The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores

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Summary

To insure an adequate supply of nutrients, omnivores choose among available food sources. This process is exemplified by the well-characterized innate aversion of omnivores to otherwise nutritious foods of imbalanced amino acid content. We report that brain-specific inactivation of GCN2, a ubiquitously expressed protein kinase that phosphorylates translation initiation factor 2 α (eIF2α) in response to intracellular amino acid deficiency, impairs this aversive response. GCN2 inactivation also diminishes phosphorylated eIF2α levels in the mouse anterior piriform cortex following consumption of an imbalanced meal. An ancient intracellular signal transduction pathway responsive to amino acid deficiency thus affects feeding behavior by activating a neuronal circuit that biases consumption against imbalanced food sources.

Introduction

Animals depend on their environment for essential nutrients, and complex behavioral patterns have evolved to ensure consumption of a balanced diet. These are especially important in case of omnivores that have to choose among a variety of available food sources. While culture and conditioning are clearly important in such choices, innate factors are also involved (Louis-Sylvestre, 1976; Eaton and Konner, 1985), and understanding the underlying molecular mechanisms is therefore of considerable interest.

A remarkable example of an innate mechanism governing food choice is presented by the fact that omnivorous animals will consume substantially less of an otherwise identical experimental meal lacking a single essential amino acid (Harper et al., 1970; Gietzen, 1993). The basis for this innate aversive response, which biases the diet against imbalanced food sources, is poorly understood. Cell ablation experiments and localized injection of the limiting amino acid have implicated the anterior piriform cortex (APC) in sensing postprandial blood amino acid levels and in initiating an acute food aversion when blood levels of even a single amino acid are disproportionately reduced by the imbalanced composition of the food (Leung and Rogers, 1971; Gietzen, 1993).

It has been reported recently that consumption of a meal of imbalanced amino acid composition selectively elevates levels of phosphorylated eIF2α in APC neurons, establishing a molecular correlate to the aversive response (Gietzen et al., 2004). Phosphorylation of eIF2α on serine 51 serves as a highly conserved signal that adapts cells to a variety of stresses (Dever, 2002; Harding et al., 2002). A protein kinase, GCN2, conserved from yeast to mammals, specifically couples the accumulation of uncharged transfer RNAs to this phosphorylation event (Hinnebusch, 1994; Berlanga et al., 1999; Harding et al., 2000; Zhang et al., 2002). GCN2 is therefore an important sensor of amino acid homeostasis inside cells, and it activates downstream rectifying responses mediated by phosphorylated eIF2α. The latter effect gene expression programs at the level of mRNA translation and transcription (Hinnebusch, 1994; Harding et al., 2000; Harding et al., 2003). GCN2 is ubiquitously expressed and is thus poised to respond to the amino acid imbalance by promoting eIF2α phosphorylation in the APC and thereby contribute to initiation of the aforementioned food aversion.

Results

To explore GCN2's role in the aversive response to a diet of imbalanced amino acid content, we used homologous recombination in ES cells to create mouse strains with a *GCN2* allele constitutively missing exon XII (*GCN2*–) or a conditional allele (*GCN2*^{conditional}) in which exon XII is flanked by *lox*P sites, which serve as targets for Cre-mediated recombination (Figure 1). Exon XII encodes a region of GCN2 that binds ATP, and its deletion eliminates kinase activity (Harding et al., 2000).

We compared the rate of food consumption by wild-type and knockout mice (*GCN2*$^{-/-}$) offered balanced chow or an otherwise identical chow lacking a single essential amino acid. Over the same time interval, wild-type mice consumed significantly less imbalanced chow than balanced chow: 28.8% ± 2.7% and 34.1% ± 1.5% less leucine-deficient chow was consumed by the wild-type mice after 1 hr ($p = 0.005$) and 4 hr ($p = 0.0003$) (Figure 2A). This aversive response was markedly blunted in the GCN2 knockout mice who consumed only 7.7% ± 3.1% and 9.4% ± 1.3% less of the leucine-deficient diet after 1 hr ($p = 0.13$) and 4 hr ($p = 0.05$). The aversive response to threonine-deficient diet was likewise blunted in the homozygous mutant mice (Figure 2A), whereas mice heterozygous for the GCN2 mutation exhibited a wild-type aversive response (data not shown). A mixed model ANOVA was performed to discriminate among the parameters tested (genotype, diet, and time), and a significant effect was observed only for genotype ($p <$

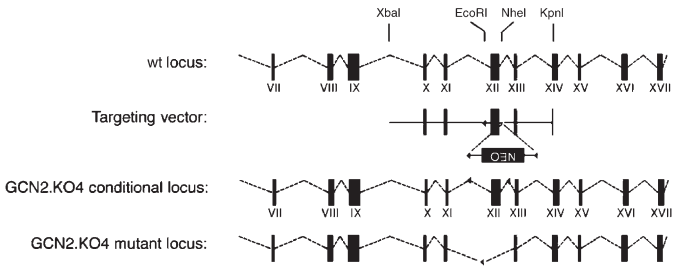

Figure 1. Targeting the *GCN2* locus

The region of the mouse *GCN2* gene (also known as *Eif2ak4*) encompassing exons VII–XVII is shown as is the structure of the targeting vector. The *lox*P sites flanking exon XII are depicted by the small triangles in the cartoon of *GCN2.KO4*^{conditional} locus, and deletion of exon XII by recombination across the two *lox*P sites is shown in the cartoon of the *GCN2.KO4* mutant locus.

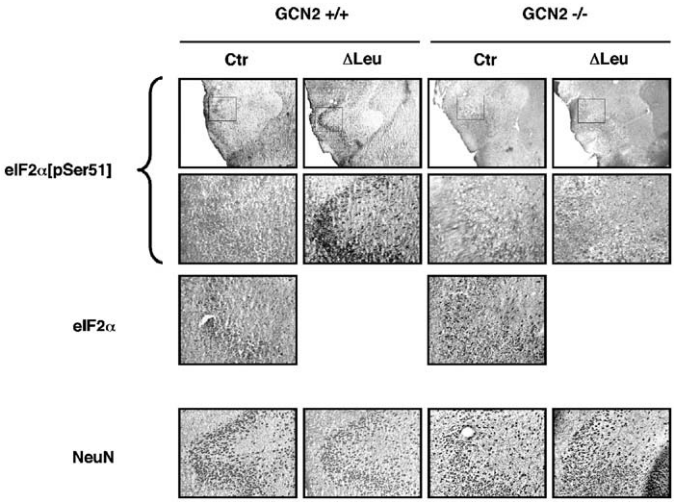

Figure 3. Mice lacking GCN2 have impaired eIF2α phosphorylation in the anterior piriform cortex

Immunohistochemistry of fixed brain sections of GCN2$^{+/+}$ and GCN2$^{-/-}$ mice fed balanced chow (Ctr) or chow lacking leucine (ΔLeu) for 20 min. The sections were stained with antisera reactive with eIF2α phosphorylated on serine 51 (eIF2α[pSer51], upper panels), total eIF2α (middle panels), or the neuronal marker NeuN (lowest panel). The region boxed in the uppermost panel is displayed at higher magnification in the panels below.

0.0001; see Table S1 in the Supplemental Data available with this article online). Serum amino acid levels were decreased to similar levels by the imbalanced diets in both genotypes (Figure 1B), indicating altered response to amino acid deficiency in mice lacking GCN2 activity.

To determine if GCN2 affects levels of phosphorylated eIF2α in the APC of mice consuming imbalanced chow, we compared the immunoreactivity of fixed brain sections from wild-type and GCN2 knockout mice with an antiserum that specifically recognizes the phosphorylated form of eIF2α. High levels of phosphorylated eIF2α were detected in the APC of wild-type mice after as little as 20 min of consumption of an imbalanced meal (as previously described [Gietzen et al., 2004]), whereas we observed no such signal in the mutant mice (Figure 3, upper panels). Total eIF2α staining showed that its expression level is not affected by genotype (Figure 3, middle panel). This observation indicates a role for GCN2 in mediating eIF2α phosphorylation in the APC of mice fed an imbalanced diet.

Consumption of an imbalanced meal also activates GCN2 in peripheral tissues and promotes eIF2α phosphorylation in the liver (Zhang et al., 2002; Anthony et al., 2004; Figure 4A). To distinguish the roles of GCN2-induced eIF2α phosphorylation in the periphery and brain on the aversive response to an imbalanced meal, we studied a conditional allele of GCN2 that is inactivated selectively in the brain by the expression of Cre recombinase from a nestin promoter (Betz et al., 1996). Introduction of this Nes::Cre transgene led to selective excision of the essential GCN2 exon XII in the brain (Figure 4B) but had no impact on eIF2α phosphorylation in the liver (Figure S1). Comparison of consumption of balanced and imbalanced chow showed that mice with brain-specific ablation of GCN2 function were also impaired in their aversive response to an imbalanced diet (Figure 4C). These observations implicate GCN2 signaling in the brain in activation of the aversive response. Together with the defect in eIF2α phosphorylation observed in the APC of GCN2 mutant mice fed imbalanced chow (Figure 3), they suggest a special role for GCN2 in neurons in that region of the brain in mediating the aversive response.

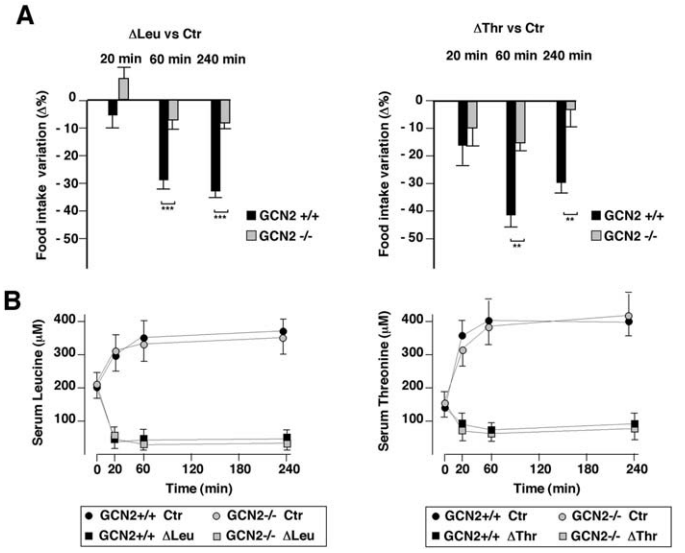

Figure 2. Mice lacking the GCN2 kinase are impaired in their aversive response to amino acid-imbalanced food

A) The relative consumption of balanced chow (Ctr) and chow lacking the indicated amino acid over the course of a meal in wild-type (GCN2$^{+/+}$) and mutant mice (GCN2$^{-/-}$) expressed as the ratio of consumption (Δ% ± SEM) of the amino acid-devoid diets (ΔLeu and ΔThr) to the consumption of the control diet (Ctr) by the same animal. These results represent the mean (±SEM) of six measurements for each diet, obtained with two different cohorts of ten males for each genotype. ANOVA analysis showed that a significant effect is observed only for genotype ($p < 0.0002$), and nonpaired t tests were used to compare the food intake variation (Δ%) between the two genotypes (***, $p < 0.01$; **, $p < 0.05$; *, $p < 0.1$).

B) Plasma leucine and threonine levels (μM) of mice fed the indicated chow.

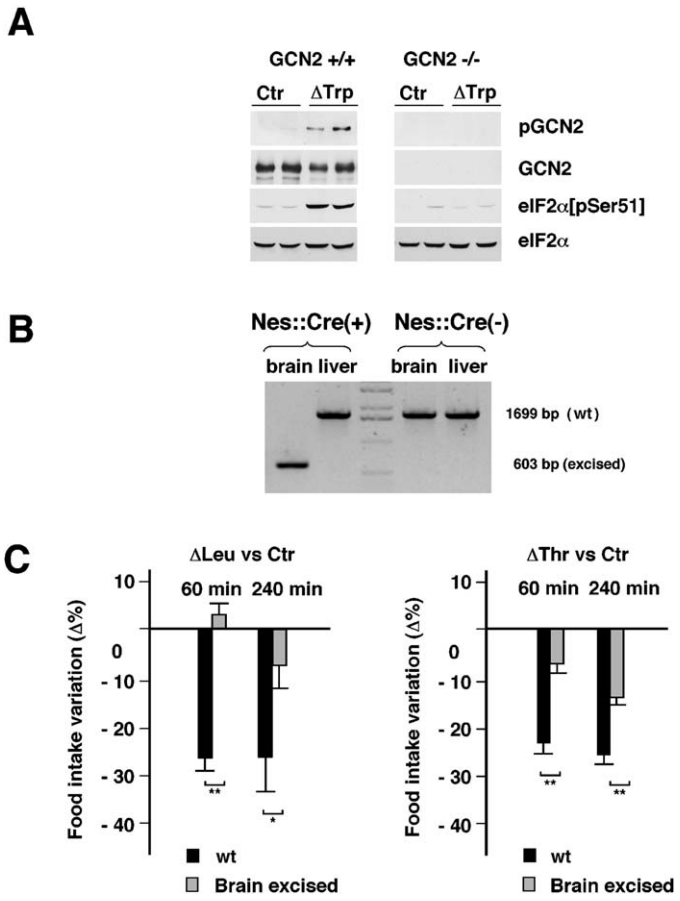

Figure 4. Selective ablation of GCN2 in the brain attenuates the aversive response to food with imbalanced amino acid content

A) Consumption of imbalanced food activates GCN2 in the liver. Shown are immunoblots of activated GCN2 (phosphorylated pGCN2), total GCN2, phosphorylated eIF2α (eIF2α [pSer51]), and total eIF2α in lysates from paired duplicates of mice of the indicated genotype after feeding on balanced chow (Ctr) or chow lacking tryptophan (ΔTrp).

B) Selective excision of the GCN2 gene in the brain of mice by localized expression of Cre recombinase from a nestin promoter (Nes::Cre). Shown are allele-specific PCR fragments of genomic DNA from the brain and liver of mice homozygous for a conditional allele of GCN2 and varying in the presence of the Nes::Cre transgene.

C) The relative consumption of balanced chow (Ctr) and chow lacking the indicated amino acid over the course of a meal in mice with (wt) and without GCN2 activity in the brain ("Brain excised"). These results represent the mean (±SEM) of 4 measurements for each diet, obtained with two different cohorts of eight males for each genotype. ANOVA analysis showed that a significant effect is observed only for genotype ($p < 0.0002$). Nonpaired t tests were used to compare the food intake variation between the two genotypes (**, $p < 0.05$; *, $p < 0.1$).

Discussion

The studies described herein assign an important role to the eIF2α kinase GCN2 in mediating aversion toward foods with an imbalanced amino acid composition. Analysis of the phenotype of mice with selective ablation of GCN2 in the brain suggests a requirement for GCN2 activity in the brain for the development of the aversive response. Furthermore, the correlation established here between GCN2 activity and the phosphorylation of its substrate, eIF2α, in the anterior piriform cortex, implicates that specific brain region in the aversive response.

Consumption of an imbalanced diet also activates GCN2 and promotes eIF2α phosphorylation in the liver (Zhang et al., 2002; Anthony et al., 2004; Figure 4A). Because of its anatomic location downstream in the portal circulation, the liver is likely to be the first organ to sense the imbalanced composition of the diet. The observation that brain-selective ablation of GCN2 had a less-pronounced negative effect on the aversive response than constitutive knockout of GCN2 function suggests that GCN2 activation in the liver (and perhaps in other organs) might also contribute to the aversive response. However, the relatively smaller number of conditionally ablated animals studied and the wider distribution of the results render this conclusion tentative.

The evidence is consistent with a role for GCN2 in a proximal step that senses the decline in serum amino acid(s) level following consumption of an imbalanced meal. Extracellular amino acid deficiency is known to cause accumulation of uncharged cognate tRNAs. These bind to and directly activate the GCN2 kinase (Dong et al., 2000), whose only known substrate is the α subunit of translation initiation factor 2. Phosphorylation of eIF2α on serine 51 inhibits the guanine exchange factor for the eIF2 complex and thereby attenuates the initiation step of protein synthesis (Hinnebusch, 1994). In addition to promoting a global decrease in protein synthesis, eIF2α phosphorylation effects a measure of gene-specific translational control that reprograms gene expression at the translational level. Furthermore, eIF2α phosphorylation translationally activates the mRNA encoding the transcription factor ATF4, thereby altering gene expression at the transcriptional level (Harding et al., 2000; Harding et al., 2003). Therefore, GCN2-induced eIF2α phosphorylation may promote the aversive response through its effects on translation and transcription in cells of the APC, in other brain regions, and in other peripheral organs.

GCN2's role in coupling amino acid deficiency to eIF2α phosphorylation is conserved in all known eukaryotes. Our findings indicate that in omnivores this ancient pathway is exploited to recognize depressions in serum amino acid levels that occur during consumption of food with an imbalanced composition of amino acids, culminating in a behavioral response that limits consumption of imbalanced foods and favors, by default, a balanced diet. Following submission of this manuscript, a paper with confirmatory findings was published (Hao et al., 2005).

Experimental procedures

Gene targeting and animal breeding

The targeting vector was constructed from PCR fragments amplified from cloned mouse embryonic stem cell genomic DNA.

The 5′ homology arm was a 6329 base pair genomic XbaI-EcoRI fragment terminating 147 base pairs 5′ of exon XII. It was ligated into a pBS plasmid containing a thymidine kinase (TK) negative selection cassette. A double-stranded oligonucleotide pair with EcoRI linkers containing a *lox*P site was introduced into the EcoRI site. This 5′ *lox*P site thus flanks exon XII on its 5′ end. The 3′ homology arm was recovered as a PCR fragment whose 5′ end is the aforementioned genomic EcoRI site and whose 3′ end is in the seventeenth codon of exon XIV at a KpnI site introduced by the oligonucleotide used in the PCR. This 4023 base pair fragment was inserted at the EcoRI-KpnI sites of the aforementioned pBS plasmid. The *lox*P-flanked Neo^R selection cassette was inserted into an intronic NheI site 530 base pairs 3′ of exon XII, as shown in Figure 1.

W4 ES cells were transfected with the targeting vector that was linearized at the KpnI site, and homologous recombination was confirmed by PCR

and Southern blotting. The targeted ES cells were transfected transiently with a Cre-recombinase expression plasmid, and derivative clones that had recombined across the 2 *lox*P sites flanking the Neo^R selection cassette (GCN2.KO4 conditional locus) as well as clones that had recombined across all three *lox*P sites (GCN2.KO4 mutant locus) were isolated. The latter, GCN2.KO4 mutant allele ($GCN2^-$) deletes a 1101 base pair fragment that encompasses exon XII, which encodes a region of the kinase domain required for ATP binding. The null phenotype associated with deletion of exon XII has been previously documented (Harding et al., 2000). In addition, splicing of exon XI to exon XIII is predicted to disrupt the reading frame of the mRNA and introduce multiple stop codons that destabilize the mRNA. Indeed, no GCN2 signal is detected by immunoblot in cells and tissues derived from $GCN2.KO4^{-/-}$ mice. The mutant allele is thus likely to be a null and is certain to direct no eIF2α kinase activity.

The mutant and corresponding wild-type or conditional alleles are detected by a two-primer PCR assay in which mGCN215S (TCT CCC AGC GGA ATC CGC ACA TCG) and mGCN218AS (T GCC ACT GTC AGA ATC TGA AGC AGG) give a wild-type band of 1665 base pairs, a conditonal band of 1699 base pairs and a mutant band of 603 base pairs.

Chimeric animals were derived by blastocyst injection of $GCN2.KO4^{+/-}$ or $GCN2.KO4^{conditional/+}$ ES cells, and the modified allele was transmitted through the germline of these chimeras by mating to C57BL/6 females. The $GCN2^-$ allele was subsequently backcrossed for 10 generations into C57BL/6, whereas the conditional allele was bred to homozygosity in the 129svev;C57BL/6 F2 background and thus maintained by intercrossing siblings. Nestin::Cre animals were obtained from Jackson lab and bred into the $GCN2.KO4^{conditional}$ background.

Male animals with $GCN2^{+/+}$ and $GCN2^{-/-}$ genotypes were derived by crossing +/– animals, whereas animals with brain-specific excision of GCN2 exon XII were derived by crossing $GCN2.KO4^{conditional/+}$; nestin::Cre(+) to $GCN2.KO4^{conditional/conditional}$.

Diets, food intake measurements, and activation of GCN2 in the liver

All animal experiments were approved by the INRA and NYU institutional animal utilization and care committee in conformance with French, European Union, and New York laws. The aversive response was studied in France, whereas animal production and activation of GCN2 in the liver was in the United States. The diets used to study the aversive response were semiliquid and manufactured in INRA using purified ingredients (Louis François, France) and free L-amino acids (Jerafrance, France; Table S2). All diets contained free L-amino acids (calculated on the base of the amino acid composition of a 20% lactoserum diet as the sole protein source). The amino acid-devoid diets were adjusted with alanine. In the leucine-devoid diet, isoleucine and valine levels were also reduced in order to keep the blood concentration of these amino acids constant after eating the leucine-devoid diet (Table S3). The tryptophan-deficient diet used to induce GCN2 in the liver was manufactured by Research Diets, New Brunswick, New Jersey. In experiments not shown, we confirmed that a tryptophan-deficient diet will also induce an aversive response.

Food intake experiments were performed on pairs (to avoid isolation stress) of 2- to 3-month-old male mice (22–28 g) in metabolic cages to avoid food spillage and coprophagy. The cohorts were constituted of ten male mice of each genotype. The room was maintained at 22°C ± 1°C. Within 2 weeks, the animals were progressively trained to a reversed light:dark cycle, with food deprivation during the light phase, and to the novelty of the metabolic cages and the semiliquid diet. After the training phase, animals weight and food consumption were measured daily. The control diet intake was measured for three days at regular intervals before submitting the mice to the amino acid-deficient diet following the same parameters; thus, each animal served as its own control. Between experiments, the experimental diet was replaced by the control diet for at least 3 days to allow for recovery. The food intake values were normalized to animal weight. These experiments were repeated with two different cohorts and three times for a given cohort.

Measurement of GCN2 activation and eIF2α phosphorylation by immunoblot (Harding et al., 2000) were performed on a detergent extract of liver from paired mice fed a control chow or the Trp-deficient or Leu-deficient diet for 2 hr.

Statistical analysis

All food intake data are expressed as mean Δ% ± SEM. An ANOVA analysis showed that a significant effect is observed only for genotype ($p < 0.0002$; Table S1). Paired t tests were used to compare the food intake between control and amino acid-devoid diets in each group, and nonpaired t tests were used to compare the food intake variations (Δ%) between the two genotypes (the p value is given in the figure).

Plasma amino acid analysis

Blood samples were drawn from the aorta of anaesthetized mice. Plasma samples were treated with sulfosalicylic acid and thiodiglycol. Free amino acid proportions were determined using ion-exchange liquid chromatography followed by postcolumn detection with ninhydrine (Bio-Tek system). The internal standard, norleucine, allowed the evaluation of sample treatment efficiency in order to correct the crude values.

Immunohistochemistry

Animals were deeply anaesthetized with pentobarbital (100 mg/kg) and perfused through the heart with 10 ml PBS followed by 200 ml 4% paraformaldehyde in PBS at 4°C. Brains were removed and cryoprotected in 15% sucrose in PBS for 24 hr at 4°C. Coronal sections of 20 μm thickness were collected on SuperFrost Plus slides and stored at –80°C. Immunolabeling was performed as previously described (Carnevalli et al., 2004). Adjacent sections were washed in PBS for 30 min at room temperature, incubated with 0.3% H_2O_2 in PBS for 30 min, washed again for 30 min, and incubated in blocking buffer for 30 min (PBS containing 0.3% Triton X-100 and 0.5% normal goat serum [Vector Laboratories, Inc.]). Incubation with either an antibody that reacts specifically with eIF2α phosphorylated on serine 51 (anti-eIF2α[P] rabbit antibody, Biosource International), with total eIF2α (rabbit antibody from Cell Signaling Technology), or with a neuronal marker monoclonal anti-NeuN mouse antibody (Chemicon; diluted 1:1000 in blocking buffer) were performed at room temperature for 24 hr. The sections were then washed with PBS for 30 min, incubated with goat (anti-rabbit or anti-mouse, respectively) biotinylated IgG (Vector Laboratories, Inc.; 1:200) for 2 hr, washed again with PBS for 30 min, and incubated with a horseradish peroxidase-coupled avidin/biotin complex (Vector Elite ABC kit) for 90 min. The substrate used was 3,3′-diaminobenzidine tetrahydrochloride (DAB). The reaction was intensified by nickel. The brain sections were imaged using a Sony camera connected to a Zeiss microscope equipped with 2.5× and 10× objectives.

Supplemental data

Supplemental data include one figure and three tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/1/4/273/DC1/.

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