

1 **Title: Obesity promotes breast epithelium DNA damage in BRCA mutation**
2 **carriers**

3
4 **Authors:** Priya Bhardwaj¹, Neil M. Iyengar^{1,2}, Heba Zahid³, Katharine M. Carter¹, Dong Jun
5 Byun⁴, Man Ho Choi⁴, Qi Sun⁵, Oleksandr Savenkov⁶, Charalambia Louka¹, Catherine Liu¹,
6 Phoebe Piloco¹, Monica Acosta¹, Rohan Bareja⁷, Olivier Elemento⁷, Miguel Foronda⁸, Lukas E.
7 Dow^{1,9}, Sofya Oshchepkova¹, Dilip D. Giri¹⁰, Michael Pollak¹¹, Xi Kathy Zhou⁶, Benjamin D.
8 Hopkins¹², Ashley M. Laughney⁹, Melissa K. Frey¹³, Lora Hedrick Ellenson¹⁰, Monica
9 Morrow¹⁴, Jason A. Spector¹⁵, Lewis C. Cantley⁹, Kristy A. Brown^{1,9*}

10 **Affiliations:**

11 ¹Department of Medicine, Weill Cornell Medicine; New York, USA.

12 ²Department of Medicine, Memorial Sloan Kettering Cancer Center; New York, USA.

13 ³Department of Medical Laboratory Technology, College of Applied Medical Science, Taibah
14 University; Medina, Saudi Arabia.

15 ⁴Center for Advanced Biomolecular Recognition, Korea Institute of Science and Technology,
16 Seoul, Korea.

17 ⁵Computational Biology Service Unit of Life Sciences Core Laboratories Center, Cornell
18 University, Ithaca; USA.

19 ⁶Department of Population Health Sciences, Weill Cornell Medicine; New York, USA.

20 ⁷Caryl and Israel Englander Institute for Precision Medicine, Weill Cornell Medicine; New York,
21 USA.

22 ⁸Department of Neurology, Memorial Sloan Kettering Cancer Center; New York, USA.

23 ⁹Sandra and Edward Meyer Cancer Center, Weill Cornell Medicine; New York, USA.

24 ¹⁰Department of Pathology, Memorial Sloan Kettering Cancer Center; New York, USA.

25 ¹¹Departments of Medicine and Oncology, McGill University; Montreal, Canada.

26 ¹²Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai;

27 New York, USA

28 ¹³Department of Obstetrics & Gynecology, Weill Cornell Medicine; New York, USA

29 ¹⁴Department of Surgery, Memorial Sloan Kettering Cancer Center; New York, USA.

30 ¹⁵Laboratory of Bioregenerative Medicine & Surgery, Weill Cornell Medicine; New York, USA.

31 *Corresponding author. Email: kab2060@med.cornell.edu

32

33 **One Sentence Summary:** Elevated bodyweight is positively associated with DNA damage in

34 breast epithelium of *BRCA* mutation carriers

35

36 **Abstract:**

37 Obesity is an established risk factor for breast cancer among women in the general
38 population after menopause. Whether elevated bodyweight is a risk factor for women with a
39 germline mutation in *BRCA1* or *BRCA2* is less clear due to inconsistent findings from
40 epidemiological studies and lack of mechanistic studies in this population. Here, we show that
41 DNA damage in normal breast epithelium of *BRCA* mutation carriers is positively correlated
42 with body mass index and with biomarkers of metabolic dysfunction. Additionally, RNA-
43 sequencing reveals significant obesity-associated alterations to the breast adipose

44 microenvironment of *BRCA* mutation carriers, including activation of estrogen biosynthesis,
45 which impacts neighboring breast epithelial cells. We found that blockade of estrogen
46 biosynthesis or estrogen receptor activity decreases DNA damage, whereas treatment with leptin
47 or insulin increases DNA damage in *BRCA* heterozygous epithelial cells. Furthermore, we show
48 that increased adiposity is associated with mammary gland DNA damage and increased
49 penetrance of mammary tumors in *Brca1*^{+/-} mice. Overall, our results provide mechanistic
50 evidence in support of a link between bodyweight and breast cancer development in *BRCA*
51 mutation carriers and suggests that maintaining a healthy bodyweight or pharmacologically
52 targeting estrogen or metabolic dysfunction may reduce the risk of breast cancer in this
53 population.

54

55 INTRODUCTION

56

57 Inheriting a pathogenic mutation in the DNA repair genes *BRCA1* or *BRCA2* is causally
58 linked to the development of breast and ovarian cancer in women (1, 2). Although there is strong
59 evidence linking obesity to the development of hormone receptor positive breast cancer after
60 menopause in the general population (3), there are conflicting results in *BRCA* mutation carriers.
61 Some studies have found that maintaining a healthy bodyweight or weight loss in young
62 adulthood is associated with delayed onset of breast cancer (4, 5). Other studies have reported
63 that adiposity or elevated bodyweight in adulthood is associated with increased cancer risk (5-9).
64 Conversely, some reports indicate that increased body mass index (BMI) in young adulthood
65 may have protective effects, and that risk is modified by menopausal status (9-11). The lack of
66 clarity on the role of bodyweight and risk of breast cancer development in *BRCA* mutation

67 carriers limits the ability of clinicians to provide evidence-based guidance on prevention
68 strategies beyond prophylactic surgical intervention.

69 Weight gain and obesity are often coupled with metabolic syndrome, insulin resistance,
70 and significant changes to adipose tissue, including that of the breast microenvironment (12-15).
71 Obesity-induced changes to breast adipose tissue includes dysregulation of hormone and
72 adipokine balance, and increased production of inflammatory mediators (16). For example,
73 estrogen biosynthesis is increased in obese breast adipose tissue due to overexpression of
74 aromatase in adipose stromal cells which catalyzes the conversion of androgens to estrogen (17-
75 19). Additionally, excessive expansion of adipocytes leads to hypoxia, lipolysis, and altered
76 adipokine production including higher leptin to adiponectin ratio (15, 20, 21). These changes to
77 the breast microenvironment may have important implications for breast carcinogenesis given
78 that breast epithelial cells are embedded in this milieu and engage in epithelium-adipose
79 crosstalk (22).

80 BRCA1 and BRCA2 are critical for their role in homologous recombination-mediated
81 repair of DNA double strand breaks (23). Mutations in either *BRCA1* or *BRCA2* cause a defect in
82 DNA repair which can lead to an accumulation of DNA damage and consequently,
83 tumorigenesis (24, 25). Studies have linked obesity or metabolic syndrome to DNA damage,
84 including in leukocytes (26), skeletal muscle (27), peripheral blood mononuclear cells (28), and
85 in pancreatic β -cells (29), but no studies have examined the relationship between obesity and
86 DNA damage in normal breast epithelial cells.

87 We show that BMI and markers of metabolic dysfunction are positively correlated with
88 DNA damage in normal breast epithelium of women carrying a *BRCA* mutation, a finding that is
89 extended to the fallopian tube of *BRCA* mutation carriers. RNA-sequencing of whole breast

90 tissue and of isolated breast epithelial organoids from *BRCA* mutation carriers, along with *ex*
91 *vivo* and *in vitro* studies with *BRCA1* and *BRCA2* mutant primary tissues and cell lines, suggests
92 several obesity-associated factors as possible drivers of DNA damage. Additionally, metformin,
93 fulvestrant, leptin neutralizing antibodies and a PI3K inhibitor reduce damage induced by the
94 obese breast microenvironment. *In vivo* studies in *Brcal* heterozygous knockout mice
95 demonstrate that high fat diet-induced obesity leads to glucose intolerance in association with
96 elevation in epithelial cell DNA damage and greater mammary tumor penetrance relative to mice
97 fed a low fat diet. The data presented provide mechanistic evidence supporting an increased risk
98 of breast cancer development in *BRCA* mutation carriers with elevated bodyweight and
99 metabolic dysfunction, and importantly, provides clinically relevant strategies for risk reduction.

100

101 **RESULTS**

102

103 **Obesity positively correlates with breast epithelial cell DNA damage in women carrying a** 104 **mutation in *BRCA1* or *BRCA2***

105 To assess levels of DNA damage in normal breast epithelium in association with
106 bodyweight in women carrying a *BRCA1* or *BRCA2* mutation, tissue microarrays were
107 constructed from non-cancerous breast tissue obtained from 72 women undergoing mastectomy.
108 The study population included *BRCA1* (n=43) and *BRCA2* (n=29) mutation carriers who had
109 documented body mass index (BMI, kg/m²) between 17.7 and 44.9 (median 23.7) at the time of
110 surgery as shown in **Table 1**. When grouping the population by BMI category of lean (BMI ≤
111 24.9 kg/m², n=46) or overweight/obese (BMI ≥ 25.0 kg/m², n=26), median age is significantly
112 higher in the overweight/obese group compared to the lean group (44.5 vs 38.5, respectively,
113 *P*=0.01). Additional clinical features elevated in the overweight/obese group compared to the

114 lean group include percent of subjects diagnosed with dyslipidemia (23.1% vs 2.2%, $P=0.01$)
115 and with hypertension (23.1% vs 4.3%, $P=0.02$). The lean group also has a greater representation
116 of pre-menopausal vs post-menopausal subjects compared to the overweight/obese group
117 ($P=0.04$). Diagnosis of diabetes, race, presence of invasive tumor, tumor subtype and *BRCA1* vs
118 *BRCA2* mutation were not significantly different between the two BMI groups (**Table 1**).

119 Immunofluorescence staining for the DNA double strand break marker γ H2AX was
120 performed with nuclear counterstain Hoechst to visualize the number of foci of DNA damage per
121 epithelial cell (**Fig. 1A**). Among *BRCA1* and *BRCA2* mutation carriers, BMI was positively
122 associated with breast epithelial cell DNA damage as quantified by # of γ H2AX foci/100 cells
123 (**Fig. 1B**). Age was also found to be significantly correlated with DNA damage (**Fig. 1C**). While
124 this correlation diminished when adjusting for BMI ($P=0.11$, **Table 2**), BMI remained positively
125 associated with DNA damage when adjusting for age ($P=0.025$, **Table 2**). Post-menopausal
126 women were found to exhibit significantly higher levels of DNA damage compared to pre-
127 menopausal women (**Fig. 1D**). Additionally, circulating levels of sex hormone binding globulin
128 (SHBG), which binds estrogen to decrease its bioavailability, were negatively correlated with
129 breast epithelial cell DNA damage (**Fig. 1E**). This negative association remains significant when
130 adjusting for both BMI and age ($P=0.047$ and $P=0.026$, respectively, **Table 2**). Elevated BMI is
131 often coupled to insulin resistance, a hallmark of metabolic dysfunction. Accordingly, fasting
132 serum levels of insulin and HOMA2 IR were positively correlated with levels of breast epithelial
133 cell DNA damage while glucose was not (**Fig. 1F-H**). Insulin and HOMA2 IR retained
134 significance after adjustments for either BMI or age ($P<0.001$ for both, **Table 2**). No correlation
135 with DNA damage was observed for circulating biomarkers of inflammation including high-
136 sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6) or with crown-like structures

137 (CLS), a histological marker of local breast adipose inflammation (30) (Fig 1. I-K). These data
138 indicate that among *BRCA* mutation carriers, elevated bodyweight is a risk factor for breast
139 epithelial cell DNA damage. Furthermore, specific obesity-associated factors including insulin
140 resistance and estrogen balance may be important drivers of this risk.

141

142

143 **Elevated bodyweight is associated with significant differences in gene expression in breast** 144 **adipose tissue and in breast epithelial cells of *BRCA* mutation carriers**

145 To identify changes associated with obesity in breast epithelial cells and in the breast
146 adipose microenvironment that may be linked to DNA damage, we conducted RNA-seq studies
147 on both isolated primary breast epithelial cells and non-cancerous whole breast tissue obtained
148 from *BRCA1* and *BRCA2* mutation carriers.

149 RNA-seq was conducted on breast tissue pieces obtained from lean ($BMI \leq 24.9 \text{ kg/m}^2$,
150 $n=64$) and overweight/obese ($BMI \geq 25 \text{ kg/m}^2$, $n=67$) *BRCA* mutation carriers. An unsupervised
151 heatmap was constructed which shows general clustering of lean cases and clustering of
152 overweight/obese cases by gene expression (Fig. 2A). 2329 genes were significantly upregulated
153 by obesity and 1866 were significantly downregulated. Ingenuity Pathway Analysis (IPA)
154 identified several pathways that were significantly altered in the overweight/obese cases which
155 include pathways associated with obesity and metabolic dysfunction, such as “Phagosome
156 Formation”, “LXR/RXR Activation”, “Tumor Microenvironment Pathway Activation”, and
157 “Estrogen Biosynthesis” (Fig. 2B). A heatmap of genes involved in estrogen regulation shows a
158 significant increase in many genes involved in the bioactivity, biosynthesis and activation of
159 estrogens, including steroid sulfatase, $3\beta\text{HSD1}$, AKR1C3 , AKR1B15 , $17\beta\text{HSD1}$ and aromatase

160 (CYP19A1) (**Fig. 2C**). Conversely, gene expression of 17 β HSD8, involved in estrogen
161 inactivation, was significantly lower in overweight/obese relative to lean cases. Moreover, there
162 were mixed effects of obesity on the expression of genes involved in estrogen catabolism to
163 hydroxylated metabolites and neutralization by COMT.

164 To explore which changes in the breast microenvironment are associated with DNA
165 damage in breast epithelial cells, we analyzed breast tissue pathway changes in relation to level
166 of epithelial cell DNA damage quantified by γ H2AX immunofluorescence staining (**Fig. 2D**;
167 n=61). The level of epithelial cell DNA damage in each case was stratified by quartiles and
168 breast tissue gene expression was compared in the highest quartile (Q4) relative to the lowest
169 quartile (Q1), independent of BMI. The top 15 canonical pathways activated in Q4 vs Q1 breast
170 tissue are shown (**Fig. 2D**) with several pathways being common to both DNA damage and BMI
171 analyses (**Fig. 2D** vs **Fig. 2B**). Although the estrogen biosynthesis pathway was found to be
172 activated in tissue from overweight/obese compared to lean cases (**Fig. 2B**, z-score=0.775, *P*
173 value=1.14x10⁻⁶), a stronger activation score is found when comparing Q4 vs Q1 (**Fig. 2D**, z-
174 score=2.646, *P* value=2.7x10⁻³), suggesting that tissue estrogen biosynthesis is highly correlated
175 with level of breast epithelial cell DNA damage, irrespective of BMI.

176 Breast epithelial organoids were isolated from *BRCA* mutation carriers who were either
177 lean (n=10) or overweight/obese (n=9) at the time of surgery. To validate and characterize the
178 isolated epithelial organoids, immunofluorescence staining was conducted for cytokeratin 8
179 (CK8) and cytokeratin 14 (CK14), characteristic markers of luminal and basal epithelial cells,
180 respectively, that are known to comprise the breast epithelium (**Fig. 2E**). 1144 genes were
181 significantly upregulated in the setting of overweight/obesity and 537 were significantly
182 downregulated compared to lean organoids. The top 20 canonical pathways identified by IPA as

183 regulated in the overweight/obese organoids are shown (**Fig. 2F**) and include activation of
184 pathways known to be associated with obesity, including “HIF1 α signaling”, “IL-8 signaling”,
185 “ERK/MAPK signaling”, and “PI3K/AKT signaling”, among others.

186 Collectively, these RNA-seq studies show that *BRCA1* and *BRCA2* mutation carriers who
187 are overweight or obese have significantly altered breast epithelial cell and breast adipose
188 microenvironment gene expression compared with lean counterparts. Moreover, our data provide
189 rationale for further exploring whether estrogen is a driver of DNA damage in the breast.

190

191

192 **Crosstalk between epithelial cells and the breast adipose microenvironment**

193 Given the significant gene expression changes identified in *BRCA* heterozygous breast
194 adipose tissue and in breast epithelial cells in association with overweight/obesity, we next
195 investigated whether the breast adipose microenvironment drives gene expression in breast
196 epithelial cells. IPA Upstream Regulator tool was used to identify regulators of gene expression
197 differences in overweight/obese relative to lean organoids. To highlight endogenous factors that
198 may be responsible for driving gene expression changes, results were filtered to show the top 20
199 secreted factors. Among these factors, beta-estradiol (an estrogen) is the top predicted upstream
200 regulator (**Table 3**). A number of additional predicated upstream organoid regulators are
201 significantly upregulated in overweight/obese breast tissue, including several interleukins (IL2,
202 IL15, and IL5), TGF β 1, CSF1, ANGPT2, and WNT5A. Some factors, such as insulin, are known
203 to be elevated in obesity, but not produced locally in breast tissue and therefore do not have an
204 observed tissue gene expression level. These data suggest that some endogenously produced

205 factors in the overweight/obese breast microenvironment may interact with neighboring breast
206 epithelial cells to induce gene expression changes and DNA damage.

207

208 **Targeting estrogen in breast tissue from *BRCA* mutation carriers reduces epithelial cell**
209 **DNA damage**

210 Next, we conducted mechanistic studies to determine whether targeting estrogen
211 signaling or biosynthesis in breast tissue would lead to decreased levels of breast epithelial cell
212 DNA damage. We first conducted immunohistochemistry (IHC) staining to verify that normal
213 epithelium from *BRCA1* and *BRCA2* mutation carriers express the estrogen receptor (ER α).
214 Epithelial cells staining positively for ER α were found throughout the epithelium among carriers
215 of *BRCA1* or *BRCA2* mutations (representative images shown in **Fig. 3A, top row**). IF staining
216 was then conducted to visualize whether γ H2AX foci co-localize with ER α positive cells.
217 Representative images are shown which highlight ER α positive cells frequently staining
218 positively for γ H2AX foci (**Fig. 3A, bottom row**). Next, we tested whether disrupting estrogen
219 signaling through use of the drug fulvestrant, which degrades the estrogen receptor, would
220 impact levels of DNA damage in the breast. Breast tissue was obtained from *BRCA* mutation
221 carriers undergoing surgery (n=7) and were plated as explants in the presence of fulvestrant
222 (100 μ M) or vehicle for 24 hours (**Fig. 3B**). Explants were formalin fixed and sectioned for
223 assessment of breast epithelial cell DNA damage by IF staining. An approximately 32.5%
224 reduction in DNA damage was observed overall after treatment with fulvestrant (**Fig. 3C**).

225 Next, we hypothesized that targeting estrogen biosynthesis in the breast by
226 downregulating aromatase expression would lead to less estrogen exposure to the epithelial cells,
227 and consequently decreased DNA damage. In support of this hypothesis, RNA-seq data from
228 *BRCA1* and *BRCA2* mutation carriers showed a positive correlation between breast adipose

229 aromatase expression and level of breast epithelial cell DNA damage (**Fig. 3D**). Importantly,
230 since aromatase expression is known to be upregulated in association with obesity, we conducted
231 additional statistical analyses to adjust for BMI and found that aromatase remained
232 independently positively correlated with DNA damage ($P=0.037$). To target estrogen
233 biosynthesis, we utilized metformin, a widely used antidiabetic drug which has also been shown
234 to decrease aromatase production in the breast via stimulation of AMP-activated protein kinase
235 (AMPK) in adipose stromal cells (31, 32). Breast tissue obtained from *BRCA* mutation carriers
236 ($n=3$) were plated as explants and treated with metformin (0-100 μ M) for 24 hours followed by IF
237 assessment of breast epithelial cell DNA damage. A dose-dependent decrease in DNA damage
238 was observed with significant differences after 75 and 100 μ M of metformin treatment (**Fig. 3E**).
239 Since metformin is known to decrease aromatase expression in adipose stromal cells surrounding
240 breast epithelial cells, we digested breast tissue to isolate the epithelial cells from their
241 microenvironment (**Fig. 3B**) and treated them with metformin for 24 hours to determine if the
242 presence of the breast microenvironment is required for the effect of metformin on DNA
243 damage. Although there was a modest trend for reduction in DNA damage with increasing doses
244 of metformin, these results were not significant (**Fig. 3F**). Consistently, tissue levels of estradiol
245 (E2) were markedly reduced in breast explants after 24-hour metformin treatment in a dose-
246 dependent manner (**Fig. 3G**). Additionally, testosterone and androstendione, which are converted
247 to E2 and estrone (E1) by aromatase, respectively, were increased in explants following
248 treatment with metformin while both E1 and E2 decreased (**Fig. 3H**). These data show that
249 metformin treatment leads to decreased estrogen biosynthesis in breast tissue in association with
250 reduction in epithelial cell DNA damage.

251

252 **Local and systemic factors contribute to DNA damage in *BRCA1* and *BRCA2* heterozygous**
253 **breast epithelial cells**

254 Our data support a paracrine interaction between adipose tissue and breast epithelial cells.
255 Having found a direct role for estradiol in mediating DNA damage in primary human tissues, we
256 next explored the role of additional obesity-associated factors, including those present in breast
257 adipose tissue conditioned media (CM), as well as recombinant leptin and insulin. To first
258 investigate whether factors derived from breast adipose tissue have the ability to directly induce
259 DNA damage in *BRCA* mutant breast epithelial cells, *BRCA1* heterozygous knockout MCF-10A
260 cells were treated with CM from reduction mammoplasty or non-tumor quadrants of mastectomy
261 tissue (**Fig. 4A**, n=36, BMI: 20.6-40.1 kg/m²). Breast adipose CM treatment was positively
262 correlated with DNA damage as a function of the patient's BMI, as measured by
263 immunofluorescence of γ H2AX foci (**Fig. 4B**). RNA-seq was performed in a subset of CM-
264 treated samples (lean and obese, n=3/group). Results demonstrate that consistent with DNA
265 damage measurements (**Fig. S1**), IPA analysis of differentially regulated genes in the obese CM
266 treated cells relative to lean showed increased activation of functions associated with DNA
267 damage and genomic instability including "Formation of micronuclei", "Chromosomal
268 instability", and "Breakage of chromosomes". Alternatively, activation of functions associated
269 with DNA repair were decreased, including "Repair of DNA" and "Checkpoint control" (**Table**
270 **4**).

271 To determine whether effects of CM on DNA damage were generalizable to *BRCA2*
272 mutation carriers, a subset of CM cases were tested in MCF-10A cells carrying a heterozygous
273 *BRCA2* mutation, generated using CRISPR-Cas9 gene editing (see Supplementary Materials and
274 Methods). A positive correlation between BMI and DNA damage was also observed in these

275 cells (**Fig. 4C**). These studies demonstrate that factors secreted by breast adipose tissue directly
276 stimulate DNA damage in breast epithelial cells. Furthermore, given the lack of estrogen
277 receptor expression in MCF-10A cells (33), these studies also highlight the existence of
278 additional factors beyond estrogen that may be contributing to DNA damage induction in the
279 setting of obesity in *BRCA1* and *BRCA2* mutant breast epithelial cells.

280 The expression of leptin, known to be directly correlated with adiposity, is significantly
281 higher in overweight/obese compared to lean breast tissue from *BRCA* mutation carriers (Table
282 S1, log₂FC= 0.61, $P=3.48 \times 10^{-6}$). A number of studies have found leptin to have pro-mitogenic
283 and anti-apoptotic effects in breast cancer cells (34-37). However, its effects on normal breast
284 epithelial cells are less well characterized. Here, we treated both *BRCA1* and *BRCA2*
285 heterozygous MCF-10A cells with leptin (400ng/mL) for 24 hours and found a significant
286 induction of DNA damage in both cell lines (**Fig. 4D**) and in primary breast epithelial cells (**Fig.**
287 **4E**). Additionally, the ability of obese CM to induce DNA damage in *BRCA1* heterozygous
288 breast epithelial cells is blocked when treating in the presence of a leptin neutralizing antibody
289 (**Fig. 4F**).

290 Next, having identified insulin as positively correlated with DNA damage in tissue
291 microarrays from *BRCA* mutation carriers, independent of BMI (**Fig. 1F, Table 2**), and as a top
292 upstream regulator of gene expression in primary breast epithelial organoids from
293 overweight/obese women (**Fig. 3A**), we conducted additional mechanistic studies to determine
294 whether insulin can directly induce DNA damage. Treatment of *BRCA1* and *BRCA2*
295 heterozygous knockout MCF-10A cells with insulin (100nM) for 24 hours resulted in a
296 significant increase in DNA damage in both cell lines (**Fig. 4G**) and in primary breast epithelial
297 cells (**Fig. 4H**). Both leptin and insulin have been shown to act via PI3K (38, 39). Treatment of

298 *BRCA1* heterozygous breast epithelial cells with a PI3K inhibitor, BKM120 (1 μ M), was
299 effective at reducing obese CM-induced DNA damage (**Fig. 4I**). These data show that factors
300 produced locally by obese breast adipose tissue or elevated with metabolic dysfunction
301 contribute to induction of DNA damage in *BRCA* heterozygous knockout breast epithelial cells.

302

303 **High fat diet feeding is associated with elevated mammary gland DNA damage and early**
304 **tumor penetrance in female *Brcal* heterozygous knockout mice**

305

306 DNA damage is a known driver of chromosomal defects that can lead to cancer.

307 However, whether obesity-associated elevation in breast epithelial cell DNA damage is linked to
308 breast cancer penetrance in the setting of a heterozygous *BRCA* mutation has not been
309 established. To investigate this question, we conducted preclinical studies utilizing mice that
310 were developed to carry a whole-body heterozygous loss in *Brcal* (*Brcal*^{+/-}) on a C57Bl/6
311 background. Four-week-old female *Brcal*^{+/-} mice were randomized to receive low fat diet
312 (LFD) or high fat diet (HFD) for 22 weeks to produce lean and obese mice, respectively (**Fig.**
313 **5A**). Mice fed HFD gained significantly more weight than LFD fed mice and weighed on
314 average 34.1g vs 23.3g, respectively, at the time of sacrifice (**Fig. 5B**). Overall adiposity was
315 also increased in association with HFD feeding as determined by greater accumulation of
316 subcutaneous and visceral fat compared to the LFD group (**Fig. S2**). To confirm that the HFD-
317 fed mice exhibit altered metabolic homeostasis in our *Brcal*^{+/-} model of diet-induced obesity,
318 glucose tolerance tests were conducted after 21 weeks on experimental diets, highlighting
319 delayed clearance of glucose from blood over 90 minutes post-intraperitoneal injection of
320 glucose in the HFD group compared to LFD-fed mice (**Fig. 5C & D**). To determine whether
321 changes observed in the mammary fat pad of *Brcal*^{+/-} mice in response to feeding were

322 analogous to those seen in the breast tissue of women in relation to obesity, RNA-seq was
323 conducted on inguinal mammary fat pads from LFD and HFD mice harvested at sacrifice (**Table**
324 **S5**). IPA was used to identify activation of the top differentially regulated canonical pathways in
325 HFD mammary fat pads relative to LFD, results of which were juxtaposed with regulation of
326 these same pathways in human breast tissue from overweight/obese vs lean *BRCA* mutation
327 carriers. The top 20 canonical pathways regulated by obesity in the mouse mammary fat pad
328 show very similar regulation patterns compared to overweight/obese human breast tissue (**Fig.**
329 **5E**), suggesting that diet-induced obesity in our *Brcal*^{+/-} mice can serve as a model system for
330 obesity in women carrying a *BRCA* mutation with respect to studies of the breast.

331 Similar to findings made in human breast tissue from *BRCA* mutation carriers, IF staining
332 for γ H2AX of *Brcal*^{+/-} mouse mammary glands at the time of sacrifice show that HFD-fed mice
333 have elevated levels of mammary gland DNA damage compared to LFD-fed mice (**Fig. 5F**).
334 Furthermore, there is a trend for a positive correlation between DNA damage and bodyweight
335 (irrespective of diet) (**Fig. 5G**) and a significant positive correlation between DNA damage and
336 mammary fat pad weight (**Fig. 5H**), suggesting that level of adiposity may be a stronger
337 predictor of DNA damage in mammary epithelium compared to whole body weight.

338 Next, we examined whether elevation in mammary gland DNA damage is associated
339 with tumorigenesis. Female *Brcal*^{+/-} mice were first made obese by HFD-feeding for 10 weeks
340 and then were implanted with a subcutaneous medroxyprogesterone acetate (MPA) pellet to
341 sensitize them to mammary tumor development upon exposure to three doses of the carcinogen
342 7,12-dimethylbenz[a]anthracene (DMBA) (**Fig. 5I**). Mammary tumors developed earlier in the
343 HFD group compared with the control LFD group (**Fig. 5J**). Additionally, 85.7% of mice in the

344 HFD group developed mammary tumors by the end of the 28-week surveillance period
345 compared to 69.2% of mice in the LFD group (**Fig. 5K**).

346

347 **Obesity is associated with DNA damage in the fallopian tube, but not ovary, of *BRCA***
348 **mutation carriers**

349 In addition to elevated breast cancer risk, women carrying a *BRCA1* or *BRCA2* mutation
350 have high lifetime risk for developing ovarian cancer (1, 2). Since weight gain is associated with
351 increased risk of ovarian cancer in *BRCA* mutation carriers (40), we extended our studies in the
352 breast to investigate the impact of elevated BMI on DNA damage in the ovarian epithelium as
353 well as in epithelial cells of the fallopian tube (**Fig. 6**). IF staining for γ H2AX was performed
354 with nuclear counter stain Hoechst to quantify number of foci of DNA damage per epithelial cell
355 in non-tumorous ovarian tissue and fallopian tube fimbria from women carrying a *BRCA1* or
356 *BRCA2* mutation undergoing prophylactic salpingo-oophorectomy. In the ovarian epithelium,
357 there was no increase in DNA damage in the overweight/obese cases (n=12) compared to the
358 lean cases (n=21) (**Fig. 6A**, $P=0.59$). However, there was a significant increase in DNA damage
359 observed in the epithelial cells of the fallopian tube from overweight/obese women (n=9)
360 compared to lean women (n=17) (**Fig. 6B**, $P=0.03$).

361

362 **DISCUSSION**

363

364 The data presented here demonstrate that BMI is positively associated with DNA damage
365 in normal breast epithelial cells in carriers of a mutation in *BRCA1* or *BRCA2*. Beyond BMI,
366 insulin and insulin resistance, as measured by HOMA2 IR, were independently associated with
367 DNA damage, irrespective of BMI or age. Accordingly, it is possible that *BRCA* mutation

368 carriers who are defined as lean by BMI, but hyperinsulinemic ('metabolically obese'), may also
369 be at risk for elevated levels of DNA damage and consequently, breast cancer development.
370 Although previous studies have shown that inflammation can lead to DNA damage in both
371 normal and cancerous cells in other tissues (41-44), our data do not support a link between local
372 or systemic inflammation and breast epithelial cell DNA damage.

373 To our knowledge, this is the first study to conduct transcriptional profiling of non-
374 cancerous breast tissue and isolated breast epithelial cells from overweight/obese vs lean *BRCA*
375 mutation carriers. While several factors and pathways associated with metabolic dysfunction
376 were shown to be upregulated in breast tissue and in epithelial cells, the identification of
377 pathways related to estrogen biosynthesis (tissue) and signaling (epithelial cells) were of
378 particular interest given the availability of clinically approved drugs that target estrogen.
379 Additionally, previous *in vitro* studies showed that treatment with estrogen and estrogen
380 metabolites induced DNA damage in *BRCA1* heterozygous breast epithelial cells (45), providing
381 further rationale for exploring the role of estrogen as a mediator of obesity-induced DNA
382 damage. Here, we show that fulvestrant, an estrogen receptor degrader, is effective at reducing
383 epithelial cell DNA damage in breast tissue explants from *BRCA* mutation carriers. However,
384 this drug is not currently approved for use in the prevention setting and the side effects may limit
385 its future use for this purpose. Alternatively, metformin is widely prescribed in patients with type
386 II diabetes and has an excellent safety profile which makes this drug an intriguing option for
387 preventative use in *BRCA* mutation carriers with excess bodyweight. We show that metformin
388 was effective at reducing breast epithelial cell DNA damage at clinically relevant concentrations
389 primarily due to effects on the breast adipose microenvironment. Previous studies have shown
390 that metformin decreases adipose stromal cell expression of aromatase through activation of

391 AMPK (31, 32). Our study extends these findings by demonstrating the downstream
392 consequence of downregulation in aromatase through mass spectrometry studies which showed
393 marked reduction in E2 in breast tissue after metformin treatment. In addition to reducing
394 estrogen exposure, previous work has shown that metformin treatment reduces endogenous
395 reactive oxygen species (ROS) and associated DNA damage (46) in a mammary epithelial cell
396 line, providing an additional possible mechanism for the effects of metformin in our studies.

397 Epidemiological studies have reported decreased risk of breast cancer in *BRCA* mutation
398 carriers in association with reduced estrogen exposure achieved via salpingo-oophorectomy
399 surgery which diminishes ovarian estrogen production or through treatment with tamoxifen, an
400 estrogen receptor antagonist in the breast (47-49). Our studies propose estrogen-mediated
401 induction of DNA damage as a possible explanation for the protective effects observed by
402 decreasing estrogen exposure in this population. Estrogen can induce DNA damage through
403 various actions as reviewed by our group and others (50, 51), including through ligand binding to
404 ER α which stimulates proliferation and potentially replication stress with ROS production as a
405 byproduct of increased cellular respiration. Additionally, the metabolism of estrogen yields
406 genotoxic metabolites, a process which produces ROS through redox cycling. These metabolites
407 can directly interact with DNA to form adducts in an ER-independent manner. Given the
408 multiple avenues through which estrogen can induced DNA damage in cells, additional studies
409 are warranted to characterize the mechanisms of estrogen-induced DNA damage in breast
410 epithelial cells from *BRCA* mutation carriers in the setting of obesity.

411 Interestingly, our RNA-seq analysis of *BRCAl* heterozygous MCF-10A cells treated with
412 obese vs lean CM not only showed increased activation of pathways associated with DNA
413 damage, but also downregulation of pathways associated with DNA repair. This raises the

414 possibility that obesity may affect DNA repair capacity, which would be especially detrimental
415 in cells already exhibiting defective DNA repair due to a mutation in *BRCA1* or *BRCA2*.
416 Additional studies exploring the relationship between obesity and DNA repair would be relevant
417 not only for *BRCA* mutation carriers, but also for the general population where obesity is
418 associated with increased breast cancer risk in post-menopausal women (3). Defective DNA
419 repair capacity would illuminate a novel mechanism through which obese non-carriers become
420 more susceptible to breast cancer.

421 Our *in vitro* studies demonstrate the ability of several obesity-associated factors,
422 including leptin and insulin, to cause DNA damage, suggesting a collective milieu of factors that
423 may contribute to the elevation in DNA damage observed in *BRCA* mutation carriers in
424 association with BMI. The ability of obese CM to induce damage in *BRCA1* heterozygous cells
425 was diminished when treating in the presence of an antibody or drug that inhibits leptin or insulin
426 signaling, respectively. Of note, since insulin signals through phosphatidylinositol 3-kinases
427 (PI3K), we utilized BKM120, a PI3K inhibitor, to disrupt insulin actions in the presence of obese
428 CM. It is possible that inhibiting PI3K signaling not only disrupted insulin signaling, but also
429 signaling of other factors associated with obesity that act through PI3K, including growth factors
430 or leptin, which collectively contributed to the observed decrease in DNA damage. Additionally,
431 growing evidence points to a role for the PI3K pathway in the DNA damage response, however,
432 these studies have been limited to cancer cells (52-55).

433 Our studies also show a link between obesity-induced DNA damage and tumor
434 development using a *Brcal*^{+/-} mouse model of diet-induced obesity. HFD-fed mice exhibited
435 elevated mammary gland DNA damage in association with decreased latency and increased
436 overall penetrance of mammary tumors when exposed to the carcinogen DMBA. These data

437 suggest that the elevation in DNA damage that we observed in association with BMI in women
438 carrying a *BRCA* mutation may also be associated with increased breast cancer penetrance. The
439 extent to which data from this mouse model can be extrapolated to humans is somewhat limited
440 given that we employed a carcinogen-induced tumor model, whereas in *BRCA* mutation carriers,
441 tumors will arise after years of exposure to both endogenous and environmental factors, some of
442 which will act as carcinogens.

443 Finally, our data show that obesity-associated DNA damage may not only be limited to
444 the breast epithelium of *BRCA* mutation carriers. Although no increase in DNA damage was
445 found in epithelial cells of the ovary in overweight/obese women undergoing prophylactic
446 salpingo-oophorectomy, we did observe a significant increase in DNA damage in the epithelial
447 cells of the fallopian tube in overweight/obese women. Our results are consistent with reports
448 from recent years which point to the fallopian tube as the likely site of origin of ovarian cancer
449 (56, 57), to be confirmed by ongoing clinical trials of risk-reducing salpingectomy with delayed
450 oophorectomy, and also highlights a potential mechanism for the link between weight gain and
451 ovarian cancer in this population.

452 A limitation of our study includes a cohort size of n=72 in our correlation study of DNA
453 damage and BMI which prevented us from analyzing effects of BMI separately in *BRCA1* and
454 *BRCA2* mutation carriers. Although both *BRCA1* and *BRCA2* are essential for DNA repair, their
455 roles in the DNA damage response are not identical and each mutation is associated with
456 different subtypes of tumor development. Larger studies assessing the relative effect of BMI on
457 DNA damage in *BRCA1* and *BRCA2* mutation carriers separately could provide additional
458 information to help personalize risk estimates. Additionally, levels of estrogens vary
459 considerably during the menstrual cycle and impact proliferation of breast epithelial cells. Our

460 studies did not account for phase of menstrual cycle when assessing DNA damage which may
461 have led to increased variability in our data, particularly considering our identification of
462 estrogen as a mediator of obesity-induced epithelial cell DNA damage.

463 Many methodological challenges exist which explain the lack of consensus in
464 epidemiological studies attempting to ascertain modifiers of breast cancer risk in *BRCA* mutation
465 carriers, as reviewed by Milne & Antinou (58). Although a number of studies have associated
466 bodyweight with increased risk of breast cancer as discussed earlier, the largest study to date to
467 contradict these findings showed protective effects of BMI on pre-menopausal breast cancer risk
468 in *BRCA* mutation carriers (11). Drawing definitive conclusions from this study is limited due to
469 the utilization of subject-reported BMI at the time of study questionnaire which is subject to
470 recall bias and utilization of calculated genetic BMI score which does not necessarily predict
471 actual observed BMI and may be influenced by dietary and environmental factors. Additionally,
472 a subset of the overweight/obese population may have received treatment for obesity-associated
473 co-morbidities like diabetes which potentially confounds risk assessment if these treatments or
474 medications reduce breast cancer risk. Overall, given the inconsistencies in reported data and
475 significant challenges in assessing modifiers of breast cancer risk in this population, the
476 consensus to date is that there is insufficient evidence to determine the effect of bodyweight on
477 breast cancer risk in *BRCA* mutation carriers (58-60). Therefore, a strength of our study is the
478 presentation of mechanistic experimental evidence which helps to elucidate the relationship
479 between bodyweight and breast cancer risk in this population.

480 Additionally, our findings provide rationale for conducting clinical trials in
481 overweight/obese *BRCA* mutation carriers to test the efficacy of pharmacological interventions
482 that target metabolic health, weight and/or estrogens. In fact, identifying which obesity-related

483 factors need to be targeted for risk reduction, if not all, will have a meaningful impact on
484 developing effective risk reduction strategies. Although recently reported results of the phase 3
485 randomized MA.32 trial (NCT01101438) found that addition of metformin to standard of care in
486 non-diabetic patients with high-risk breast cancer did not significantly improve invasive disease-
487 free survival vs placebo (61), it remains to be determined if metformin in the preventative setting
488 would be effective at reducing risk of breast cancer, particularly among *BRCA* mutation carriers
489 and those with metabolic dysfunction. Our studies point towards the potential of metformin in
490 this setting, as it has been shown to reduce weight, as well as cause decreases in circulating
491 levels of insulin, leptin and estrogens (62-64). These studies would help clarify whether
492 accumulation of DNA damage over time is reversible or if targeted interventions prevent
493 accumulation of further damage. Positive results would offer clinicians actionable evidence-
494 based prevention strategies for patients in this high-risk population who opt to delay or forgo
495 risk-reducing surgery.

496

497

498 **MATERIALS AND METHODS**

499

500 **Study Design**

501

502 The objective of this study was to gain insight into the role of obesity and metabolic
503 dysfunction on breast cancer penetrance among carriers of germline mutations in *BRCA1* and
504 *BRCA2* and to identify clinically relevant prevention strategies. Clinical samples including both
505 archival tissues and prospectively collected tissues from *BRCA* mutation carriers, as well as cell
506 lines engineered to carry a *BRCA1* or *BRCA2* heterozygous knockout mutation and *Brca1*^{+/-}
507 mouse models were utilized in support of this objective. All studies utilizing human tissues were

508 conducted in accordance with protocols approved by the Institutional Review Boards of
509 Memorial Sloan Kettering Cancer Center (MSKCC) under protocol #10-040 and Weill Cornell
510 Medicine under protocols #1510016712, 1004010984-01 and 1612017836. Informed consent
511 from each subject was obtained by study investigators prior to tissue collection. Animal
512 experiments were conducted in accordance with an approved Institutional Animal Care and Use
513 Committee protocol (#2018-0058) at Weill Cornell Medicine.

514 Studies utilizing archival tissues were coded and DNA damage was analyzed in a blinded
515 fashion. Studies utilizing prospectively collected tissues and *in vitro* treatment studies were not
516 blinded, however, DNA damage was analyzed by immunofluorescence staining using
517 methodology to limit bias as described in the section “*Confocal microscopy & quantification of*
518 *γ H2AX foci*” below. Sample size power calculations were performed for human breast tissue
519 microarray construction (BMI vs DNA damage study) and in animal studies. Any sample
520 exclusion criteria are described in the sections below or in the figure legends.

521

522 **Human breast tissue microarray construction & study population**

523 Archival paraffin blocks of embedded non-tumorous breast tissue were obtained from 72
524 women carrying a *BRCA1* (n=42) or *BRCA2* (n=30) mutation who had previously undergone
525 prophylactic or therapeutic mastectomy at MSKCC from 2011-2016. **Table 1** describes the
526 clinical characteristics of the study population which were extracted from electronic medical
527 records. BMI was calculated using height and weight recorded prior to surgery (kg/m^2) and
528 menopausal status was determined per criteria established by the National Comprehensive
529 Cancer Network ((65). A pathologist reviewed hematoxylin & eosin-stained sections from each
530 block to identify areas enriched in breast epithelium. Cores measuring 1.5mm in diameter from

531 identified epithelial areas of each case were incorporated into paraffin blocks for the construction
532 of tissue microarrays. Each tissue microarray was constructed with cases representing an equal
533 distribution of clinical characteristics, including *BRCA1* or *BRCA2* mutation status and BMI.
534 Unstained sections were cut from each tissue microarray and used for quantification of breast
535 epithelial cell DNA damage by immunofluorescence staining as described in the section below.
536

537 **Assessment of DNA damage by immunofluorescence staining**

538 To quantify epithelial cell DNA damage, immunofluorescence staining of the DNA
539 double strand break marker γ H2AX was conducted on human tissue sections, mouse mammary
540 gland tissue sections, or plated cells. Antibodies/reagents that were used include: primary
541 γ H2AX (p Ser139) antibody (Novus Biologicals #NB100-74435 unless otherwise stated) at
542 1:300 dilution, Goat anti-Mouse Alexa Fluor 546 secondary antibody (Life technologies
543 #A11030) at 1:1000 dilution, Hoechst 33342 nuclear stain (Santa Cruz Biotechnology #SC-
544 495790) at 1:1000 dilution, CAS block (Life Technologies #008120), M.O.M (Mouse-on-
545 Mouse) immunodetection kit (Vector Laboratories # BMK-2202), and ProLong Gold Antifade
546 Mountant (Invitrogen # P36934). Full staining procedures for tissue sections, plated cells, and
547 co-localization studies can be found in the Supplementary Materials and Methods.

548

549 *Confocal microscopy & quantification of γ H2AX foci*

550 Tissue slides or plated epithelial cells stained with γ H2AX and Hoechst were imaged
551 using a Zeiss LSM 880 confocal microscope. Confocal settings were not changed across samples
552 within each experiment. Areas to image were first selected based on identification of regions rich
553 in breast epithelial cells as determined by Hoechst staining prior to viewing the γ H2AX channel

554 to limit any potential bias in image selection. Images were exported to the image analysis
555 software Imaris (Oxford Instruments) for semi-automated quantification of γ H2AX foci per 100
556 cells. Imaris analysis settings were programmed to identify and quantify total cell number in
557 each image and to identify number of γ H2AX foci co-localizing with nuclei. All Imaris-analyzed
558 images were visually inspected by investigators to ensure appropriate identification of γ H2AX
559 foci and exclusion of background staining. A minimum of 100 cells per case or condition were
560 analyzed and DNA damage was reported as # of γ H2AX foci per 100 cells. Any sample with less
561 than 100 cells detected were excluded.

562

563 **Quantification of blood biomarkers**

564 Fasting blood was collected from patients prior to surgery. Serum was separated by
565 centrifugation, aliquoted, and stored at -80°C . Enzyme-linked immunosorbent assay was used to
566 measure serum levels of insulin (Merckodia, Uppsala, Sweden), hsCRP, glucose, SHBG, and IL-6
567 (R&D Systems, Minneapolis, MN) following the manufacturer's protocols.

568

569 **RNA-Seq studies & computational analysis:**

570 RNA-Sequencing (RNA-Seq) was conducted on samples in 4 studies including: breast
571 tissue from *BRCA* mutation carriers, isolated breast epithelial organoids from *BRCA* mutation
572 carriers, breast adipose tissue conditioned media (CM)-treated *BRCA1* heterozygous MCF-10A
573 cells, and *Brcal*^{+/-} mouse mammary fat pads. Details on RNA extraction, sequencing
574 methodology, and computational analyses can be found in the Supplementary Materials and
575 Methods.

576

577 **Isolation of primary breast epithelial cells and breast explant studies**

578 For *ex vivo* tissue explant studies and isolation of breast epithelial cells, breast tissue was
579 obtained from women undergoing breast mammoplasty or mastectomy surgeries at Weill Cornell
580 Medicine and MSKCC from 2017-2021. Surgical specimens were transferred from the operating
581 room to a pathologist who evaluated the breast tissue to confirm that the tissue distributed for
582 experimentation was normal and uninvolved with any quadrant where a tumor may have been
583 present. The tissue was then brought to the laboratory and utilized in the experiments as
584 described below.

585

586 *Isolation of breast epithelial cells*

587 Approximately 25mL of breast tissue was utilized in each organoid preparation with care
588 taken dissect out overly fibrous areas or visible blood vessels. The tissue was finely minced and
589 mixed with complete Ham's F12 media (Corning #10-080-CV, supplemented with 10% FBS and
590 1% penicillin/streptomycin) containing a digestion mix of 10mg/mL collagenase type 1 (Sigma
591 Aldrich #C0130) and 10 μ g/mL hyaluronidase (Sigma Aldrich #H3506) in a total volume of
592 50mL. The tissue was digested overnight on a rotator at 37°C and then centrifuged. The
593 supernatant containing free lipid and adipocytes was discarded and the pellet was washed and
594 reconstituted with in media followed by incubation at 4°C for 1 hour to ensure inhibition of
595 enzyme activities. After centrifugation, the pellet was treated with red cell lysis buffer (Sigma
596 Aldrich #11814389001), pelleted, reconstituted in media, and then ran through a 100 μ M filter
597 followed by 40 μ M filter. Breast epithelial organoids were collected from the top of the 40 μ M
598 filter in mammary epithelial cell growth medium with added supplements (PromoCell #C-

599 21010). Isolated mammary epithelial organoids were snap frozen in liquid nitrogen for RNA
600 extraction and RNA-sequencing or plated for *in vitro* studies.

601

602 *Ex vivo metformin and fulvestrant explant studies*

603 To examine the role of breast adipose tissue estrogen in mediation of DNA damage in
604 *BRCA* mutant epithelial cells, breast explants were treated with drugs targeting estrogen
605 signaling (fulvestrant) or production (metformin). 1 cm breast tissue explants were cut from
606 breast tissue transferred after surgery and were plated in replicate in a 12-well dish. Metformin
607 studies: Breast explants from n=3 subjects were cultured in complete Ham's F12 media (10%
608 FBS, 1% penicillin/streptomycin) supplemented with either vehicle (methanol) or metformin
609 hydrochloride (25-100 μ M, Sigma #PHR1084). Fulvestrant studies: Breast explants from n=7
610 subjects were cultured in basal mammary epithelial cell growth media + 0.1% BSA containing
611 either vehicle (ethanol) or 100uM fulvestrant (Sigma #I4409).

612 After 24 hours of treatment at 37°C in a 5% CO₂ incubator, explants were snap frozen in
613 liquid nitrogen and formalin fixed and paraffin embedded. Tissue sections were cut from each
614 paraffin block for assessment of breast epithelial cell DNA damage by immunofluorescence
615 staining.

616

617 *Collection of breast adipose tissue conditioned media*

618 Conditioned media (CM) was generated from breast tissue obtained from n=36 women
619 with BMIs that range from lean to obese (20.6 – 49.1 kg/m²). Ten 1 cm explant pieces of breast
620 adipose tissue were cut from each case with a focus on fatty areas containing no visible blood
621 vessels. The pieces were weighed and placed on a 10cm dish with 10mL of basal (phenol red

622 free, serum free, and supplement mix free) mammary epithelial cell growth media (PromoCell
623 #C-21215) containing 0.1% BSA. The explants were incubated at 37°C for 24 hours. After
624 incubation the breast adipose tissue CM was collected and centrifuged at 300xg. The supernatant
625 was aliquoted and stored -80°C for use in *in vitro* treatment studies.

626

627 ***In vitro* studies in MCF-10A cells**

628 Non-cancerous breast epithelial cell line MCF-10A carrying a *BRCA1* heterozygous
629 mutation (185delAG/+) was purchased from Horizon Discovery and have been previously
630 described (66). MCF-10A cells carrying a *BRCA2* heterozygous mutation (6174delT/+) were
631 generated in-house using CRISPR/Cas9 gene editing (additional details provided in the
632 Supplementary Materials and Methods). Cells were cultured in DMEM/F12 (Invitrogen #11330-
633 032) supplemented with 5% FBS, 1% penicillin/streptomycin and the following growth factors:
634 20ng/mL EGF, 0.5mg/mL hydrocortisone, 100ng/mL cholera toxin, and 10µg/mL insulin (all
635 purchased from Sigma Aldrich). Cells were serum starved for 16 hours prior to treatments.

636 In CM studies, CM was thawed on ice from each case and diluted to a final concentration
637 of 25% CM. In leptin studies, cells were treated with 400ng/mL of human recombinant leptin
638 (Sigma #L4146). In leptin neutralization studies, obese CM was pre-incubated with a leptin
639 neutralizing antibody (Lep ab, 13.3µg/mL, Fisher Scientific #AF398) for 1 hour at 4°C and then
640 cells were treated with lean or obese CM alone or obese CM + Lep ab. In insulin studies, cells
641 were treated with 100nM insulin (Sigma #I1882). To block insulin signaling, cells were pre-
642 treated with the PI3K inhibitor BKM120 (1µM, MedChemExpress #HY-70063) for 1 hour and
643 then then treated with obese CM + BKM120. All treatments were conducted in replicates or

644 triplicates for 24 hours unless otherwise stated. After treatment, all wells were fixed with ice cold
645 methanol followed by γ H2AX IF staining.

646

647 ***Brca1*^{+/-} mice studies**

648 *Generation of Brca1^{+/-} mice*

649 To determine if obesity impacts mammary gland DNA damage and tumor penetrance in
650 the setting of a *Brca* mutation, *Brca1* heterozygous (*Brca1*^{+/-}) mice were generated on a
651 C57BL/6 background as described in the Supplementary Materials and Methods.

652

653 *Diet-induced obesity & mammary gland DNA damage*

654 At 4 weeks of age, 24 female *Brca1*^{+/-} mice were randomized to one of two groups
655 (n=12/gp). One group was fed 10 kcal% low fat diet (LFD, 12450Bi, Research Diets) and the
656 second group was fed 60 kcal% high fat diet (HFD, D12492i, Research Diets) *ad libitum* for 22
657 weeks until sacrifice. One week prior to sacrifice all mice were fasted overnight for 12 hours and
658 underwent glucose tolerance tests to confirm obesity-induced metabolic dysfunction as
659 previously described. In brief, baseline glucose measurements were taken from tail vein blood
660 drop collection using a handheld glucose meter (Bayer Contour). Mice then received an
661 intraperitoneal injection of 1g/kg glucose and tail vein blood glucose levels were recorded at 15-
662 30 minute intervals over 90 minutes. Following the final measurement respective experimental
663 diets were re-started *ad libitum* for an additional week prior to sacrifice. Mice were euthanized
664 via CO₂ inhalation and mammary gland tissue was collected and snap frozen (inguinal fat pads)
665 for RNA-Seq or fixed (thoracic fat pads) in 10% neutral buffered formalin overnight prior to
666 paraffin embedding and sectioning for histological assessment of DNA damage.

667

668 *MPA/DMBA tumor model*

669 To investigate how obesity impacts mammary gland tumor development in *Brcal*^{+/-}
670 mice the same diet-induced obesity model as described above was utilized. At 4 weeks of age, 27
671 female *Brcal*^{+/-} mice were randomized to one of two groups (n=13-14/gp). One group was fed
672 LFD and the second group was fed HFD for the duration of the study. At 14 weeks of age (after
673 10 weeks on experimental diets) all mice were surgically implanted with a 40mg
674 medroxyprogesterone acetate (MPA) pellet (90-day continuous release, Innovative Research of
675 America, #NP-161) placed subcutaneously. At 15, 16, and 17 weeks of age all mice were dosed
676 with 1mg/22g bodyweight of the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) delivered
677 by oral gavage in corn oil once per week for 3 consecutive weeks. Mammary tumor development
678 and growth were monitored weekly by palpating all 5 mammary gland pairs and recording tumor
679 presence and size with caliper measurements for 28 weeks following the last dose of DMBA.
680 Mice were euthanized at the end of the 28-week surveillance period or earlier based on ethical
681 endpoints, including tumor burden reaching 1.5cm. Mice that did not recover from pellet
682 implantation surgery or displayed morbidity unrelated to mammary tumors were excluded from
683 the study.

684

685 **Quantitative steroid analysis in breast explants**

686 Quantification of steroid levels (E2, E1, testosterone, and androstendione) in snap frozen
687 breast adipose tissue explants treated with metformin was performed using gas chromatography-
688 mass spectrometry (GC-MS)-based steroid profiling as previously described (67, 68). Detailed
689 protocol included in the Supplementary Materials.

690

691 **Statistical analysis**

692 To assess significant differences in baseline clinical characteristics and categorical
693 variables the Fisher exact test was used. To test the strength of correlation between DNA damage
694 and continuous variables, nonparametric Spearman's rank correlation coefficient was used with
695 two-tailed *P* value to determine significance of correlations. A multivariable linear model was
696 used to test the association between the level of DNA damage and clinical characteristics
697 adjusting for BMI or age. Two-tailed Mann Whitney test was performed on clinical data testing
698 significant differences between two groups. Two-tailed student's t-test was used *in vitro*
699 treatment studies and in mouse studies comparing two groups. All results were performed using
700 R (version 4.0.5) or GraphPad Prism 9. Results with a *P*-value < 0.05 were considered
701 statistically significant.

702

703 **Supplementary Materials**

704 **Materials and Methods**

705 **Fig. S1.** Breast adipose conditioned media from obese women stimulate more DNA damage in
706 *BRCA1*^{+/-} MCF-10A cells compared to conditioned media from lean women.

707 **Fig. S2.** *Brcal*^{+/-} mice fed high fat diet have significantly greater accumulation of body fat
708 compared to *Brcal*^{+/-} mice fed low fat diet

709 **Fig. S3.** Generation of MCF-10A cells carrying a *BRC A2* heterozygous mutation

710 **Data file S1:** Tables S1-5: Full list of differentially expressed genes in presented RNA-seq
711 studies (multi-tab Excel file).

712 **Data file S2:** Original data for experiments presented

713 References (69-80)

714

715

716 **References**

- 717 1. K. B. Kuchenbaecker, J. L. Hopper, D. R. Barnes, K.-A. Phillips, T. M. Mooij, M.-J.
718 Roos-Blom, S. Jervis, F. E. Van Leeuwen, R. L. Milne, N. Andrieu, D. E. Goldgar, M. B.
719 Terry, M. A. Rookus, D. F. Easton, A. C. Antoniou, L. McGuffog, D. G. Evans, D.
720 Barrowdale, D. Frost, J. Adlard, K.-R. Ong, L. Izatt, M. Tischkowitz, R. Eeles, R.
721 Davidson, S. Hodgson, S. Ellis, C. Nogues, C. Lasset, D. Stoppa-Lyonnet, J.-P. Fricker,
722 L. Faivre, P. Berthet, M. J. Hooning, L. E. Van Der Kolk, C. M. Kets, M. A. Adank, E.
723 M. John, W. K. Chung, I. L. Andrulis, M. Southey, M. B. Daly, S. S. Buys, A. Osorio, C.
724 Engel, K. Kast, R. K. Schmutzler, T. Caldes, A. Jakubowska, J. Simard, M. L.
725 Friedlander, S.-A. Mclachlan, E. Machackova, L. Foretova, Y. Y. Tan, C. F. Singer, E.
726 Olah, A.-M. Gerdes, B. Arver, H. Olsson, Risks of Breast, Ovarian, and Contralateral
727 Breast Cancer for BRCA1 and BRCA2 Mutation Carriers. *JAMA* **317**, 2402-2416 (2017).
- 728 2. S. Chen, G. Parmigiani, Meta-Analysis of BRCA1 and BRCA2 Penetrance. *Journal of*
729 *Clinical Oncology* **25**, 1329-1333 (2007).
- 730 3. B. Lauby-Secretan, C. Scoccianti, D. Loomis, Y. Grosse, F. Bianchini, K. Straif, Body
731 Fatness and Cancer — Viewpoint of the IARC Working Group. *New England Journal of*
732 *Medicine* **375**, 794-798 (2016).
- 733 4. M. C. King, J. H. Marks, J. B. Mandell, G. New York Breast Cancer Study, Breast and
734 ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* **302**,
735 643-646 (2003).

- 736 5. J. Kotsopoulos, O. I. Olopade, P. Ghadirian, J. Lubinski, H. T. Lynch, C. Isaacs, B.
737 Weber, C. Kim-Sing, P. Ainsworth, W. D. Foulkes, A. Eisen, P. Sun, S. A. Narod,
738 Changes in body weight and the risk of breast cancer in BRCA1 and BRCA2 mutation
739 carriers. *Breast Cancer Research* **7**, (2005).
- 740 6. E. Bruno, A. Oliverio, A. Paradiso, A. Daniele, S. Tommasi, D. A. Terribile, A.
741 Filippone, M. Digennaro, B. Pilato, K. Danza, D. Guarino, C. Rossi, M. M. Rossi, E.
742 Venturelli, M. Giussani, B. Peissel, P. Pasanisi, Lifestyle Characteristics in Women
743 Carriers of BRCA Mutations: Results From an Italian Trial Cohort. *Clin Breast Cancer*
744 **21**, e168-e176 (2021).
- 745 7. A. Nkondjock, A. Robidoux, Y. Paredes, S. A. Narod, P. Ghadirian, Diet, lifestyle and
746 BRCA-related breast cancer risk among French-Canadians. *Breast Cancer Res Treat* **98**,
747 285-294 (2006).
- 748 8. S. Abbas, A. Siddique, N. Shahid, R. T. Khan, W. Fatima, Breast cancer risk associated
749 with BRCA1/2 variants in the Pakistani population. *Breast Cancer* **26**, 365-372 (2019).
- 750 9. P. Manders, A. Pijpe, M. J. Hooning, I. Kluijft, H. F. Vasen, N. Hoogerbrugge, C. J. van
751 Asperen, H. Meijers-Heijboer, M. G. Ausems, T. A. van Os, E. B. Gomez-Garcia, R. M.
752 Brohet, Hebon, F. E. van Leeuwen, M. A. Rookus, Body weight and risk of breast cancer
753 in BRCA1/2 mutation carriers. *Breast Cancer Res Treat* **126**, 193-202 (2011).
- 754 10. S. J. Kim, T. Huzarski, J. Gronwald, C. F. Singer, P. Møller, H. T. Lynch, S. Armel, B.
755 Y. Karlan, W. D. Foulkes, S. L. Neuhausen, L. Senter, A. Eisen, C. Eng, S. Panchal, T.
756 Pal, O. Olopade, D. Zakalik, J. Lubinski, S. A. Narod, J. Kotsopoulos, P. Ainsworth, L.
757 Bordeleau, N. Tung, E. Friedman, W. Meschino, C. Snyder, K. Metcalfe, E. Warner, B.
758 Rosen, R. Demsky, J. N. Weitzel, K. Panabaker, F. Couch, S. Manoukian, B. Pasini, M.

759 B. Daly, L. Steele, H. Saal, T. Fallen, M. Wood, W. Mckinnon, E. Lemire, A. E.
760 Chudley, K. Serfas, C. Elser, S. T. Vadaparampil, O. Ginsburg, C. A. Cullinane, J. L.
761 Blum, T. Ross, C. Mauer, A. Kwong, C. Cybulski, J. Mccuaig, D. Rayson, C. Isaacs,
762 Prospective evaluation of body size and breast cancer risk among BRCA1 and BRCA2
763 mutation carriers. *International Journal of Epidemiology* **47**, 987-997 (2018).

764 11. F. Qian, S. Wang, J. Mitchell, L. Mcguffog, D. Barrowdale, G. Leslie, J. C. Oosterwijk,
765 W. K. Chung, D. G. Evans, C. Engel, K. Kast, C. M. Aalfs, M. A. Adank, J. Adlard, B.
766 A. Agnarsson, K. Aittomäki, E. Alducci, I. L. Andrulis, B. K. Arun, M. G. E. M.
767 Ausems, J. Azzollini, E. Barouk-Simonet, J. Barwell, M. Belotti, J. Benitez, A. Berger,
768 A. Borg, A. R. Bradbury, J. Brunet, S. S. Buys, T. Caldes, M. A. Caligo, I. Campbell, S.
769 M. Caputo, J. Chiquette, K. B. M. Claes, J. Margriet Collée, F. J. Couch, I. Coupier, M.
770 B. Daly, R. Davidson, O. Diez, S. M. Domchek, A. Donaldson, C. M. Dorfling, R. Eeles,
771 L. Feliubadaló, L. Foretova, J. Fowler, E. Friedman, D. Frost, P. A. Ganz, J. Garber, V.
772 Garcia-Barberan, G. Glendon, A. K. Godwin, E. B. Gómez Garcia, J. Gronwald, E.
773 Hahnen, U. Hamann, A. Henderson, C. B. Hendricks, J. L. Hopper, P. J. Hulick, E. N.
774 Imyanitov, C. Isaacs, L. Izatt, Á. Izquierdo, A. Jakubowska, K. Kaczmarek, E. Kang, B.
775 Y. Karlan, C. M. Kets, S.-W. Kim, Z. Kim, A. Kwong, Y. Laitman, C. Lasset, M. Hyuk
776 Lee, J. Won Lee, J. Lee, J. Lester, F. Lesueur, J. T. Loud, J. Lubinski, N. Mebirouk, H. E.
777 J. Meijers-Heijboer, A. Meindl, A. Miller, M. Montagna, T. M. Mooij, P. J. Morrison, E.
778 Mouret-Fourme, K. L. Nathanson, S. L. Neuhausen, H. Nevanlinna, D. Niederacher, F.
779 C. Nielsen, R. L. Nussbaum, K. Offit, E. Olah, K.-R. Ong, L. Ottini, S. K. Park, P.
780 Peterlongo, G. Pfeiler, C. M. Phelan, B. Poppe, N. Pradhan, P. Radice, S. J. Ramus, J.
781 Rantala, M. Robson, G. C. Rodriguez, R. K. Schmutzler, C. G. Hutten Selkirk, P. D.

- 782 Shah, J. Simard, C. F. Singer, J. Sokolowska, D. Stoppa-Lyonnet, C. Sutter, Y. Yen Tan,
783 R. M. Teixeira, S. H. Teo, M. B. Terry, M. Thomassen, M. Tischkowitz, A. E. Toland, K.
784 M. Tucker, N. Tung, C. J. Van Asperen, K. Van Engelen, E. J. Van Rensburg, S. Wang-
785 Gohrke, B. Wappenschmidt, J. N. Weitzel, D. Yannoukakos, M. H. Greene, M. A.
786 Rookus, D. F. Easton, G. Chenevix-Trench, A. C. Antoniou, D. E. Goldgar, O. I.
787 Olopade, T. R. Rebbeck, D. Huo, Height and Body Mass Index as Modifiers of Breast
788 Cancer Risk in BRCA1/2 Mutation Carriers: A Mendelian Randomization Study. *JNCI:*
789 *Journal of the National Cancer Institute* **111**, 350-364 (2019).
- 790 12. S. M. Grundy, Obesity, Metabolic Syndrome, and Cardiovascular Disease. *The Journal*
791 *of Clinical Endocrinology & Metabolism* **89**, 2595-2600 (2004).
- 792 13. K. Sun, C. M. Kusminski, P. E. Scherer, Adipose tissue remodeling and obesity. *Journal*
793 *of Clinical Investigation* **121**, 2094-2101 (2011).
- 794 14. H. Sell, C. Habich, J. Eckel, Adaptive immunity in obesity and insulin resistance. *Nat*
795 *Rev Endocrinol* **8**, 709-716 (2012).
- 796 15. P. Bhardwaj, K. A. Brown, Obese Adipose Tissue as a Driver of Breast Cancer Growth
797 and Development: Update and Emerging Evidence. *Front Oncol* **11**, 638918 (2021).
- 798 16. K. A. Brown, Metabolic pathways in obesity-related breast cancer. *Nature Reviews*
799 *Endocrinology* **17**, 350-363 (2021).
- 800 17. E. R. Simpson, S. R. Davis, Minireview: Aromatase and the Regulation of Estrogen
801 Biosynthesis—Some New Perspectives. *Endocrinology* **142**, 4589-4594 (2001).
- 802 18. X. Wang, E. R. Simpson, K. A. Brown, Aromatase overexpression in dysfunctional
803 adipose tissue links obesity to postmenopausal breast cancer. *The Journal of Steroid*
804 *Biochemistry and Molecular Biology* **153**, 35-44 (2015).

- 805 19. H. Zahid, K. Subbaramaiah, N. M. Iyengar, X. K. Zhou, I. C. Chen, P. Bhardwaj, A.
806 Gucalp, M. Morrow, C. A. Hudis, A. J. Dannenberg, K. A. Brown, Leptin regulation of
807 the p53-HIF1alpha/PKM2-aromatase axis in breast adipose stromal cells: a novel
808 mechanism for the obesity-breast cancer link. *Int J Obes (Lond)* **42**, 711-720 (2018).
- 809 20. F. Liu, J. He, H. Wang, D. Zhu, Y. Bi, Adipose Morphology: a Critical Factor in
810 Regulation of Human Metabolic Diseases and Adipose Tissue Dysfunction. *Obesity*
811 *Surgery* **30**, 5086-5100 (2020).
- 812 21. K. A. Brown, Metabolic pathways in obesity-related breast cancer. *Nat Rev Endocrinol*
813 **17**, 350-363 (2021).
- 814 22. N. Marino, R. German, X. Rao, E. Simpson, S. Liu, J. Wan, Y. Liu, G. Sandusky, M.
815 Jacobsen, M. Stoval, S. Cao, A. M. V. Storniolo, Upregulation of lipid metabolism genes
816 in the breast prior to cancer diagnosis. *npj Breast Cancer* **6**, (2020).
- 817 23. K. Yoshida, Y. Miki, Role of BRCA1 and BRCA2 as regulators of DNA repair,
818 transcription, and cell cycle in response to DNA damage. *Cancer Science* **95**, 866-871
819 (2004).
- 820 24. A. R. Venkitaraman, Cancer Susceptibility and the Functions of BRCA1 and BRCA2.
821 *Cell* **108**, 171-182 (2002).
- 822 25. E. Kowalska, S. A. Narod, T. Huzarski, S. Zajaczek, J. Huzarska, B. Gorski, J. Lubinski,
823 Increased Rates of Chromosome Breakage in BRCA1 Carriers Are Normalized by Oral
824 Selenium Supplementation. *Cancer Epidemiology, Biomarkers & Prevention* **14**, 1302-
825 1306 (2005).

- 826 26. M. Zaki, W. Basha, H. T. El-Bassyouni, S. El-Toukhy, T. Hussein, Evaluation of DNA
827 damage profile in obese women and its association to risk of metabolic syndrome,
828 polycystic ovary syndrome and recurrent preeclampsia. *Genes Dis* **5**, 367-373 (2018).
- 829 27. C. M. Dungan, B. D. Peck, R. G. Walton, Z. Huang, M. M. Bamman, P. A. Kern, C. A.
830 Peterson, In vivo analysis of γ H2AX+ cells in skeletal muscle from aged and obese
831 humans. *The FASEB Journal* **34**, 7018-7035 (2020).
- 832 28. J. Fieres, M. Fischer, C. Sauter, M. Moreno-Villanueva, A. Burkle, P. H. Wirtz, The
833 burden of overweight: Higher body mass index, but not vital exhaustion, is associated
834 with higher DNA damage and lower DNA repair capacity. *DNA Repair (Amst)* **114**,
835 103323 (2022).
- 836 29. V. S. Y. Tay, S. Devaraj, T. Koh, G. Ke, K. C. Crasta, Y. Ali, Increased double strand
837 breaks in diabetic β -cells with a p21 response that limits apoptosis. *Scientific Reports* **9**,
838 (2019).
- 839 30. P. G. Morris, C. A. Hudis, D. Giri, M. Morrow, D. J. Falcone, X. K. Zhou, B. Du, E.
840 Brogi, C. B. Crawford, L. Kopelovich, K. Subbaramaiah, A. J. Dannenberg,
841 Inflammation and Increased Aromatase Expression Occur in the Breast Tissue of Obese
842 Women with Breast Cancer. *Cancer Prevention Research* **4**, 1021-1029 (2011).
- 843 31. K. A. Brown, N. I. Hunger, M. Docanto, E. R. Simpson, Metformin inhibits aromatase
844 expression in human breast adipose stromal cells via stimulation of AMP-activated
845 protein kinase. *Breast Cancer Research and Treatment* **123**, 591-596 (2010).
- 846 32. N. U. Samarajeewa, S. Ham, F. Yang, E. R. Simpson, K. A. Brown, Promoter-specific
847 effects of metformin on aromatase transcript expression. *Steroids* **76**, 768-771 (2011).

- 848 33. H. D. Soule, T. M. Maloney, S. R. Wolman, W. D. Peterson, Jr., R. Brenz, C. M.
849 McGrath, J. Russo, R. J. Pauley, R. F. Jones, S. C. Brooks, Isolation and characterization
850 of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res*
851 **50**, 6075-6086 (1990).
- 852 34. C. N. Perera, H. G. Chin, N. Duru, I. G. Camarillo, Leptin-regulated gene expression in
853 MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor
854 growth and progression. *Journal of Endocrinology* **199**, 221-233 (2008).
- 855 35. N. K. Saxena, P. M. Vertino, F. A. Anania, D. Sharma, Leptin-induced Growth
856 Stimulation of Breast Cancer Cells Involves Recruitment of Histone Acetyltransferases
857 and Mediator Complex to CYCLIN D1 Promoter via Activation of Stat3. *Journal of*
858 *Biological Chemistry* **282**, 13316-13325 (2007).
- 859 36. A. L. Strong, J. F. Ohlstein, B. A. Biagas, L. V. Rhodes, D. T. Pei, H. A. Tucker, C.
860 Llamas, A. C. Bowles, M. F. Dutreil, S. Zhang, J. M. Gimble, M. E. Burow, B. A.
861 Bunnell, Leptin produced by obese adipose stromal/stem cells enhances proliferation and
862 metastasis of estrogen receptor positive breast cancers. *Breast Cancer Research* **17**,
863 (2015).
- 864 37. N. Yin, D. Wang, H. Zhang, X. Yi, X. Sun, B. Shi, H. Wu, G. Wu, X. Wang, Y. Shang,
865 Molecular Mechanisms Involved in the Growth Stimulation of Breast Cancer Cells by
866 Leptin. *Cancer Research* **64**, 5870-5875 (2004).
- 867 38. H.-K. Park, R. S. Ahima, Leptin signaling. *F1000Prime Reports* **6**, (2014).
- 868 39. B. D. Hopkins, M. D. Goncalves, L. C. Cantley, Insulin–PI3K signalling: an
869 evolutionarily insulated metabolic driver of cancer. *Nature Reviews Endocrinology* **16**,
870 276-283 (2020).

- 871 40. S. J. Kim, J. Lubinski, T. Huzarski, P. Moller, S. Armel, B. Y. Karlan, L. Senter, A.
872 Eisen, W. D. Foulkes, C. F. Singer, N. Tung, L. Bordeleau, S. L. Neuhausen, O. I.
873 Olopade, C. Eng, J. N. Weitzel, R. Fruscio, S. A. Narod, J. Kotsopoulos, G. Hereditary
874 Ovarian Cancer Clinical Study, Weight Gain and the Risk of Ovarian Cancer in BRCA1
875 and BRCA2 Mutation Carriers. *Cancer Epidemiol Biomarkers Prev* **30**, 2038-2043
876 (2021).
- 877 41. F. Colotta, P. Allavena, A. Sica, C. Garlanda, A. Mantovani, Cancer-related
878 inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*
879 **30**, 1073-1081 (2009).
- 880 42. L. M. Coussens, Z. Werb, Inflammation and cancer. *Nature* **420**, 860-867 (2002).
- 881 43. L. B. Meira, J. M. Bugni, S. L. Green, C.-W. Lee, B. Pang, D. Borenshtein, B. H.
882 Rickman, A. B. Rogers, C. A. Moroski-Erkul, J. L. Mcfaline, D. B. Schauer, P. C.
883 Dedon, J. G. Fox, L. D. Samson, DNA damage induced by chronic inflammation
884 contributes to colon carcinogenesis in mice. *Journal of Clinical Investigation*, (2008).
- 885 44. O. Kiraly, G. Gong, W. Olipitz, S. Muthupalani, B. P. Engelward, Inflammation-Induced
886 Cell Proliferation Potentiates DNA Damage-Induced Mutations In Vivo. *PLOS Genetics*
887 **11**, e1004901 (2015).
- 888 45. K. I. Savage, K. B. Matchett, E. M. Barros, K. M. Cooper, G. W. Irwin, J. J. Gorski, K. S.
889 Orr, J. Vohhodina, J. N. Kavanagh, A. F. Madden, A. Powell, L. Manti, S. S. Mcdade, B.
890 H. Park, K. M. Prise, S. A. Mcintosh, M. Salto-Tellez, D. J. Richard, C. T. Elliott, D. P.
891 Harkin, BRCA1 Deficiency Exacerbates Estrogen-Induced DNA Damage and Genomic
892 Instability. *Cancer Research* **74**, 2773-2784 (2014).

- 893 46. C. Algire, O. Moiseeva, X. Deschênes-Simard, L. Amrein, L. Petrucelli, E. Birman, B.
894 Viollet, G. Ferbeyre, M. N. Pollak, Metformin Reduces Endogenous Reactive Oxygen
895 Species and Associated DNA Damage. *Cancer Prevention Research* **5**, 536-543 (2012).
- 896 47. N. D. Kauff, S. M. Domchek, T. M. Friebe, M. E. Robson, J. Lee, J. E. Garber, C.
897 Isaacs, D. G. Evans, H. Lynch, R. A. Eeles, S. L. Neuhausen, M. B. Daly, E. Matloff, J.
898 L. Blum, P. Sabbatini, R. R. Barakat, C. Hudis, L. Norton, K. Offit, T. R. Rebbeck, Risk-
899 Reducing Salpingo-Oophorectomy for the Prevention of BRCA1- and BRCA2-
900 Associated Breast and Gynecologic Cancer: A Multicenter, Prospective Study. *Journal of*
901 *Clinical Oncology* **26**, 1331-1337 (2008).
- 902 48. S. A. Narod, J. S. Brunet, P. Ghadirian, M. Robson, K. Heimdal, S. L. Neuhausen, D.
903 Stoppa-Lyonnet, C. Lerman, B. Pasini, P. de los Rios, B. Weber, H. Lynch, G. Hereditary
904 Breast Cancer Clinical Study, Tamoxifen and risk of contralateral breast cancer in
905 BRCA1 and BRCA2 mutation carriers: a case-control study. Hereditary Breast Cancer
906 Clinical Study Group. *Lancet* **356**, 1876-1881 (2000).
- 907 49. T. R. Rebbeck, H. T. Lynch, S. L. Neuhausen, S. A. Narod, L. Van'T Veer, J. E. Garber,
908 G. Evans, C. Isaacs, M. B. Daly, E. Matloff, O. I. Olopade, B. L. Weber, Prophylactic
909 Oophorectomy in Carriers of BRCA1 or BRCA2 Mutations. *New England Journal of*
910 *Medicine* **346**, 1616-1622 (2002).
- 911 50. C. E. Caldon, Estrogen Signaling and the DNA Damage Response in Hormone
912 Dependent Breast Cancers. *Frontiers in Oncology* **4**, (2014).
- 913 51. P. Bhardwaj, C. C. Au, A. Benito-Martin, H. Ladumor, S. Oshchepkova, R. Moges, K. A.
914 Brown, Estrogens and breast cancer: Mechanisms involved in obesity-related

- 915 development, growth and progression. *The Journal of Steroid Biochemistry and*
916 *Molecular Biology* **189**, 161-170 (2019).
- 917 52. T. T. Huang, E. J. Lampert, C. Coots, J. M. Lee, Targeting the PI3K pathway and DNA
918 damage response as a therapeutic strategy in ovarian cancer. *Cancer Treat Rev* **86**,
919 102021 (2020).
- 920 53. A. Juvekar, H. Hu, S. Yadegarynia, C. A. Lyssiotis, S. Ullas, E. C. Lien, G. Bellinger, J.
921 Son, R. C. Hok, P. Seth, M. B. Daly, B. Kim, R. Scully, J. M. Asara, L. C. Cantley, G. M.
922 Wulf, Phosphoinositide 3-kinase inhibitors induce DNA damage through nucleoside
923 depletion. *Proc Natl Acad Sci U S A* **113**, E4338-4347 (2016).
- 924 54. R. J. Cardnell, Y. Feng, L. Diao, Y. H. Fan, F. Masrourpour, J. Wang, Y. Shen, G. B.
925 Mills, J. D. Minna, J. V. Heymach, L. A. Byers, Proteomic markers of DNA repair and
926 PI3K pathway activation predict response to the PARP inhibitor BMN 673 in small cell
927 lung cancer. *Clin Cancer Res* **19**, 6322-6328 (2013).
- 928 55. A. Juvekar, L. N. Burga, H. Hu, E. P. Lunsford, Y. H. Ibrahim, J. Balmana, A.
929 Rajendran, A. Papa, K. Spencer, C. A. Lyssiotis, C. Nardella, P. P. Pandolfi, J. Baselga,
930 R. Scully, J. M. Asara, L. C. Cantley, G. M. Wulf, Combining a PI3K inhibitor with a
931 PARP inhibitor provides an effective therapy for BRCA1-related breast cancer. *Cancer*
932 *Discov* **2**, 1048-1063 (2012).
- 933 56. J. Ducie, F. Dao, M. Considine, N. Olvera, P. A. Shaw, R. J. Kurman, I.-M. Shih, R. A.
934 Soslow, L. Cope, D. A. Levine, Molecular analysis of high-grade serous ovarian
935 carcinoma with and without associated serous tubal intra-epithelial carcinoma. *Nature*
936 *Communications* **8**, (2017).

- 937 57. S. I. Labidi-Galy, E. Papp, D. Hallberg, N. Niknafs, V. Adleff, M. Noe, R. Bhattacharya,
938 M. Novak, S. Jones, J. Phallen, C. A. Hruban, M. S. Hirsch, D. I. Lin, L. Schwartz, C. L.
939 Maire, J.-C. Tille, M. Bowden, A. Ayhan, L. D. Wood, R. B. Scharpf, R. Kurman, T.-L.
940 Wang, I.-M. Shih, R. Karchin, R. Drapkin, V. E. Velculescu, High grade serous ovarian
941 carcinomas originate in the fallopian tube. *Nature Communications* **8**, (2017).
- 942 58. R. L. Milne, A. C. Antoniou, Modifiers of breast and ovarian cancer risks for BRCA1
943 and BRCA2 mutation carriers. *Endocrine-Related Cancer* **23**, T69-T84 (2016).
- 944 59. T. M. Friebel, S. M. Domchek, T. R. Rebbeck, Modifiers of Cancer Risk in BRCA1 and
945 BRCA2 Mutation Carriers: A Systematic Review and Meta-Analysis. *JNCI: Journal of*
946 *the National Cancer Institute* **106**, dju091-dju091 (2014).
- 947 60. A. M. Coletta, S. K. Peterson, L. A. Gatus, K. J. Krause, S. M. Schembre, S. C. Gilchrist,
948 B. Arun, Y. N. You, M. A. Rodriguez-Bigas, L. L. Strong, K. H. Lu, K. Basen-Engquist,
949 Diet, weight management, physical activity and Ovarian & Breast Cancer Risk in women
950 with BRCA1/2 pathogenic Germline gene variants: systematic review. *Hereditary*
951 *Cancer in Clinical Practice* **18**, (2020).
- 952 61. P. J. Goodwin, B. E. Chen, K. A. Gelmon, T. J. Whelan, M. Ennis, J. Lemieux, J. A.
953 Ligibel, D. L. Hershman, I. A. Mayer, T. J. Hobday, J. M. Bliss, P. Rastogi, M. Rabaglio-
954 Poretti, S. D. Mukherjee, J. R. Mackey, V. G. Abramson, C. Oja, R. Wesolowski, A. M.
955 Thompson, D. W. Rea, P. M. Stos, L. E. Shepherd, V. Stambolic, W. R. Parulekar, Effect
956 of Metformin vs Placebo on Invasive Disease-Free Survival in Patients With Breast
957 Cancer. *JAMA* **327**, 1963 (2022).
- 958 62. P. J. Goodwin, R. J. O. Dowling, M. Ennis, B. E. Chen, W. R. Parulekar, L. E. Shepherd,
959 M. J. Burnell, R. Vander Meer, A. Molckovsky, A. Gurjal, K. A. Gelmon, J. A. Ligibel,

- 960 D. L. Hershman, I. A. Mayer, T. J. Whelan, T. J. Hobday, P. Rastogi, M. Rabaglio-
961 Poretti, J. Lemieux, A. M. Thompson, D. W. Rea, V. Stambolic, Effect of metformin
962 versus placebo on metabolic factors in the MA.32 randomized breast cancer trial. *NPJ*
963 *Breast Cancer* **7**, 74 (2021).
- 964 63. P. J. Goodwin, W. R. Parulekar, K. A. Gelmon, L. E. Shepherd, J. A. Ligibel, D. L.
965 Hershman, P. Rastogi, I. A. Mayer, T. J. Hobday, J. Lemieux, A. M. Thompson, K. I.
966 Pritchard, T. J. Whelan, S. D. Mukherjee, H. I. Chalchal, C. D. Oja, K. S. Tonkin, V.
967 Bernstein, B. E. Chen, V. Stambolic, Effect of metformin vs placebo on and metabolic
968 factors in NCIC CTG MA.32. *J Natl Cancer Inst* **107**, (2015).
- 969 64. I. Pimentel, B. E. Chen, A. E. Lohmann, M. Ennis, J. Ligibel, L. Shepherd, D. L.
970 Hershman, T. Whelan, V. Stambolic, I. Mayer, T. Hobday, J. Lemieux, A. Thompson, P.
971 Rastogi, K. Gelmon, D. Rea, M. Rabaglio, S. Ellard, M. Mates, P. Bedard, L. Pitre, T.
972 Vandenberg, R. J. O. Dowling, W. Parulekar, P. J. Goodwin, The Effect of Metformin vs
973 Placebo on Sex Hormones in Canadian Cancer Trials Group MA.32. *J Natl Cancer Inst*
974 **113**, 192-198 (2021).
- 975 65. W. J. Gradishar, B. O. Anderson, S. L. Blair, H. J. Burstein, A. Cyr, A. D. Elias, W. B.
976 Farrar, A. Forero, S. H. Giordano, L. J. Goldstein, D. F. Hayes, C. A. Hudis, S. J. Isakoff,
977 B. M. Ljung, P. K. Marcom, I. A. Mayer, B. McCormick, R. S. Miller, M. Pegram, L. J.
978 Pierce, E. C. Reed, K. E. Salerno, L. S. Schwartzberg, M. L. Smith, H. Soliman, G.
979 Somlo, J. H. Ward, A. C. Wolff, R. Zellars, D. A. Shead, R. Kumar, P. National
980 Comprehensive Cancer Network Breast Cancer, Breast cancer version 3.2014. *J Natl*
981 *Compr Canc Netw* **12**, 542-590 (2014).

- 982 66. H. Konishi, M. Mohseni, A. Tamaki, J. P. Garay, S. Croessmann, S. Karnan, A. Ota, H.
983 Y. Wong, Y. Konishi, B. Karakas, K. Tahir, A. M. Abukhdeir, J. P. Gustin, J. Cidado, G.
984 M. Wang, D. Cosgrove, R. Cochran, D. Jelovac, M. J. Higgins, S. Arena, L. Hawkins, J.
985 Lauring, A. L. Gross, C. M. Heaphy, Y. Hosokawa, E. Gabrielson, A. K. Meeker, K.
986 Visvanathan, P. Argani, K. E. Bachman, B. H. Park, Mutation of a single allele of the
987 cancer susceptibility gene BRCA1 leads to genomic instability in human breast epithelial
988 cells. *Proc Natl Acad Sci U S A* **108**, 17773-17778 (2011).
- 989 67. S. Han, T. Baba, S. Yanai, D. J. Byun, K. I. Morohashi, J. H. Kim, M. H. Choi, GC-MS-
990 based metabolic signatures reveal comparative steroidogenic pathways between fetal and
991 adult mouse testes. *Andrology* **9**, 400-406 (2021).
- 992 68. J. Y. Moon, K. M. McNamara, J. J. Lee, B. C. Chung, H. Sasano, M. H. Choi, Improved
993 detectability of sex steroids from frozen sections of breast cancer tissue using GC-triple
994 quadrupole-MS. *J Steroid Biochem Mol Biol* **178**, 185-192 (2018).
- 995 69. N. L. Bray, H. Pimentel, P. Melsted, L. Pachter, Near-optimal probabilistic RNA-seq
996 quantification. *Nat Biotechnol* **34**, 525-527 (2016).
- 997 70. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for
998 RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
- 999 71. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M.
1000 Chaisson, T. R. Gingeras, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**,
1001 15-21 (2013).
- 1002 72. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis,
1003 R. Durbin, S. Genome Project Data Processing, The Sequence Alignment/Map format
1004 and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

- 1005 73. C. Trapnell, A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L.
1006 Salzberg, J. L. Rinn, L. Pachter, Differential gene and transcript expression analysis of
1007 RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* **7**, 562-578 (2012).
- 1008 74. T. Derrien, R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, G. Guernec, D.
1009 Martin, A. Merkel, D. G. Knowles, J. Lagarde, L. Veeravalli, X. Ruan, Y. Ruan, T.
1010 Lassmann, P. Carninci, J. B. Brown, L. Lipovich, J. M. Gonzalez, M. Thomas, C. A.
1011 Davis, R. Shiekhatar, T. R. Gingeras, T. J. Hubbard, C. Notredame, J. Harrow, R. Guigo,
1012 The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene
1013 structure, evolution, and expression. *Genome Res* **22**, 1775-1789 (2012).
- 1014 75. X. Liu, H. Holstege, H. van der Gulden, M. Treur-Mulder, J. Zevenhoven, A. Velds, R.
1015 M. Kerkhoven, M. H. van Vliet, L. F. Wessels, J. L. Peterse, A. Berns, J. Jonkers,
1016 Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of
1017 human BRCA1-mutated basal-like breast cancer. *Proc Natl Acad Sci U S A* **104**, 12111-
1018 12116 (2007).
- 1019 76. H. Liu, C. J. Murphy, F. A. Karreth, K. B. Emdal, F. M. White, O. Elemento, A. Toker,
1020 G. M. Wulf, L. C. Cantley, Identifying and Targeting Sporadic Oncogenic Genetic
1021 Aberrations in Mouse Models of Triple-Negative Breast Cancer. *Cancer Discov* **8**, 354-
1022 369 (2018).
- 1023 77. F. Schwenk, U. Baron, K. Rajewsky, A cre-transgenic mouse strain for the ubiquitous
1024 deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids*
1025 *Res* **23**, 5080-5081 (1995).

- 1026 78. A. Katti, M. Foronda, J. Zimmerman, B. Diaz, M. P. Zafra, S. Goswami, L. E. Dow, GO:
1027 a functional reporter system to identify and enrich base editing activity. *Nucleic Acids*
1028 *Res* **48**, 2841-2852 (2020).
- 1029 79. M. P. Zafra, E. M. Schatoff, A. Katti, M. Foronda, M. Breinig, A. Y. Schweitzer, A.
1030 Simon, T. Han, S. Goswami, E. Montgomery, J. Thibado, E. R. Kasthuber, F. J.
1031 Sanchez-Rivera, J. Shi, C. R. Vakoc, S. W. Lowe, D. F. Tschaharganeh, L. E. Dow,
1032 Optimized base editors enable efficient editing in cells, organoids and mice. *Nat*
1033 *Biotechnol* **36**, 888-893 (2018).
- 1034 80. J. Y. Moon, K. M. McNamara, J. J. Lee, B. C. Chung, H. Sasano, M. H. Choi, Improved
1035 detectability of sex steroids from frozen sections of breast cancer tissue using GC-triple
1036 quadrupole-MS. *J Steroid Biochem Mol Biol* **178**, 185-192 (2018).

1037

1038

1039 **Acknowledgments:**

1040 We thank Dr. David Otterburn and Dr. Leslie Cohen, Department of Surgery, and Dr.
1041 Paula Ginter, Department of Pathology, at Weill Cornell Medicine for facilitating access
1042 to reduction mammoplasty tissue. Studies were conducted with the support and facilities
1043 provided by the Microscopy and Image Analysis Core Facility, Genomics Resources
1044 Core Facility, and the Research Animal Resource Center at Weill Cornell Medicine (New
1045 York, NY). All schematics were created in BioRender.

1046

1047 **Funding:**

1048 National Cancer Institute of the National Institutes of Health grant 1R01CA215797
1049 (KAB)

1050 National Cancer Institute of the National Institutes of Health grant F31CA236306 (PB)
1051 Anne Moore Breast Cancer Research Fund (KAB)

1052 National Breast Cancer Foundation grant ECF-16-004 (KAB)

1053 National Center for Advancing Translational Sciences of the National Institutes of Health
1054 grant KL2-TR-002385 (MKF)

1055 National Institute of General Medical Sciences of the National Institutes of Health grant
1056 T32GM007739 to the Weill Cornell/Rockefeller/Sloan Kettering Tri-Institutional MD-
1057 PhD Program (MA)

1058 The content is solely the responsibility of the authors and does not necessarily represent
1059 the official views of the National Institutes of Health or other funding agencies.

1060

1061 **Author contributions:**

1062 Conceptualization: KAB, NMI
1063 Methodology: KAB, PB, NMI, HZ, DJB, MHO, QS, RB, MF, LED, DDG, XKZ
1064 Investigation: PB, HZ, KMC, DJB, CL, CL, PP, MA, MF, SO, MP, BH, MKF
1065 Formal analysis: KAB, PB, QS, OS, RB, XKZ
1066 Resources: KAB, MHC, OE, AML, LHE, MM, JAS, LCC
1067 Funding acquisition: KAB, PB
1068 Project administration: KAB
1069 Supervision: KAB
1070 Writing – original draft: PB, KAB
1071 Writing – review & editing: All authors
1072

1073 **Competing interests:**

1074 NMI receives consulting fees from Novartis, Pfizer, and Seattle Genetics; Honoraria from
1075 Curio Science, Cardinal Health, OncLive, IntrinsiQ Health; Research funding (to
1076 institution) from Novartis, SynDevRx, National Cancer Institute, American Cancer
1077 Society, Breast Cancer Research Foundation, and the Conquer Cancer Foundation. LED
1078 is a scientific advisor and holds equity in Mirimus Inc. and has received consulting fees
1079 and/or honoraria from Volastra Therapeutics, Revolution Medicines, Repare
1080 Therapeutics, Fog Pharma, and Frazier Healthcare Partners. BDH is a founder and
1081 consultant for Faeth Therapeutics.

1082

1083

1084

1085

1086

1087

1088

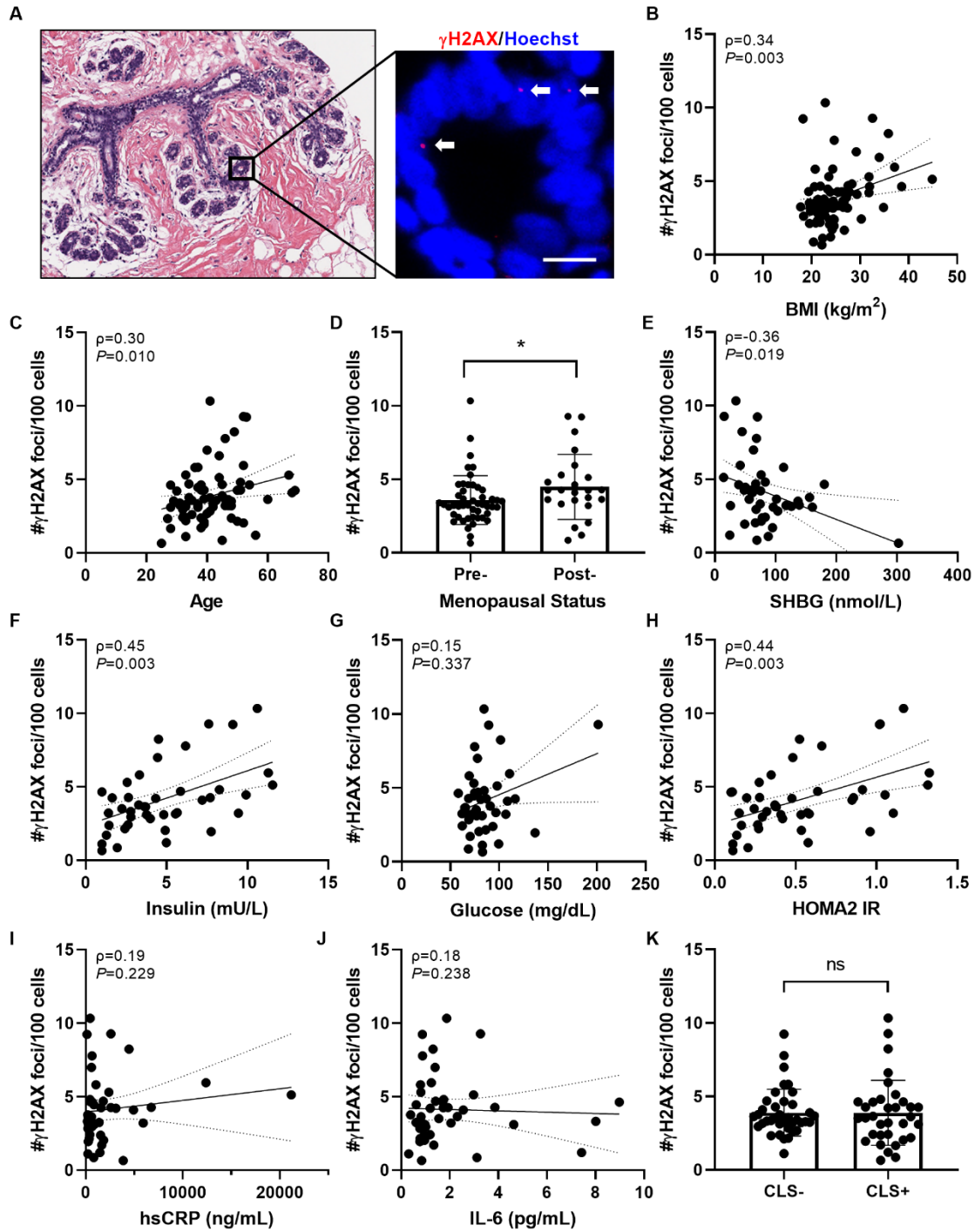


Fig.1

1089
1090

1091 **Fig. 1. BMI and additional clinical characteristics are positively correlated with DNA**

1092 **damage in breast epithelium of women carrying a *BRCA* mutation**

1093 (A) Representative image of tissue microarray section of normal breast epithelium shown by
1094 H&E stain (left) and by IF staining (right) for γ H2AX (red, arrows) co-localizing with Hoechst
1095 (blue), scale bar=10 μ M. (B-C) Correlation between epithelial cell DNA damage as measured by
1096 # γ H2AX foci/100 cells with clinical characteristics including BMI and age. (D) Average DNA
1097 damage in the study population grouped by menopausal status: pre-menopausal, n=48 and post-
1098 menopausal, n=24. (E-J) Epithelial cell DNA damage correlated with circulating serum
1099 biomarkers in a subset of the study population with available fasting serum at the time of surgery
1100 (n=43). (K) Average DNA damage in the study population when grouped by those exhibiting
1101 histological breast adipose tissue inflammation defined as presence of crown-like structures
1102 (CLS) vs those with no CLS present (i.e. CLS- vs CLS+). Two-tailed Mann Whitney test was
1103 used to determine significant differences in grouped comparisons and data is presented as mean
1104 +/- SD. and Correlation between variables were assessed by Spearman's rank correlation
1105 coefficient (ρ). Associated *P* value and ρ are shown for continuous variables with 95%
1106 confidence intervals. **P*<0.05; ns, not significant; n=72 unless otherwise stated.

1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122

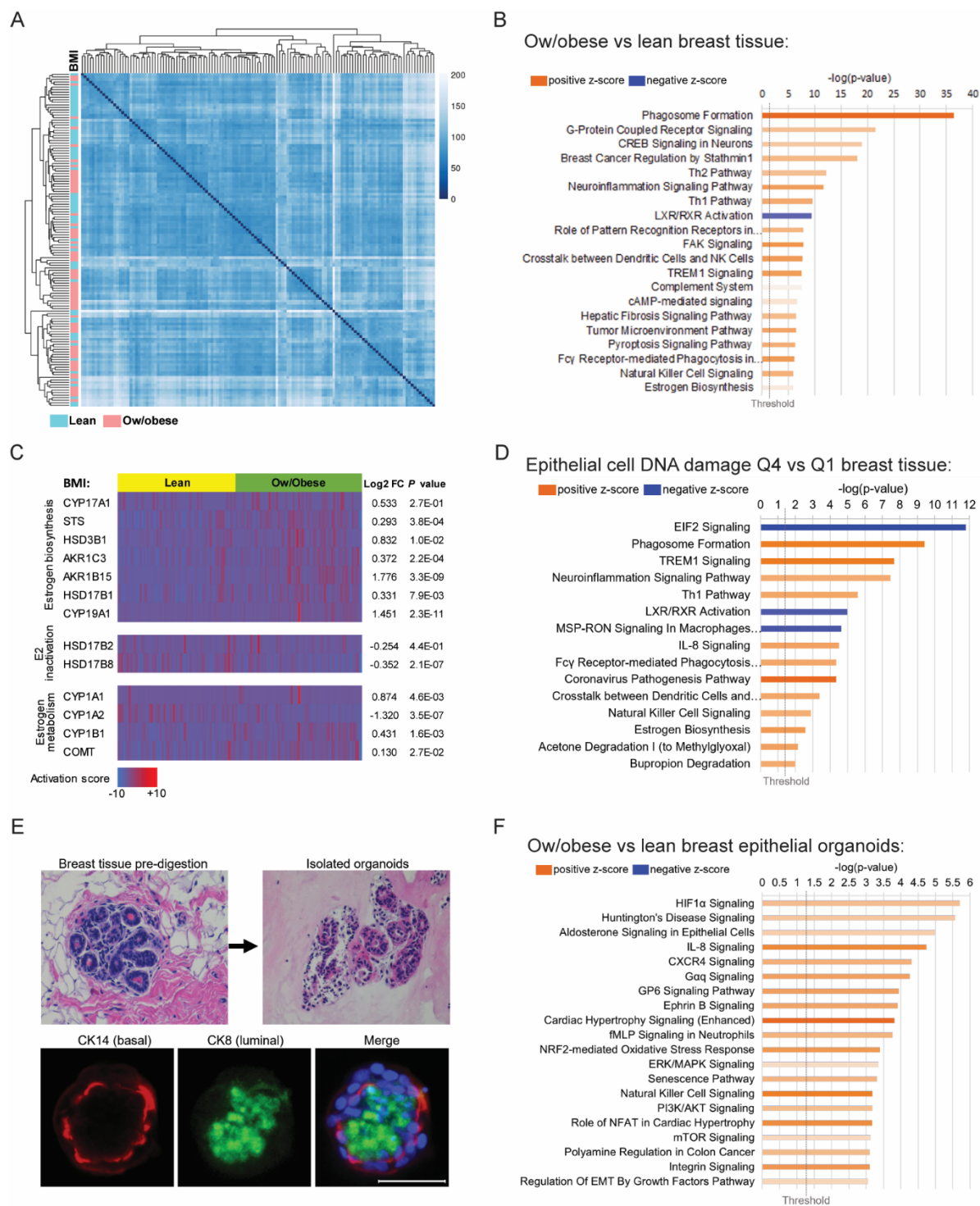


Fig. 2

1123
 1124
 1125 **Fig. 2. Elevated bodyweight is associated with significant changes in gene expression in**
 1126 **breast adipose tissue and in breast epithelial cells of *BRCA* mutation carriers**

1127 (A) Unsupervised heatmap of whole breast tissue gene expression by RNA-seq in *BRCA*
1128 mutation carriers identified by BMI category of lean (n=64, blue) or overweight/obese (n=67,
1129 pink). (B) IPA analysis of RNA-Seq data showing activation (z-score) of the top 20 canonical
1130 pathways regulated in breast tissue from overweight/obese *BRCA* mutation carriers compared to
1131 lean carriers with an absolute value z-score of >0.5 . (C) Heatmap of RNA-seq gene expression
1132 data generated from breast tissue of *BRCA* mutation carriers grouped by BMI category of lean
1133 (yellow) or overweight/obese (Ow/obese, green) showing selected genes associated with
1134 estrogen biosynthesis, estradiol (E2) inactivation, and estrogen metabolism. Corresponding gene
1135 expression (\log_2FC) and *P* values are shown in Ow/obese relative to lean tissue. (D) DNA
1136 damage in breast epithelial cells was quantified in tissue sections from n=61 patients from whom
1137 corresponding whole breast tissue RNA-seq data was also available. The cases were stratified by
1138 quartile of DNA damage and the breast tissue gene expression from cases with the highest level
1139 of DNA damage (quartile 4, Q4) were compared to cases with the lowest level (quartile 1, Q1) of
1140 DNA damage. Top 15 canonical pathways regulated in Q4 vs Q1 with an absolute value z-score
1141 of >2.0 are shown. (E) Representative H&E-stained images of a breast tissue section before
1142 digestion and epithelial organoids after isolation are shown. Organoids stain positively for
1143 luminal marker cytokeratin 8 (CK8, green) and basal marker cytokeratin 14 (CK14, red) as
1144 shown by IF staining merged with Hoechst (blue). Scale bar= 50 μ M. (F) IPA analysis of RNA-
1145 seq gene expression data showing activation of the top 20 canonical pathways regulated in
1146 primary breast epithelial organoids from of overweight/obese (Ow/obese) *BRCA* mutation
1147 carriers (n=9) relative to lean carriers (n=10) with an absolute value z-score of >1.0 is shown.
1148 The length of the bars on all canonical pathway graphs are determined by the Fisher's Exact Test
1149 *P* value with entities that have a $-\log(p\text{-value}) >1.3$ shown.

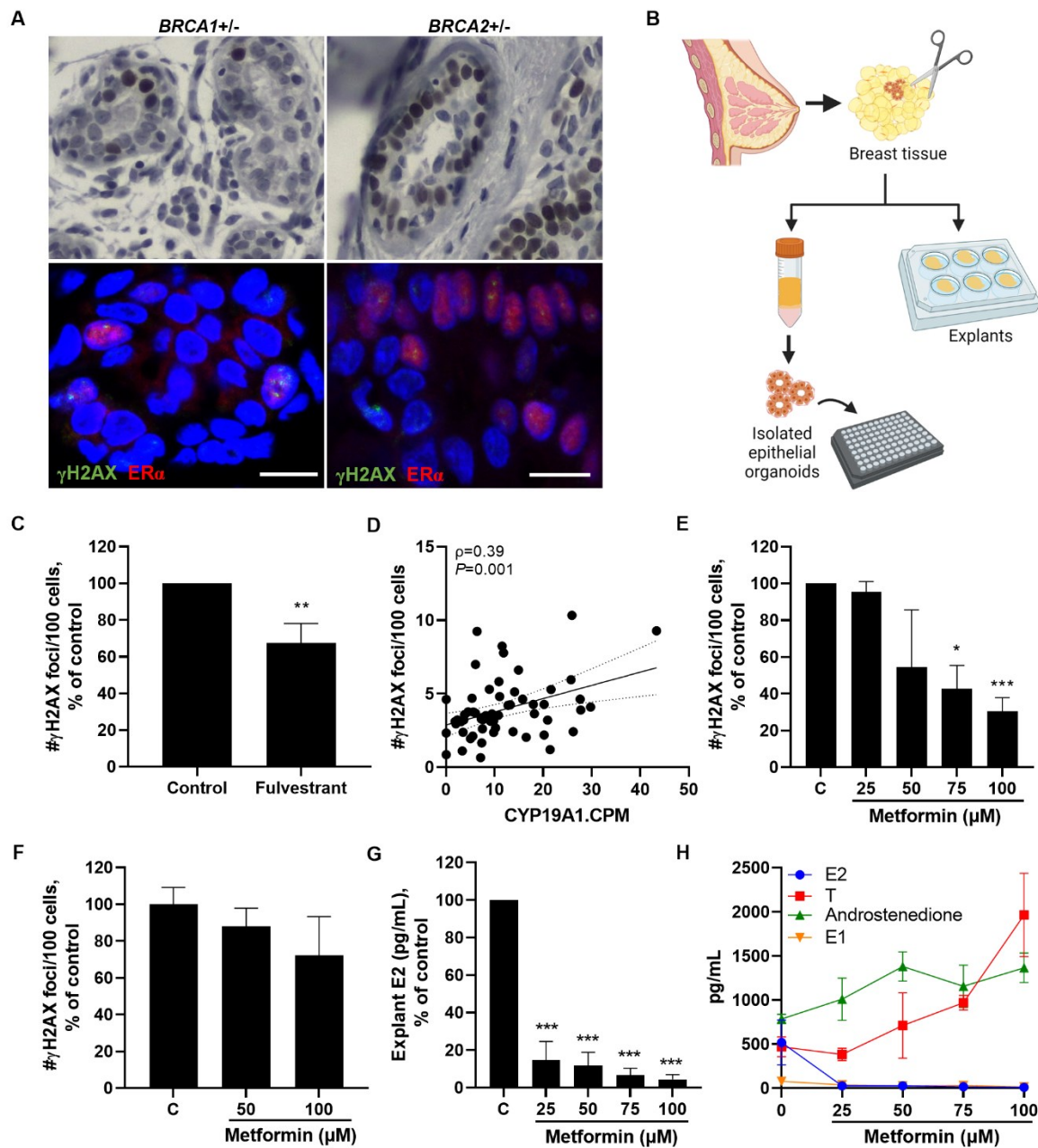


Fig. 3

1150
1151

1152 **Fig. 3. Targeting estrogen signaling or production in breast tissue decreases epithelial cell**
1153 **DNA damage in in women carrying a mutation in *BRCA1* or *BRCA2***

1154 (A) Representative IHC staining of ER α expression in breast epithelium from carriers of a
1155 *BRCA1* or *BRCA2* mutation (top panel). Representative IF staining showing co-localization of
1156 # γ H2AX foci (green) with ER α positive cells (red) (bottom panel), scale bar=10 μ M. (B)
1157 Experimental schematic showing collection of breast tissue and plating of explants or isolation of
1158 primary breast epithelial organoids for treatment studies. (C) Breast epithelial cell DNA damage
1159 assessed by IF (# γ H2AX foci/100 cells) in *ex vivo* breast adipose tissue explants from *BRCA*
1160 mutation carriers treated with fulvestrant (100nM) for 24 hours (pooled average of n=7 patients).
1161 (D) Aromatase expression in breast tissue from *BRCA* mutation carriers (RNA-seq counts per
1162 million, CPM) correlated with level of breast epithelial cell DNA damage in corresponding tissue
1163 sections (n=61). Spearman's rank correlation coefficient (ρ) and associated *P* value are shown
1164 with 95% confidence intervals. (E) Breast epithelial cell DNA damage in *ex vivo* breast adipose
1165 tissue explants from *BRCA* mutation carriers treated with metformin (0-100 μ M) for 24 hours
1166 (pooled average of n=3 patients). (F) DNA damage in isolated primary breast epithelial cells
1167 from *BRCA* mutation carriers treated with metformin (0-100 μ M) for 24 hours (representative of
1168 n=2 experiments). (G) Average 17 β -estradiol (E2) levels and (H) overlay of E2, testosterone (T),
1169 androstenedione, and estrone (E1) levels in *ex vivo* breast adipose explants after 24-hour
1170 treatment with metformin (pooled average of n=3 patients). Student's t-test was used to
1171 determine significant differences from control unless otherwise stated. Data is presented as mean
1172 +/- SEM. **P* <0.05, ***P* <0.01, ****P* <0.001.

1173
1174
1175
1176
1177
1178
1179
1180

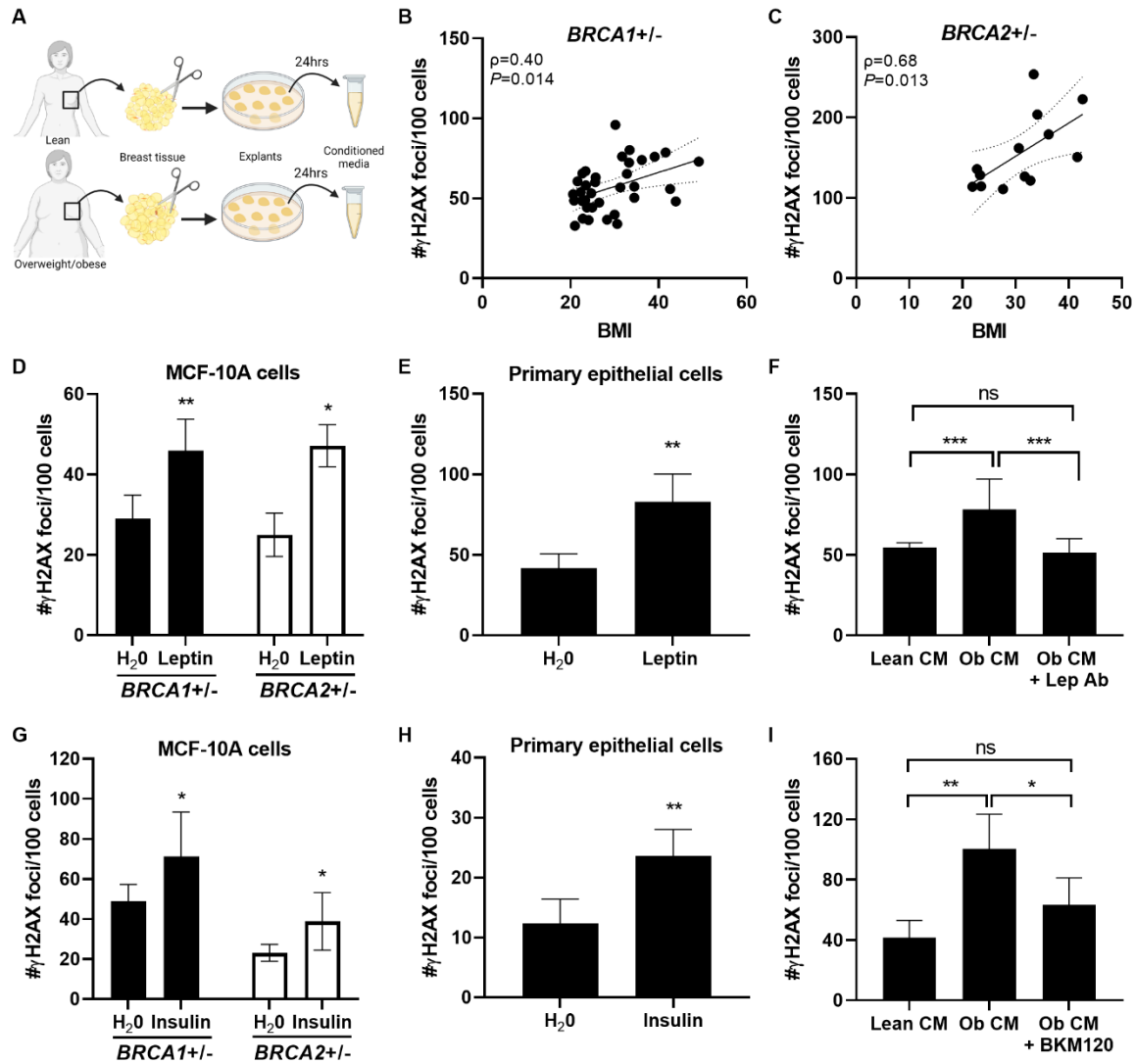


Fig. 4

1181
 1182
 1183 **Fig. 4. Obesity-induced changes to the local breast adipose microenvironment promotes**
 1184 **DNA damage in *BRCA1* and *BRCA2* heterozygous breast epithelial cells**
 1185 (A) Experimental schematic showing the collection of breast adipose tissue conditioned media
 1186 (CM) from lean and overweight/obese women. (B) MCF-10A cells were treated with CM for 24
 1187 hours. DNA damage assessed by IF (# γ H2AX foci/100 cells) is shown correlated with BMI in
 1188 *BRCA1*+/- (n=36 CM cases) and (C) *BRCA2*+/- (n=13 CM cases) MCF-10A cells. Spearman's

1189 rank correlation coefficient (ρ) and associated P value are shown along with 95% confidence
1190 intervals. **(D)** DNA damage in *BRCAl*^{+/-} and *BRCa2*^{+/-} MCF-10A cells and in **(E)** primary
1191 *BRCAl*^{+/-} breast epithelial cells treated with leptin (400ng/ μ l) for 24 hours. **(F)** DNA damage in
1192 *BRCAl*^{+/-} MCF-10A cells after 24-hour treatment with lean CM, obese (ob) CM, or ob CM in
1193 the presence of a leptin neutralizing antibody (Lep Ab). **(G)** DNA damage in *BRCAl*^{+/-} and
1194 *BRCa2*^{+/-} MCF-10A cells and in **(H)** primary *BRCa2*^{+/-} breast epithelial cells treated with
1195 insulin (100nM) for 24 hours. **(I)** DNA damage in *BRCAl*^{+/-} MCF-10A cells after 24-hour
1196 treatment with lean CM, ob CM, or ob CM in the presence of PI3K inhibitor BKM120 (1 μ M).
1197 Student's t-test was used to determine significant differences in **(D-I)**. All experiments in MCF-
1198 10A cells were conducted a minimum of two times with representative results from one
1199 experiment shown. Data in primary cells were generated from cells treated in triplicate. Data is
1200 presented as mean \pm SD. * P < 0.05, ** P < 0.01, *** P < 0.001, ns= not significant.

1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222

1223
1224

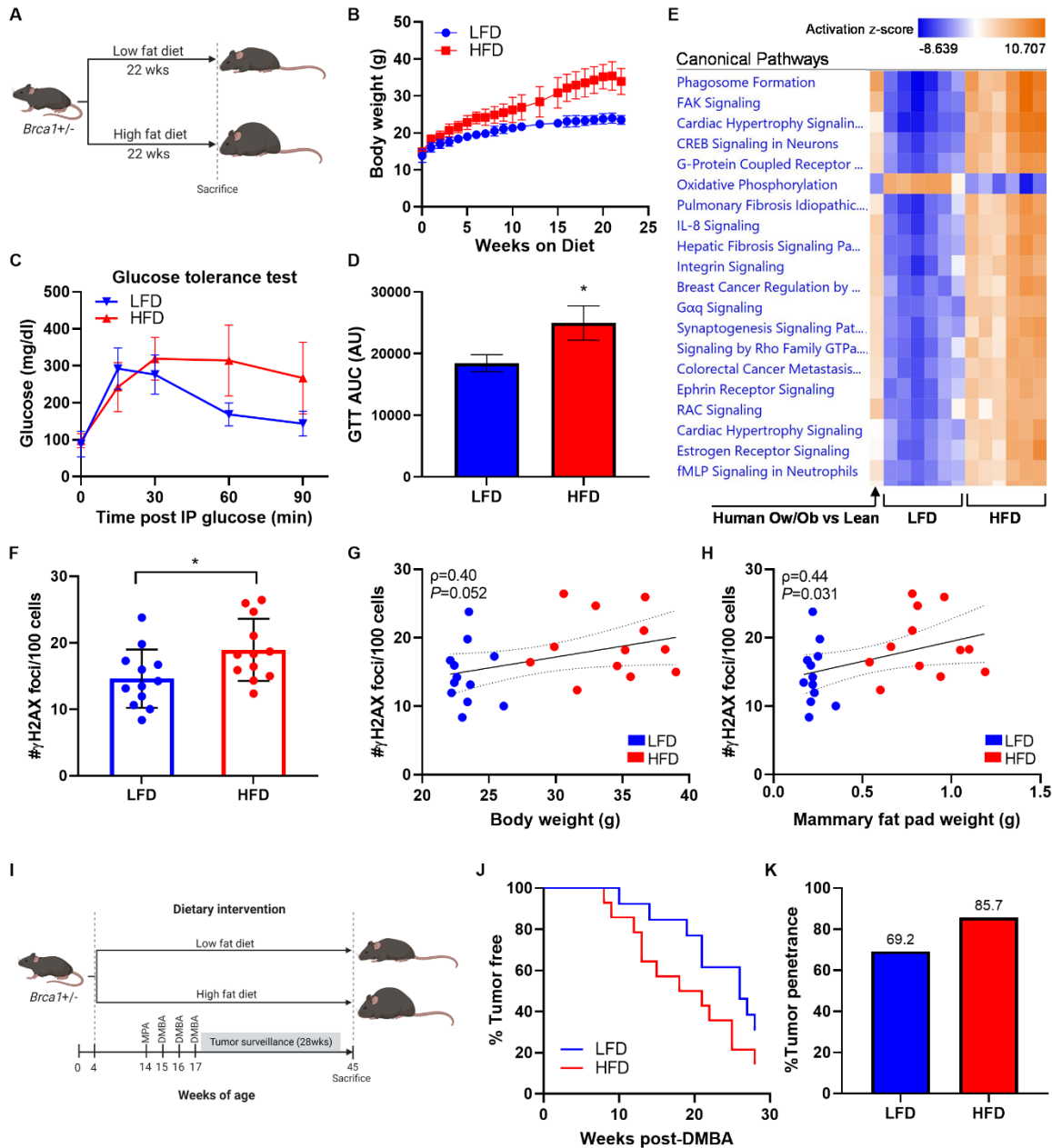


Fig. 5

1225
1226
1227

Fig. 5. High fat diet feeding leads to elevated mammary gland DNA damage in association

1228

with increased mammary tumor penetrance and decreased tumor latency in *Brca1*^{+/-} mice

1229 (A) Experimental schematic of diet-induced obesity in female *Brcal*^{+/-} mice (n=12/gp). (B)
1230 Average body weight of mice fed low fat diet (LFD) or high fat diet (HFD) over 22 wks. (C)
1231 Glucose tolerance test conducted one week prior to sacrifice and (D) area under curve (AUC)
1232 calculation for each group (mean +/- SEM). (E) RNA-Seq was conducted on whole mammary
1233 fat pad tissue from HFD and LFD mice (n=6/gp). Activation of top 20 canonical pathways
1234 regulated in mammary fat pads from HFD mice compared to LFD mice are shown adjacent to
1235 corresponding pathway regulation in breast tissue from overweight (Ow)/obese vs lean women
1236 carrying a *BRC*A mutation (n=64-67/gp). (F) DNA damage assessed by IF (# γ H2AX foci/100
1237 cells) in mammary glands at the time of sacrifice. (G) Correlation between mammary gland
1238 DNA damage and mouse body weight and (H) mammary fat pad weight among all mice.
1239 Spearman's rank correlation coefficient (ρ) and associated *P* values are shown along with 95%
1240 confidence intervals. (I) Experimental schematic of MPA/DMBA-induced tumorigenesis model
1241 in female *Brcal*^{+/-} mice randomized to LFD or HFD groups (n=13-14/gp). (J) Mammary tumor
1242 development in LFD and HFD mice shown as % of mice tumor free over the 28-week
1243 surveillance period. (K) Overall mammary tumor penetrance at the end of the surveillance period
1244 shown as % of mice in each group that developed a mammary tumor. Student's t-test was used to
1245 determine significance unless otherwise stated. Data is presented as mean +/- SD unless
1246 otherwise stated. **P* <0.05.

1247
1248
1249
1250
1251
1252
1253
1254
1255
1256

1257
1258
1259
1260

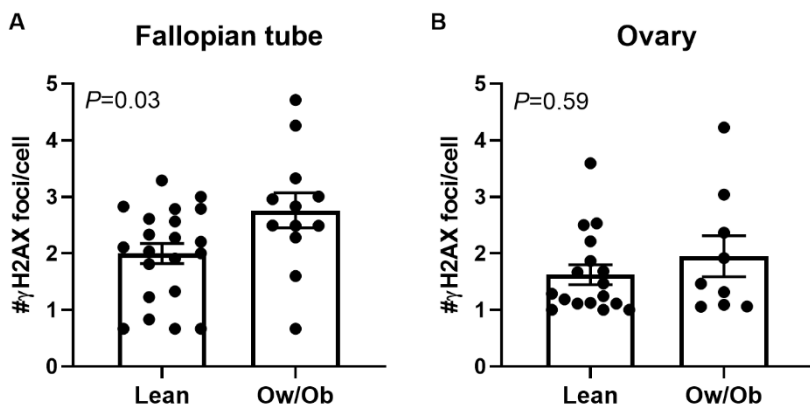


Fig. 6

1261
1262
1263
1264
1265
1266
1267
1268

Fig. 6. BMI is associated with DNA damage in the fallopian tube but not ovary

(A) DNA damage assessed by IF (# γ H2AX foci/cell) in epithelial cells of the ovary and in (B) epithelial cells of fallopian tube fimbriae in *BRCA* mutation carriers grouped by BMI category of lean (n=17-21/gp) or overweight (Ow)/obese (Ob) (n=9-12). Two-tailed Mann Whitney test was used to determine significant differences (*P* value) between groups. Data is presented as mean +/-SEM.

1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281

1282
1283
1284
1285

Table 1. Baseline characteristics of study population based on BMI category

Variables	All (n = 72)	Lean (n = 46)	Overweight/Obese (n = 26)	P
BMI, median (range)	23.7 (17.7-44.9)	21.8 (17.7-24.7)	28.8 (25.3-44.9)	<0.01
BRCA mutation, n (%)				0.46
BRCA1	42 (58.3%)	29 (63.0%)	14 (53.8%)	
BRCA2	30 (41.7%)	17 (37.0%)	12 (46.2%)	
Age, median (range)	40 (25-69)	38.5 (25-60)	44.5 (28-69)	0.01
Diabetes, n (%)				0.36
No	71 (98.6%)	46 (100.0%)	25 (96.2%)	
Yes	1 (1.4%)	0 (0%)	1 (3.8%)	
Dyslipidemia, n (%)				0.01
No	65 (90.3%)	45 (97.8%)	20 (76.9%)	
Yes	7 (9.7%)	1 (2.2%)	6 (23.1%)	
Hypertension, n (%)				0.02
No	64 (88.9%)	44 (95.7%)	20 (76.9%)	
Yes	8 (11.1%)	2 (4.3%)	6 (23.1%)	
Menopausal status, n (%)				0.037
Pre-	48 (66.7%)	35 (76.1%)	13 (50.0%)	
Post-	24 (33.3%)	11 (23.9%)	13 (50.0%)	
Race, n (%)				0.19
Asian	1 (1.4%)	1 (2.2%)	0 (0.0%)	
Black	2 (2.8%)	2 (4.3%)	0 (0.0%)	
Other	2 (2.8%)	0 (0.0%)	2 (7.7%)	
White	59 (81.9%)	36 (78.3%)	23 (88.5%)	
Missing	8 (11.1%)	7 (15.2%)	1 (3.8%)	
Invasive tumor present, n (%)				1
No	40 (55.6%)	26 (56.5%)	14 (53.8%)	
Yes	32 (44.4%)	20 (43.5%)	12 (46.2%)	
Tumor subtype, n(%)				1
HR+	23 (31.9%)	15 (32.6%)	8 (30.8%)	
HER2+	1 (1.4%)	1 (2.2%)	0 (0.0%)	
TNBC	10 (13.9%)	6 (13.0%)	4 (15.4%)	
N/A	38 (52.8%)	24 (52.2%)	14 (53.8%)	

Abbreviations: BMI, body mass index; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer

1286
1287
1288

1289
1290
1291

Table 2. Association of clinical features and blood biomarkers with DNA damage, adjusting for age or BMI

Variables	Correction	P	Correction	P
BMI			Age	0.025
Age	BMI	0.115		
SHBG (nmol/L)	BMI	0.047	Age	0.026
Insulin (mU/L)	BMI	<0.001	Age	<0.001
HOMA2 IR	BMI	<0.001	Age	<0.001

Abbreviations: BMI, body mass index; SHBG, steroid hormone binding globulin, HOMA2 IR, homeostatic model assessment 2 for insulin resistance

1292
1293
1294

Table 3. Predicted upstream regulators of gene expression differences in breast epithelial organoids from overweight/obese *BRCA* mutation carriers relative to lean carriers and associated gene expression in whole breast tissue

Organoid upstream regulator	Predicted activation state	Activation z-score	P-value of overlap	Breast tissue log2FC	P-value
beta-estradiol	Activated	4.728	2.2E-10	see Fig. 2C	
IL2	Activated	3.402	3.1E-02	0.563	2.3E-01
GDF2	Activated	3.217	4.9E-03	-0.081	9.8E-01
IL15	Activated	3.152	1.5E-03	0.299	4.1E-05
TNFSF11	Activated	3.125	3.2E-02	-0.757	9.7E-02
Insulin	Activated	3.113	6.1E-03		
IL4	Activated	3.016	1.9E-03	-0.25	7.4E-01
TGFB1	Activated	2.942	6.0E-09	0.455	2.2E-08
hydrogen peroxide	Activated	2.839	3.1E-03		
IL3	Activated	2.674	7.6E-04	-0.122	9.7E-01
CSF1	Activated	2.602	8.9E-03	0.35	1.4E-06
Lh	Activated	2.598	1.7E-03		
dinoprost (PGF2 α)	Activated	2.569	2.9E-02		
IL5	Activated	2.496	5.5E-03	0.173	6.9E-01
ATP	Activated	2.443	8.7E-03		
MDK	Activated	2.433	2.9E-02	-0.34	4.4E-03
AGT	Activated	2.345	4.2E-03	-0.56	9.6E-04
ANGPT2	Activated	2.329	1.1E-03	0.38	9.2E-05
WNT5A	Activated	2.292	1.7E-03	0.184	1.3E-01
pyruvic acid	Activated	2.156	1.5E-03		

1295
1296
1297
1298

1299
1300

Table 4. Activation of diseases or functions associated with DNA damage or DNA repair in *BRCA1*+/- epithelial cells treated with breast adipose tissue condition media derived from obese women relative to lean women

Categories	Diseases or functions annotation	P-value	Predicted activation state	Activation z-score	# Molecules
Cellular Assembly and Organization	Formation of micronuclei	2.53E-06	Increased	2.756	9
DNA Replication, Recombination & Repair	Chromosomal aberration	5.37E-06	Increased	2.853	31
	Chromosomal instability	2.43E-08	Increased	2.603	19
	Breakage of chromosomes	2.88E-05	Increased	2.488	11
Cell Cycle; DNA Replication, Recombination & Repair	Checkpoint control	1.99E-06	Decreased	-2.756	15
	Spindle checkpoint	9.33E-07	Decreased	-2.035	12
DNA Replication, Recombination & Repair	Repair of DNA	4.36E-09	Decreased	-3.334	47
	Double-stranded DNA break repair of tumor cell lines	9.94E-06	Decreased	-2.241	14
	Metabolism of DNA	2.10E-10	Decreased	-2.09	54

1301
1302
1303
1304
1305
1306
1307
1308