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1 **Energy balance modulation impacts epigenetic reprogramming, ERα and**
2 **ERβ expression, and mammary tumor development in MMTV-neu**
3 **transgenic mice**
4
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ABSTRACT

The association between obesity and breast cancer risk and prognosis is well established in ER-positive disease but less clear in HER2-positive disease. Here, we report preclinical evidence suggesting weight maintenance through calorie restriction may limit risk of HER2-positive breast cancer. In female MMTV-HER2/neu transgenic mice, we found that ERα and ERβ expression, mammary tumorigenesis and survival are energy balance-dependent in association with epigenetic reprogramming. Mice were randomized to receive a calorie restriction (CR), overweight (OW)-inducing, or diet-induced obesity (DIO) regimen (n = 27/group). Subsets of mice (n = 4/group/time point) were euthanized after 1, 3 and 5 months to characterize diet-dependent metabolic, transcriptional, and epigenetic perturbations. Remaining mice were followed up to 22 months. Relative to the OW and DIO regimens, CR decreased body weight, adiposity, and serum metabolic hormones as expected, and also elicited an increase in mammary ERα and ERβ expression. Increased DNA methylation accompanied this pattern, particularly at CpG dinucleotides located within binding or flanking regions for the transcriptional regulator CCCTC-binding factor (CTCF) of *ESR1* and *ESR2,* consistent with sustained transcriptional activation of ERα and ERβ. Mammary expression of the DNA methylation enzyme *DNMT1* was stable in CR mice but increased over time in OW and DIO mice, suggesting CR obviates epigenetic alterations concurrent with chronic excess energy intake. In the survival study, CR elicited a significant suppression in spontaneous mammary tumorigenesis. Overall, our findings suggest a mechanistic rationale to prevent or

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99 reverse excess body weight as a strategy to reduce HER2-positive breast cancer

100 risk.

101

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102 **INTRODUCTION**

103 Breast cancer is the most frequently diagnosed noncutaneous neoplasm
104 among women in the United States and is the leading cause of cancer death
105 among women worldwide (1,2). Approximately 30% of all breast cancers lack
106 estrogen receptor alpha (ERα), which confers a worse prognosis in comparison
107 to ERα-positive breast tumors (3). Furthermore, the expression of estrogen
108 receptor beta (ERβ), a putative tumor suppressor, is also lost in most ERα-
109 negative breast tumors (4). ERβ is the more prevalent ER in normal mammary
110 tissue, but its expression is reduced during tumor formation. Numerous studies
111 have linked greater breast tumor ERβ expression with an improved prognosis (5,
112 6). However, the degree to which ERα and ERβ expression is impacted by
113 dietary energy balance and/or controlled epigenetically in breast tumorigenesis
114 remains unclear.

115 Obesity, a result of chronic positive dietary energy balance, is an
116 established risk factor for postmenopausal breast cancer and may also enhance
117 risk in premenopausal women with additional breast cancer risk factors, including
118 type 2 diabetes (7, 8). In addition, excess energy intake and increased adiposity
119 have been associated with greater breast tumor size, increased progression
120 markers, and therapy resistance in both pre- and postmenopausal women (9-11).
121 In contrast, the maintenance of a negative energy balance via calorie restriction
122 (CR) prevents weight gain and inhibits the development of several types of
123 cancer, including ER-positive and ER-negative breast cancers, in numerous
124 animal models (12, 13). CR also has been associated with changes in several

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125 serum and tissue biomarkers in women causally linked with reduced breast
126 cancer risk (14, 15).
127 Although links between energy metabolism, epigenetic regulation of gene
128 expression, and several chronic diseases have been previously established, the
129 relationship between dietary energy balance, epigenetics and breast cancer is
130 poorly understood (16, 17). DNA methylation levels are regulated in part by DNA
131 (cytosine-5)-methyltransferase 1 (*DNMT1*), which predominantly serves to
132 maintain genomic DNA methylation during DNA replication (17). Thus, during
133 times of high cell proliferation such as during development, *DNMT1* is highly
134 expressed. However, *DNMT1* can become deregulated throughout the life course
135 in response to metabolic, inflammatory and environmental disturbances, and this
136 dysregulation has been linked with aberrant DNA methylation and cancer (16,
137 18-20).
138 The effects of DNA methylation are dependent upon the location of methyl
139 groups in the genomic landscape. In general, promoter methylation results in
140 transcriptionally silent genes. However, methylation in the transcription region of
141 genes is often positively correlated with gene expression (21, 22). In addition,
142 methylation that prevents a repressor from binding DNA can correspond to
143 increased gene expression (23). CCCTC-binding factor (CTCF) is an 11-zinc
144 finger protein and highly conserved transcription factor with enhancer-blocking
145 activity (24). DNA methylation at CpG (5'-C-phosphate-G-3') dinucleotides is
146 inversely correlated with CTCF occupancy (23, 25). We posit that the diet-
147 methylation-CTCF axis may serve as a critical sensory system that regulates

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148 gene transcription via energy balance-associated changes in DNA methylation at
149 or near CTCF binding sites.
150 The genetically engineered Mouse Mammary Tumor Virus (MMTV)-neu
151 mouse model is characterized by mammary gland overexpression of the
152 oncogene human epidermal growth factor receptor (HER2). MMTV-neu mice
153 initially have histologically normal, ERα-positive mammary glands that
154 subsequently develop regions of ductal carcinoma *in situ* with ERα expression
155 lost in most cells. If untreated, these mice ultimately develop ERα-negative,
156 HER2-positive mammary adenocarcinomas before 24 months of age (26). In the
157 present study, we tested the hypothesis that dietary energy balance modulation
158 alters ERα and/or ERβ expression, DNA methylation and tumor incidence in
159 female MMTV-neu mice.
160

161 **MATERIALS AND METHODS**

162 ***In vivo* studies in MMTV-neu transgenic mice**

163 All animal studies and procedures were approved and monitored by the
164 University of Texas Institutional Animal Care and Use Committee. Female 6- to
165 8-week-old MMTV-neu mice (JAX stock #002376, *n* = 86) were purchased from
166 Jackson Laboratory (Bar Harbor, ME, USA) and fed a modified AIN-93G
167 semipurified diet, defined as the overweight (OW)-inducing diet for this study
168 (catalog #D12450B, Research Diets, Inc., New Brunswick, NJ, USA) *ad libitum*
169 for 1 week of acclimation.
170

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171 **Baseline mice:** Following acclimation, a subset of mice (*n* = 5) were fasted for
172 6 hours and then euthanized by CO_2 asphyxiation followed by cervical dislocation.
173 Blood was collected by cardiac puncture, allowed to coagulate for 30 minutes at
174 room temperature, and centrifuged at 10,000 x *g* for 5 minutes; serum was
175 removed and stored at -80°C for subsequent analyses. Mammary tissues were
176 collected for further molecular and pathological analysis.
177
178 **Time point study**: A subset of 36 mice were singly housed and randomized
179 (*n* = 12/diet group) to one of the following three diet treatment groups (each
180 modified from the OW diet, which is AIN-93G semipurified diet formulation) for a
181 5-month time point study: 1) calorie restriction regimen (CR), a low-fat, low-
182 carbohydrate diet (#D0302702); 2) OW diet, a high-carbohydrate, low-fat diet
183 providing 3.8 kcal/g (#D12450B); or 3) diet-induced obesity regimen (DIO), a
184 high-carbohydrate, high-fat diet providing 5.2 kcal/g (#D12492), all from
185 Research Diets, Inc. CR mice were administered their diet formulation as daily
186 aliquots of food that provided 70% of the kcal but 100% of the vitamins, minerals,
187 essential fatty acids and amino acids relative to the OW group. Mice were
188 weighed weekly. After 1, 3 and 5 months on diet, mice were analyzed for
189 percent body fat using quantitative magnetic resonance spectroscopy (Echo
190 Medical Systems, Houston, TX, USA). At each of these same time points 4 mice
191 per diet group were killed, and serum and nontumor-bearing mammary tissue
192 were collected as described above. No tumors developed in any mice in the time
193 point study.

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194

195 **Survival study**: The remaining subset of 45 mice were singly housed and
196 randomized (n = 15/diet group) to the CR, OW or DIO diet regimens and were
197 followed for survival for up to 22 months. The survival curve for each diet group
198 illustrates time-to-event data, with the event consisting of either death or the
199 presence of a mammary tumor > 1.0 cm in any direction, the IACUC approved
200 maximal tumor size (27). Non-mammary tumor-related deaths were censored.
201 Mice were palpated for mammary tumors weekly. Once detected, tumor
202 diameters were measured in two dimensions twice weekly with electronic
203 calipers. When tumor diameter reached 1.0 cm in either dimension (or after 22
204 months of study in the absence of tumor), mice were killed. Tumor and/or distal
205 mammary tissue were collected and processed. One half of each collected tissue
206 sample was fixed in 10% neutral buffered formalin for 24 hours, transferred to
207 70% ethanol for at least 24 hours, embedded in paraffin, and cut into 4 μm thick
208 sections for hematoxylin and eosin (H&E) staining or immunohistochemical
209 analysis. The other half was placed in a cryotube, flash frozen in liquid nitrogen
210 and stored at -80°C for subsequent molecular analyses. Blood was collected by
211 cardiac puncture and serum was isolated for analysis.
212

213 **Analyses of circulating energy balance-related hormones and 17β-estradiol**
214 Serum samples from all 5 mice at baseline, and all 4 mice per diet group
215 at the 1-, 3- and 5-month time points were collected after mice were fasted to
216 reduce variability of metabolic hormones. Estrous cycle was not assessed.

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217 Serum samples were analyzed for leptin, insulin, insulin-like growth factor (IGF)-1,
218 and adiponectin by Luminex-based bead array assay (Millipore, Billerica, MA,
219 USA) read on MagPix multianalyte detection system (BioRad, Hercules, CA,
220 USA). The mean inter-assay coefficient of variation (C.V.) of multiplexed bead-
221 based assays for metabolic hormone detection has been shown to be <15% in
222 published studies (28). Serum 17β-estradiol was measured by ELISA (Alpha
223 Diagnostics, San Antonio, TX, USA).
224

225 **Real-time quantitative reverse transcription (qRT)-PCR analyses of ERα**
226 **and ER*β***

227 Total RNA was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis,
228 MO, USA) according to manufacturer's instructions from the flash-frozen
229 mammary tissue samples collected at baseline (*n* = 5 mice) and each of the 3
230 time points (*n* = 4 mice/diet group/time point). RNA was also extracted from
231 nontumor-bearing, flash-frozen mammary tissue collected from mice in the
232 survival study upon their termination (between 14 and 22 months). RNA
233 concentration was spectrophotometrically determined using a nanodrop (Thermo
234 Scientific, Logan, UT, USA), and quality was confirmed using an Agilent 2100
235 Bioanalyzer (Santa Clara, CA, USA). RNA was reverse transcribed with
236 Multiscribe RT (Applied Biosystems, Carlsbad, CA, USA) and resulting cDNA
237 were assayed in triplicate using Taqman® Gene Expression Assays for ERα,
238 ERβ and *DNMT1* (Applied Biosystems). PCR reactions were monitored by a
239 ViiA™7 Real time PCR system (Applied Biosciences). Gene expression data

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240 were normalized to the housekeeping gene β-actin and analyzed using the delta
241 delta cycle threshold method.
242
243 **Histopathologic and immunohistochemical analyses**
244 Tumors were examined for histopathological markers of tumor progression,
245 including vascularity (presence of blood vessels) and proliferation (number of
246 mitotic figures per field) in H&E sections by a board-certified veterinary
247 pathologist. Vascularity was graded in a blinded fashion on a categorical score
248 for the entire slide (0 = no intratumoral blood vessels present, 1 = low number of
249 vessels present, 2 = medium number of vessels present, 3 = high number of
250 vessels present). Mitotic figures were counted in 5 non-overlapping fields of view,
251 and a mean number of mitotic figures was determined for each mouse. Values
252 from each mouse were used to calculate mean vascularity score and mitotic
253 figures for each diet group.
254 Immunohistochemical staining of mammary tissue was performed (n = 4
255 mice/diet group) using a primary antibody for ERα (Catalog #sc542, Santa Cruz
256 Biotechnology, Santa Cruz, CA, USA) at 1:500 and ERβ (Abcam #3576,
257 Cambridge, MA, USA) at 1:100. The secondary antibody was horseradish
258 peroxidase-labeled anti-rabbit antibody (DAKO Cytomation, Carpinteria, CA,
259 USA).
260

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261 **DNA methylation analysis**

262 DNA was extracted from a random sample (*n* = 3/group) of mammary
263 tissues from baseline mice and CR and OW mice in the 5-month time point and
264 survival study using UltraPure™ phenol:chloroform:isoamyl alcohol per
265 manufacturer's instructions (Life Technologies). Library preparation and
266 sequencing for baseline, CR and OW in the 5-month time point and survival
267 study were performed at UT MD Anderson Cancer Center's DNA Methylation
268 Analysis Core and Science Park Next-Generation Sequencing Facility, according
269 to published protocols as previously described (29) (Supplementary Table S1).
270 Samples from DIO mice were not analyzed given the cost of RRBS and the
271 similarities between OW and DIO mice in ERα and ERβ mRNA and protein
272 expression. Gene promoter regions were calculated based on RefSeq gene
273 annotations with regions starting 1 kb upstream of the annotated transcription
274 start site (TSS) and extending 500 base pairs downstream of TSS. Differential
275 methylation was calculated by filtering samples based on read coverage ≥ 20,
276 then performed at the single base level. Methylkit R package was used to apply
277 logistic regression and the likelihood ratio test. Observed p-values were adjusted
278 with the success likelihood index method (SLIM). CpG dinucleotides that
279 exhibited differential methylation patterns between CR and OW groups were
280 cross-referenced with annotated gene regulatory regions within and surrounding
281 *ESR1* and *ESR2* in *Mus musculus* outlined by Ensembl (30). To generate a heat
282 map, we identified CpG dinucleotides with significantly higher methylation in CR
283 survival vs. OW survival mice that also had percent methylated DNA values

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284 available for baseline and CR and OW mice at the 5-month time point.
285 Differentially methylated CpG dinucleotides were clustered using hierarchical
286 clustering with complete linkage and a Euclidian distance measure.
287 Corresponding dendrogram and heat maps for promoter, intron, exon and other
288 were produced using the *heatmap.2* function from the gplots package in R
289 (version 3.3.1).
290

291 **Differential expression analysis using RNA-seq and Ingenuity Pathway**
292 **Analysis (IPA)**

293 RNA was extracted as described above. RNA libraries were prepared
294 using the Illumina TruSeq Stranded Total RNA Sample Preparation kit according
295 to manufactures instructions. The libraries were sequenced using a 2x76 bases
296 paired end protocol on the Illumina HiSeq 2000 instrument. The reads were
297 mapped to mouse genome (mm10) by TopHat (version 2.0.7) (31). The number
298 of fragments in each known gene from RefSeq database (32) (UCSC Genome
299 Browser 2013) was enumerated using HTSeq-count from HTSeq package
300 (version 0.5.3p9) (HTSeq). The differential expression between conditions was
301 statistically assessed by R/Bioconductor package EdgeR (version 1.10.1). Genes
302 with false discovery rate ≤ 0.05 were called significant. For pathway analysis,
303 genes with differential expression in tissue from CR vs. OW mice after 5 months
304 on diet and/or in survival study mice were integrated into IPA pathway designer
305 (Qiagen, Venlo, Netherlands) to draw connections of regulatory relationships
306 using validated scientific findings.
307

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308 **Statistical analyses**

309 Values are presented as mean ± standard deviation (s.d.). For all tests,
310 GraphPad Prism software was used (GraphPad Software Inc., La Jolla, CA), and
311 $P < 0.05$ was considered statistically significant. Differences between diet groups
312 in body weight were analyzed by repeated measures analysis of variance
313 (ANOVA) followed by Tukey's post hoc test. Differences between diet groups in
314 insulin, leptin, adiponectin, IGF-1, 17β-estradiol and mammary ERα and ERβ
315 mRNA and protein expression at each time point were analyzed by one-way
316 ANOVA followed by Tukey's post hoc test. Kaplan-Meier survival curves were
317 plotted, and the difference in overall survival between the groups was analyzed
318 by the log-rank test.

319

320 **RESULTS**

321 **Dietary energy balance modulation impacts body weight, body composition**
322 **and serum metabolic hormones**

323 Female MMTV-neu mice were randomized to receive dietary energy
324 balance modulation via CR, overweight-inducing (OW), or diet-induced obesity
325 (DIO) diet regimens, and were monitored as part of a 1, 3 and 5 month time point
326 study (n = 4 mice/diet for each time point) or for up to 22 months in a survival
327 study (n = 15 mice/diet; hereafter referred to as "survival study mice"). CR, OW,
328 and DIO diet regimens resulted in lean, overweight, and obese phenotypes,
329 respectively. After 1 month of diet treatment, differences in average caloric intake
330 (Supplementary Figure S1) produced differences in mean body weight (Figure

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331 1A), with CR < OW < DIO. This continued for up to 15 months in the survival
332 study mice, after which tumor development in OW and DIO mice made body
333 weight measurements unstable. CR mice had significantly lower percent body fat
334 (Figure 1B) compared with DIO mice after 1, 3 and 5 months on diet. OW mice
335 had intermediate percent body fat between CR and DIO.
336 Compared with CR mice, OW and DIO mice were metabolically
337 dysregulated as assessed by energy balance-related hormones (Table 1). DIO
338 mice, compared with CR mice, had higher serum levels of insulin, leptin, insulin-
339 like growth factor (IGF-1) and 17β-estradiol, and lower serum levels of
340 adiponectin, consistent with obesity-associated metabolic perturbations. OW
341 mice generally displayed intermediate levels of these metabolic factors after 1, 3
342 and 5 months on diet.
343

344 **Dietary energy balance modulation impacts mammary ERα and ERβ**
345 **expression**

346 Mammary tissue was collected from time point study mice after 1, 3 and 5
347 months, and from survival study mice between 14 and 22 months upon their
348 termination. Tissues were analyzed for ERα and ERβ expression and values are
349 reported as relative to expression in mammary tissues collected from a baseline
350 sample of 5 mice killed before initiation of dietary modulation. CR mice,
351 compared with OW and DIO mice, had significantly higher mammary mRNA
352 levels of ERα after 3 and 5 months on diet and in survival study mice (Figure 2A).
353 In addition, mammary ERα protein levels were decreased in OW and DIO mice,

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354 compared to CR mice, by 1 month and continued throughout study (Figures 2B
355 and F).
356 Relative to baseline, mammary ERβ mRNA levels were increased (at least
357 4-fold, on average) in tissues from CR mice, but decreased in tissues from OW
358 and DIO mice (Figure 2C). At the 1-month time point and every time point
359 thereafter, CR mice had significantly greater levels of both ERβ mRNA and ERβ
360 protein compared with OW and DIO mice (Figures 2C, D, and F). No differences
361 between OW and DIO mice in ERα expression, ERβ expression, or the ratio of
362 ERα to ERβ were found. Significant diet-dependent differences in the ERα to
363 ERβ ratio were detected at each time point in tissues collected from CR vs. OW
364 mice, as well CR vs. DIO mice (Figure 2E).
365
366 **Dietary energy balance modulation impacts DNA methylation within *ESR1***
367 **and near *ESR2***
368 Using reduced representation bisulfite sequencing (RRBS), we analyzed
369 diet effects on the methylation status of DNA isolated from the mammary tissue
370 of baseline, CR and OW mice at the 5-month time point and in survival study
371 mice. Due to the similarities between OW and DIO mice in ERα and ERβ mRNA
372 and protein expression, samples from DIO mice were not analyzed.
373 Mammary DNA methylation was generally higher in CR mice than OW
374 mice, particularly in CpG dinucleotides at CTCF binding sites or flanking regions
375 within *ESR1* and near *ESR2* (Supplementary Table S2). In mammary tissue
376 collected in the survival study from CR mice, compared with OW mice, the

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377 percentage of methylated DNA was significantly different (CR > OW) at 3 distinct
378 intronic CpG dinucleotides (Chr10:4710028, Chr10:4710036, Chr10:4710084) at
379 an annotated CTCF binding site within *ESR1*, which encodes ERα. Diet-
380 dependent effects on ERα DNA methylation were not observed in samples from
381 the 5-month time point (Figure 3A and Supplementary Table S3).

382 In mammary tissue from CR mice compared with OW mice, collected at
383 either the 5-month time point or the survival study, 5 CpG dinucleotides near
384 *ESR2* (the gene encoding ERβ) had significantly higher methylation within or
385 near a CTCF binding site (Figure 3B). Specifically, significant diet-dependent
386 effects on DNA methylation were detected in samples from the survival study at 3
387 CpG dinucleotides near *ESR2* (Chr 12:76080926, downstream of *ESR2*; and Chr
388 12:76184418 and Chr 12:76184436, both upstream) that all fell ± 1 kb of a CTCF
389 binding site, which we define as a CTCF flanking region. We also observed
390 significantly higher methylation in CR mice at the 5-month time point at 2 other
391 sites (Chr 12:76086754 and Chr 12:76086771, both downstream) that fell within
392 an annotated CTCF binding site (Figure 3B and Supplementary Table S4).
393

394 **Dietary energy balance modulation impacts ERα and ERβ expression in**
395 **part through epigenetic mechanisms**

396 We investigated global diet-dependent differences in mammary DNA
397 methylation in CR versus OW mice. After 5 months on diet, OW mice had 511
398 CpG dinucleotides with significantly higher methylation and 248 CpG
399 dinucleotides with significantly lower methylation compared with CR mice.

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400 Furthermore, survival study mice possessed more pronounced diet-dependent
401 differences in mammary DNA methylation. OW survival study mice had 651 CpG
402 dinucleotides with significantly higher methylation and 6901 with significantly
403 lower methylation compared with CR. Often, these genes with higher methylation
404 in CR survival compared to OW survival had higher methylation levels in baseline
405 and in CR and OW mice at the 5-month time point, supporting a deviance in OW
406 survival methylation (Figures 4A and B).

407 Mammary gland RNA-seq analysis demonstrated that DNA (cytosine-5)-
408 methyltransferase 1 (*DNMT1*) expression was significantly higher in OW
409 compared with CR survival study mice (Supplementary Table S5). To validate
410 RNA-seq results, we analyzed *DNMT1* mRNA levels by qRT-PCR in the
411 mammary tissue of CR, OW and DIO mice at all time points (Figure 4C). *DNMT1*
412 mRNA levels were not significantly different among CR, OW and DIO mice after
413 1, 3 and 5 months on diet. However, in survival study mice, mammary tissues
414 from OW and DIO mice had significantly higher *DNMT1* mRNA levels compared
415 with mammary tissues from CR mice.

416 RNA-seq analysis in non-tumor mammary tissue from the survival study
417 also identified several components of the signal transducer and activator of
418 transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B
419 cells (NF-κB), E2F transcription factor (E2F), and insulin signaling pathways, that
420 can impact expression of *DNMT1* and were significantly different in OW than CR
421 mice (Supplementary Table S5). Each of these pathways can impact expression
422 of *DNMT1* (33-35). Thus, the metabolic and inflammation-related perturbations

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423 measured in OW and DIO mice (relative to CR mice) may underlie the observed
424 diet-dependent changes in expression of *DNMT1.*
425
426 **CR inhibits MMTV-neu mammary tumor development**
427 Compared with OW and DIO diet regimens, CR was associated with
428 significantly increased survival (Figure 5A). Survival was comparable between
429 OW and DIO mice. After 22 months of study, only 1 OW mouse and 2 DIO mice,
430 compared with 11 CR mice, remained alive and tumor-free. Among the tumor-
431 bearing mice, the mammary tumors from OW (n = 12) and DIO (n = 11) mice,
432 compared with CR mice (n = 3), were generally more vascular and consisted of
433 more proliferating cells (Figure 5B).
434
435 **DISCUSSION**
436 This study assessed whether dietary energy balance modulation, ranging
437 from lean (CR) to overweight (OW) to obesity (DIO), alters mammary ER
438 expression, epigenetic reprogramming and/or mammary tumor development in
439 female MMTV-neu transgenic mice. We first characterized diet-dependent
440 metabolic perturbations in subsets of diet-treated mice euthanized at baseline, 1,
441 3 and 5 months on study. CR mice, relative to OW and DIO mice, had decreased
442 body weight, adiposity, and obesity-associated serum metabolic hormones,
443 including insulin, leptin, IGF-1, and 17β-estradiol, as well as increased
444 adiponectin, after 1 month of diet treatment, consistent with previous studies (36,
445 37).

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446 We show for the first time that CR (but not OW and DIO regimens)
447 preserves mammary ERα and ERβ expression in MMTV-neu mice. Loss of ERα
448 and ERβ, and increased ERα to ERβ, as observed in OW and DIO mice, have
449 each been linked with poor breast cancer prognosis in clinical studies (38, 39).
450 Our finding that the ERα to ERβ ratio, a prognostic indicator in breast cancer, can
451 be manipulated by energy balance modulation has not, to our knowledge, been
452 previously reported.
453 We and others have shown that obesity can induce aberrant DNA
454 methylation of genes involved in growth factor and inflammatory signaling (29,
455 40). To assess whether the energy balance-dependent effects on ERα and ERβ
456 expression are controlled epigenetically, we characterized epigenetic alterations
457 in mammary tissue DNA from baseline mice, from CR and OW mice after 5
458 months on diet, and from survival study mice after 14 to 22 months of diet. In
459 survival study mice differentially methylated CpG dinucleotides were observed in
460 a CTCF binding site within the *ESR1* gene and in CTCF binding sites or flanking
461 regions (± 1kb of a CTCF binding site) upstream and downstream of *ESR2,*
462 consistent with sustained transcriptional activation of ERα and ERβ. Mammary
463 expression of *DNMT1* was stable in CR mice but increased over time in OW and
464 DIO mice, suggesting CR prevented epigenetic reprogramming of *DNMT1* that
465 occurs with excess energy intake and weight gain. The effect of altered *DNMT1*
466 expression likely impacts the expression of many genes, including *ESR1* and
467 *ESR2*. However, a plausible mechanism for the sustained expression of the
468 *ESR1* and *ESR2* genes in response to CR is the maintenance of *DNMT1*

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469 expression and DNA methylation (Figure 6). This can prohibit CTCF binding,
470 thereby preventing allosteric repression and decreased interactions between
471 enhancers and promoters (24). Previous studies have shown that DNA
472 methylation can impede CTCF binding, positively influencing transcription via a
473 loss of repression of specific genes, such as *GAD1* (41) and *XAF1* (42), and
474 increased enhancer-promoter interactions in *IGF2* (43) and *c-MYC* (44).

475 We also assessed the effects of dietary energy balance modulation on
476 mammary tumor development in a cohort of mice randomized to the three diets
477 (*n* = 15/diet). CR, relative to the OW and DIO regimens, resulted in significantly
478 increased survival in MMTV-neu mice in association with increased mammary
479 ERα and ERβ expression and DNA methylation at or near CTCF binding sites.
480 Specifically, of the 15 MMTV-neu mice fed the CR diet for up to 22 months, only
481 3 developed spontaneous mammary tumors, while the median survival of the
482 OW and DIO groups was less than 15 months (Figure 5A). To our knowledge,
483 this study is the first to demonstrate the anticancer effects of a chronic CR
484 regimen (compared with OW or DIO) in MMTV-neu mice. However, Mizuno *et al.*
485 found that intermittent calorie restriction decreased mammary tumor incidence in
486 MMTV-neu mice (45). Our findings of the anticancer effects of CR, compared
487 with OW or DIO regimens, are consistent with reports of a link between dietary
488 energy balance modulation and mammary tumorigenesis in other preclinical
489 models of mammary cancer (46, 47).

490 Two previous publications compared DIO versus chow diets (similar to our
491 OW regimen, Supplementary Table S6) on spontaneous mammary

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492 tumorigenesis in MMTV-neu mice. Cleary *et al.* reported that DIO and chow-fed
493 mice had similar mammary tumor development and survival, consistent with our
494 observation of no significant difference in tumor development or survival in OW
495 versus DIO mice (48). In contrast, Chen *et al.* reported a significant diet-
496 dependent difference (chow > DIO) in survival rates (49). Our findings with CR
497 may help reconcile these apparently conflicting results, since we found highly
498 significant differences in survival in CR mice relative to OW and DIO mice. Chen
499 *et al.* linked the procancer effects of DIO, relative to chow, in their study to
500 increased signaling through the IKKβ, mTOR, and VEGF pathways which
501 stimulate proliferation and survival. We have previously established that DIO
502 increases, and CR decreases, circulating IGF-1, insulin and their downstream
503 signals through the IGF-1 and insulin receptor tyrosine kinases (36, 47). IGF-1 is
504 a potent mitogen, which promotes signaling through the IKKβ, mTOR, and VEGF
505 pathways, ultimately promoting growth and also inhibiting apoptosis. In women
506 circulating IGF-1 is positively associated with terminal duct lobular unit involution,
507 mammographic density, and breast cancer risk (50, 51).
508 In the present study, we found that after 1 month of diet treatment CR
509 mice, relative to OW and DIO mice, had decreased serum IGF-1 and insulin
510 (Table 1). However, the DIO and chow-fed mice in the Cleary study did not differ
511 in IGF-1. Thus, one possible explanation for the observed differential tumor
512 responses may involve the diet-dependent effect (or lack thereof) on systemic
513 metabolism, particularly growth factors and their downstream signals. Differential
514 tumor latencies across the studies may also contribute to the apparent study-

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515 specific differences in tumor responses to DIO, as the median survival for the
516 Cleary study chow-fed mice and our OW mice were comparable (~18 months
517 and ~15 months, respectively), in contrast to the Chen study chow-fed mice (~7.5
518 months).

519 Interactions between dietary energy balance modulation and DNA
520 methylation may influence the expression of key breast cancer-related genes,
521 such as ERα, ERβ, via diet-induced changes in *DNMT1* expression and DNA
522 methylation at or near CTCF binding sites. Figure 6 integrates findings from RNA-
523 seq analysis (Supplementary Table S5) using IPA to illustrate a proposed model
524 of a diet responsive network contributing to altered gene expression related to
525 several transcription factor pathways (e.g. E2F, STAT3, and NF-κB) that serve as
526 regulators of *DNMT1*. Inflammation promotes *DNMT1* expression, which has
527 been shown to positively correlate with IL-6 expression in tumors and blood (52,
528 53). Overexpression of *DNMT1* may be a mechanism utilized by cancer cells to
529 evade regulation by rendering tumor suppressors transcriptionally inactive (17,
530 54). Thus, mediators of obesity-associated inflammation may promote increased
531 expression of *DNMT1,* thereby contributing to aberrant DNA methylation and
532 transcription of breast cancer-related genes such as ERα and ERβ.

533 To our knowledge, there are no previous reports regarding the
534 maintenance of mammary ERα and ERβ positivity with a CR regimen. As
535 mentioned previously our CR mice had lower levels of energy balance-related
536 metabolic factors, including IGF-1, insulin, and leptin, consistent with numerous
537 studies in the literature (12, 15, 47). As illustrated in Figure 6, these factors and

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538 their downstream signaling pathways can also impact *DNMT1*, but our study is
539 limited in the ability to establish their precise role. Future studies are planned to
540 assess the possible links between systemic metabolic factors, such as insulin,
541 IGF-1, leptin, and 17β-estradiol and the increased mammary ERα- and ERβ-
542 associated DNA methylation at or near CTCF binding sites in CR mice.
543 Alternatively, the decreased levels of 17β-estradiol observed in CR mice could
544 contribute to prolonged survival, independent of epigenetic events, as high levels
545 of 17β-estradiol can promote cell proliferation and tumor progression (39).
546 Additional study limitations include: a) restricting RRBS and RNA-seq analysis to
547 CR and OW mice (although OW and DIO mice were similar regarding ERα and
548 ERβ mRNA and protein expression and survival); and b) not measuring physical
549 activity (although we have previously shown that CR, relative to control or DIO
550 diets, does not increase locomotor activity in mice) (55).
551 In conclusion, we found in MMTV-neu transgenic mice that mammary
552 tumor development and ERα and ERβ expression are dietary energy balance-
553 dependent in association with epigenetic reprogramming. Specifically, a diet-
554 *DNMT1*-methylation axis may serve as a primary regulator of gene transcription
555 via diet-induced changes in DNA methylation at or near CTCF binding sites.
556 These preclinical findings suggest that interventions reducing the impact of
557 excess weight on epigenetic dysregulation of ER may represent a new strategy
558 for the prevention or control of HER2-positive breast cancer in overweight and
559 obese women.
560

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567 and methylation analysis.

568

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757

758 **Figure Legends**

759 **Figure 1**. Dietary energy balance modulation affects body weight and percent
760 body fat in MMTV-neu mice. (**A**) Body weight (mean ± s.d.) in mice receiving
761 calorie restriction (CR), overweight-inducing (OW), or diet-induced obesity (DIO)
762 diet regimens over 15 months (n = 27 mice/diet). Heterogeneity in body weights
763 after 15 months increased as mice became moribund therefore weight data
764 beyond this point are not shown. Statistical differences in body weights between
765 groups were determined by repeated measures analysis of variance (ANOVA).
766 (**B**) Percent body fat assessed by quantitative magnetic resonance spectroscopy
767 (mean ± s.d.) at 1, 3 and 5 months on diet (n = 4 mice/diet/time point). Statistical
768 differences in body fat between groups were determined by one-way ANOVA.

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769 OW or DIO vs. CR: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. OW vs. DIO: ♦$P < 0.05$,
770 ♦♦$P < 0.01$, ♦♦♦$P < 0.001$

771

772 **Figure 2.** Dietary energy balance modulation affects mammary ERα and ERβ
773 gene and protein expression. Relative mammary ERα (**A**) mRNA and (**B**) protein
774 levels (mean ± s.d.) in CR, OW, or DIO mice at the 1-, 3- and 5-month time point
775 and at survival ($n = 4$ mice/diet/time point). Data are presented as relative to
776 baseline levels ($n = 5$ mice). Relative mammary ERβ (**C**) mRNA and (**D**) protein
777 levels (mean ± s.d.) in CR, OW, and DIO mice at 1, 3 and 5 months on diet and
778 at survival ($n = 4$ mice/diet/time point). Protein levels were analyzed by IHC, with
779 percent ERα and ERβ positive nuclei determined and results presented as
780 relative to baseline levels ($n = 5$ mice). (**E**) The ERα to ERβ ratio was calculated
781 by dividing the percent positive nuclei for ERα by percent positive nuclei for ERβ.
782 (**F**) Representative images of ERα and ERβ IHC at baseline and for each diet
783 group at 5 months on diet. Statistical differences in ERα or ERβ mRNA and
784 protein between diet groups were determined by one-way ANOVA. OW or DIO
785 vs. CR: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

786

787 **Figure 3.** Dietary energy balance modulation affects DNA methylation of the
788 intron regions of *ESR1* and upstream and downstream of *ESR2*. (**A**) *ESR1* intron
789 methylation (mean percent DNA methylation ± s.d.) in the mammary tissue of CR
790 and OW mice at the 5-month time point and survival study mice. DNA
791 methylation at three distinct CpG dinucleotides (Chr 10: 4710028, Chr 10:

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792 4710036, and Chr 10: 4710084), which all fall within a CTCF binding site, are
793 shown. (**B**) Mean percent DNA methylation ± s.d. at CpG dinucleotides upstream
794 (Chr 12: 76184418, Chr 12: 76184436) and downstream (Chr 12: 76080926, Chr
795 12: 76086754, Chr 12: 76086771) of *ESR2* in the mammary tissue of mice
796 maintained on CR or OW diet regimens for 5 months or through survival are
797 shown. These all fall within a CTCF binding site or CTCF flanking region as
798 indicated. Statistical differences determined by logistic regression and the
799 likelihood ratio test, p-values were adjusted with the success likelihood index
800 method. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
801
802 **Figure 4.** Differences in dietary energy balance impact genome wide
803 methylation patterns in the mammary tissue. (**A**) Heat map and clustering
804 dendrogram of mammary tissue DNA methylation. CpG dinucleotides with
805 significant differences in methylation (CR survival vs. OW survival) are shown
806 and clustered within genomic location, i.e. promoter, exon, intron, other. Baseline,
807 CR and OW after 5 months on diet and CR and OW survival study mice are each
808 represented (n = 3/group, with one column per n shown). The methylation
809 frequency percentage ranges from 0 to 100. A value of '0' is completely
810 unmethylated and '100' is fully methylated. Statistical differences (CR vs. OW
811 survival) determined by logistic regression and the likelihood ratio test, p-values
812 were adjusted with the success likelihood index method. (**B**) Representation of
813 genomic locations of differentially methylated CpG dinucleotides, 6.5% mapped
814 to a promoter region, 34.1% mapped to a gene exon, 25.6% mapped to an intron

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815 region, and 33.8% did not map to a promoter, exon or intron and are classified as
816 'other.' (**C**) Mammary *DNMT1* mRNA levels, measured by qualitative real time
817 PCR, in mice maintained on CR, OW, and DIO diet regimens for 1, 3 and 5
818 months or through survival. Data are presented as relative to baseline levels (n =
819 4 mice). Statistical differences in *DNMT1* expression determined by one-way
820 ANOVA *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
821
822 **Figure 5.** Dietary energy balance modulation affects survival, tumor vascularity
823 and proliferation. In MMTV-neu mice (**A**) Kaplan-Meier survival curves for CR,
824 OW and DIO mice (n = 15 mice/diet) over a 22-month period. Mice with
825 nontumor-related deaths (1 CR, 2 OW, and 2 DIO mice) were censored.
826 Statistical differences in survival rate determined by log-rank test (**B**)
827 Photomicrographs of mammary tumor sections stained with hematoxylin and
828 eosin and displayed at 10x or 40x magnification. Among the tumor-bearing mice,
829 the mammary tumors from OW (n = 12) and DIO (n = 11) mice, compared with
830 CR mice (n = 3) were more vascular (group mean ± s.d. vascularity scores: 2.6 ±
831 0.18, 2.2 ± 0.25, and 1.5 ± 1.5, respectively) and consisted of more proliferating
832 cells (group mean ± s.d. number of mitotic figures, indicated by black arrowhead:
833 2.6 ± 0.5, 2.4 ± 0.3 and 0.5 ± 0.4, respectively). Statistical differences in
834 vascularity and mitotic figures determined by one-way ANOVA. *$P < 0.05$, **$P <$
835 0.01, ***$P < 0.001$.
836

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837 **Figure 6.** Proposed model of an energy balance-responsive network associated
838 with *DNMT1* regulation, DNA methylation and transcriptional regulation of ERα
839 and ERβ. Obesity and associated energy excess results in several metabolic
840 perturbations that are ameliorated by calorie restriction. To identify regulatory
841 relationships between *DNMT1* and other genes that were differentially expressed
842 in our RNA-seq analysis, we used the Path Designer function of Ingenuity
843 Pathway Analysis (IPA). We found transcription factors STAT3, NF-kB and E2F
844 to have direct relationships with *DNMT1* activation. Upstream of these
845 relationships, IPA also linked growth factors such as insulin and EGF, as well as
846 cytokines including TNF, IL-1β and IL-8, to the E2F, STAT3 and NF-kB signaling
847 pathways. We propose that one consequence of obesity-associated growth
848 factor and cytokine signaling is increased DNMT1 activation, which in turn can
849 modulate DNA methylation and impact transcription of important genes in breast
850 cancer such, as ERα and ERβ. Methylated CpG dinucleotides are indicated by
851 red circles, unmethylated CpG dinucleotides are indicated by white circles.
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Table 1. Energy balance impacts serum metabolic hormones

	Months on Diet		CR	OW	DIO
Insulin (ng/mL)	**Baseline**	0.83 ± 0.40			
	1		0.5 ± 0.3	0.7 ± 0.1	1.4 ± 0.7
	3		0.3 ± 0.1	0.8 ± 0.6	1.0 ± 0.6
	5		0.2 ± 0.1	0.7 ± 0.2	1.8 ± 0.5***,♦♦
Leptin (ng/mL)	**Baseline**	0.94 ± 0.38			
	1		0.7 ± 0.5	2.8 ± 2.7	4.4 ± 3.4
	3		0.6 ± 0.5	4.1 ± 2.4*	6.0 ± 2.7*
	5		0.2 ± 0.2	3.2 ± 2.0	9.2 ± 6.1*
Adiponectin (ng/mL)	**Baseline**	2.02 ± 0.32			
	1		3.8 ± 0.5	3.1 ± 0.7	2.8 ± 0.3*
	3		4.5 ± 0.9	3.1 ± 0.3*	2.4 ± 0.6**
	5		4.3 ± 0.9	2.6 ± 0.2**	2.3 ± 0.5**
IGF-1 (ng/mL)	**Baseline**	463.2 ± 88.7			
	1		232.2 ± 96.7	396.5 ± 70.3*	425.4 ± 36.8*
	3		187.8 ± 26.5	344.4 ± 56.4*	503.9 ± 112.2***, ♦
	5		194.9 ± 26.4	376.2 ± 28.5***	452.0 ± 57.7***, ♦
17 β -estradiol (pg/mL)	**Baseline**	209.8			
	1		155.6 ± 50.9	180.4 ± 68.0	227.9 ± 36.7
	3		148.7 ± 25.2	262.4 ± 42.1*	260.1 ± 58.8*
	5		117.4 ± 17.4	198.1 ± 22.3*	210.4 ± 54.5*

861 Differences between diet groups in insulin, leptin, adiponectin, IGF-1, and 17β-
862 estradiol at each time point were analyzed by one-way ANOVA followed by
863 Tukey's post hoc test. OW or DIO vs. CR: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
864 OW vs. DIO: ♦$P < 0.05$, ♦♦$P < 0.01$, ♦♦♦$P < 0.001$.

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A

Figure 1

Body weight (g)

60
50
40
30
20
10
0

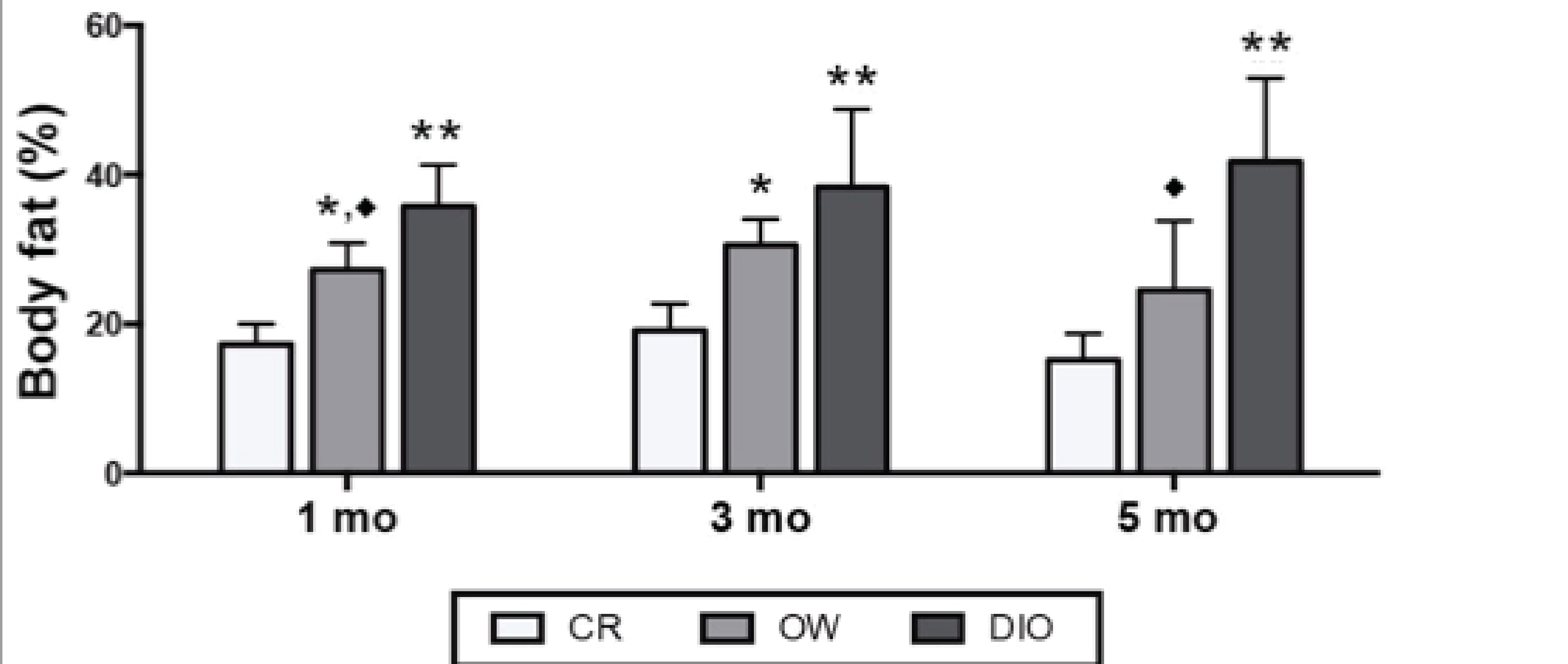
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Months on diet

***

***,◆◆◆

CR OW DIO



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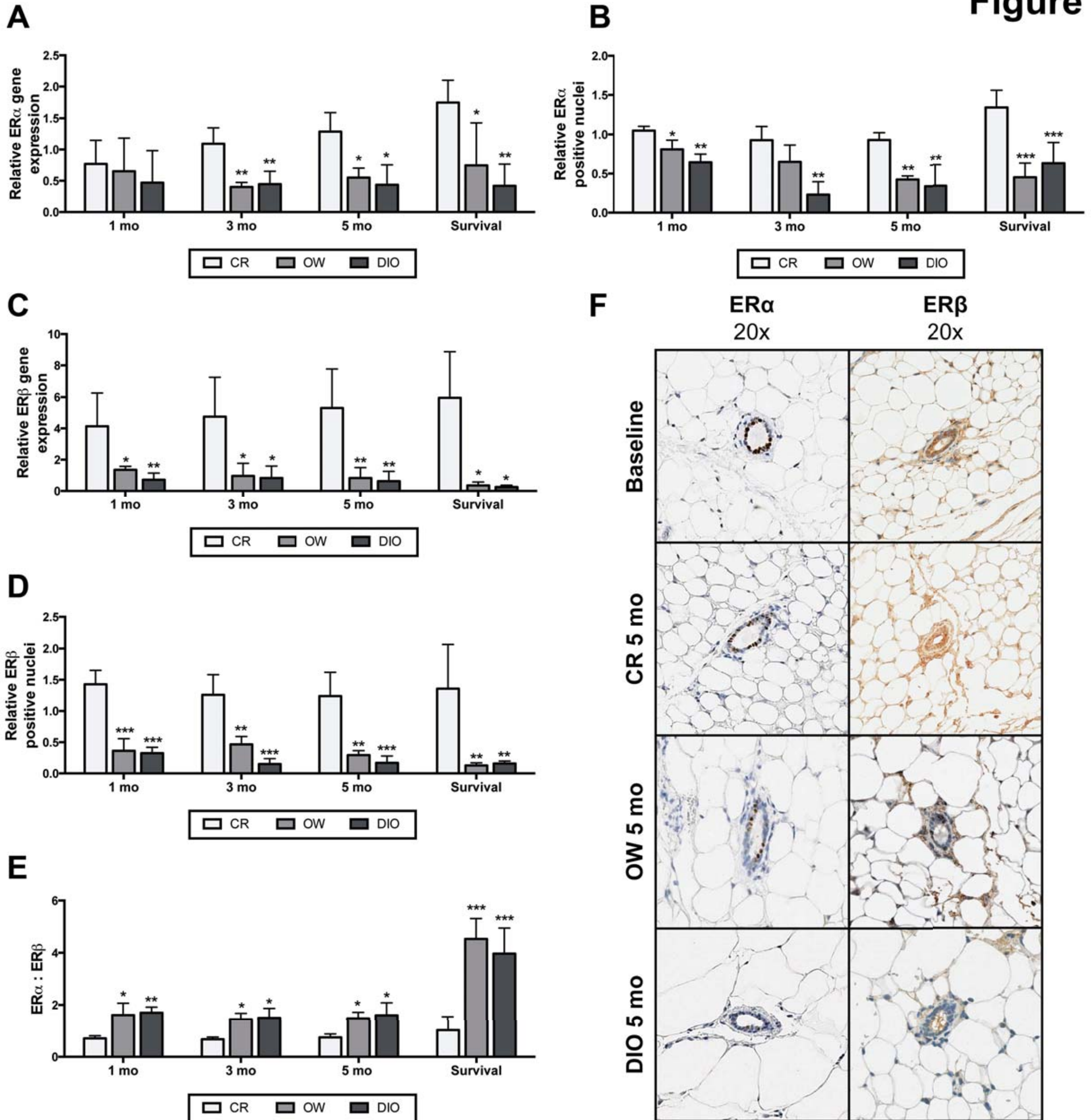

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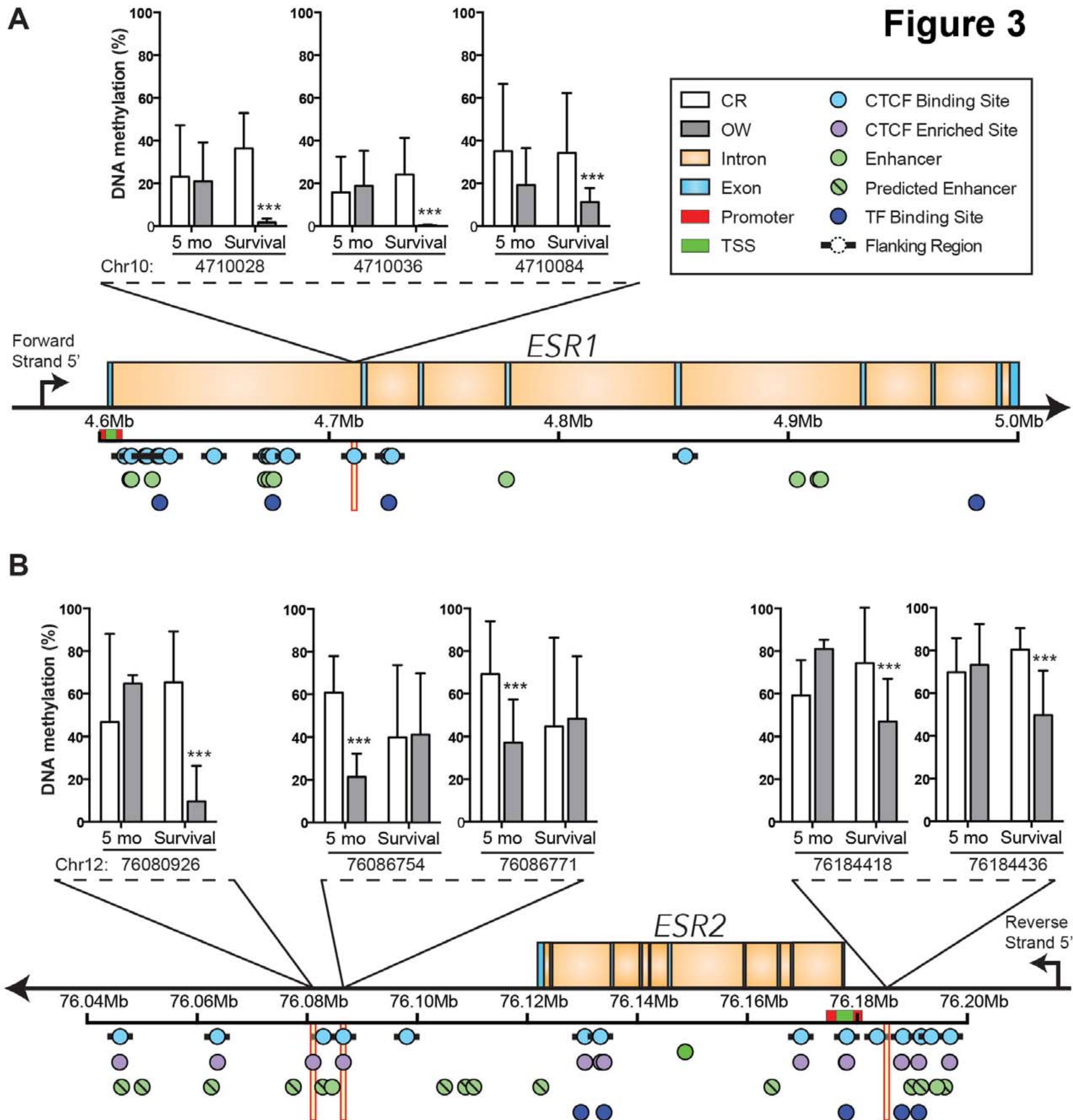

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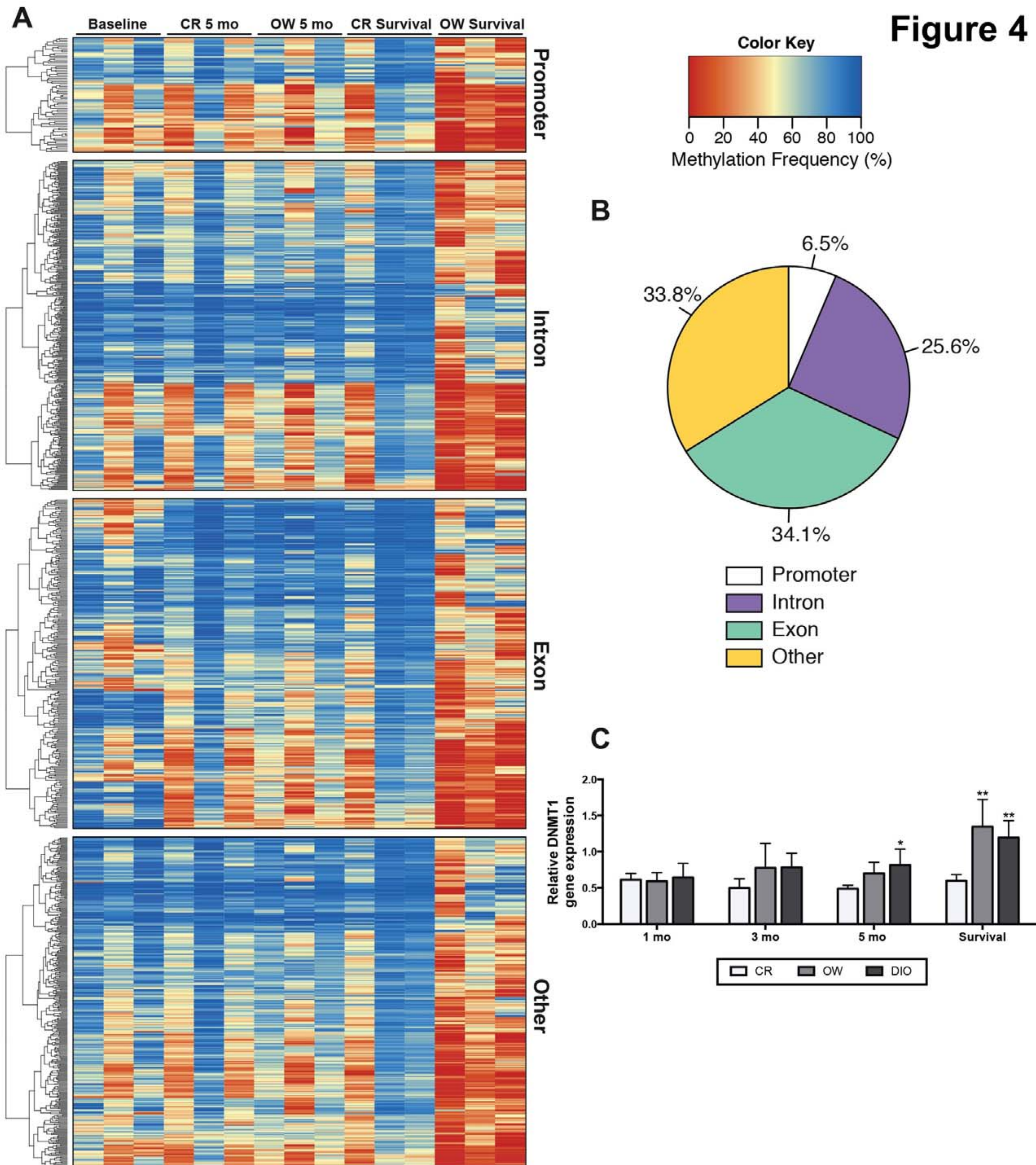

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Figure 5

A

Survival (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22
Months on diet
***
***
CR OW DIO

B

CR OW DIO
10x
40x

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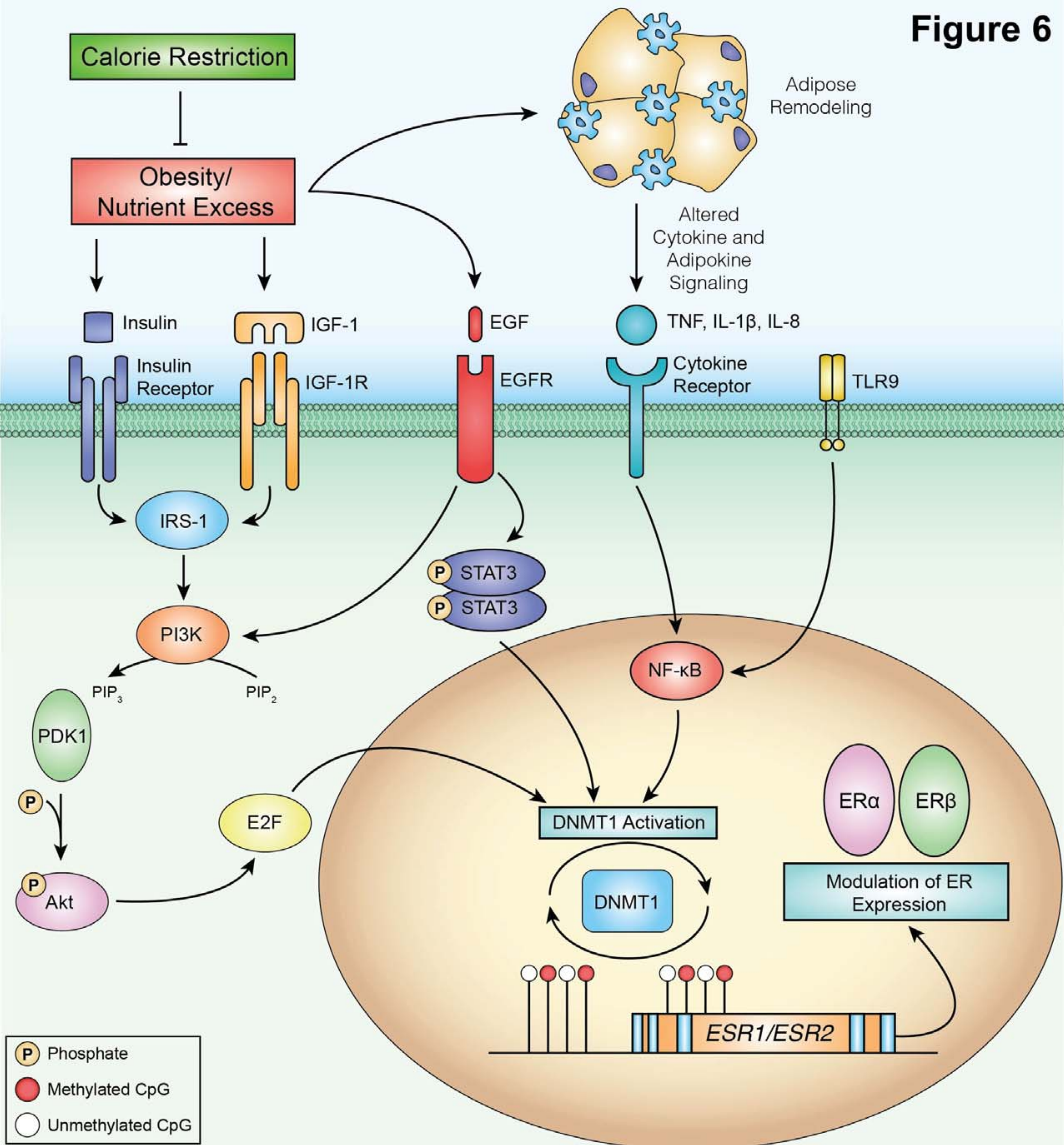

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Energy balance modulation impacts epigenetic reprogramming, ER α and ERβ expression and mammary tumor development in MMTV-neu transgenic mice

Emily L. Rossi, Sarah M Dunlap, Laura W. Bowers, et al.

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