# Supporting Information

# Estrogen receptor inhibition enhances cold-induced adipocyte beiging and glucose tolerance

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# Supporting Figure Legends

**Figure S1. Related to Figure 1.** Four-month-old female ERαWT (WT) and ERαKO (KO) mice were subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A)** Representative photographs of inguinal (IGW) and perigonadal (PGW) white adipose depots of WT and KO females. **(B)** Weight of indicated fat depots in WT and KO females: SWAT - IGW and intrascapular (ISCW); VWAT - PGW, retroperitoneal (RPW) and mesenteric (MWAT); and intrascapular BAT, $n \geq 8$. **(C)** Food intake was measured by weighing crude normal chow per mouse per cage, $n \geq 8$. **(D)** Lean mass by NMR, $n \geq 8$. **(E)** Weight of indicated organs in WT and KO females, $n \geq 7$. **(F)** Serum cholesterol and triglyceride levels of WT and KO females, $n \geq 4$. **(G)** Insulin tolerance tests were performed in WT and KO females at RT (a week prior to cold-exposure) and immediately after cold-exposure. Mice were fasted, i.p. injected with 0.3mU/g insulin, and their glucose levels were monitored, $n \geq 8$. Inset – areas under curve. **(H)** Serum insulin levels of WT and KO females, $n \geq 5$. Error bars indicate S.E.M. Statistical significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p < 0.01$; and one-way ANOVA test, $ $p < 0.01$.

**Figure S2. Related to Figure 1.** Four-month-old female ERαWT (WT) and ERαKO (KO) mice were subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A)** Representative H&E-stained histological sections of VWAT, $n \geq 8$. **(B)** UCP1 immunohistochemistry - representative sections of SWAT. **(C)** Relative mRNA levels, quantified by qPCR, of brown/beige adipocyte markers expressed in VWAT (PGW) of WT and KO females at cold, $n \geq 6$. **(D)** Relative mRNA levels, quantified by qPCR, of UCP1 in SWAT (IGW) of WT and KO females at RT and cold, $n \geq 5$. **(E)** Body temperature (rectal probe) of WT and KO females at RT and cold, $n \geq 9$. Scale bars = 100 µm. Error bars indicate S.E.M. Statistical significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p < 0.01$; and one-way ANOVA test, $ $p < 0.01$.

**Figure S3. Related to Figure 2.** Two-month-old female mice underwent sham operation or ovariectomy (OVX), 3 months later, both groups were either subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A)** Representative photographs of IGW and PGW adipose depots of Sham and OVX females. **(B)** Weight of indicated fat depots in Sham and OVX females: SWAT - IGW and ISCW; VWAT - PGW, RPW and MWAT; and intrascapular BAT, $n \geq 8$. **(C)** Food intake was measured by weighing crude normal chow per mouse per cage, $n \geq 6$. **(D)** Liver weights of Sham and OVX females, $n \geq 8$. **(E)** Lean mass by NMR, $n \geq 6$. **(F)** Weight of indicated organs in Sham and OVX females, $n \geq 8$. **(G)** Serum cholesterol and triglyceride levels of Sham and OVX females, $n \geq 8$. **(H)** Insulin tolerance tests were performed in 6-month-old Sham and OVX females at RT (a week prior to cold-exposure) and immediately after cold-exposure. Mice were fasted, i.p. injected with 0.75mU/g insulin, and their glucose levels were monitored, $n \geq 10$. Inset – areas under curve. **(I)** Serum insulin levels of Sham and OVX females, $n \geq 5$. Error bars indicate S.E.M. Statistical significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p < 0.01$, NS – not significant; and one-way ANOVA test, \$ $p < 0.01$.

**Figure S4. Related to Figure 2.** Two-month-old female mice underwent sham operation or ovariectomy (OVX), 3 months later, both groups were either subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A-B)** Representative H&E-stained histological sections of VWAT (A) and BAT (B), $n \geq 8$. **(C)** UCP1 immunohistochemistry - representative sections of SWAT. **(D)** Relative mRNA levels, quantified by qPCR, of brown/beige adipocyte markers expressed in VWAT (PGW) at cold, $n \geq 10$. **(E)** Relative mRNA levels, quantified by qPCR, of UCP1 in SWAT (IGW) of Sham and OVX females at RT and cold, $n \geq 7$. **(F)** Body temperature (rectal probe) of Sham and OVX females at RT and cold, $n \geq 11$. **(G)** Triglyceride levels in the livers of Sham and OVX females at RT and cold, $n \geq 8$. Scale bars = 100 μm. Error bars indicate

66 S.E.M. Statistical significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p <0.01$, NS
67 – not significant; and one-way ANOVA test, $ $p < 0.01$.

68 **Figure S5. Related to Figure 3.** **(A-D)** SV cells were isolated from SWAT of two-month-old
69 females. **(A-B)** Confluent cells were induced with white adipogenic media in the presence of
70 vehicle or Fulvestrant. A week later, cells were stained with Oil Red O (A) or immunostained for
71 UCP1 (B). Nile Red stains lipid droplets. **(C-D)** Confluent cells were induced with beige
72 adipogenic media in the presence of vehicles, Estradiol and/or Fulvestrant. A week later, beige
73 cells were activated with Forskolin. Beiging was assessed by relative mRNA levels, quantified by
74 qPCR, of brown/beige adipocyte markers, $n \geq 6$ (C) or by UCP1 immunostaining (D). Nile Red
75 stains lipid droplets. **(E-H)** Acute administration: Four-month-old WT females were given a
76 vehicle or 40 mg/kg/injection Fulvestrant as described in Figure 3D, then they were subjected to
77 cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(E)** Body weight, $n \geq 7$. **(F)** Weight of
78 indicated fat depots in vehicle-treated and Fulvestrant-treated females: SWAT - IGW and ISCW;
79 VWAT - PGW, RPW and MWAT; and intrascapular BAT, $n \geq 7$. **(G)** Food intake was measured
80 by weighing crude normal chow per mouse per cage, $n \geq 6$. **(H)** Representative H&E-stained
81 histological sections of SWAT, $n \geq 7$. Scale bars = 100 μm. Error bars indicate S.E.M. Statistical
82 significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p <0.01$; and one-way ANOVA
83 test, $ $p < 0.01$.

84 **Figure S6. Related to Figure 4.** Chronic administration: Four-month-old WT ICR(CD1) females
85 were given a vehicle or 40 mg/kg/injection Fulvestrant for a month as described in Figure 4A, then
86 they were subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A-C)** ERα
87 immunofluorescence of vehicle-treated and Fulvestrant-treated females at RT (prior to cold-
88 exposure) - representative sections of SWAT (A), VWAT (B) and BAT (C). Negative controls

89 (lower lane) – representative sections from ERαKO females that lack ERα expression. **(D-G)** Body
90 weight (D), fat mass by NMR (E), fat content by NMR (F) and lean mass by NMR (G) were
91 monitored during vehicle and Fulvestrant administration and following cold-exposure, n≥11. **(H)**
92 Food intake was measured by weighing crude normal chow per mouse per cage, n≥11. **(I)** Weight
93 of indicated organs in vehicle-treated and Fulvestrant-treated females, n≥6. Scale bars = 100 µm.
94 Error bars indicate S.E.M. Statistical significance assessed by two-tailed student's t-test, * p <
95 0.05, ** p <0.01; and one-way ANOVA test, $ p < 0.01. (Fulvestrant only) – statistically
96 significant only in Fulvestrant-treated mice.

97 **Figure S7. Related to Figure 5.** Chronic administration: Four-month-old WT ICR(CD1) females
98 were given a vehicle or 40 mg/kg/injection Fulvestrant for a month as described in Figure 4A, then
99 they were subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C). **(A)** Relative
100 mRNA levels, quantified by qPCR, of UCP1 in SWAT (IGW) of vehicle-treated and Fulvestrant-
101 treated females at RT and cold, n≥6. **(B)** The body temperatures (rectal probe) of vehicle-treated
102 and Fulvestrant-treated females were monitored upon cold-exposure, n≥12. Error bars indicate
103 S.E.M. Statistical significance assessed by two-tailed student's t-test, * p < 0.05, ** p <0.01; and
104 one-way ANOVA test, $ p < 0.01.

105 **Figure S8. Related to Figure 6. (A-L)** Chronic administration in a high-fat diet (HFD) model: At
106 the age of two-month-old onwards, WT female mice were fed with high-fat high-sucrose diet. Two
107 months later, the females were given a vehicle or 40 mg/kg/injection Fulvestrant for a month as
108 described in Figure 6A, then they were subjected to cold-exposure (6°C) for 7 days or maintained
109 at RT (23°C). **(A)** Body weight was monitored from the onset of HFD, during vehicle and
110 Fulvestrant administration and following cold-exposure, n≥21. **(B)** Fat content by NMR, n≥17.
111 **(C)** Lean mass by NMR, n≥17. **(D)** Representative photographs of IGW adipose depots of vehicle-

112 treated and Fulvestrant-treated females on HFD at RT and cold. **(E)** Weight of indicated fat depots
113 in vehicle-treated and Fulvestrant-treated females on HFD: SWAT - IGW and ISCW; VWAT -
114 PGW, RPW and MWAT; and intrascapular BAT, n≥7. **(F)** Liver weights of vehicle-treated and
115 Fulvestrant-treated females on HFD, n≥7. **(G)** Serum cholesterol and triglyceride levels of vehicle-
116 treated and Fulvestrant-treated females on HFD, n≥7. **(H)** Serum insulin levels of vehicle-treated
117 and Fulvestrant-treated females on HFD, n≥7. **(I)** Representative histological sections of SWAT -
118 H&E-stained at RT (upper lane), n≥7, and UCP1 immunofluorescence at cold (lower lane). **(J)**
119 Relative mRNA levels, quantified by qPCR, of UCP1 in SWAT (IGW) and BAT of vehicle-treated
120 and Fulvestrant-treated females on HFD at RT and cold, n≥7. **(K)** The body temperatures (rectal
121 probe) of vehicle-treated and Fulvestrant-treated females on HFD were monitored upon cold-
122 exposure, n≥7. **(L)** Triglyceride levels in the livers of vehicle-treated and Fulvestrant-treated
123 females on HFD at RT and cold, n≥7. Scale bars = 100 µm. Error bars indicate S.E.M. Statistical
124 significance assessed by two-tailed student's t-test, * $p < 0.05$, ** $p < 0.01$, NS – not significant;
125 and one-way ANOVA test, $ $p < 0.01$. Beige cells - Confluent cells were induced with beige
126 adipogenic media in the presence of vehicle or Fulvestrant for a week.

127 **Figure S9. Related to Figure 7. (A-E)** SV cells were isolated from SWAT of two-month-old WT
128 females. Confluent cells were treated daily with a vehicle or Fulvestrant for 8-48hr, in the absence
129 of beige adipogenic reagents – termed undifferentiated cells. **(A)** Relative changes in cAMP
130 concentrations in undifferentiated cells upon induction with or without Forskolin. Every dot
131 represents an average of technical triplicates of one biological sample. The relative changes in
132 cAMP concentrations are calculated as differences between treatments, n≥7. **(B)** Relative mRNA
133 levels, quantified by qPCR, of UCP1, β-adrenergic receptors, β-adrenergic receptor kinases and
134 PPARγ2, n≥5. **(C, E)** Vehicle-treated or Fulvestrant-treated undifferentiated cells were

135 immunostained for AdRβ3 (C) or PCNA (E) expression. Arrows indicate $PCNA^+$ cells and the
136 numbers represent their percentage. **(D)** Relative mRNA levels, quantified by qPCR, of UCP1 and
137 AdRβ3 in undifferentiated cells (taken from Fig. S9B) and beige cells (taken from Fig. 7B), n≥5.
138 **(F)** Confluent cells were treated daily with a vehicle or Fulvestrant for 48hr, and then induced with
139 beige adipogenic media in the presence of β3 agonist without Fulvestrant. Beiging was assessed
140 by UCP1 immunostaining. Nile Red stains lipid droplets. **(G-H)** Vehicle-treated and Fulvestrant-
141 treated females (G, see Figure 3) as well as ERαWT (WT) and ERαKO (KO) females (H, see
142 Figure 1) were subjected to cold-exposure (6°C) for 7 days or maintained at RT (23°C).
143 Representative histological sections of SWAT, which were immunostained for AdRβ3 and
144 Perilipin. **(I)** Relative mRNA levels, quantified by qPCR, of AdRβ3 and AdRβK2 in SWAT of
145 vehicle-treated and Fulvestrant-treated females, n≥6. **(J-K)** Four-month-old WT females were pre-
146 treated with a vehicle or 40 mg/kg/injection Fulvestrant, followed by administration of 1
147 mg/kg/day β3 agonist as described in Figure 7G. Relative mRNA levels, quantified by qPCR, of
148 brown/beige adipocyte markers expressed in SWAT (IGW) (J), and BAT (K), n≥7. Scale bars =
149 100 μm. Error bars indicate S.E.M. Statistical significance assessed by two-tailed student's t-test,
150 * $p < 0.05$, ** $p < 0.01$; and one-way ANOVA test, $ $p < 0.01$. In (a), results are based on a matched
151 standard curve and a linear regression analysis.

152 **Figure S10. Summary. (A)** In this study, we studied mouse models of inhibited ERα signaling:
153 1) a genetic approach - whole-body ERαKO mice; 2) a surgical approach - OVX females; 3) a
154 pharmacological approach - treatment with the estrogen receptor antagonist – Fulvestrant, and
155 exposed them to cold. Upon cold-exposure, we detected adipocyte beiging in control mice (e.g.
156 WT, Sham or vehicle-treated) as expected, however, mouse models of inhibited ERα showed an
157 enhanced response. This response included increased emergence of multilocular beige cells and

elevated gene expression of beige/brown adipocyte markers in WAT and BAT. By using a primary beige cell culture, we demonstrated that this enhanced beiging is cell-autonomous. **(B)** Although under normal circumstances of room temperature, ERαKO, OVX and mice on HFD are obese and diabetic, cold-exposure resulted in significantly improved metabolism. As a result of cold-exposure, we observed: reduced adiposity and hyperglycemia, increased glucose and insulin tolerance, and corrected diabetes-associated hypercholesterolemia, hypertriglyceridemia and hepatosteatosis. **(C)** Hypothesis – estrogen receptor inhibition enhances adipocyte beiging by "sensitizing" the cells to the beiging process upon stimulation. Stimuli of beiging may be elicited by cold-exposure or adrenergic signals. Our preliminary results support such a hypothesis – Fulvestrant pre-treatment increased cAMP levels and AdRβ3 expression in both immature and mature cells. This allowed increased beige cell differentiation or activation via AdRβ3. The mechanism that underlies enhanced cold-induced adipocyte beiging following ERα inhibition is yet to be elucidated.

179 **Table S1. qPCR primer sequences.**

| Gene | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| Acot2 | ATGGTGGCCTCGTCTTTCG | GAGCGGCGGAGGTACAAAC |
| Adrβ1 | CTCGTCCGTCGTCTCCTTCTAC | GTCGATCTTCTTTACCTGTTTTTGG |
| Adrβ2 | TTGCAGTGGATCGCTATGTTG | TGACCACTCGGGCCTTATTCT |
| Adrβ3 | GGCCCTCTCTAGTTCCCAG | TAGCCATCAAACCTGTTGAGC |
| Adrβk1 | AGCGAGTACCCAAGATGAAGAACA | CACTGCCACGCTGGATCA |
| Adrβk2 | GGACAGAAGTCGTTACAGCTAATTCA | ACTCCCAAGCTGCTGTCAAA |
| Cidea | TGCTCTTCTGTATCGCCCAGT | GCCGTGTTAAGGAATCTGCTG |
| Cox7a1 | CAGCGTCATGGTCAGTCTGT | AGAAAACCGTGTGGCAGAGA |
| β-Actin | GCGCATCCTCTTCCT | GGAGCAATGATCTTGATC |
| Eva1 | CCACTTCTCCTGAGTTTACAGC | GCATTTTAACCGAACATCTGTCC |
| Oplah | CTTCACGCACGTCTCCTTGT | GCATCTGCACAGGCCGTAT |
| PGC1α | AGCCGTGACCACTGACAACGAG | GCTGCATGGTTCTGAGTGCTAAG |
| PPARγ2 | ACAGCAAATCTCTGTTTATGC | GCTTGATGTCAAAGGAATGCG |
| PRDM16 | CAGCACGGTGAAGCCATTC | GCGTGCATCCGCTTGTG |
| Slc29a1 | CACCAGCCTCAGGACAGGTAT | GTCCAGGCGGTTTGTGAAA |
| UCP1 | ACTGCCACACCTCCAGTCATT | CTTTGCCTCACTCAGGATTGG |

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193 **Table S2. Unilocularity Index.**

| **Cold-exposed:** | **ERαWT females** | **ERαKO females** |
|---|---|---|
| SWAT (Fig. 1G) | 0.5418 ± 0.022 | 0.4047 ± 0.0255 ** |
| VWAT (Fig. S2A) | 0.6684 ± 0.0202 | 0.6063 ± 0.0373 $^{NS}$ |
| **Cold-exposed:** | **Sham females** | **OVX females** |
| SWAT (Fig. 2G) | 0.5528 ± 0.0194 | 0.401 ± 0.0368 * |
| VWAT (Fig. S4A) | 0.6726 ± 0.0196 | 0.6281 ± 0.0336 $^{NS}$ |
| **Cold-exposed:** | **Vehicle-treated females (acute)** | **Fulvestrant-treated females (acute)** |
| SWAT (Fig. 3G) | 0.474 ± 0.0188 | 0.3573 ± 0.0297 * |
| VWAT (Fig. S5F) | 0.6756 ± 0.0186 | 0.6342 ± 0.0197 $^{NS}$ |
| **Cold-exposed:** | **Vehicle-treated females (chronic)** | **Fulvestrant-treated females (chronic)** |
| SWAT (Fig. 5A) | 0.4966 ± 0.0199 | 0.3378 ± 0.0205 *** |
| VWAT (Fig. 5B) | 0.6746 ± 0.0239 | 0.4338 ± 0.0218 *** |
| **Cold-exposed:** | **Vehicle-treated females (HFD)** | **Fulvestrant-treated females (HFD)** |
| SWAT (Fig. S8I) | 0.6828 ± 0.0171 | 0.6444 ± 0.0273 $^{NS}$ |
| **β3 agonist-treated:** | **Vehicle-treated females** | **Fulvestrant-treated females** |
| SWAT (Fig. 7H) | 0.4718 ± 0.013 | 0.27 ± 0.024 *** |
| **β3 agonist-treated:** | **ERαWT females** | **ERαKO females** |
| SWAT (Fig. 7J) | 0.488 ± 0.0134 | 0.377 ± 0.0261 ** |

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195 Microscopic photos of H&E histological sections were analyzed using ImageJ and the MRI
196 Adipocytes Tools plugin. The fraction of area covered by large lipid droplets was defined as the
197 unilocularity index (further details in Materials & Methods). The values indicate average
198 unilocularity indices per experimental group ± S.E.M, n=20-40 random photos per experimental
199 group. Statistical significance assessed by two-tailed student's t-test, * $p < 10^{-2}$, ** $p < 10^{-3}$, *** p
200 $< 10^{-6}$, $^{NS}$ – not significant.

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