

Dietary Energy Balance Modulates Signaling through the Akt/Mammalian Target of Rapamycin Pathways in Multiple Epithelial Tissues

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Abstract

The prevalence of obesity, an established risk factor for several types of cancer, has increased steadily over the past several decades in the United States. New targets and strategies for offsetting the effect of obesity on cancer risk are urgently needed. In the present study, we examined the effect of dietary energy balance manipulation on steady-state signaling in multiple epithelial tissues, with a focus on the Akt and mammalian target of rapamycin (mTOR) pathways. For these experiments, male FVB/N and C57BL/6 and female ICR mice were maintained on a control (10 kcal% fat) diet, a diet-induced obesity (DIO; 60 kcal% fat) regimen, or a 30% calorie restriction (CR) regimen for 15 to 17 weeks. Relative to the control group, the DIO regimen increased, whereas CR decreased, circulating insulin-like growth factor-I (IGF-I) as has previously been reported. Western blot analyses showed that the DIO regimen enhanced, whereas CR inhibited, activation of Akt and mTOR, regardless of epithelial tissue or genetic background. In contrast, activation of AMP-activated protein kinase was modulated by dietary energy balance manipulation in the liver but not in the epidermis or dorsolateral prostate. Western blot analyses of epidermal extracts taken from ICR mice also revealed reduced activation of both the IGF-I receptor and epidermal growth factor receptor in CR mice, compared with control mice or mice maintained on the DIO regimen. Taken together, these novel findings suggest that dietary energy balance modulates signaling through cell-surface receptors (i.e., IGF-I receptor and epidermal growth factor receptor), affecting activation of multiple downstream pathways including Akt and mTOR, thus providing important dietary and pharmacologic targets for disrupting the obesity-cancer link.

The prevalence of obesity has dramatically increased over the past 40 years in the United States, with nearly two thirds of adult Americans currently considered as overweight and nearly one third as obese (1). The obesity rates in children and adolescents are alarmingly increasing as well (1). Epidemiologic studies have established obesity as an important risk factor for several types of epithelial cancers. For example, the American Cancer Society conducted a large prospective study of the relationship between obesity and cancer and es-

timated that 20% of total cancer deaths in women and 14% of cancer deaths in men are attributable to excess body weight (2). Insights into the mechanisms underlying the increased cancer risk associated with obesity are urgently needed to develop new strategies for preventing and treating obesity-related cancers.

In experimental model systems, calorie restriction (CR), which induces negative energy balance and prevents or reverses obesity, is arguably the most potent dietary-based intervention for preventing cancer (3). CR has been shown to inhibit formation of spontaneous neoplasias in several knockout and transgenic mouse models, to suppress tumor growth in tumor transplant models, and to inhibit radiation-induced and chemically induced carcinogenesis in a variety of rodent cancer models (3-8). In contrast, tumor development is generally enhanced in rodent models of diet-induced obesity (DIO; refs. 5, 9-12). Despite the well-established anticancer effects of CR, no mechanism of inhibition has been clearly identified.

We and others have previously established that reductions in circulating insulin-like growth factor-I (IGF-I) are associated with the anticancer effects of CR in specific model systems (3). In addition, with the exception of a short-term IGF-I infusion study (13), restoration of circulating IGF-I levels in mice on CR diets has been shown to ablate many of the antitumor effects of CR in multiple tumor models (14, 15). In contrast, DIO can

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lead to insulin resistance and increased circulating IGF-I (5). We have also reported that A-Zip/F-1 mice, which lack white adipose tissue but are diabetic, display elevated IGF-I levels and, like obese mice, are highly susceptible to several types of epithelial cancers (16). Taken together, these data suggest a critical role for circulating levels of growth factors, such as IGF-I, in the regulation of dietary energy balance effects on carcinogenesis.

The possible involvement of IGF-I in cancer was first suspected when *in vitro* studies consistently showed that IGF-I enhanced the growth of a variety of cancer cell lines (17, 18). A role for IGF-I in cancer was further confirmed when human breast (19), colon (20), and lung tumors (21) were shown to over-express IGF-I, the IGF-I receptor (IGF-IR), or both. Additional epidemiologic evidence identified an association between elevated circulating levels of IGF-I and increased risk of several epithelial cancers in humans (22, 23). Increased signaling through the IGF-IR leads to enhanced suppression of apoptosis, increased mitogenesis, and cell cycle progression (24, 25).

Evidence suggests that many of these IGF-I-related effects on cellular growth and metabolism involve signaling through the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway (26-28), one of the most commonly altered pathways in human tumors (29-33). For example, over-expression of *IGF-I* in the epidermis of HK1.IGF-I and BK5.IGF-I transgenic mice led to a dramatic increase in sensitivity to tumor development using the two-stage skin carcinogenesis protocol, a well-established model for epithelial carcinogenesis (26, 34). Thus, elevated tissue levels of IGF-I and enhanced signaling through the IGF-IR led to enhanced susceptibility to tumorigenesis. The ability of elevated tissue IGF-I levels to promote skin tumors is due, at least in part, to activation of the phosphatidylinositol 3-kinase/Akt signaling pathway, which has been shown to regulate epithelial cell proliferation (28, 35, 36). When either wild-type or myristoylated mouse *Akt* was overexpressed in epidermal basal cells under control of the *BK5* promoter, susceptibility to two-stage skin carcinogenesis was further enhanced (36). Western blot analyses performed on protein lysates prepared from either *Akt* transgenic mouse model showed enhanced signaling through the Akt/mTOR pathways, with heightened activation of downstream effectors of both Akt and mTOR (36).

A similar pattern of increased activation of Akt/mTOR signaling in the skin epidermis of the fatless but diabetic A-Zip/F-1 mice was associated with the increased skin and mammary tumor susceptibility observed in these mice (16). Collectively, these data further support the hypothesis that elevated IGF-IR signaling and, in particular, activation of the Akt/mTOR pathways may contribute to increased susceptibility to epithelial carcinogenesis. AMP-activated protein kinase (AMPK), which acts as a nutrient-dependent regulator of mTOR (37), may also be involved. During nutrient deprivation conditions, AMPK can be activated by upstream kinases and function to repress activation of mTOR, thus reducing cellular energy expenditure (38-44).

In the present study, we used well-established dietary regimens to induce positive and negative energy balance in mice to further explore potential mechanisms underlying the energy balance and cancer link (5, 45-48). Biochemical analyses were performed on multiple epithelial tissues from three commonly used mouse strains to determine diet-induced changes

in steady-state cell signaling. The results indicate that dietary energy balance manipulation modulates signaling through the Akt and mTOR pathways in all three tissues examined (i.e., epidermis, liver, and dorsolateral prostate). Furthermore, modulation of these signaling pathways seemed to be primarily mediated via alterations in signaling through the IGF-IR and the epidermal growth factor receptor (EGFR). Finally, phosphorylation of AMPK in response to either a positive or a negative energy balance seemed to be tissue dependent. Overall, the current data identify the Akt/mTOR signaling pathways as potential targets for cancer prevention and, in particular, for prevention of obesity-related cancers.

Materials and Methods

Chemicals and biologicals

Antibody against phospho-EGFR (Y1086) was purchased from Abcam, whereas antibodies against phospho-IGF-I/insulin receptor (Y1135/1136), phospho-Akt (S473), Akt, phospho-mTOR (S2448), mTOR, phospho-p70S6K (T389), p70S6K, phospho-4E-BP1 (T37/46), phospho-S6 ribosomal (S235/236), phospho-GSK-3 β (S9), cyclin D1, phospho-AMPK (T172), phospho-extracellular signal-regulated kinase (Erk)-1/2 (T202,Y204), and phospho-Src (Y416) were all purchased from Cell Signaling Technology, Inc. Anti- β -actin, as well as anti-rabbit and anti-mouse secondary antibodies, was obtained from Sigma Chemical Co. Antibodies against phospho-IRS-1 (Y989), phospho-IRS-1 (Y632), phospho-IRS-1 (Y465), and phospho-IRS-1 (Y941) were purchased from Santa Cruz Biotechnology and combined to generate an anti-phospho-IRS-1 antibody cocktail.

Animals

Male FVB/N and C57BL/6 mice (30 per genetic background, 3-4 wk of age) were purchased from National Cancer Institute and singly housed for the duration of the experiment. Thirty-two female ICR mice (3-4 wk of age) were purchased from Harlan Teklad and group-housed for the duration of the experiment.

Diet regimens

All diets were purchased in pellet form from Research Diets, Inc. On arrival, mice were placed on a 10 kcal% fat (control) diet (AIN-76A semipurified diet, fed *ad libitum*; diet D12450B) for a 1-wk equilibration period and then randomized into three dietary treatment groups (10 mice per group): (a) control diet (10 kcal% fat), fed *ad libitum*; (b) DIO (high-fat) diet (60 kcal% fat; D12492), fed *ad libitum*; and (c) 30% CR diet (D03020702). These diets have previously been described (4). For the study conducted in ICR mice, female mice were maintained on the diets described above (eight mice per group) and an additional dietary treatment was introduced: 15% CR diet (D03020703). Mice receiving either CR diet were given a daily aliquot equivalent to either 70% or 85% of the daily amount of total energy consumed by the control diet group. Both CR diets were adjusted to provide 100% of all vitamins, minerals, fatty acids, and amino acids relative to the control group. Under group-housing conditions, mice receiving the 30% CR diet were placed in a cage divider system for 2 h and allowed to consume their daily food allotment. Average body mass and food consumption were determined weekly for each dietary treatment group. With the exception of ICR mice, which received their diet regimens for 15 wk, all mice were maintained on their diet regimens for 17 wk. All groups were terminated by CO₂ asphyxiation and tissues and blood were collected, processed, and stored as described below.

Preparation of protein lysate

Immediately after the mice were terminated, the dorsal skin was shaved and then a depilatory agent was applied for 30 s and then removed. The skin was excised and the epidermal tissue was scraped from the excised skin using a razor blade into prepared lysis buffer

[0.5% Triton X-100, 1% NP40, 10% glycerol, 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EGTA, 1.5 mmol/L MgCl₂, 10% Sigma inhibitor cocktail, 10% phosphatase inhibitor cocktail I, and 10% phosphatase inhibitor cocktail II] and homogenized using an 18-gauge needle. Epidermal scrapings from all mice in each dietary group were pooled (10 mice per group for FVB/N and C57BL/6 and 8 mice per group for ICR). The liver and dorsolateral prostate were removed from FVB/N and C57BL/6 mice, frozen in liquid nitrogen, and then ground with a mortar and pestle. Once in powder form, liver and prostate tissues were homogenized using an 18-gauge needle in the lysis buffer described above. Again, both liver and prostate tissues from 10 mice were pooled per dietary group. The epidermal, liver, and prostate homogenates were then centrifuged at 14,000 rpm for 15 min, and the supernatant was aliquoted for use for Western blot analysis.

Western blot analysis

For analysis of receptor tyrosine kinase activation and phosphorylation of Akt/mTOR signaling molecules, 100 µg of epidermal lysate were electrophoresed in 4% to 15% SDS-polyacrylamide gels according to the method of Laemmli (49). The separated proteins were then electrophoretically transferred onto nitrocellulose membranes and blocked with 5% bovine serum albumin in TBS with 1% Tween 20 (TTBS). Blots were then incubated overnight with the antibodies described above in 5% bovine serum albumin in TTBS. Blots were washed with TTBS thrice for 15 min each and then incubated in anti-rabbit and anti-mouse secondary antibody in 5% bovine serum albumin in TTBS for 2 h. Blots were washed again with TTBS thrice for 15 min each, and then the protein bands were visualized by enhanced chemiluminescence (Pierce). Protein quantification was then determined using an alpha imager system. Each blot was repeated, producing nearly identical results.

Serum IGF-I analysis

Blood was collected by cardiac puncture immediately following CO₂ asphyxiation (10 mice per diet group for FVB/N and C57BL/6 and 8 mice per diet group for ICR), allowed to sit at room temperature for 2 h, and then spun at 7,500 rpm for 7 min. Supernatant was then collected and spun again under the same conditions. The final supernatant was collected, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Total mouse serum IGF-I concentration was then measured using a 25-µL sample with a RIA kit (Diagnostic Systems Laboratories, Inc.).

Results

Effect of dietary manipulation on body weight distribution, feed consumption, and serum IGF-I levels in FVB/N and C57BL/6 male mice

FVB/N and C57BL/6 male mice were randomized into three dietary treatment groups (10 per group): (a) control (10 kcal% fat), (b) DIO regimen (60 kcal% fat), and (c) lean regi-

men (30% CR) and maintained on these diets for 17 weeks. As shown in Tables 1 and 2, there were no statistically significant differences in body mass among the three groups at the start of the study. The weight distribution of both FVB/N and C57BL/6 mice on the CR diet began to diverge from mice of either strain receiving the other two diets within 2 weeks of diet commencement. In contrast, 8 weeks of experimental diet consumption was necessary to separate the control group from the DIO group in mice from either genetic background (data not shown). Following 17 weeks on diet, the average body mass of mice maintained on the CR diet was significantly reduced, whereas the average body mass of mice maintained on the DIO (high-fat) diet was significantly increased relative to FVB/N and C57BL/6 mice on the control diet (see again Tables 1 and 2; Student's *t* test, *P* < 0.05).

Average feed consumption was determined for each diet regimen across the 17-week experiment. As shown in Tables 1 and 2, mice maintained on the CR diet consumed 30% less feed relative to the control in both FVB/N and C57BL/6 mice, corresponding to a 30% reduction in caloric intake. Although feed consumption was reduced in FVB/N and C57BL/6 mice maintained on the DIO (high-fat) regimen relative to the control groups (statistically significant reduction only occurred in FVB/N mice), total energy consumption (kcal) was significantly increased by >20% in DIO mice of either genetic background (data not shown; Student's *t* test, *P* < 0.05) due to the higher caloric density of the DIO diet.

Serum analyses of total IGF-I were done to further characterize the effects of dietary manipulation on circulating IGF-I levels. As shown in Tables 1 and 2, serum IGF-I levels were significantly different among the diet groups in both FVB/N and C57BL/6 mice, with the greatest differences occurring between mice on the CR and DIO regimens. FVB/N mice on the CR diet exhibited a 55% reduction in total circulating IGF-I levels relative to FVB/N mice on the DIO diet, whereas C57BL/6 mice on the CR diet exhibited a 79% reduction in total circulating IGF-I levels relative to C57BL/6 mice on the DIO diet. These data, in combination with the weight distribution data, indicate that both FVB/N and C57BL/6 mice respond similarly to dietary energy balance manipulation.

Effect of dietary energy balance manipulation on the activation of Akt and mTOR in multiple epithelial tissues

In an effort to explore the signaling pathways involved in the dietary energy balance effects on epithelial carcinogenesis, we carried out Western blot analyses on protein lysates prepared

Table 1. Weight distribution, food consumption, and serum IGF-I levels of FVB male mice maintained on control, high-fat, and lean diet regimens (10 mice per group)

Experimental diet	Mass, start of study (g)	Mass, end of study (g)	Average food consumption (g)	Serum IGF-I, end of study (ng/mL)
10 kcal% fat	27.2 ± 0.4	41.9 ± 0.9 ^a	26.8 ± 0.45 ^a	742 ± 62.23 ^a
60 kcal% fat	25.5 ± 0.3	46.7 ± 0.9 ^b	24.2 ± 0.42 ^b	987 ± 107.85 ^b
30% CR	26.8 ± 0.3	26.8 ± 0.3 ^c	19.0 ± 0.30 ^c	440 ± 65.47 ^c

NOTE: The data represent mean ± SE. Superscript letters indicate that values are significantly different from each other.

Table 2. Weight distribution, food consumption, and serum IGF-I levels of C57BL/6 male mice maintained on control, high-fat, and lean diet regimens (10 mice per group)

Experimental diet	Mass, start of study (g)	Mass, end of study (g)	Average food consumption (g)	Serum IGF-I, end of study (ng/mL)
10 kcal% fat	17.3 ± 0.3	37.5 ± 0.8 ^a	23.3 ± 0.43 ^a	615 ± 52.25 ^a
60 kcal% fat	17.7 ± 0.3	47.0 ± 0.8 ^b	22.9 ± 0.88 ^a	987 ± 92.53 ^b
30% CR	16.9 ± 0.4	21.1 ± 0.3 ^c	16.2 ± 0.37 ^b	208 ± 32.38 ^c

NOTE: The data represent mean ± SE. Superscript letters indicate that values are significantly different from each other.

from pooled epidermal, hepatic, and dorsolateral prostate tissues collected from FVB/N and C57BL/6 male mice maintained on the different diets as described above (tissue samples were pooled from 10 mice per diet group). As shown in Fig. 1, CR reduced, whereas the DIO diet increased, activation (as assessed by phosphorylation status) of both Akt and mTOR in all three epithelial tissues examined, relative to mice maintained on the control diet. These data show that dietary energy balance manipulation altered steady-state signaling to Akt and mTOR in the epidermis, liver, and dorsolateral prostate.

Effect of dietary energy balance modulation on signaling downstream of Akt and mTOR in multiple epithelial tissues

In light of the observed effects of dietary energy balance modulation on steady-state activation of Akt and mTOR, we next examined several downstream signaling molecules. As shown in Fig. 2, CR consistently led to decreased phosphorylation of downstream effectors of both Akt and mTOR (e.g., GSK-3 β , p70S6K, and 4E-BP1, respectively) in the epidermis, liver, and dorsolateral prostate, as compared with mice maintained on either the control or the DIO diet. Cyclin D1 levels were also reduced in all three tissues by CR. CR was also shown to inhibit activation of S6 ribosomal protein in the epidermis and liver; however, S6 ribosomal protein was not examined in prostate due to the limited amount of tissue available. Thus, CR, relative to the control and DIO diets, consistently inhibited signaling downstream of Akt and mTOR, independent of genetic background or epithelial tissue examined, suggesting that CR functions to suppress Akt and mTOR signaling in multiple epithelial tissues.

Although the tissues from mice on the CR diet consistently showed reduced signaling through Akt, mTOR, and downstream molecules relative to tissues from mice on the control and DIO diets, differences between the latter two groups were less apparent in some cases. This lack of consistent differences between the control and DIO diet regimens may be due to the fact that both regimens induced positive energy balance and weight gain. The differences in body weight and adiposity are much greater between the CR and control mice than the differences between the DIO and control mice.

Role of AMPK in the regulation of mTOR signaling

To explore the role of AMPK in the regulation of mTOR signaling in tissues from mice on the various diets, we carried out Western blot analyses to examine its activation status. As shown in Fig. 3, activation of AMPK, as measured by phosphorylation at Thr¹⁷², was similar in protein lysates from epi-

dermis and prostate across all three diets. This was also true for both genetic backgrounds (i.e., FVB/N and C57BL/6). In contrast, phosphorylation of AMPK was elevated in protein lysates from liver of mice on the CR diet relative to mice on either the control or the DIO diet. Again, this was true for either genetic background. These results suggest that the effects of dietary energy balance modulation on AMPK signaling may be tissue dependent. The lack of dietary energy effects on epidermal AMPK was confirmed in subsequent studies using ICR mice (see below).

Effect of dietary energy balance manipulation on the activation of mitogen-activated protein kinase and c-Src

In light of the finding that dietary energy balance differentially modulated activation of Akt and mTOR, we carried out Western blot analyses of Erk1/2 and c-Src phosphorylation status with the same protein lysates from epidermis, liver, and dorsolateral prostate used to assess Akt/mTOR and AMPK signaling. As shown in Fig. 4, CR reduced, whereas the DIO regimen enhanced, phosphorylation of both of these signaling molecules in all three epithelial tissues.

Effects of dietary energy balance manipulation on EGFR, IGF-IR, and Akt/mTOR signaling in the epidermis of female ICR mice

In an effort to further confirm the effect of dietary energy balance manipulation on signaling via the Akt and mTOR pathways, we randomized female ICR mice into four dietary treatment groups (eight mice per group): (a) control (10 kcal% fat), (b) DIO regimen (60 kcal% fat), (c) 15% CR, and (d) 30% CR. As shown in Table 3, the DIO (high-fat) diet significantly increased body mass, whereas both 15% and 30% CR significantly decreased body mass, relative to mice on the control diet, following 15 weeks of dietary manipulation. Serum IGF-I levels in ICR mice also correlated with body mass and caloric consumption. Thus, female ICR mice seemed to respond to dietary energy balance manipulation in a fashion similar to that observed in the male FVB/N and C57BL/6 mice.

To further examine differences in cellular signaling resulting from dietary manipulation, epidermal lysates were collected and pooled from eight mice maintained on the various diets for 15 weeks and then analyzed by Western blot analysis. As shown in Fig. 5, dietary energy balance manipulation differentially regulated activation and signaling through both the EGFR and IGF-IR. In this regard, CR (both at 30% and 15% CR) reduced steady-state activation (i.e., phosphorylation) of the EGFR, IGF-IR, and IRS-1. In addition, CR (especially 30%

CR) led to a significant reduction in the activation of Akt, mTOR, and downstream effectors of mTOR (i.e., p70S6K and S6 ribosomal protein), as compared with mice on the control or DIO (high-fat) diet. When activation of AMPK was examined, no effect of dietary energy balance manipulation was observed. These latter results are similar to those shown in Fig. 3 for both FVB/N and C57BL/6. Together, these data suggest that dietary energy balance alters signaling in epidermis

through both the EGFR and IGF-IR, which then leads to changes in Akt and mTOR signaling, and these changes are independent of AMPK.

Discussion

The current study was designed to examine potential mechanisms involved in the effects of dietary energy balance

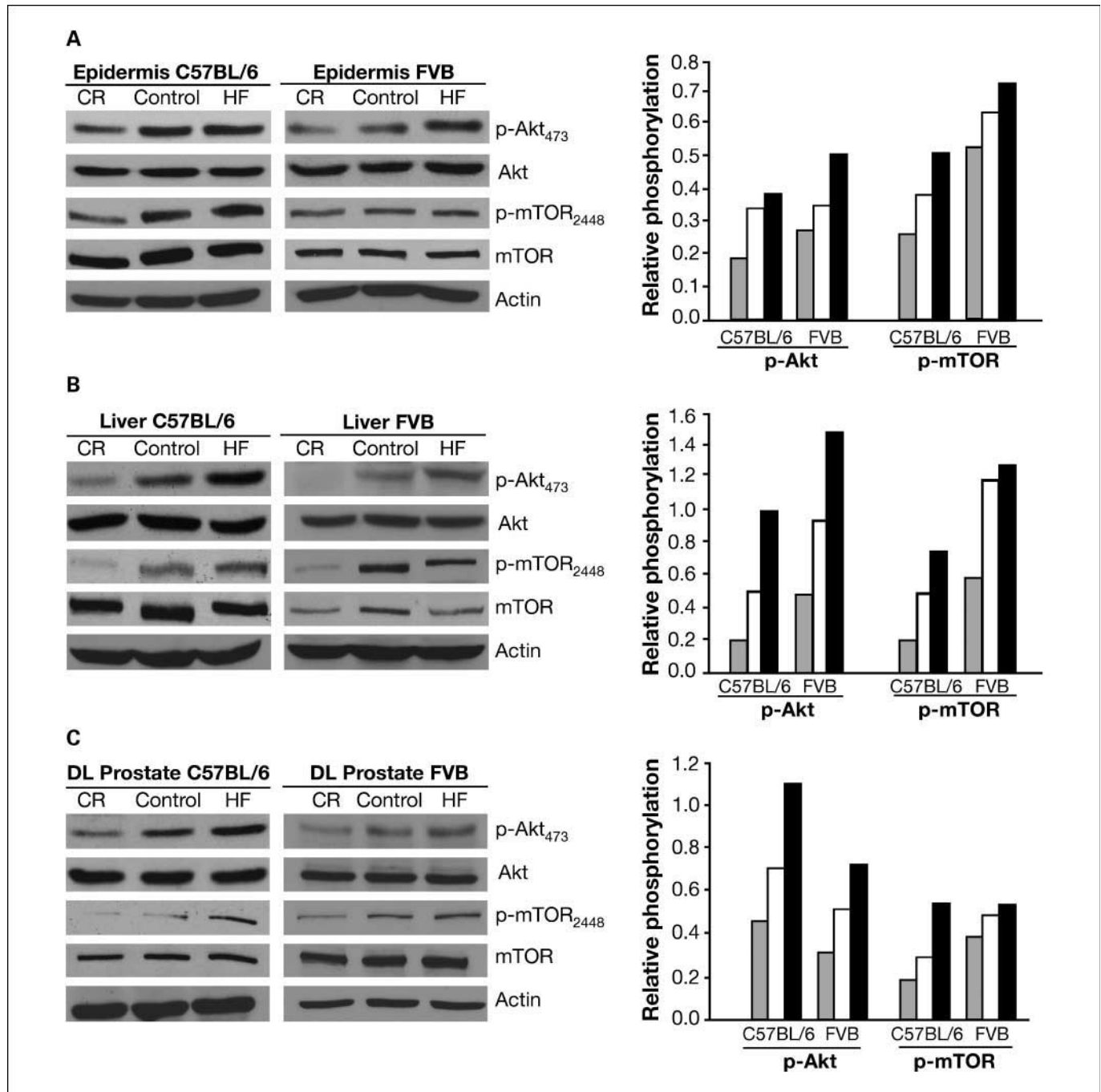


Fig. 1. Effect of dietary energy balance manipulation on the activation of Akt and mTOR in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and dorsolateral prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 wk (10 per group). Western blot analyses were then conducted to examine activation of Akt and mTOR in various tissues: epidermis (A), liver (B), and dorsolateral (DL) prostate (C). Quantification was then done: gray columns, CR; white columns, control; black columns, high-fat/DIO (HF). Western blots for each pooled tissue sample were repeated with nearly identical results.

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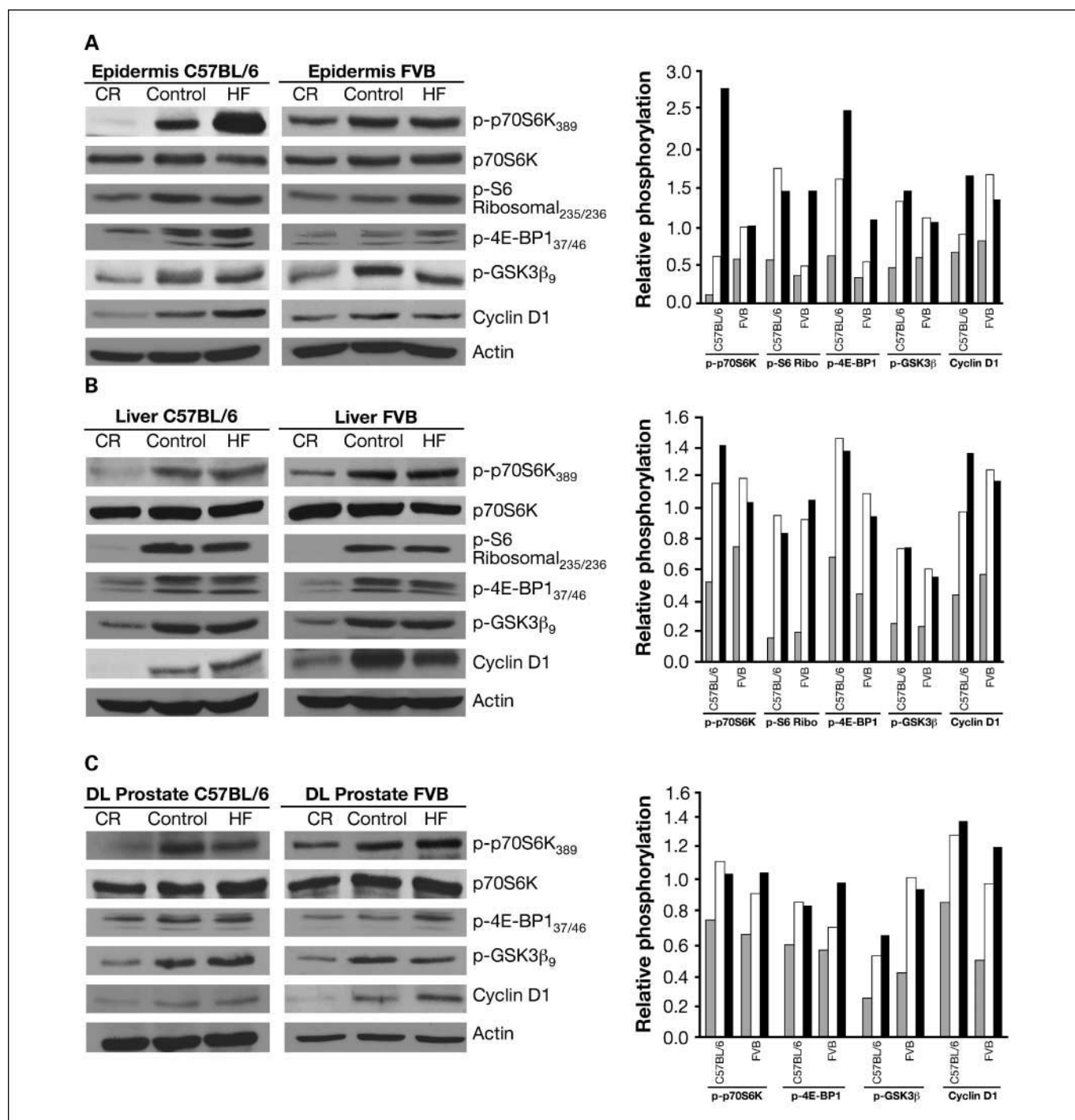


Fig. 2. Effect of dietary energy balance manipulation on the activation of Akt and mTOR downstream signaling in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and dorsolateral prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 wk (10 per group). Western blot analyses were then conducted to examine activation of Akt and mTOR downstream signaling molecules in various tissues: epidermis (A), liver (B), and dorsolateral (DL) prostate (C). Quantification was then done: gray columns, CR; white columns, control; black columns, high-fat/DIO. Western blots were repeated for each pooled tissue sample with nearly identical results.

on cellular signaling and epithelial carcinogenesis. Numerous studies have examined the dietary energy balance-cancer link, although few have provided a mechanistic explanation for the observed effects. We employed commonly used regimens for dietary manipulation to examine alterations in steady-state cellular signaling that occur in multiple epithelial tissues.

Body weight distribution data generated in the current study paralleled those reported in recent publications, thus validating the model systems used in the current investigation (5, 45-48). Consistent with the data published in earlier studies (5, 45), we also found that positive energy balance significantly increased, whereas negative energy balance

significantly decreased, the levels of circulating IGF-I in FVB/N and C57BL/6 mice, relative to the respective controls. Similar effects of dietary energy balance on serum IGF-I levels were observed in ICR mice maintained on the different diets. Thus, the effects of dietary energy balance on serum IGF-I levels were independent of genetic background or gender. As noted in the introduction, changes to globally active circulatory proteins, such as IGF-I, may mediate the effects of dietary

energy balance on epithelial carcinogenesis (3, 12). In addition, we found significant effects on steady-state growth factor signaling pathways associated with dietary energy balance modulation. In particular, we found that negative energy balance decreased, whereas positive energy balance increased, signaling through the IGF-IR and EGFR. Furthermore, downstream signaling via several pathways, including Akt and mTOR, was affected in a similar manner. Overall, the current data suggest that dietary energy balance across the spectrum from negative (CR) to positive (DIO) modulates major growth factor signaling pathways linked to tumor development and tumor progression in multiple epithelial tissues.

As noted in the introduction, evidence in the literature suggests a role for circulating IGF-I in modulating tumorigenesis (50, 51). Further evidence comes from using liver IGF-I-deficient mice (50-53). In this regard, deletion of the *IGF-I* gene in hepatocytes leads to a 75% reduction in circulating IGF-I levels (53), allowing for examination of the effect of reduced circulating IGF-I on carcinogenesis in multiple tissues in the absence of dietary manipulation. This LID mouse model has been used to study the effect of reduced circulating IGF-I on mammary tumor development with both chemical induction and transgenic approaches (52). Additional studies were conducted to examine the effect of reduced circulating IGF-I levels on growth and metastasis of Colon 38 adenocarcinoma cells following orthotopic transplantation (50). Both of these studies showed significant effects of reduced circulating IGF-I on tumor growth (inhibition) although the underlying mechanism (s) for these effects was not explored.

When serum IGF-I levels were restored in LID mice by recombinant human IGF-I supplementation, the inhibitory effects on colon cancer were abolished. More recently, we have examined the effect of reductions in circulating IGF-I on two-stage skin carcinogenesis, a well-established model for epithelial carcinogenesis (51). In these studies, LID mice were highly resistant to two-stage skin carcinogenesis. Mechanistic studies showed that LID mice had a reduced responsiveness to 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal hyperplasia and epidermal proliferation. Furthermore, biochemical studies showed that LID mice exhibited reduced activation of both the IGF-IR and EGFR, as well as downstream signaling through Akt and mTOR, following TPA treatment compared with wild-type mice. These data suggest a possible mechanism whereby reduced circulating IGF-I attenuates activation of Akt and mTOR, thus reducing the response of epidermal cells to tumor promotion. Furthermore, these findings support the hypothesis that reduced circulating IGF-I levels contribute to the anticancer effects of CR in multiple tissues.

In an effort to determine if modulation of circulating IGF-I levels through manipulation of dietary energy balance led to altered Akt/mTOR signaling, as suggested by the studies with LID mice, we examined the status of critical signaling molecules in the Akt and mTOR pathways in the epidermis, liver, and dorsolateral prostate from both FVB/N and C57BL/6 mice maintained on disparate dietary regimens. As shown in Fig. 1, positive energy balance enhanced, whereas negative energy balance inhibited, activation of both Akt and mTOR, regardless of tissue or genetic background. Furthermore, the inhibitory effects of CR were confirmed when phosphorylation status or protein level of downstream effectors of both Akt and mTOR was examined. Of particular interest is the

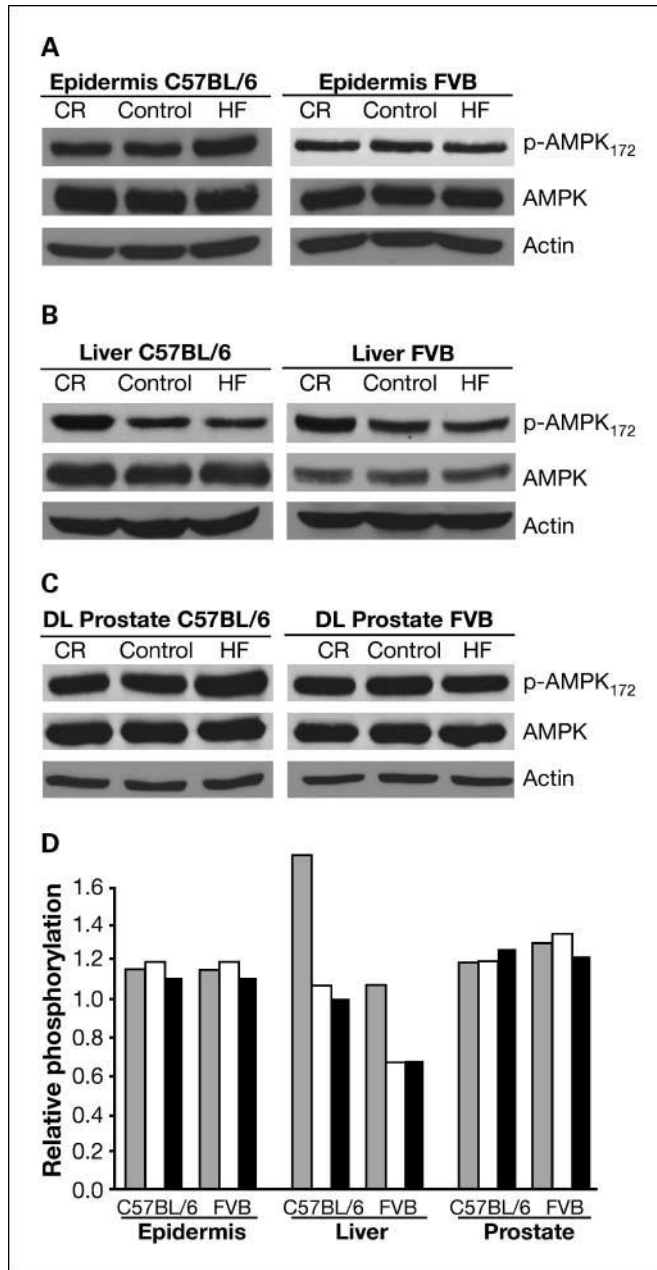


Fig. 3. Effect of dietary energy balance manipulation on the activation of AMPK in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and dorsolateral prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 wk (10 per group). Western blot analyses were then conducted to examine activation of AMPK in various tissues: epidermis (A), liver (B), and dorsolateral (DL) prostate (C). D, quantification of AMPK activation in multiple tissues. Gray columns, CR; white columns, control; black columns, high-fat/DIO. Western blots for each pooled tissue sample were repeated with nearly identical results.

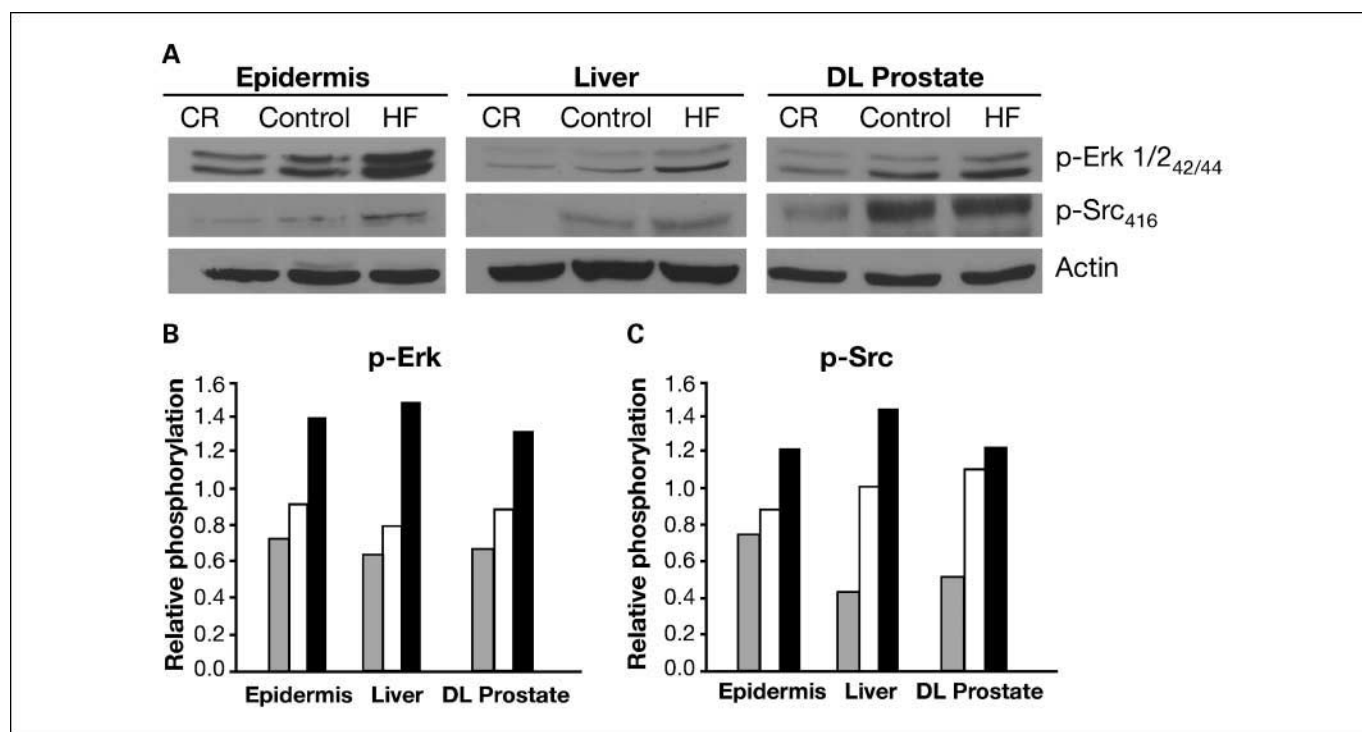


Fig. 4. Effect of dietary energy balance manipulation on the activation of mitogen-activated protein kinase and c-Src. Pooled protein lysates were prepared from epidermis, liver, and dorsolateral prostate excised from C57BL/6 mice maintained on the three dietary regimens for 17 wk (10 per group). *A*, Western blot analyses were then conducted to examine the effect of dietary energy balance on the activation of Erk1/2 and Src in epidermis, liver, and dorsolateral (DL) prostate. Quantification was then done: gray columns, CR; white columns, control; black columns, high-fat/DIO. *B*, quantification of Erk1/2 phosphorylation in multiple tissues. *C*, quantification of c-Src phosphorylation in multiple tissues. Western blots for each pooled tissue sample were repeated with nearly identical results.

effect of dietary energy balance on cyclin D1 levels across the three tissues. In general, cyclin D1 levels were reduced by CR relative to the control and DIO diet groups across the three tissues.

These findings are consistent with earlier studies linking reduced levels of cyclin D1 to energy restriction in both a mouse mammary cell line and in uninvolved, premalignant and malignant rat mammary tissues following treatment with methylnitrosourea (54, 55). Cyclin D1 levels are known to be regulated downstream of both Akt and mTOR (56-59) as well as downstream of other signaling pathways activated by growth factor receptor signaling (60-62). As can be seen in Fig. 2, phosphorylation of GSK-3 β , which is immediately downstream of Akt, was modulated by dietary energy balance in all three tissues in a manner consistent with Akt phosphor-

ylation status and cyclin D1 levels. The importance of cyclin D1 in epithelial carcinogenesis in mouse epidermis has been shown in a number of studies with transgenic mouse models (63-65) as well as cyclin D1 knockout mice (66).

Further Western blot analyses were performed to determine if the effects of dietary energy balance on steady-state signaling to the mTOR pathway were controlled by AMPK, a known upstream nutrient-sensing regulator of mTOR signaling (37). As shown in Fig. 3, AMPK activation was not affected by dietary manipulation in protein lysates from either the epidermis or the dorsolateral prostate; however, CR led to activation of AMPK in the liver. Notably, these results in the liver differ from previously published data in which hepatic AMPK phosphorylation was found to be unchanged in C57BL/6

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Table 3. Weight distribution, food consumption, and serum IGF-I levels of ICR female mice maintained on control, high-fat, normal, and lean diet regimens (eight mice per group)

Experimental diet	Mass, start of study (g)	Mass, end of study (g)	Average food consumption (g)	Serum IGF-I, end of study (ng/mL)
10 kcal% fat	30.8 \pm 0.50	40.3 \pm 1.06 ^a	25.6 \pm 0.3 ^a	786 \pm 86.26 ^a
60 kcal% fat	32.2 \pm 0.56	55.9 \pm 1.28 ^b	31.5 \pm 0.4 ^b	917 \pm 84.19 ^a
15% CR	30.9 \pm 1.07	34.9 \pm 0.84 ^c	21.5 \pm 0.2 ^c	542 \pm 88.31 ^b
30% CR	30.8 \pm 0.66	23.3 \pm 0.52 ^d	18.1 \pm 0.2 ^d	311 \pm 42.5 ^c

NOTE: The data represent mean \pm SE. Superscript letters indicate that values are significantly different from each other.

male mice in response to CR (67). There are several differences, however, between the study by Gonzalez et al. (67) and our current study: (a) mice were maintained on a 35% CR dietary regimen; (b) CR mice were fed on an alternate-day feeding regimen; and (c) food consumption of control mice was actually restricted by 10%. In our current study, dietary energy balance manipulation seemed to alter signaling to mTOR in epidermis and prostate in a manner independent of AMPK activation, although in liver AMPK does seem to play a role. Further work will be necessary to explore how changes in dietary energy balance affect AMPK activity *in vivo* in specific tissues.

The data from our recent studies using LID mice suggested that reduced circulating IGF-I levels affected signaling through both the IGF-IR and EGFR in epidermis of mice during tumor promotion (51). Therefore, Western blot analyses were carried out on epidermal protein lysates from mice maintained on control, DIO, and CR diets. For these studies, we used female ICR mice and also included a 15% CR group. Notably, we found that dietary energy balance affected signaling through the IGF-IR and EGFR in epidermis, consistent with its effects on downstream signaling (see Figs. 1A, 2A, and 4). This effect on receptor tyrosine kinase activation is

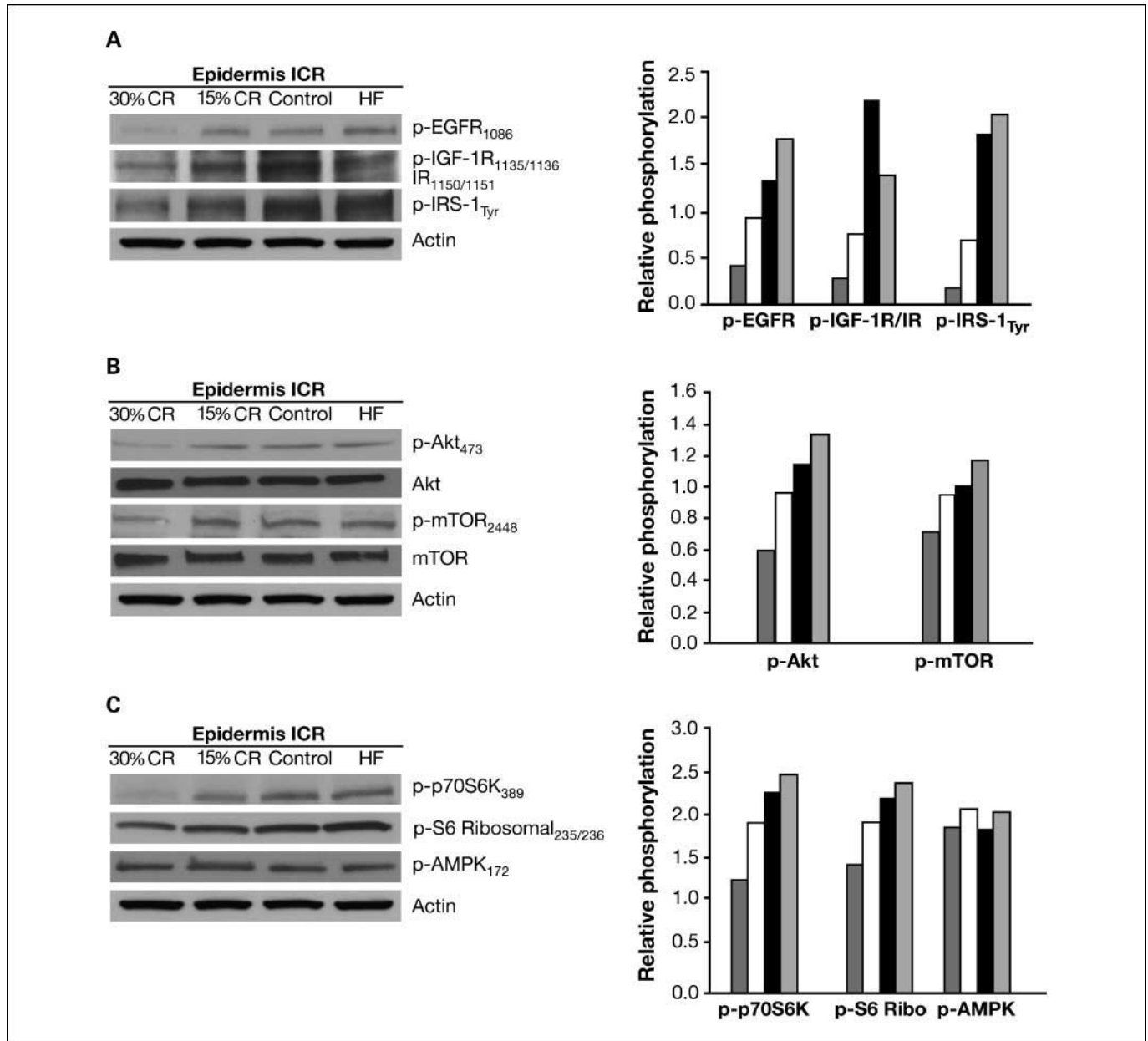


Fig. 5. Effect of dietary energy balance manipulation on the activation of epidermal signaling pathways in ICR female mice. Female ICR mice (eight per group) were maintained on the 30% CR (dark gray columns), 15% CR (white columns), control (black columns), and high-fat/DIO (light gray columns) diets for 15 wk. Pooled epidermal lysates were prepared following sacrifice for Western blot analysis. **A**, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal IGF-IR and EGFR activation. **B**, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal Akt and mTOR activation. **C**, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal Akt and mTOR downstream signaling. Western blots for each pooled tissue sample were repeated with nearly identical results.

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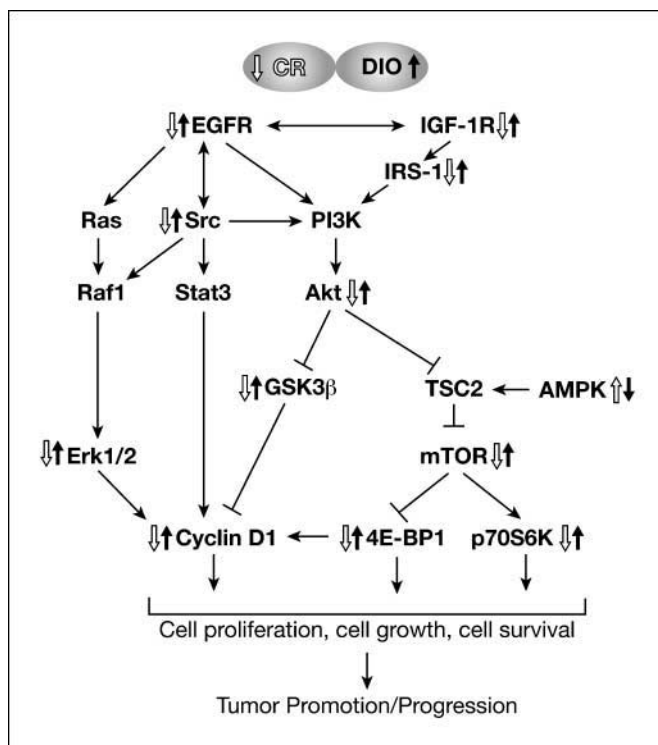


Fig. 6. Proposed mechanism by which dietary energy balance modulates cellular signaling and epithelial carcinogenesis. Positive energy balance increases, whereas negative energy balance decreases, levels of circulating IGF-I, resulting in differential activation of the IGF-IR. An alteration in signaling through the IGF-IR subsequently affects signaling through the EGFR, due to receptor cross talk. As a result of this increased or decreased signaling to cell-surface receptor tyrosine kinases, signaling through downstream pathways is differentially regulated. Data from the current study show that signaling downstream of Akt and mTOR is differentially modulated by dietary energy balance. In this regard, obesity (▲) enhances, whereas CR (◻) decreases, phosphorylation of Akt, mTOR, and their downstream effectors. Of particular importance is the differential effect of dietary energy balance on cyclin D1 levels. Signaling downstream of both Akt and mTOR has been shown to regulate cyclin D1. Further analysis of receptor tyrosine kinase downstream signaling shows that dietary energy balance differentially regulates activation of both Erk and c-Src, which subsequently regulate cyclin D1. Modulation of the Akt and mTOR signaling pathways, in combination with altered signaling through other growth factor signaling pathways, provides a mechanistic explanation for the effect of dietary energy balance on epithelial carcinogenesis.

strikingly similar to the effects seen in LID mice and may explain, in part, the mechanism by which dietary energy balance alters signaling to Akt and mTOR as well as other downstream signaling pathways (Erk1/2 and c-Src). Collectively, these data suggest that serum IGF-I levels may regulate signaling through both the IGF-IR and EGFR possibly by modulating cross talk between these two cell-surface receptor tyrosine kinases. In support of this latter idea, we previously reported the development of HK1.IGF-I transgenic mice wherein expression of *IGF-I* is targeted to epidermis using

the *human keratin 1 (HK1)* promoter (34, 68). Following treatment with TPA, there was a significant increase in EGFR activation in epidermis of HK1.IGF-I transgenic mice compared with wild-type mice. These data suggested that tissue levels of IGF-I and, presumably, activation state of the IGF-IR could influence the overall activation level of the EGFR. A number of mechanisms have recently been proposed whereby cross talk between the EGFR and IGF-IR may occur (69-73). Current experiments are exploring possible mechanisms whereby circulating IGF-I levels, as modulated by dietary energy balance manipulation, influence cross talk between the IGF-IR and the EGFR.

In conclusion, we have shown that dietary energy balance modulation alters signaling through the Akt and mTOR pathways in multiple epithelial tissues of mice, regardless of genetic background. The mechanism for the effect of dietary energy balance on signaling to the Akt and mTOR pathways seems, at least in part, to be mediated by changes in serum IGF-I levels, which then alters signaling through the IGF-IR and EGFR. The role of AMPK in regulating mTOR signaling *in vivo* during energy balance modulation is less clear and may be highly tissue specific. Further work using *in vivo* model systems will be important in this regard. Earlier work reported by Birt and colleagues showed an attenuation of TPA-induced activator protein-1 activation (74-76) and Erk activation in mice on 40% CR diets. We found that dietary energy balance also modulated steady-state activation of both Erk1/2 and c-Src (Fig. 4). Both of these signaling pathways are known to be downstream of receptor tyrosine kinases such as the IGF-IR and EGFR (60-62).

Finally, Xie et al. (77) reported reduced phosphatidylinositol 3-kinase and ras signaling in response to TPA in skin of SENCAR mice on CR diets as compared with controls. In this study, phosphorylation of Akt in epidermis following TPA treatment was reduced by CR and to a greater extent by CR plus exercise. Collectively, these published findings and the data currently presented support the hypothesis that dietary energy balance modulates signaling downstream of cell-surface receptors. A summary of our current results and its implication for epithelial carcinogenesis is shown in Fig. 6. The observation that dietary energy balance manipulation leads to altered signaling through both Akt and mTOR in multiple epithelial tissues via modulation of cell-surface receptor tyrosine kinase signaling is novel. These findings provide the basis for future translational studies targeting the Akt/mTOR pathway via combinations of lifestyle (i.e., moderate calorie restriction regimens) and pharmacologic approaches for the prevention and control of obesity-related epithelial cancers in humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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