grown in galactose medium (Fig. 7), indicating that the SNF1/MIG1 pathway is not involved in the regulation of amino acid permeases under CCR.

Other nutrient-regulated intracellular protein kinases involved in signaling during conditions of low glucose or amino acids are GCN2 (4, 5) and PAS kinases (22). We next tested whether GCN2 and PAS kinase pathways were involved in the CCR-induced increase in amino acid permease activity. The uptake of both \( ^{14} \text{C} \) arginine and leucine was measured in gcn2Δ and psk2Δ strains. There was no reduction in the uptake of either amino acid in gcn2Δ or psk2Δ compared with parent BY4741 yeast grown in galactose medium (Fig. 7).

The TOR pathway is another important nutrient-regulated signaling pathway in yeast. Unlike SNF, GCN2, or PAS kinases, TOR (a member of the phosphatidylinositide kinase family) is linked to the availability of nitrogen (4, 6, 7, 9). Furthermore, apart from TAT2 and HIP1 that were shown to be down-regulated in response to short term nitrogen starvation, no other amino acid permease is known to be regulated by the TOR1 signaling pathway in yeast (7). After exhausting the possibility of the involvement of the nutritional transducers known to regulate CCR in yeast, we tested the idea that TOR1 might be involved in the functional up-regulation of amino acid permease activity observed when yeast were grown in galactose medium. In the tor1Δ strain, grown in glucose medium, uptake of \( L \) \( ^{14} \text{C} \)-labeled arginine and leucine was increased between 2 and 4-fold compared with the parent BY4741 strain (Fig. 7). However, deletion of TOR1 from the parent BY4741 strain eliminated the CCR-induced \( L \) \( ^{14} \text{C} \)-leucine and arginine uptake (Fig. 7).

These results demonstrate that the phosphatidylinositide kinase signaling pathway regulates the functional activation of neutral and cationic amino acid permease genes under CCR.

**DISCUSSION**

We have made the novel discovery that transport of neutral, cationic, and anionic amino acids is regulated by CCR at the protein expression and functional levels. By measuring \( L \) -leucine uptake in a series of strains sequentially deleted of amino acid permease genes, we have been able to assign the neutral amino acid permeases whose activity is increased as a result of CCR. Thus we have shown for the first time that BAP2, GNP1, and TAT1 are all under the regulation of CCR, as are the cationic amino acid permeases. Our results indicate that the most likely mechanism of the increased amino acid permease activity under CCR is the increase in the translation of the permease mRNA. We have further discovered that regulation of amino acid permeases by CCR is not via the SNF1 nutritional transduction pathway, generally associated with CCR, but a transducer more commonly associated with NCR, namely the TOR pathway (4).

**Possible Metabolic Consequences of Increased Amino Acid Intake under CCR**—Amino acid uptake from the medium through the yeast permeases is an important determinant of the behavior of the yeast and...
plays an important role in protein synthesis and other processes of cell metabolism (12). Our results demonstrate that the uptake of all three classes of amino acids, neutral, cationic, and anionic is increased by CCR. These amino acids form common intermediates for the major catabolic pathways such as acetyl CoA (leucine, isoleucine, lysine), pyruvate (alanine, serine), or 2-oxoglutarate (arginine, glutamine, and glutamate). Thus amino acid supply and utilization could be paramount when yeast metabolism shifts from fermentative, in high glucose, to respiratory in glucose-substituted medium. In the light of the above observations, a prima facie case for the utility of the observed increase in the amino acid uptake could be made. During the conditions of CCR the yeast metabolism shifts from fermentative to respiratory (as demonstrated by a 15-fold increase in oxygen consumption in yeast grown in galactose compared with glucose), and carbon is shunted to the mitochondrial tricarboxylic acid cycle, thus increasing the electron transport and respiration (2, 10). A concomitant increase in the uptake and degradation of amino acids could fuel the production of direct and indirect substrates (e.g. fumarate, α-ketoglutarate, and pyruvate) for the tricarboxylic acid cycle. The three major neutral amino acid permeases identified as candidates for regulation by CCR are all involved in the transport of a wide variety of substrates (12, 21). For example, GNP1 is the most efficient L-glutamine transporter in yeast (12, 23) that also transports amino acids such as L-leucine and threonine. In S. cerevisiae, amino acids with a nitrogen side chain, e.g. glutamine and asparagine, serve as an important source of nitrogen, critical for cell growth and viability. Also, branched chain amino acids such as L-leucine and L-isoleucine, both substrates for BAP2 and TAT1, can be utilized in amino acid oxidation via the tricarboxylic acid cycle.

Regulation of Gene Expression under CCR—CCR in yeast is a well known regulator for a variety of genes such as MLS1 (malate synthase (24)), ACS1 (acetyl-CoA synthase (25)), and ICL1 (isocitrate lyase (26)). Of the genes known to be regulated by CCR in yeast, all have a nucleotide motif (CCRTYSRNCCG, the CSRE motif) that acts as a signal for genes under the control of CCR. Our search of the Saccharomyces genome data base did not return any matches for this motif on amino acid permease genes, indicating that the carbon catabolite repression of amino acid permeases is perhaps mediated by other mechanisms. The presence of a novel regulatory motif, specific for amino acid permeases remains open and would require further analysis of the upstream promoter regions.

Nutrient-sensing Protein Kinases and Regulation of Amino Acid Permeases—Our results also eliminate the involvement of various nutrient-sensing protein kinases in yeast (4) in the activation of amino acid permease activity due to CCR. Deletion of MIG1, responsible for repression of many genes that are dispensable to yeast cells growing on high levels of glucose (27) via the SNF pathway, did not alter activation of amino acid permease activity because of CCR, eliminating the involvement of the AMP-activated protein kinase pathway. Similarly, the GCN2 pathway, activated in the presence of low nutrients (glucose or amino acids) (4), is not likely to play a role in the amino acid permease activation due to CCR (Fig. 7). Furthermore, deleting PKS2, one parologue of the two genes encoding the PAS kinase recently implicated in galactose utilization in yeast (22), did not alter the increase amino acid permease activation because of CCR. Galactose fails to support yeast growth in PSK1/PSK2 double mutants (although PSK2 mutants are also challenged but to a lesser extent) presenting a temperature-sensitive galactose utilization phenotype (22). The lack of effect of psk2Δ upon amino acid permease activation because of CCR suggests, minimally, that this phenomenon is not likely to be because of metabolic changes initiated by alteration in galactose metabolism.

By this process of elimination we have made the novel discovery that the signaling of the activation of neutral and cationic amino acid permeases because of CCR is via TOR1 and not through the SNF1/MIG1, GCN2, or PAS kinase pathways. It has been known for some time that a number of amino acid permeases, both neutral (GAP1, TAT2) and cationic (HIP1, CAN1, LYP1), are regulated by NCR (6, 7, 12). A considerable amount of information also exists on the mechanisms of NCR-mediated transcriptional regulation as well as sorting and degradation of these permeases (7, 28–30). GAP1 is up-regulated upon nitrogen starvation via a Ser/Thr nitrogen permease reactivator kinase (NPR1) (6, 19). However, no information is available on the effects of long term glucose substitution or starvation on yeast amino acid permeases, particularly in the context of nutrient signaling mechanisms. That TOR1 is involved in the regulation of NCR-mediated regulation of amino acid permease is well established (6, 9, 31); what is surprising is that amino acid permeases may also be regulated through the TOR1 pathway as a result not of NCR but of CCR.

The precise sequence of events involved in the TOR-mediated CCR-induced increase in amino acid transporter activity remain to be discovered. The fact that the CCR-induced amino acid uptake is abolished in tor1Δ strain, however, clearly implicates TOR1 in this process. It is also clear that a major contributory mechanism that may explain the increase of amino acid transport activity under CCR is increase in the protein translation for the permeases involved. A TOR-mediated functional up-regulation of amino acid permeases in yeast as a result of CCR may therefore provide a useful experimental model to investigate the coordination of nitrogen and carbohydrate metabolism and the likely complex interaction of various nutritional transducers.

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