SUPPLEMENTARY DATA

A synergistic anti-obesity effect by a combination of capsinoids and cold temperature through the promotion of beige adipocyte biogenesis

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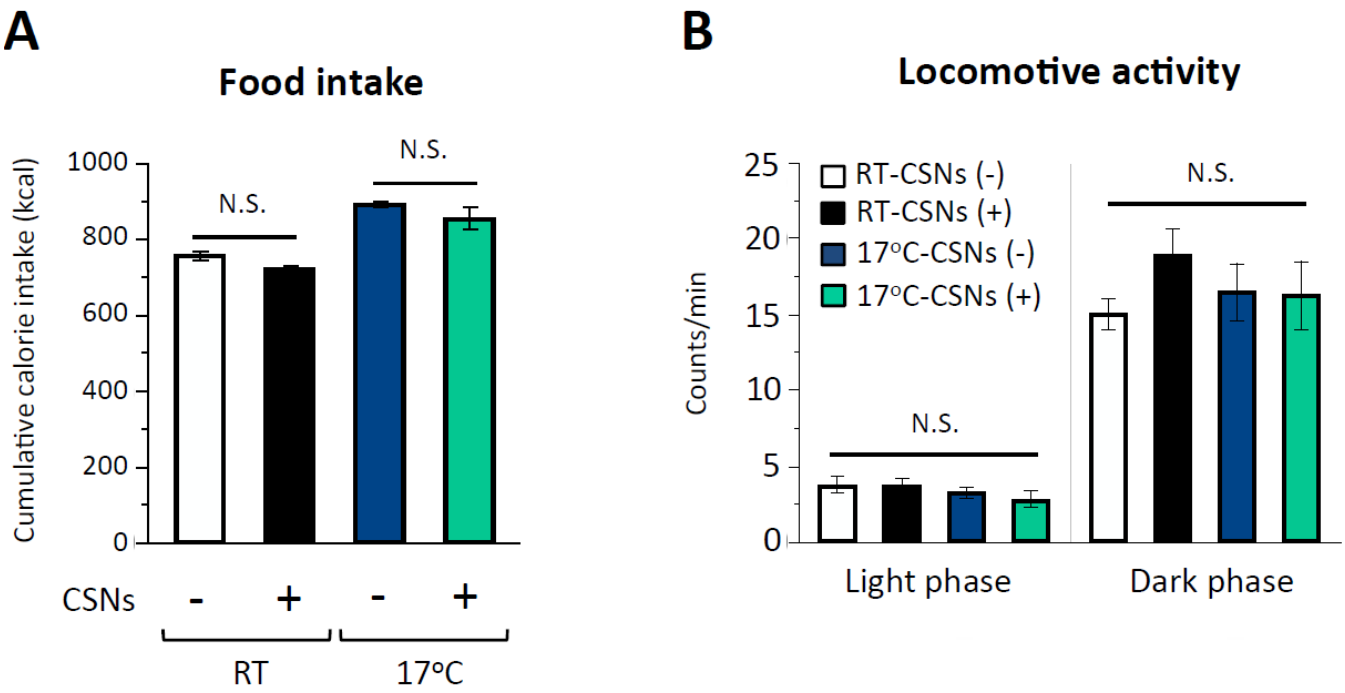
Supplementary Figure S1.
Capsinoids treatment did not affect food intake and locomotor activity.
(A) Total cumulative food intake during 8 weeks cohort of C57BL/6J mice (n=6). Mice were fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 8 weeks under ambient temperature (RT) or 17°C. Data are expressed as the mean ± SEM. N.S., not significant.

(B) Locomotive activity was measured in mice in (A) during the light phase and dark phase. Data are expressed as the mean ± SEM. N.S., not significant.



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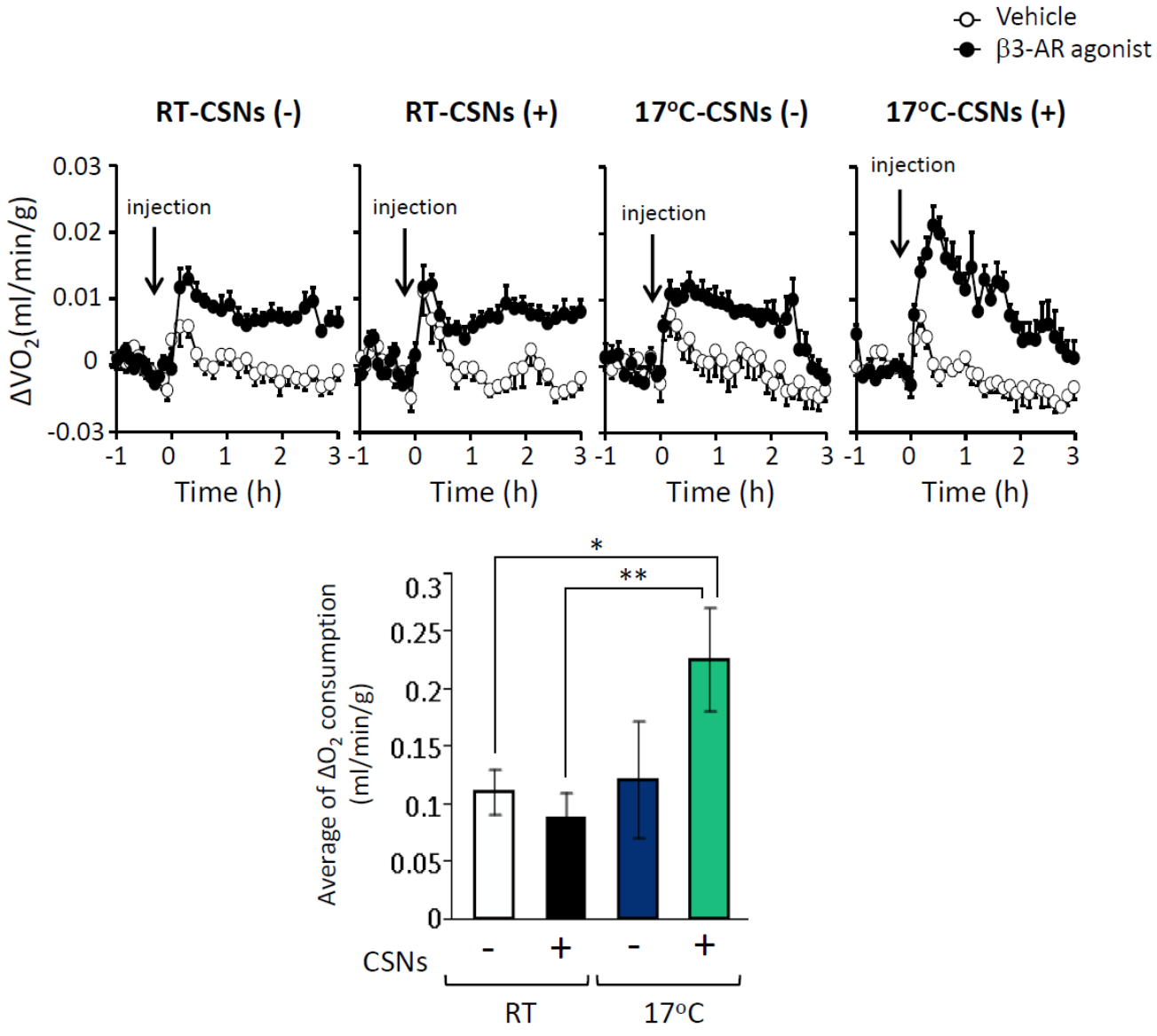
Supplementary Figure S2.
Capsinoids treatment under 17°C increased whole-body energy expenditure in response to to β3-adrenergic activation.
Whole body O_2 consumption rate (ml/min/g) was measured in C57BL/6J male mice fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) under ambient temperature (RT) or 17°C (n=6). To stimulate BAT-thermogenesis, mice were injected with vehicle (saline) or the β3-AR agonist (CL316243) at a dose of 0.01 mg/kg BW. O_2 consumption rate was monitored for 3 hours after the injection. Bottom; quantification of changes in VO_2 in response to CL316243. These changes were calculated by subtracting the VO_2 of vehicle-treated mice from the VO_2 of β3-AR agonist-treated mice. *p < 0.05, **p < 0.01.



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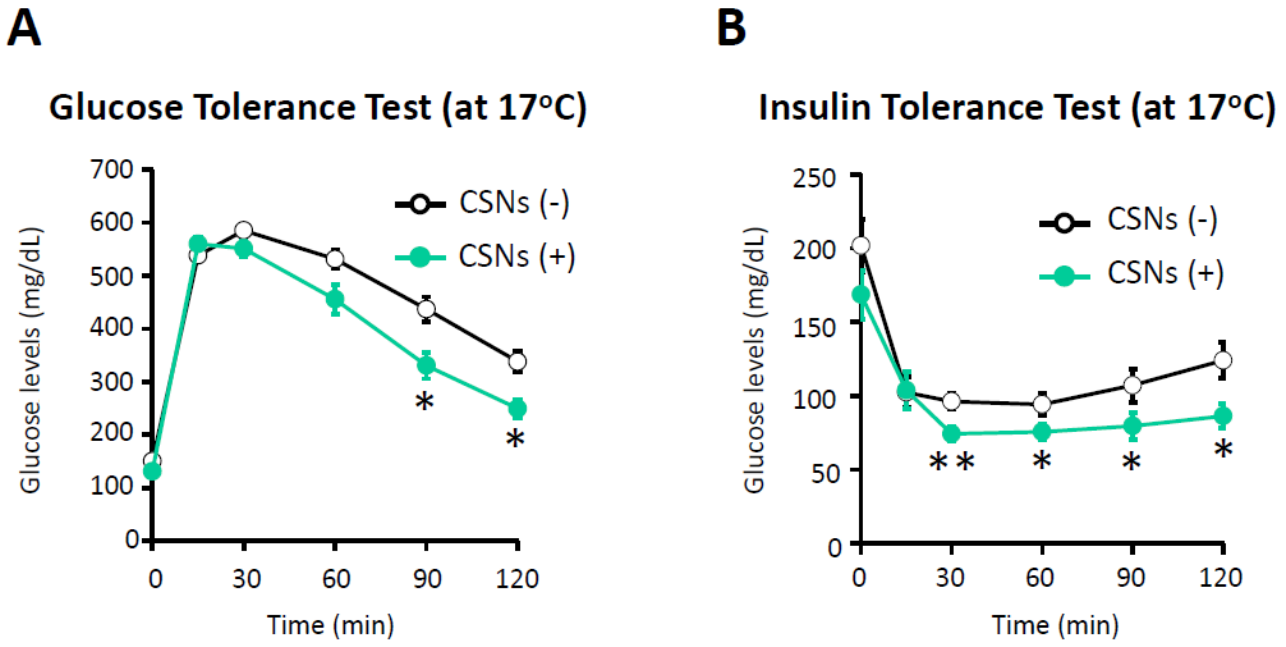
Supplementary Figure S3.
Capsinoids treatment under 17°C improved systemic glucose homeostasis.
(A) Glucose tolerance test (GTT) was performed in mice fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 6 weeks under 17˚C (n=6). Blood glucose level was measured over the course of 120 min after injecting glucose at a dose of 2 g/kg BW. Data are expressed as the mean ± SEM. *p < 0.05 relative to vehicle treated (CSNs-) group.

(B) Insulin tolerance test (ITT) was performed in mice fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 7 weeks under 17˚C (n=6). Blood glucose level was measured over the course of 120 min after injecting insulin at a dose of 0.75 U/kg BW. Data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01.



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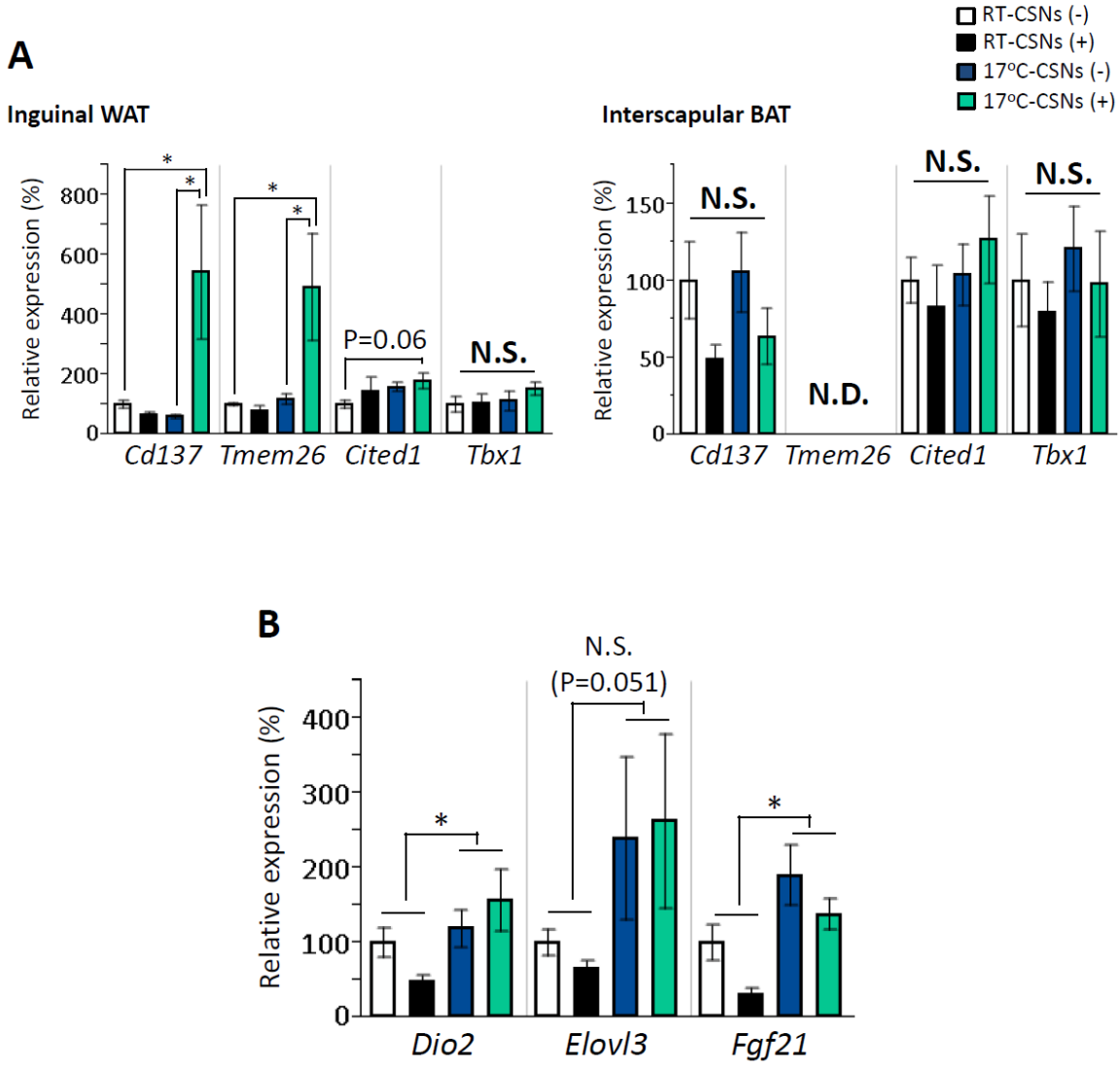
Supplementary Figure S4.
Capsinoids and mild cold exposure synergistically increased the expression of beige adipocyte-selective genes in inguinal WAT.

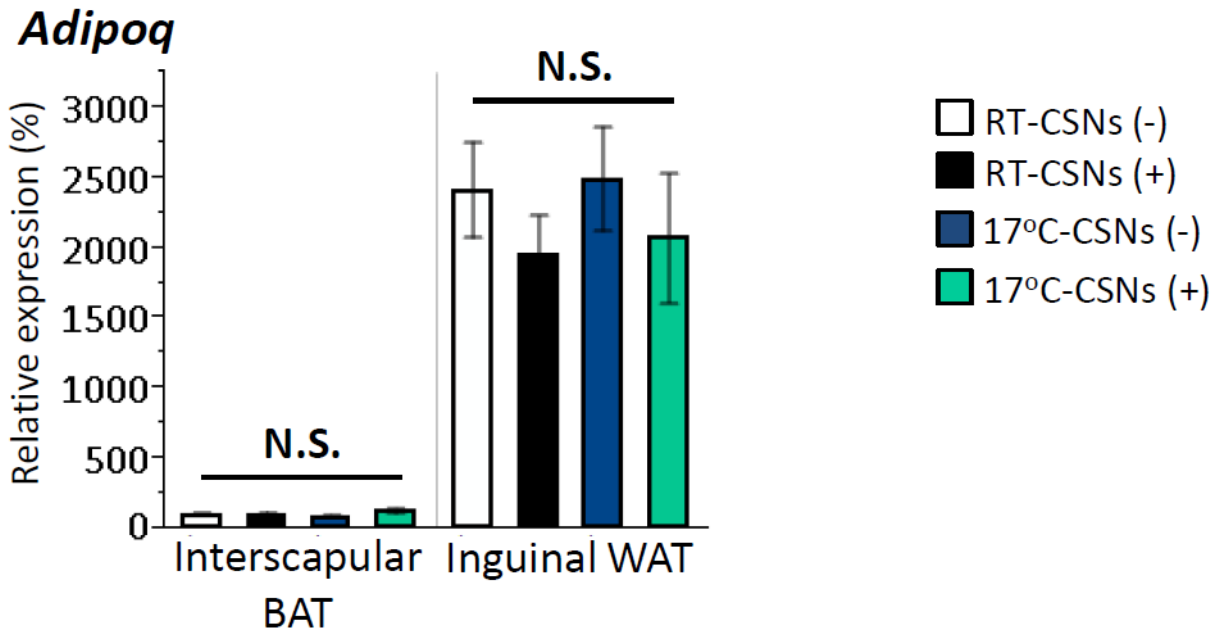
(A) Relative mRNA expression levels of *Cd137*, *Tmem26*, *Cited1*, and *Tbx1* were measured by qRT-PCR in the inguinal WAT (left) and in the interscapular BAT (right) of mice kept under RT or 17°C (n=6). Mice were fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 8 weeks. Data are expressed as the mean ± SEM. *p < 0.05. N.S., not significant.

(B) Relative mRNA expression levels of *Dio2*, *Elovl3*, and *Fgf21* were measured by qRT11 PCR in the interscapular BAT of mice in (A). Data are expressed as the mean ± SEM. *p < 0.05. N.S., not significant.



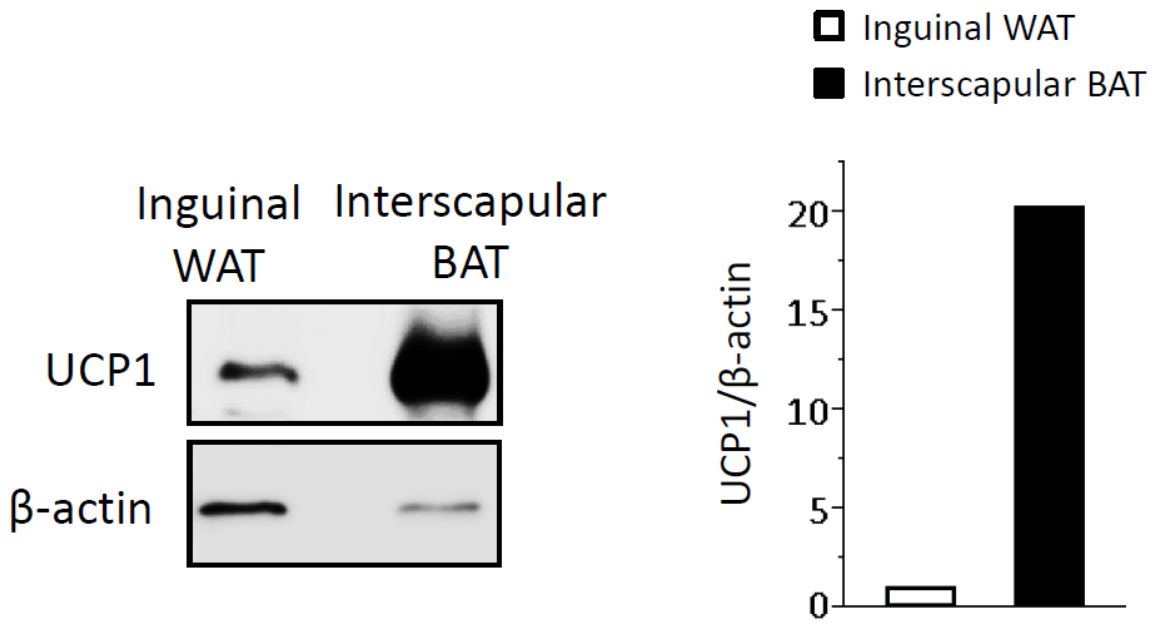
SUPPLEMENTARY DATA

Supplementary Figure S5.
Expression levels of thermogenic genes in inguinal WAT and interscaplar BAT.
Relative mRNA expression levels of *Adipoq* were measured by qRT-PCR in the inguinal WAT and in the interscapular BAT of mice kept under RT or 17°C (n=6). Mice were fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 8 weeks. Data are expressed as the mean ± SEM. N.S., not significant.



SUPPLEMENTARY DATA

Supplementary Figure S6.
UCP1 protein expression in inguinal WAT and interscaplar BAT.
UCP1 protein levels were analyzed by Western blotting in the inguinal WAT and the interscapular BAT from mice kept under RT or 17°C (n=6)**.** β-actin was used as a loading control. Right; quantification of density of UCP-1/β-actin.



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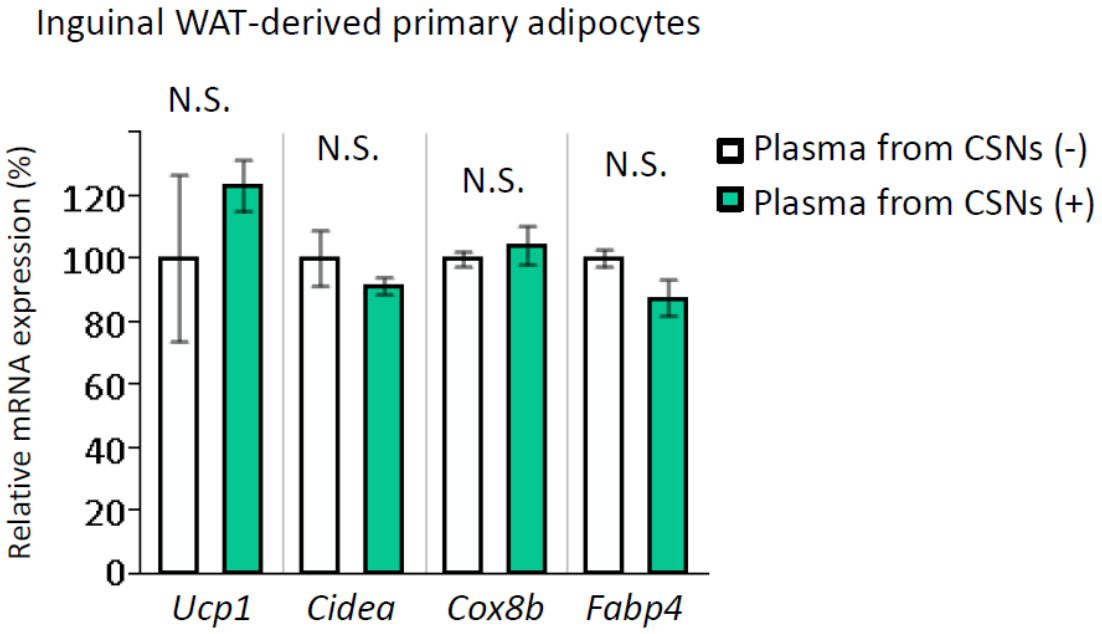
Supplementary Figure S7.
Capsinoids-induced circulation factors do not contribute to the development of beige adipocytes.
Relative mRNA expression levels of *Ucp1*, *Cidea*, *Cox8b*, and *Pgc1α* were measured by qRT31 PCR in the differentiated primary inguinal adipocytes from C57BL/6J mice (n=3). The adipocytes were treated with 2% plasma from mice fed HFD with or without 0.3% CSNs at 17°C for 8 weeks. Data are expressed as the means ± SEM.



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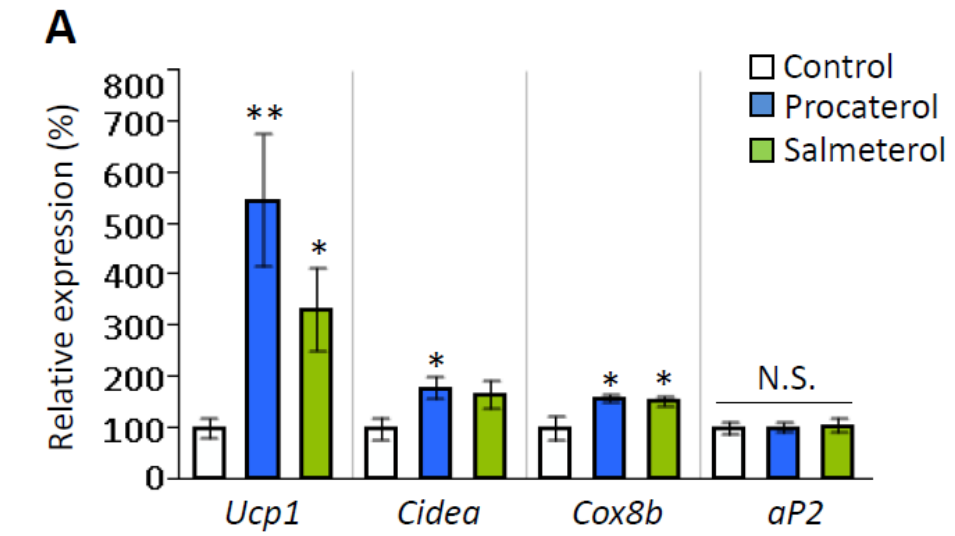
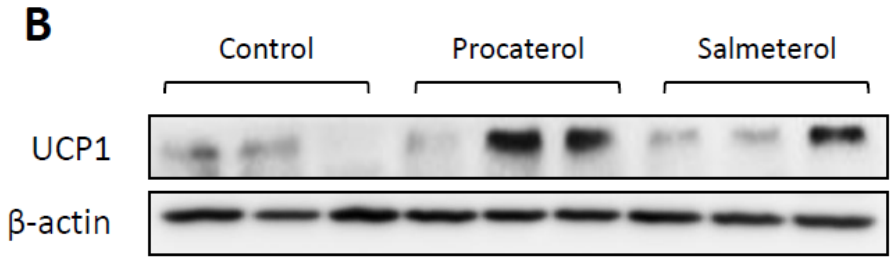
Supplementary Figure S8.

β2-AR agonists recruited beige adipocyte biogenesis.

(A) Relative mRNA expression levels of of *Ucp1*, *Cidea*, *Cox8b*, and *aP2* were measured by qRT-PCR in the inguinal WAT of mice injected with vehicle (saline) or the β2-AR agonists (procaterol or salmeterol) at a dose of 1 mg/kg/day under ambient temperature for 1 week (n=6). *p < 0.05, **p < 0.001. N.S., not significant.

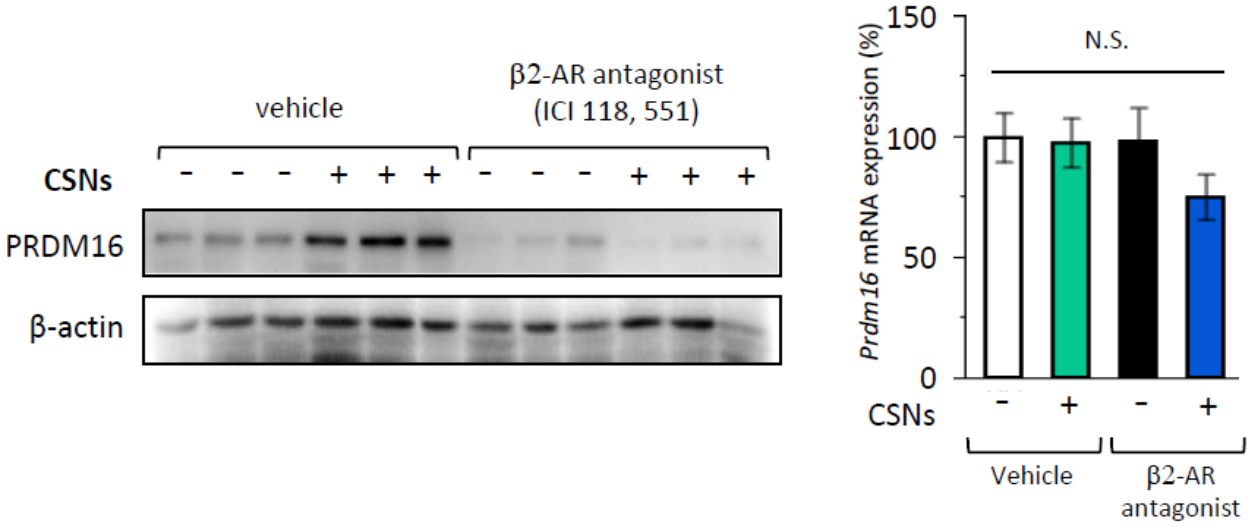
(B) UCP1 protein expression was analyzed by Western blotting in the inguinal WAT of mice shown in (A). β-actin was used as a loading control.





SUPPLEMENTARY DATA

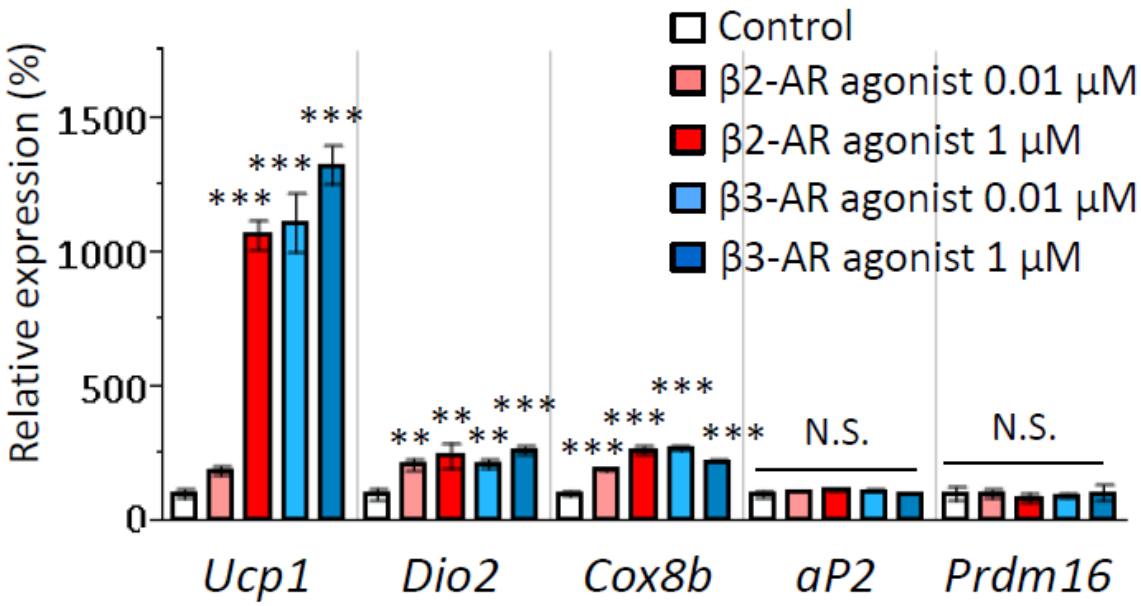
Supplementary Figure S9.
β2-AR antagonist blocked the capsinoids-induced PRDM16 protein accumulation without affecting its mRNA expression.
Mice were fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 4 weeks under 17˚C (n=6). The mice were daily injected with vehicle (saline) or the β2-AR antagonist (ICI 118,551) at a dose of 2 mg/kg/day. Endogenous PRDM16 protein expression in the inguinal WAT of these mice was analyzed by Western blotting (left). β-actin was used as a loading control. Relative *Prdm16* mRNA expression was measured by qRT-PCR (right). Data are expressed as the mean ± SEM. N.S., not significant.



SUPPLEMENTARY DATA

Supplementary Figure S10.
A β3-AR agonist did not induce PRDM16 accumulation in cultured inguinal WAT-derived primary adipocytes.

Inguinal WAT-derived primary preadipocytes were differentiated in the presence or absence of the β2-AR agonist (formoterol) or the β3-AR agonist (CL316243) at doses of 0.01 and 1 μM. The relative mRNA expression levels of *Ucp1*, *Dio2*, *Cox8b*, and *aP2* were measured by qRT-PCR. Data are expressed as the mean ± SEM. **p < 0.01, ***p < 0.001 relative to the vehicle-treated (control) group.



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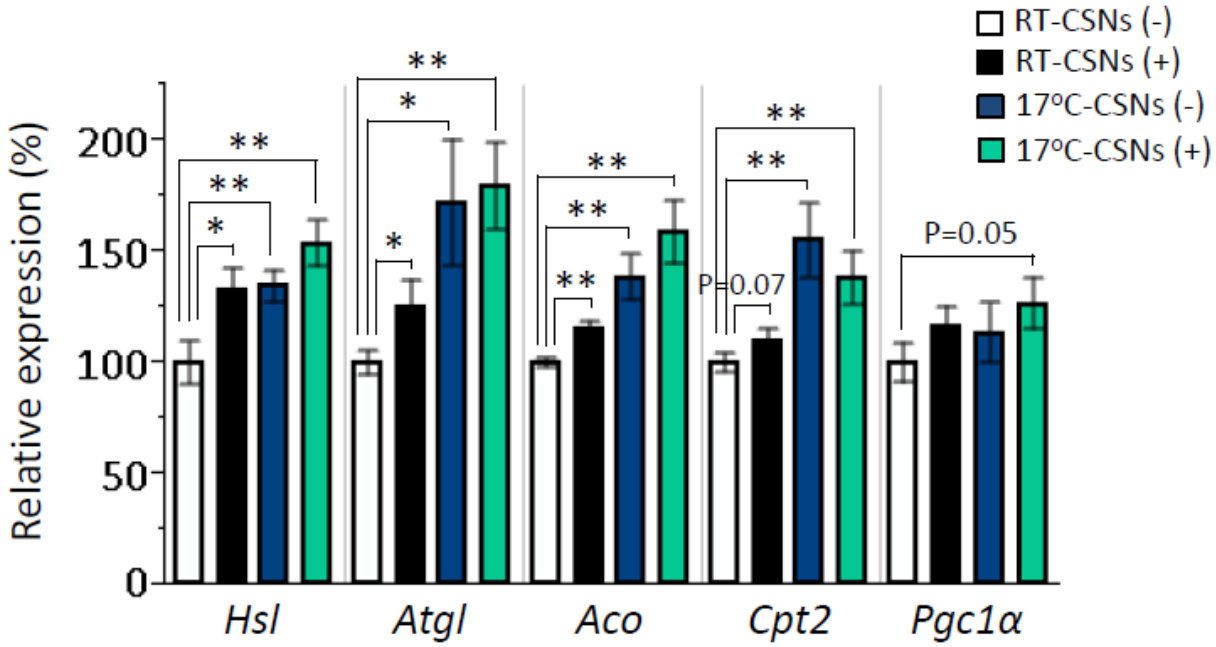
Supplementary Figure S11.
Capsinoids increased the expression of fatty acid oxidative genes in the liver
Relative mRNA expression levels of *Hsl*, *Atgl*, *Aco*, *Cpt2*, and *Pgc1α* were measured by qRT PCR in the inguinal WAT (left) and in the interscapular BAT (right) of mice kept under RT or 17°C (n=6). Mice were fed a high-fat diet supplemented with 0.3% capsinoids (CSNs+) or vehicle (CSNs-) for 8 weeks. Data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01.



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Supplementary Table 1. Compositions of the experimental diets (%)

	HFD	HFD + CSNs (0.3%)
Casein	20	20
Sucrose	10	10
Corn starch	22.48	22.48
α-corn starch	7.47	7.47
L-cysteine	0.3	0.3
Cellulose	5	5
Lard	30	30
Mineral mix	3.5	3.5
Vitamin mix	1	1
Choline bitartrate	0.25	0.25
Capsinoids	-	0.3

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Supplementary Table 2. Primers used for real-time PCR analysis

Gene	Sense	Antisense	Entrez Gene ID
Aco	CATTGGCATCGTGAGAACAG	AGCAAATCTGATGGCTTTGA	11430
Adipoq	CTGACGACACCAAAAGGGCTCAG	GCCTGCCATCCAACCTGCACAA	11450
Atgl	GGAGACCAAGTGGAACATCTCA	AATAATGTTGGCACCTGCTTCA	66853
β1AR	CCGAAAGCAGGTGAATGCAA	AGCCAGTAAGCCATACTAAGCCACA	11554
β2AR	CATTGATGTGTTGTGCGTCA	ACTCGGGCCTTATTCTTGGT	11555
β3AR	CCTTCCGTCGTCTTCTGTGT	AGCCATCAAACCTGTTGAGC	11556
Cd137	CGTGCAGAACTCTGTGATAAC	GTCCACCTATGCTGGAGAA	21942
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT	12683
Cited1	AACCTTGGAGTGAAGGATCGC	GTAGGAGAGCCTATTGGAGATGT	12705
Cox8B	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC	12869
Cpt2	CAGCACAGCATCGTACCCA	TCCCAATGCCGTTCTCAAAAT	12896
Dio2	AATTATGCCTCGGAGAAGACCG	GGCAGTTGCCTAGTGAAAGGT	13371
Elovl3	TTCTCACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC	12686
Fabp4	ACACCGAGATTTCCTTAAACTG	CCATCTAGGGTTATGATGCTCTTCA	11770
Fgf21	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCACTGTTCC	56636
Gapdh	CTGAGGACCAGGTTGCTCC	ACCACCCTGTGCTGTAGCC	14433
Hsl	TGTGGCACAGACCTCTAAAT	GGCATATCCGCTCTC	16890
Pgc1α	CACTACAGACACCGCACACA	AGGCTTCATAGCTGTCGTACC	19017
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG	70673
Tbp	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG	21374
Tbx1	CTGTGGGACGAGTTCAATCAG	TTGTCATCTACGGGCACAAAG	21380
Tmem26	TTCCTGTTGCATTCCCTGGTC	GCCGGAGAAAGCCATTTGT	327766
Ucp1	CACCTTCCCCCTGGACACT	CCCTAGGACACCTTTATACCT	22227