

**Reduced signaling of PI3K-Akt and RAS-MAPK pathways are the key targets for weight loss-induced cancer prevention by dietary calorie restriction and/or physical activity**

**Joseph Standard<sup>1</sup>, Yu Jiang<sup>1</sup>, Miao Yu<sup>1</sup>, Xiaoyu Su<sup>1</sup>, Zhihui Zhao<sup>2</sup>, Jianteng Xu<sup>1</sup>, Jie Chen<sup>1</sup>, Brenee King<sup>1</sup>, Lizhi Lu<sup>3</sup>, John Tomich<sup>4</sup>, Richard Baybutt<sup>5</sup>, Weiqun Wang<sup>1</sup>**

<sup>1</sup>Department of Human Nutrition, <sup>4</sup>Department of Biochemistry, Kansas State University, Manhattan, KS 66506

<sup>2</sup>Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, Shanghai, China 201403

<sup>3</sup>Institute of Animal Husbandry & Veterinary Science, Zhejiang Academy of Agricultural Sciences, Hangzhou, China 310021

<sup>5</sup>Department of Applied Health Science, Wheaton College, Wheaton, IL 60187

Corresponding author: Prof. Weiqun Wang, Department of Human Nutrition, Kansas State University, Manhattan, KS 66506. Phone: 785-532-0153; Fax: 785-532-3132; E-mail: [wwang@ksu.edu](mailto:wwang@ksu.edu)

## Abstract

Weight control through either dietary calorie restriction (DCR) or exercise has been associated with cancer prevention in animal models. However, the underlying mechanisms are not fully defined. Bioinformatics using genomics, proteomics, and lipidomics were employed to elucidate the molecular targets of weight control in a mouse skin cancer model. SENCAR mice were randomly assigned into 4 groups for 10 weeks: ad lib-fed sedentary control, ad lib-fed exercise (AE), exercise but pair-fed isocaloric amount of control (PE), and 20% DCR. Two hours after topical TPA treatment, skin epidermis was analyzed by Affymetrix for gene expression, DIGE for proteomics, and lipidomics for phospholipids. Body weights were significantly reduced in both DCR and PE but not AE mice versus the control. Among 39,000 transcripts, 411, 67, and 110 genes were significantly changed in DCR, PE, and AE, respectively. The expression of genes relevant to PI3K-Akt and Ras-MAPK signaling was effectively reduced by DCR and PE but not AE as measured through GenMAPP software. Proteomics analysis identified ~120 proteins, with 27 proteins significantly changed by DCR, including upregulated apolipoprotein A-1, a key antioxidant protein that decreases Ras-MAPK activity. Of the total 338 phospholipids analyzed by lipidomics, 57 decreased by PE including 5 phosphatidylinositol species that serve as PI3K substrates. Although a full impact has not been determined yet, it appears the reduction of both Ras-MAPK and PI3K-Akt signaling pathways are cancer preventive targets that have been consistently demonstrated by three bioinformatics approaches.

**Keywords:** Bioinformatics, Weight Control, Cancer Prevention, Dietary Calorie Restriction, Exercise

# 1    **Introduction**

2    Obesity has been identified as a risk factor for many chronic diseases including cancer. Since  
3    1960, obesity rates have doubled in US men and nearly tripled in US women, with both groups at  
4    rates over 30% of the population.<sup>1,2</sup> Obesity and overweightness contribute to cancer deaths in up  
5    to 14% for men and 20% for women over age 50, with higher rates of mortality for overweight  
6    cancer patients.<sup>3</sup> With rising rates of obesity and chronic diseases, many studies have focused on  
7    various interventions to prevent obesity-related chronic diseases. In the early 20th century,  
8    dietary calorie restriction (DCR) was shown to prevent tumor growth, and it has become the  
9    most robust nutritional intervention for cancer prevention in animal models. As sedentary  
10   behavior has become endemic worldwide, exercise has also been identified as another lifestyle  
11   behavior that could reduce body weight and chronic disease risk. Weight control through DCR  
12   and/or exercise may be responsible, at least in animal models, for cancer prevention.

13            DCR is a weight control measure that decreases caloric intake of fats and carbohydrates  
14   while maintaining protein, fiber, and micronutrients. Typical studies have withheld calories  
15   between 20 and 40%.<sup>4</sup> After a pilot study in 1909 by Moreschi, DCR was shown to prevent  
16   cancer in a variety of animal models and to be effective in reducing both chemically induced and  
17   spontaneous tumors.<sup>5</sup> Additionally, increased energy expenditure through exercise is another  
18   form of weight control that may contribute to cancer prevention. Exercise has been strongly  
19   associated with cardiovascular health benefits, but the cancer preventive effect of exercise is less  
20   consistent. Exercise has demonstrated convincing evidence for cancer prevention in colorectal<sup>6</sup>  
21   and breast cancer.<sup>7</sup> Additionally, exercise may prevent prostate cancer progression as well as  
22   endometrial or lung cancer.<sup>8</sup> Voluntary wheel running exercise provided cancer prevention by  
23   decreasing tumor size in a mouse skin cancer model.<sup>9,10</sup> Some studies, however, have not shown

1 a consistent protection by exercise.<sup>11-13</sup> Furthermore, previous studies in our lab had mixed  
2 results of exercise for cancer prevention, with protection only seen with iso-caloric intake.<sup>14</sup>  
3 Moore et al. demonstrated that a negative energy balance, as opposed to exercise alone, was  
4 responsible for inhibiting intestinal polyps in APC<sup>Min</sup> mice.<sup>15</sup> Thus, to help differentiate between  
5 exercise and caloric restriction upon cancer signaling pathways, our study compared exercised  
6 mice with or without iso-caloric intake.

7 A variety of studies have been conducted to discover the biological mechanisms of  
8 weight control for cancer prevention. Many studies have focused on endocrine hormones and  
9 adipokines that are modified by increased adiposity. IGF-1 and leptin are two key factors that  
10 have been widely studied for cancer promotion. Increased IGF-1 levels from obesity have been  
11 associated with increased cancer risks. IGF-1 receptor has been shown to activate downstream  
12 pathways such as: MAPK, PI3K-Akt, and JAK/STAT which contribute to proliferation, anti-  
13 apoptosis and gene transcription, respectively.<sup>16-17</sup> Additionally, adipocyte-secreted adipokines  
14 such as leptin have been shown to promote cancer cell signaling.<sup>18-19</sup>

15 While traditional studies have been limited to examining a few select genes, proteins, or  
16 fat signaling phospholipids of interest to gauge the cancer preventive effect of weight control,  
17 recently developed -omics tools have enabled us to employ a more global approach to examine  
18 the etiology of cancer development. This study utilized a well-established mouse skin cancer  
19 model by 12-0-tetradecanoylphorbol-13-acetate (TPA) as a cancer promoter. Genomics,  
20 proteomics, and lipidomics are tools that are used to examine the interplay of profiling changes  
21 of gene, protein, and phospholipid expression on the development of cancer. The goal of this  
22 study was to implement genomic, proteomic and lipidomic methods to provide new information  
23 toward a mechanistic understanding of cancer prevention by weight control.

## 1    **Materials and methods**

### 2    **Animals and treatment**

3    Six-week old female SENCAR mice were purchased from NIH (Frederick, MD). Mice  
4    underwent a two-week training period to adjust to the new environment and treadmill exercise.  
5    Mice were housed individually at  $24 \pm 1$  °C with a 12:12 light-dark cycle and given water ad  
6    libitum. Mice were divided into four treatment groups consisting of sedentary ad libitum-fed  
7    controls (control), ad-libitum-fed exercise (AE), pair-fed exercise (PE), and 20% DCR. Ad  
8    libitum controls and ad libitum exercise mice were allowed to freely access food, while the pair-  
9    fed exercise group was match-fed to the controls' consumption. The basal AIN-93 and 20%  
10    DCR diets were made by Harland Teklad (Madison, WI). The 20% DCR diet that withheld  
11    calories from fat and carbohydrate is shown in Table 1. A speed adjustable rodent treadmill  
12    (Boston Gears, Boston, MA) was used for mice in exercise treatment groups. After two weeks  
13    training, the exercise groups ran on the treadmill at 13.4 m/min, 60 min per day and 5 days a  
14    week for 10 weeks. This exercise level has been rated as moderate intensity.<sup>20</sup> Weekly body  
15    weights and food consumption measurements were taken.

16            At the end of the experiment, the mice were topically treated with 6.4 nmol TPA in 200  
17     $\mu$ L acetone on the dorsal skin, which was shaved two days before treatment. The sham treatment  
18    was acetone without TPA. There is not much difference between the controls with or without  
19    acetone-treatment. Considering the huge bioinformatics data generated by omics tools, this study  
20    was focused on TPA- verse sham-treated data for cancer risk-associated results. Mice were  
21    sacrificed 2 hours after TPA treatment, and the dorsal skin samples were snap-frozen in liquid  
22    nitrogen and stored at -70 °C until further analyses.

## 1    **Microarray Analysis**

2    Microarray analysis was performed as described in our previous studies.<sup>14,21</sup> Briefly, labeled  
3    cRNA was applied to an Affymetrix GeneChip Mouse Genome 430 2.0 Array containing 39,000  
4    transcripts and 45,101 probe sets (Santa Clara, CA). The images were quantified by using  
5    GeneChip operating software 1.0 (GCOS 1.0; Affymetrix, Santa Clara, CA). The raw image  
6    readings were analyzed using Simpleaffy package from BioConductor at  
7    <http://www.bioconductor.org>. The data were normalized using either MAS or RMA algorithms.  
8    The genes that were differentially expressed between treatment groups were identified by using  
9    pair-wise comparison. Data were filtered by using 1.5-fold difference considered statistically  
10   significant at  $p < 0.05$ .

11            Cytoscape v2.6.0 coupled with plug-in BiNGO v2.0 was used to map the predominant  
12   gene ontology categories of the differentially expressed genes.<sup>22</sup> The GO annotations p-values  
13   were obtained by hypergeometric statistical test for cluster verse whole annotation. The test was  
14   adjusted by Benjamin and Hochberg false discovery rate, which is included in the BiNGO  
15   package. The dataset consisting of the significantly altered genes was loaded into GenMAPP2.0  
16   (Gene Map Annotator and Pathway Profiler, [www.genmapp.org](http://www.genmapp.org)) to analyze the effect of target  
17   gene expression on specific pathways. As reported previously,<sup>14, 22</sup> RT-PCR on select genes was  
18   tested to confirm the microarray results (data not shown).

## 19   **Proteomics Analysis**

20   Mass spectrometry analysis for landmark identification: mouse skin tissues were homogenized,  
21   and the protein concentration was determined by utilizing Protein RC DC assay (Bio-Rad,  
22   Hercules, CA). The protein lysis was purified by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules,

CA). SDS-PAGE was performed using a precast 8-20% gradient gel (Bio-Rad, Hercules, CA). The spots of interest were excised and subjected to in-gel digestion using proteomics grade trypsin (Sigma, St. Louis, MO). The digested peptides were analyzed on a MALDI TOF/TOF instrument (Bruker, MA) using  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) as matrix. Peak annotation was carried out automatically using software Proteinscape supplied by the instrument manufacturer (Bruker, MA). The m/z-lists were submitted to MASCOT to search the NCBI protein sequence database.

For analysis of the 2-D gels: sample labeling with cyanine minimal dyes was carried out according to the manufacturer's instructions (GE healthcare, Piscataway, NJ). Protein lysates (25  $\mu$ g) were used for CyDye labeling and the ratio of protein to CyDye is 1  $\mu$ g protein: 5 pmol CyDye. The internal standard was always labeled with Cy2, and the samples were labeled with Cy3 and Cy5 alternatively. Isoelectric focusing was carried out on a PROTEAN IEF Cell following manufacture's instructions (Bio-Rad, Hercules, CA). SDS PAGE was conducted using a precast 8-20% gradient gel (Bio-Rad, Hercules, CA). After protein separation, the gels with Cydye labeled proteins were scanned using a Typhoon 9410 scanner (GE Healthcare, NJ) with a resolution of 50  $\mu$ m. Spot detection was performed on the gel images using the DeCyder 6.5 software. Before the matching process, up to 20 landmarks were defined. After a match, the cycle of reviewing and confirming the matches and re-matching was repeated manually until there were no new level 1 mismatches. The differences between the two groups were analyzed by t-test, which is provided by Decyder 6.5. The gels containing non-labeled protein were stained with Coomassie blue for protein identification. Western blot analysis for select proteins was conducted to confirm the proteomics results (data not shown).

## **Lipidomics Analysis**

Phospholipid analysis was performed as described in our previous publications.<sup>22, 23</sup> In short, each frozen dorsal skin tissue was ground with liquid nitrogen. One gram of tissue was mixed with 2 ml solvent [chloroform/methanol (1:2) + 0.01% butylated hydroxytoluene], 1 ml of chloroform, and 1 ml of water. The mixture was centrifuged for 15 min at 1,000 rpm, and the lower layer was extracted. One ml of chloroform was added and the mixture was centrifuged for 15 min at 1,000 rpm followed by collection of the lower layer. The two lower layer extracts were combined for phospholipid analysis using an automated ESI/MS-MS.<sup>22, 23</sup> Phospholipid analysis was able to determine phospholipid classes/subclasses such as Phosphatidic acid, PI, PC, lysoPC, alk(en)yl/acyl phosphocholine (ePC), PE, lysoPE, alk(en)yl/acyl phosphoethanolamine (ePE), phosphatidylserine (PS), alk(en)yl/acyl phosphoserine, sphingomyelin (SM), and ceramide PE. Phospholipid identification was based upon total mass/charge and fragment mass/charge consistent with the head group.

### **Statistical Analysis**

Body weights were compared between treatment groups using one-way analysis of variance (ANOVA). Microarray and proteomics spots were considered statistically significant at 1.5-fold change using the student t-test with significance at  $p < 0.05$ . Cytoscape v2.6.0 coupled with plugin BiNGO v2.0 was used for mapping the predominant gene ontology categories and the significantly altered genes were loaded into GenMAPP (Gene Map Annotator and Pathway Profiler, [www.genmapp.org](http://www.genmapp.org)) to analyze the specific pathways. We also provide information on protein descriptions and biological pathways using Affymetrix NetAffx web site, GeneSpring, and GenMapp relevant software platforms and databases. Lipidomics analysis was performed using one-way ANOVA and F test for significance, with pairwise comparison by the least significant difference method.



## 1    **Results**

### 2    **Body Weight Effects**

3    Body weight changes during the experiment are shown in Figure 1. The control group and AE  
4    groups demonstrated weight gains over the course of the study with an average body weight of  
5    27 g. Both PE and DCR mice groups had significantly lower body weights of 22 and 19 g,  
6    respectively. The DCR treatment group had the most pronounced weight loss, followed by PE.

### 7    **Effect of Exercise on Food Intake**

8    Table 2 shows food intake in all groups except for DCR mice that were fed 80% of the  
9    control and finished all the food daily without any variation for comparison. When  
10   compared with the sedentary control, PE but not AE had reduced food intake  
11   significantly in weeks 2, 3, 6, and 11. The overall food intake was also statistically  
12   reduced in PE verse the control.

### 13   **Effect of Weight Loss on Gene Expression Profile**

14   Among the 39,000 genes analyzed, 411 transcripts by DCR, 67 transcripts by PE, and 110  
15   transcripts by AE were significantly changed compared with the control. The gene sets identified  
16   by microarray analysis that were significantly changed by weight control were further  
17   categorized using GO annotations. The GO categories were analyzed using BiNGO, a program  
18   which helps visualize functional themes. Figure 2 shows the visualization of significantly  
19   changed gene network by Cytoscape. The shading of the node indicates the degree of statistical  
20   significance: black node > grey node > white node. There is an overall impact of gene change by

1 DCR > PE > AE. In comparison of the two exercised groups, PE had stronger impact than AE in  
2 biological processes such as cell death and cell differentiation, more molecular function gene  
3 expression in area such as DNA and protein binding, and little change in cellular components. In  
4 comparison of two weight loss groups, DCR had more impact on gene expression than PE in  
5 biological events related to stress, cell growth and homeostasis, metabolism, and many organelle  
6 differences in the cellular components section. DCR had distinctly more genetic changes in  
7 nearly all the GO categories as opposed to AE. Furthermore, Figure 3 shows examples of the  
8 pathway analysis by GenMapp, indicating the Raf MAP-Kinase pathway was significantly  
9 down-regulated by 0.64-fold and 0.66-fold change in PE and DCR groups, respectively, but  
10 upregulated by 1.55-fold in AE.

#### 11 **Effect of Weight Loss on Protein Expression Profile**

12 Using proteomic techniques, we were able to identify approximately 120 proteins. Among  
13 which, 22 proteins were significantly changed by DCR treatment. Some of the proteins had  
14 multiple spots including albumin (2), carbonic anhydrase 3 (3), enolase 3:beta muscle (3), and  
15 ATP Synthase (H<sup>+</sup> transporting, mitochondrial F1 complex) (2). Table 3 lists the 10 proteins that  
16 were up-regulated and 12 proteins that were down-regulated by DCR.

#### 17 **Effect of Weight Loss on Phospholipid Expression Profile**

18 Among 338 phospholipid species analyzed, 57 species were significantly changed by exercise.  
19 Compared to sedentary controls, most phosphatidylinositol (PI), ether phosphatidylcholine  
20 (ePC), and some lysophosphatidylcholine (lysoPC) molecular species decreased significantly in  
21 exercise with pair feeding mice. It should be noted that five PI groups decreased in PE as

opposed to AE. Figure 4 illustrates five PI species that were significantly decreased in PE but not AE.

### Discussion

The current study demonstrated that both DCR and PE groups were effective in significantly lowering body weight as compared to control animals, with DCR having the most impact on body weight. Weight loss in PE group was not as pronounced as 20% DCR, possibly because the exercise was not strenuous enough to reach 20% calories burned in exercise.<sup>14</sup> The body weight finding in this study confirms previous studies that have shown decreased food intake for a negative energy balance through PE and DCR may cause weight loss.<sup>14,21</sup> It appears that negligible change of food intake in the AE group is responsible for the weight gain that was comparable to the sedentary control. Thus, DCR or negative food intake with exercise in PE group seems more effective than AE and control groups in reducing body weight.

It is usually expected that exercise should have prompted an elevation in food intake in compensation of energy expenditure. Some studies in humans have shown that moderate to high-intensity exercise leads to increased appetite and subsequent increase in food intake as well as body weight.<sup>24,25</sup> However, our findings showed that food intake was reduced slightly in AE group and significantly in PE group compared to the controls. This is in agreement with a human study that found exercise decreased food intake in part through increased postprandial GLP-1, leading to a stronger satiety response.<sup>26</sup> Furthermore, two studies conducted in rodent models also reported a decreased food consumption by a moderate exercise.<sup>27,28</sup> The mechanisms by which exercise may reduce food intake have been associated with improved sensitivity to leptin resistance.<sup>29</sup> In regard to the difference of food intake between AE and PE group, some mice in

1 PE group could finish all the food, which means these mice were able to consume more if given  
2 free access to diet. This is probably one of the reasons that PE group had less food intake  
3 compared with AE group. In addition, fresh pellet diet was kept in freezer that usually contains  
4 about 10% of moisture. For the control group, mice were fed weekly and food intake was  
5 measured weekly, while PE and AE mice were fed daily and food intake was weighted daily. It  
6 could be true there were more water loss in weekly-fed diet than that in daily-fed diet, which  
7 might account, at least in part, for an overestimation of food intake in the controls. Based on the  
8 current results, the decrease of body weight for exercise group was attributed mainly by less food  
9 intake rather than more energy expenditure. Future studies determining energy expenditure of  
10 each mouse and then incorporating it with food intake for body weight control are warranted.

11 The gene expression data indicate that caloric restriction obstructs cancer signaling  
12 pathways. The 2-hr period of TPA treatment was selected based upon a time-course study, in  
13 which AP-1:DNA binding and c-Jun mRNA were optimally induced at 2 hours after phorbol  
14 ester treatment.<sup>30</sup> Our previous studies also confirmed the expression of tumor promotion-related  
15 genes was induced in response to 2-hr TPA after adjustment by the basal levels in acetone-  
16 treated TPA-free skin epidermis.<sup>14,21</sup> However, it would be ideal if various time points including  
17 some late response genes were detected. The approaches to gene expression were distinguishable  
18 from our previous studies,<sup>14,21</sup> since a new gene map annotator and pathway profiler was applied  
19 and since all the three bioinformatics approaches including microarray, proteomics, and  
20 lipidomics were simultaneously analyzed in this study. Out of the 39,000 transcripts measured,  
21 559 genes were significantly different in the exercise treatment groups as compared to the  
22 controls. A total of 411 genes were changed by DCR, 67 genes by PE, and 110 genes by AE,  
23 illustrating the largest total number of genes changed occurred through DCR. To better grasp the

specific molecular targets of DCR, BiNGO software was employed to analyze gene functions such as cellular components and regulations of biological processes like apoptosis, cell proliferation, and cell differentiation. DCR had the most change in functions related to cellular components and biological processes, while PE demonstrated moderate change in these functions versus AE. There was a progressive increase in gene change by treatment groups (DCR > PE > AE). While both DCR and PE modulated weight control, the genetic response shows a major distinction between DCR and PE. Many genes related to MAPK and PI3K signaling significantly decreased in both DCR and PE groups. DCR fostered more pronounced protection in genes related to pro-cancer pathways. For example, insulin-like growth factor binding protein 3, which is associated with IGF-1 activity, was reduced by 0.53-fold change in PE and 0.40-fold change in DCR. In looking for specific cellular pathways impacted by diet, we utilized GenMAPP software and found that DCR and PE both reduced the Braf/MAPK pathway involved in enhancing cellular proliferation, whereas this pathway was up-regulated by AE. While further studies will need to be conducted to elucidate the differences between DCR and PE gene ontology, our data clearly shows their effects on the Ras-MAPK pathway.

Using 2D DIGE proteomics, we identified approximately 120 protein spots, which corresponded to 86 proteins within 2D gel. Among these proteins identified, we found that 22 proteins were significantly changed in DCR versus the control. The cellular functions of the proteins analyzed were common to pathways such as energy metabolism/glycolysis and cellular stress response. In analyzing the data, we identified proteins that may be key targets for cancer prevention and promotion such as Apolipoprotein A-1 (APOA1) which was up-regulated by DCR (Table 3). APOA1 has been studied extensively for its cancer protective properties through reducing inflammation.<sup>31</sup> Over-expression of APOA1 mimetic peptides was associated with

1 increased survival rates and inhibition of size and number of tumors in a mouse ovarian cancer  
2 model. APOA1 may foster cancer protection via binding lysophosphatidic acid (LPA), a  
3 proinflammatory lipoprotein that leads to cellular proliferation through the Ras-Rho GTPase  
4 crosstalk for cancer promotion.<sup>31,32</sup> The inhibitory effect of APOA1 on Ras-MAPK pathway  
5 provides a possible link between the cancer prevention observed in microarray and proteomics  
6 data. Additionally, oxidized phospholipids contribute to pro-inflammation,<sup>33</sup> and it is postulated  
7 that APOA1 may reduce this inflammatory response and exert pro-cancer effect through  
8 reducing MAPK activity.<sup>31</sup> In contrast, gelsolin-like capping protein (capG), is an oncogenic  
9 protein down-regulated by DCR. Bahassi et al. demonstrated that capG is important for tumor  
10 cell motility and cell proliferation, therefore down-regulation of capG by the AP-1 transcription  
11 factor complex may contribute to cancer prevention.<sup>34</sup> Increased cell motility in cancer is  
12 correlated with increased Ras-MAPK activity,<sup>32</sup> as a result the down-regulation of capG may aid  
13 in the cancer preventive effects of DCR. Overall, it appears that DCR decreases cellular  
14 pathways related to cancer promotion including Ras-MAPK and PI3K-Akt signaling pathways,  
15 reduction of inflammation, and modulation of phospholipids by DCR for cancer prevention.<sup>22</sup>

16 Cancer prevention was further demonstrated through reduction of PI phospholipid species  
17 via PE treatment. Our lipidomics data indicated that weight control through PE reduced 5 PI  
18 species as opposed to the AE and control groups. The predominant form of PI in mouse tissue is  
19 PI 38:4.<sup>22,35</sup> PI species can be substrates of PI3K involved in cellular signaling pathways related  
20 to cancer promotion via downstream PI3K-Akt signaling. This decrease in PI phospholipids is  
21 consistent with previous data which indicates that PIs are decreased in exercised groups.<sup>14</sup> PE  
22 had the most pronounced reduction in PI substrates compared to AE or the control. Our lab has  
23 also shown that exercise-induced reduction in PI phospholipids leads to protection from further

1 downstream cancer promoting events through PI3K-related signaling.<sup>22</sup> We did not detect  
2 phospholipid profile in DCR group. When compared with AE, PE had shown a reduced food  
3 intake. The results of phospholipid change in PE may be, at least in part, suggested an impact of  
4 calorie restriction.

5 A major hormone decreased as a result of PI signaling following DCR or PE is IGF-1.<sup>36,37</sup>  
6 Our lab has shown that IGF-1 restoration within PE mice reversed the reduction of PI  
7 phospholipids and PI-associated PI3K.<sup>23</sup> Hence, it appears that IGF-1 is a key growth factor that  
8 promotes cancer development in overweight conditions, in part through promoting PI  
9 phosphorylation by PI3K and downstream Akt activity which contribute to anti-apoptosis.<sup>38,39</sup>  
10 Hence, increased IGF-1-Akt anti-apoptosis, or inhibition of apoptosis, may lead to cancer  
11 promotion through turning off protective nuclear apoptosis genes. Through demonstrating that  
12 PIs were increased in the phospholipid membrane, our study helps to better understand the  
13 mechanism for IGF-1 and increased PI3K-Akt activity. Morimura *et al.* demonstrated that IGF-1  
14 promotes colocalization of IGF-1 receptor and PIP3, which is a PI phosphorylated by PI3K.<sup>39</sup>  
15 This colocalization of IGF-1 receptor and PIs could explain how PIs could amplify the signal for  
16 anti-apoptosis from IGF-1. Reduced IGF-1 levels could lead to decreased localization of PIs and  
17 subsequent reduction of downstream PI3K signaling. Figure 5 illustrates the possible  
18 mechanism through which weight control could lead to reduced IGF-1 levels, PI3K activity, and  
19 anti-apoptosis. Thus, our phospholipidomics data further illustrates the importance of the PI3K-  
20 Akt pathway in cancer prevention following weight control.

21 Our current data demonstrate the cellular signaling pathways of Ras-MAPK and PI3K-  
22 Akt as key cancer preventive targets based on three independent bioinformatics approaches. A  
23 limitation of our study was that the proteomics analysis did not detect many protein kinases, as

we may have expected. A more recently developed technique of phosphoproteomics may aid in identifying more kinases related to signaling pathways.

Taken together, this study identified PI3K-Akt and Ras-MAPK as two major pathways related to weight control and cancer prevention seen through all three bioinformatics approaches. Microarray data showed that the Ras-MAPK pathway was down-regulated in DCR and PE, but increased in AE. Our proteomics data showed that APOA1 and capG are proteins that are modified for cancer prevention by Ras-MAPK. APOA1 leads to a decrease in proinflammatory response that may be helpful for cancer prevention through modulating Ras-MAPK. CapG was also a protein reduced by DCR that is indicative of reduced Ras-MAPK and PI3K-Akt activity. Finally, lipidomics data showed reduced levels of PI species with isocaloric exercise (PE), suggesting how weight control can reduce the PI3K-Akt pathway and its downstream effects on apoptosis. The three areas of bioinformatics utilized give us a more global overview for the protective effect of weight control through both isocaloric exercise and calorie restriction on cancer prevention. It seems that weight control helps to prevent against cancer through reduction in hormones from excess body fat such as IGF-1 and/or leptin and their further downstream molecular targets as concluded in Figure 5.

**Author Contributions:** ZZ, LL, RB and WW participated in the experimental design. JS, YJ, XS, JC, MY, JX and JT performed the experimental procedure and/or data analysis. JS, YJ, BK and WW wrote the manuscript. All authors reviewed and edited the manuscript.

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Table 1. Experimental Diet Composition

Diet Components <sup>a</sup>	Control Diet	20% DCR <sup>b</sup>
Corn Oil	5.0	3.7 <sup>c</sup>
Casein	20.0	20.0
DL Methionine	0.3	0.3
Dextrose	15.0	12.3 <sup>d</sup>
Dextrin	49.9	37.1 <sup>d</sup>
Fiber	5.0	5.0
AIN-93 Mineral Mix	3.5	3.5
AIN-93 Vitamin Mix	1.0	1.0
Choline bitartrate	0.25	0.25
Total amount of food	100.0	82.0

<sup>a</sup>AIN-93 custom made diet by Harlan Teklad (Madison, WI)

<sup>b</sup>DCR mice were fed 0.82 g of diet for every gram consumed by control mice

<sup>c</sup>Dietary calorie restriction from fat source

<sup>d</sup>Dietary calorie restriction from carbohydrate source



1 **Table 2.** Effects of exercise on food intake (g/week/mouse)\*

	Control	AE	PE
Week 1	25.6±2.2 <sup>a</sup>	24.6±2.6 <sup>a</sup>	24.3±2.7 <sup>a</sup>
Week 2	29.5±2.4 <sup>a</sup>	28.4±2.7 <sup>a</sup>	24.5±1.3 <sup>b</sup>
Week 3	31.5±4.7 <sup>a</sup>	28.9±3.4 <sup>a</sup>	26.2±2.0 <sup>b</sup>
Week 4	28.6±3.5 <sup>a</sup>	27.6±5.8 <sup>a</sup>	26.4±2.1 <sup>a</sup>
Week 5	27.7±3.1 <sup>a</sup>	26.9±6.2 <sup>a</sup>	26.5±1.6 <sup>a</sup>
Week 6	35.2±5.4 <sup>a</sup>	33.2±2.9 <sup>a</sup>	26.5±1.2 <sup>b</sup>
Week 7	29.4±3.4 <sup>a</sup>	28.3±3.3 <sup>a</sup>	27.0±0.6 <sup>a</sup>
Week 8	29.8±4.2 <sup>a</sup>	28.3±3.8 <sup>a</sup>	27.8±1.3 <sup>a</sup>
Week 9	29.5±3.4 <sup>a</sup>	28.9±3.8 <sup>a</sup>	27.9±1.6 <sup>a</sup>
Week 10	31.3±4.1 <sup>a</sup>	30.9±4.5 <sup>a</sup>	30.5±0.7 <sup>a</sup>
Week 11	35.8±6.5 <sup>a</sup>	35.4±3.4 <sup>a</sup>	27.4±2.2 <sup>c</sup>
Week 12	27.6±3.8 <sup>a</sup>	26.8±5.1 <sup>a</sup>	27.1±1.4 <sup>a</sup>

2 \*Data are means ± SD, n = 15-18. Means in a row without a common letter differ, p < 0.05.

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Table 3. Proteins that were up-regulated or down-regulated by Dietary Calorie Restriction (DCR) versus the Control utilizing a 2D-DIGE gel.<sup>a</sup>

Proteins Up-Regulated by DCR <sup>b</sup>	Proteins Down-Regulated by DCR <sup>c</sup>
6-phosphogluconolactonase	PDZ and LIM domain protein
trise phosphate isomerase	myosin light chain (phosphorytable)
kininogen 1 precursor	myosin A2 catalytic light chain
albumin	enolase 3: beta muscle
ornithine aminotransferase	gelsolin-like capping protein (capG)
carbonic anhydrase 3	carbonic anhydrase 3
apolipoprotein A-1	ATP Synthase (H <sup>+</sup> transporting, F1)
heat shock protein (cystallin related)	aldose reductase
Flavin reductase (NADPH-dependent)	UGP2 protein
peroxiredoxin 6	phosphoglycerate kinase
	aconitase 2
	adenylate kinase isoenzyme 1

<sup>a</sup>Proteins were scanned using a Typhoon 9410 scanner with resolution of 50  $\mu$ m

<sup>b</sup>Up-regulated proteins had fold-change of  $\geq 1.50$ ,  $p < 0.05$

<sup>c</sup>Down-regulated proteins had fold-change of  $\geq 1.50$ ,  $p < 0.05$

Figure Legends:

**Figure 1.** Body Weight Effects. Data are shown for mice in the four groups: sedentary control (Control), ad-libitum exercise (AE), pair-fed exercise (PE), and dietary calorie restriction (DCR). Values are represented as mean  $\pm$  SE, n=13-17. Means without a common letter differ,  $p < 0.05$ .

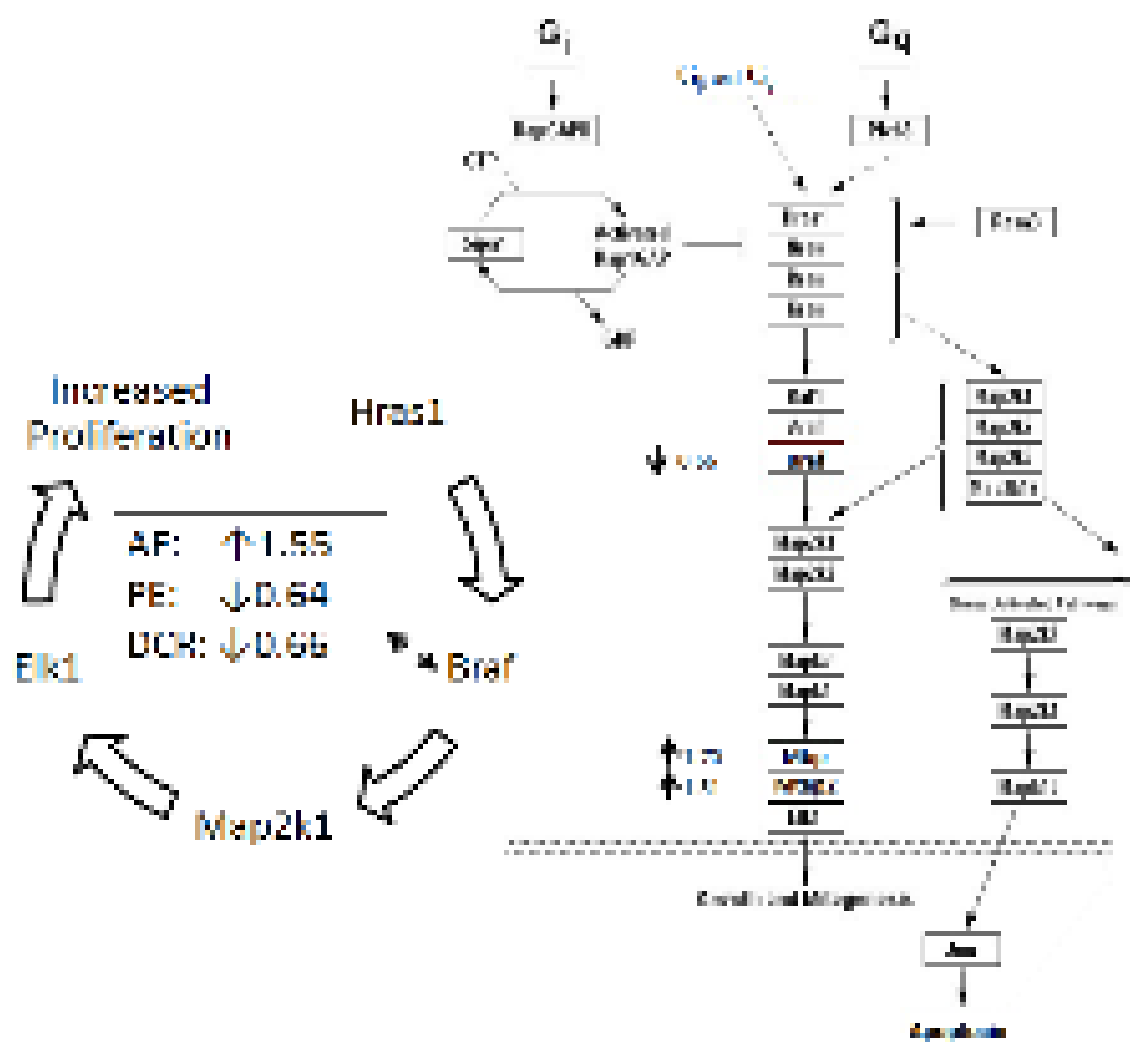
**Figure 2.** BiNGO software representation showing Gene Ontology (GO) for significantly changed gene expression in (A) AL+Exe versus Control, (B) PF+Exe versus Control, (C) DCR versus Control. The coloring of the node indicates the degree of a statistical difference in gene expression of treatment group versus control at black node > grey node > white node.

**Figure 3.** The Hras-Braf MAP-Kinase pathway visualized using GenMAPP software. Right panel: representative gene expression in Hras-Braf MAP-Kinase pathway for DCR verse the control, indicating 0.66-fold down-expression of Braf but 1.70- and 1.51-fold over-expression of Mbp (myelin basic protein) and Mtab2 (methanol cobalamin methyltransferase 2), respectively. Left panel: summary overview of Braf expression in AE, PE, and DCR verse the control, representing 1.55-fold increase in AE, but 0.64- and 0.66-fold decrease in PE and DCR, respectively.

**Figure 4.** Molar percentage of phosphatidylinositol species between AE, PE, and control as analyzed by ESI/MS-MS. Values are shown as mean $\pm$ SE, n=8-15. Means between treatment groups without a common letter differ,  $p < 0.05$ .

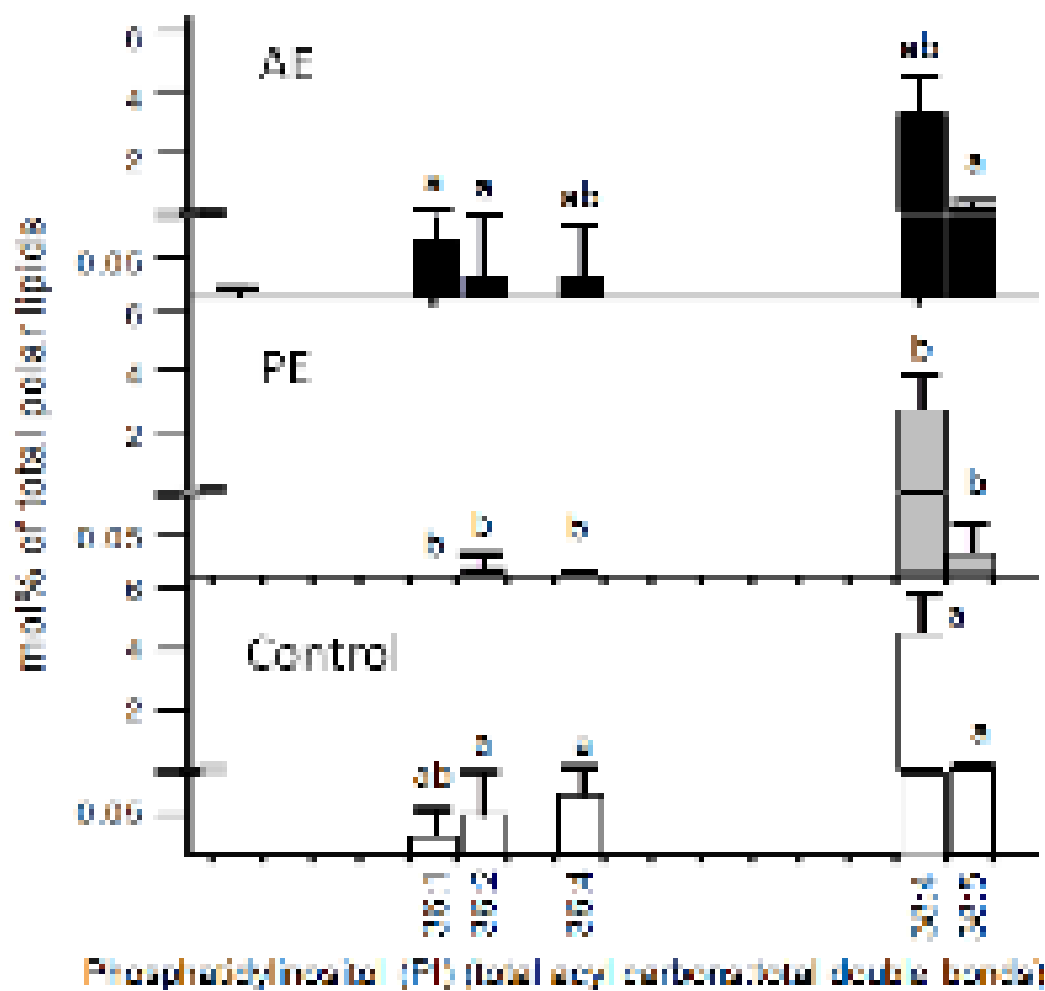
**Figure 5.** Overview of the molecular pathways through which weight control may impact for cancer prevention.





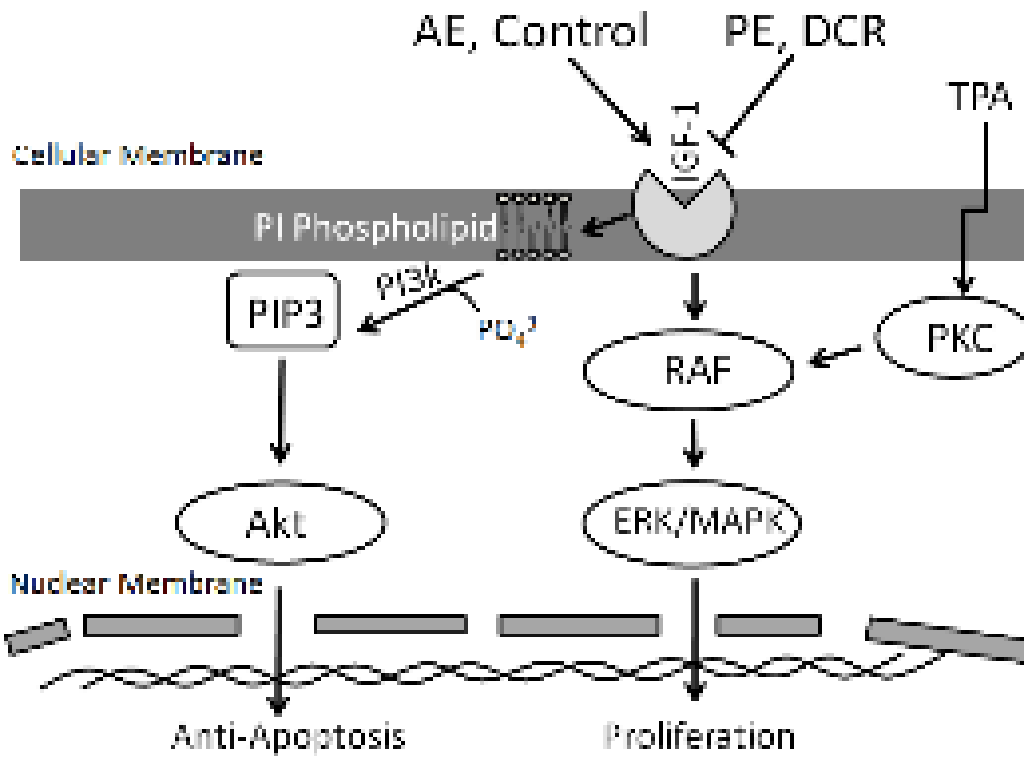
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