Supplemental Information for

Adenosine/A2B receptor signaling ameliorates the effects of ageing and counteracts obesity

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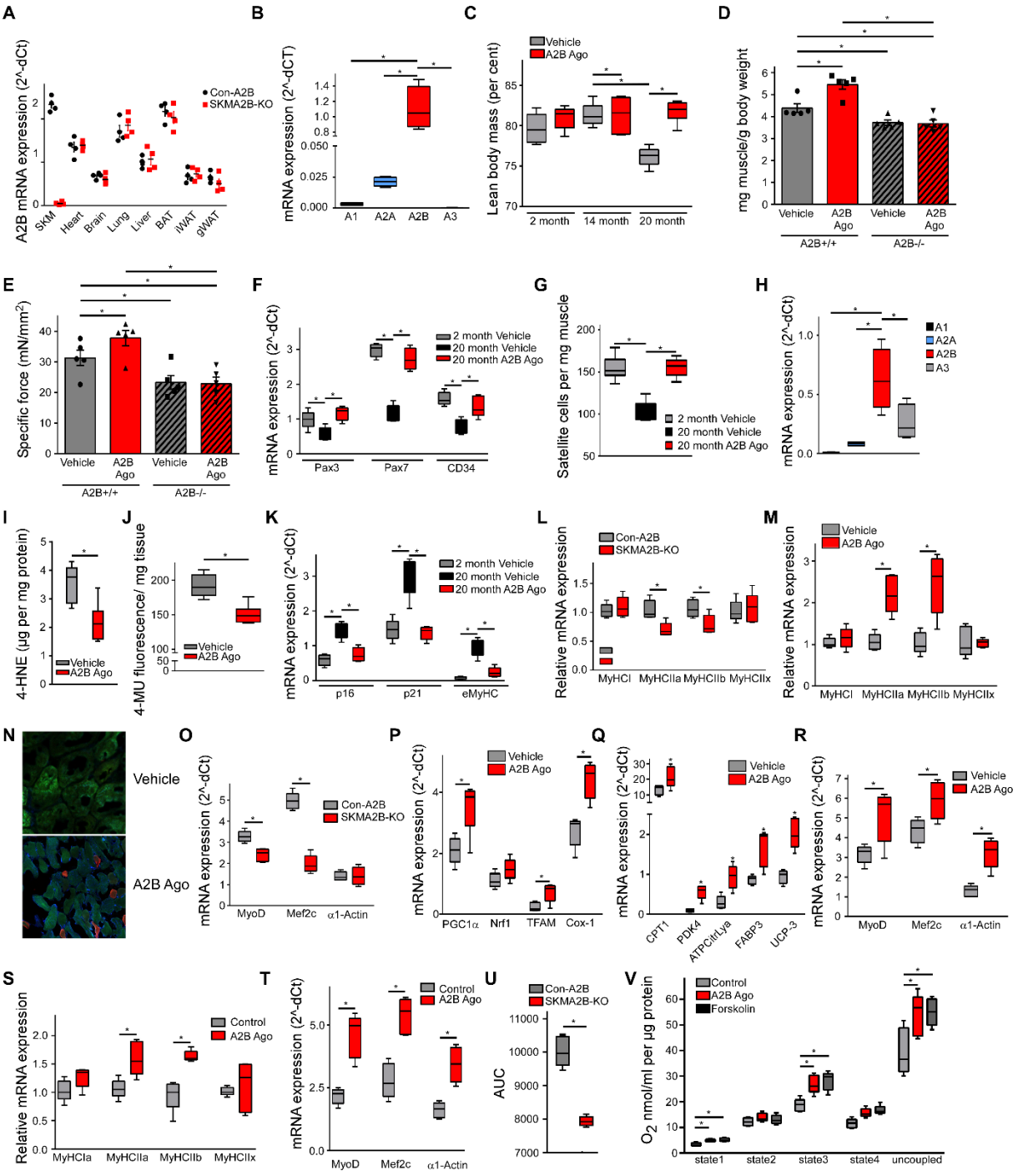

Figure S1. Related to Figure 1. A2B signaling in skeletal muscle.

(A) Expression of A2B in mouse tissues (n=4). **(B)** Expression of AdoRs in C2C12 (n=5). **(C)** Lean mass of mice treated with A2B agonist for 4 weeks. (n=6). **(D,E)** Muscle mass per body weight (**D**) or specific muscle force (**E**) of A2B+/+ and A2B-/- mice treated with A2B agonist for 4 weeks (n=5). **(F)** Satellite cell marker genes in EDL of aged mice treated with A2B agonist for 4 weeks (n=6). **(G)** Satellite cell number in SKM of mice treated with A2B agonist (n=6). **(H)** Expression of AdoRs in primary satellite cells (n=6). **(I,J)** 4-HNE (**I**) and senescence-associated Beta-galactosidase activity (**J**) in SKM of aged mice treated with A2B agonist for 4 weeks (n=6). **(K)** Senescence marker genes in SKM of aged mice treated with A2B agonist for 4 weeks (n=6). (**L,M**) MyHC isoform expression in SKMA2B-KO and Con-A2B mice (**L**) and mice treated with A2B agonist for 4 weeks (n=6). (**N**) Representative MyHCI, MyHCIIa and MyHCIIb stain of SKM from mice treated with A2B agonist for 4 weeks. (**O**) Differentiation marker genes in SKM of SKMA2B-KO and Con-A2B mice (n=6). **(P-R)** Expression of mitochondrial (**P**), oxidative metabolism (**Q**) and muscle differentiation marker genes (**R**) of mice treated with A2B agonist for four weeks (n=6). **(S,T)** Expression of MyHC isoforms (**S**) or myogenic maker genes (**T**) in C2C12 treated with A2B agonist (300 nM) (n=4). (**U**) Oxygen consumption of SKMA2B-KO and Con-A2B mice at 23° C (n=6). **(V)** Oxygen consumption of C2C12 stimulated with A2B agonist (300 nM) or Forskolin (1 µM) after addition of different substrates (state1: endogenous; state2: ADP; state3: succinate; state4: oligomycin; uncoupled: FCCP). (n=4). * $P < 0.05$. Data are shown as mean + SEM (A,D,E) or Boxplot (with median) and whiskers (1.5x interquartile range) (B,C,F-M,O-V) and analysed with two-tailed student's t-test (I,J,L,M,O-U) or ANOVA with Newman-Keuls post-hoc test (B-H; K,V).

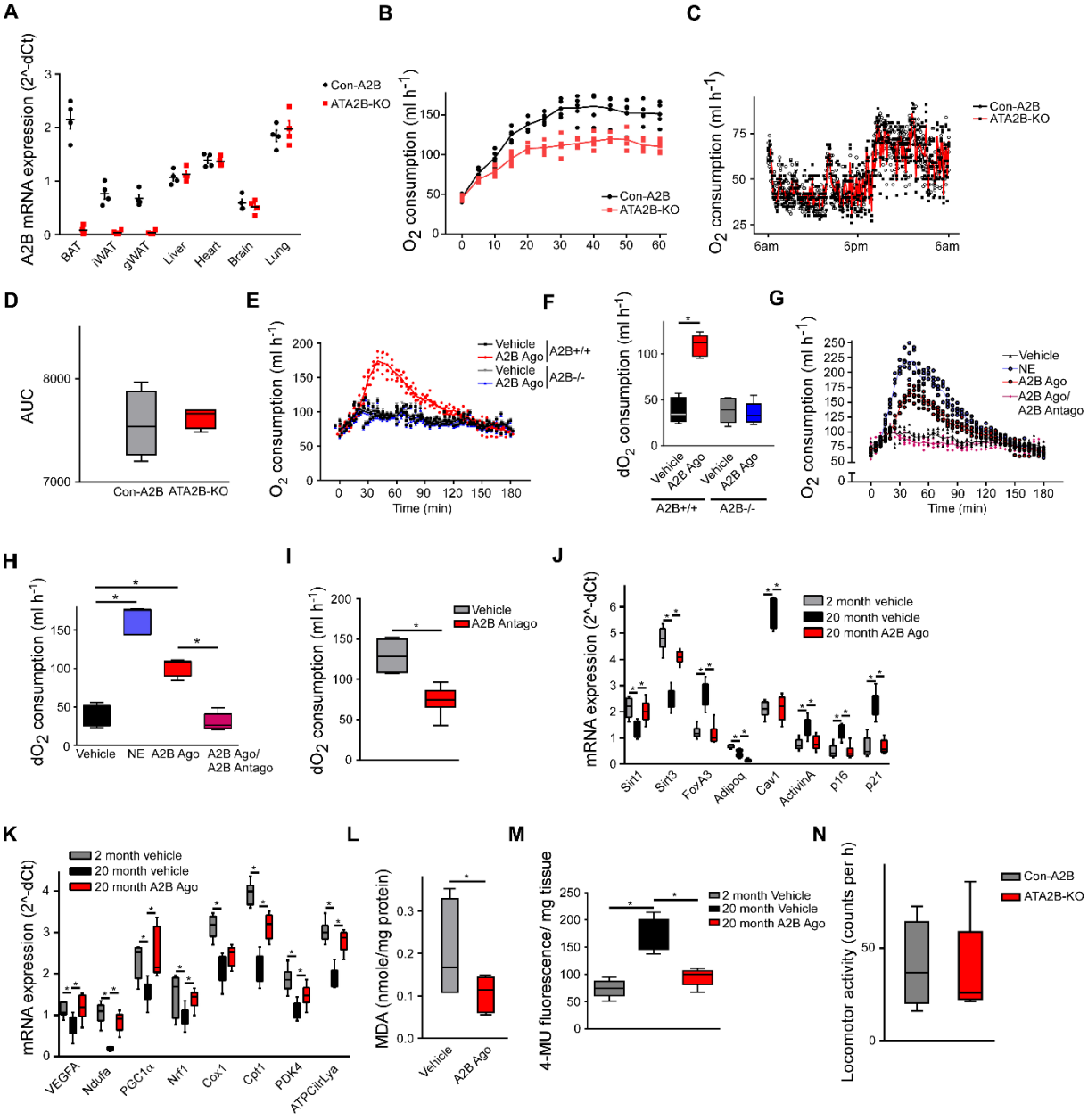

Figure S2. Related to Figure 2. A2B-mediated BAT activation.

(A) Expression of adenosine receptor A2B in various tissues of Con-A2B and ATA2B-KO mice (n=4). **(B)** Oxygen consumption of ATA2B-KO and Con-A2B at 4°C (n=5). (**C**,**D**) 24h oxygen consumption (**C**) and area under the curve (**D**) of ATA2B-KO and Con-A2B mice at 30°C (n=5). (**E**,**F**) Time course (**E**) and relative increase (**F**) of oxygen consumption from A2B+/+ and A2B-/- mice at 23°C after injection of A2B agonist (1 mg/kg). (**G**,**H**) Time course (**G**) and relative increase (**H**) of oxygen consumption from wildtype mice at 23°C after injection of NE (1 mg/kg) or A2B agonist (1 mg/kg) in the absence and presence of A2B antagonist (PSB603; 1mg/kg)(n=5). (**I**) Relative oxygen consumption of mice treated with A2B antagonist (PSB603; 1mg/kg) prior to cold exposure (n=5). (**J**,**K**) Expression of ageing associated genes in young and aged wildtype mice treated with A2B agonist (1 mg/kg). **(L,M)** Abundance of malondialdehyde (**L**) and senescence-associated Beta-galactosidase activity (**M**) in BAT of aged wildtype mice treated with and without A2B agonist (1 mg/kg) (n=7). (N) Locomotor activity of ATA2B-KO and Con-A2B mice at 23° (n=6). * $P < 0.05$. Data are shown as mean + SEM (A-C,E,G) or Boxplot (with median) and whiskers (1.5x interquartile range) (D,F,H-N) and analysed with two-tailed student's t-test (D,I,L,N) or ANOVA with Newman-Keuls post-hoc test (F,H,J,K,M).

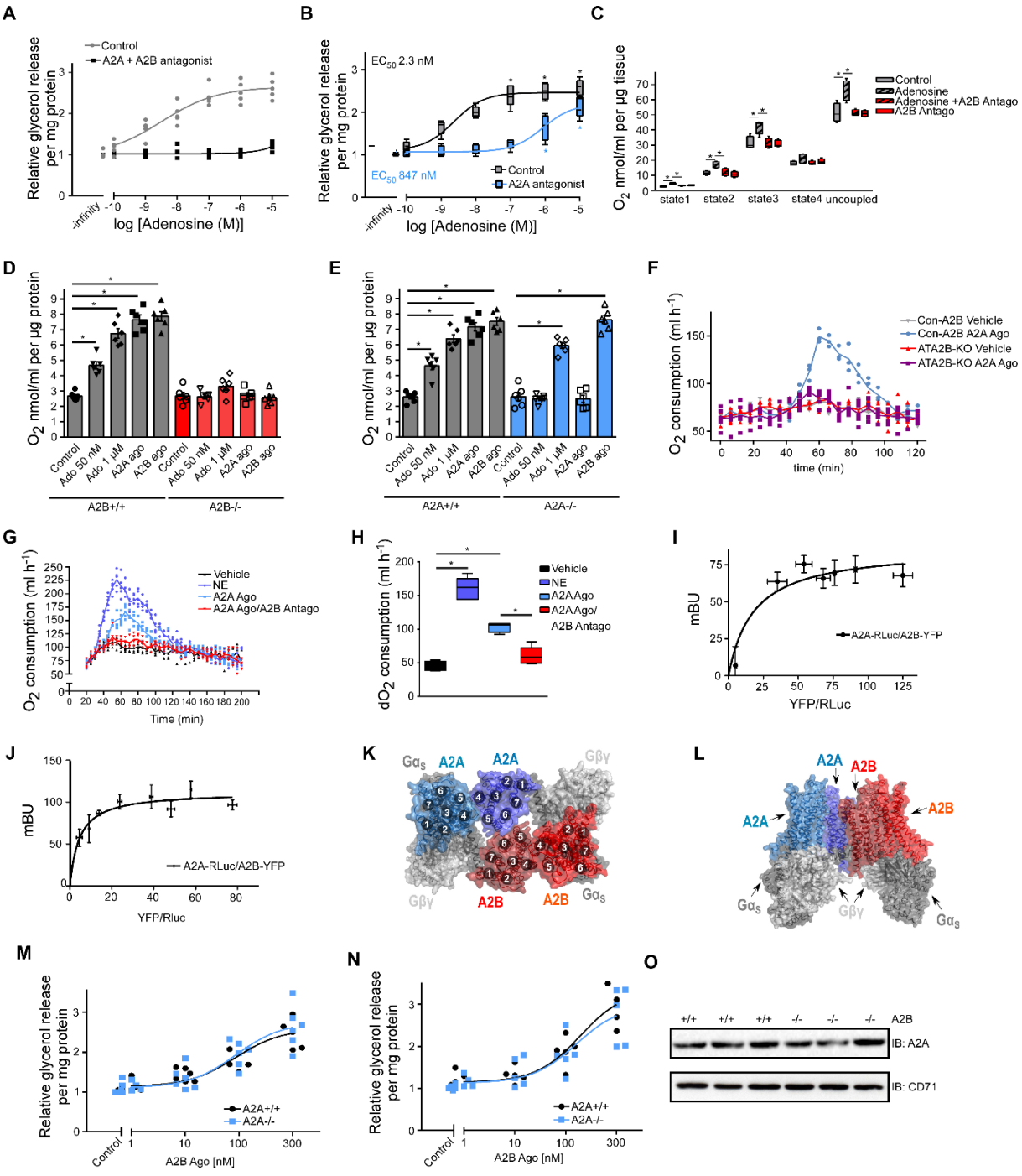

Figure S3. Related to Figure 3. A2B heterodimerization.

(A) Dose-response analysis of brown adipocytes treated with increasing concentrations of adenosine in the presence or absence of A2A and A2B antagonist (MSX-2, 300 nM; PSB603, 150 nM) (n=6). (**B**) Dose-response analysis of brown adipocytes treated with increasing concentrations of adenosine in the presence or absence of A2A antagonist (MSX-2, 300 nM). (**C**) Oxygen consumption of BAT explants treated with adenosine (1 µM) in the presence and absence of A2B antagonist PSB603 (150 nM) (n=6). **(D,E)** Oxygen consumption of A2B-/- and A2B+/+ brown adipocytes (**D**) or A2A+/+ and A2A-/- brown adipocytes (**E**) treated with and without adenosine (50 nM; 1 µM), A2A agonist (CGS21680; 150 nM) or A2B agonist (Bay 60-6583; 300 nM) (n=6). **(F)** Oxygen consumption of ATA2B-KO and Con-A2B mice treated with A2A agonist (CGS21680; 1mg/kg) (n=5). (**G**,**H**) Oxygen consumption (**G**) and relative increase in oxygen consumption (**H**) of wildtype mice injected with vehicle, NE (1 mg/kg), A2A agonist (CGS21680; 1mg/kg) or A2A agonist after pretreatment with A2B antagonist (PSB603; 1 mg/kg) (n=5). (**I**,**J**) BRET analysis of A2B and A2A in hMADS (**I**) and C2C12 (**J**) cells. (n=4). (**K**,**L**) *in silico* model of the extracellular side (**K**) and perpendicular to the membrane (**L**) of A2B/A2A interaction. (**M,N**) Relative lipolysis of A2A+/+ or A2A-/- BAT (**M**) and BA (**N**) treated with increasing concentrations of A2B agonist (n=6). (**O**) Representative immunoblot of A2A and CD71 from wildtype and A2B-deficient BAT membrane isolations. Data are shown as mean + SEM (A,D-G,M,N), mean + SD (I,J) or Boxplot (with median) and whiskers (1.5x interquartile range) (B,C,H) and analyzed with two-tailed student's t-test (B,M,N) or ANOVA with Newman-Keuls post-hoc test (C-E,H).

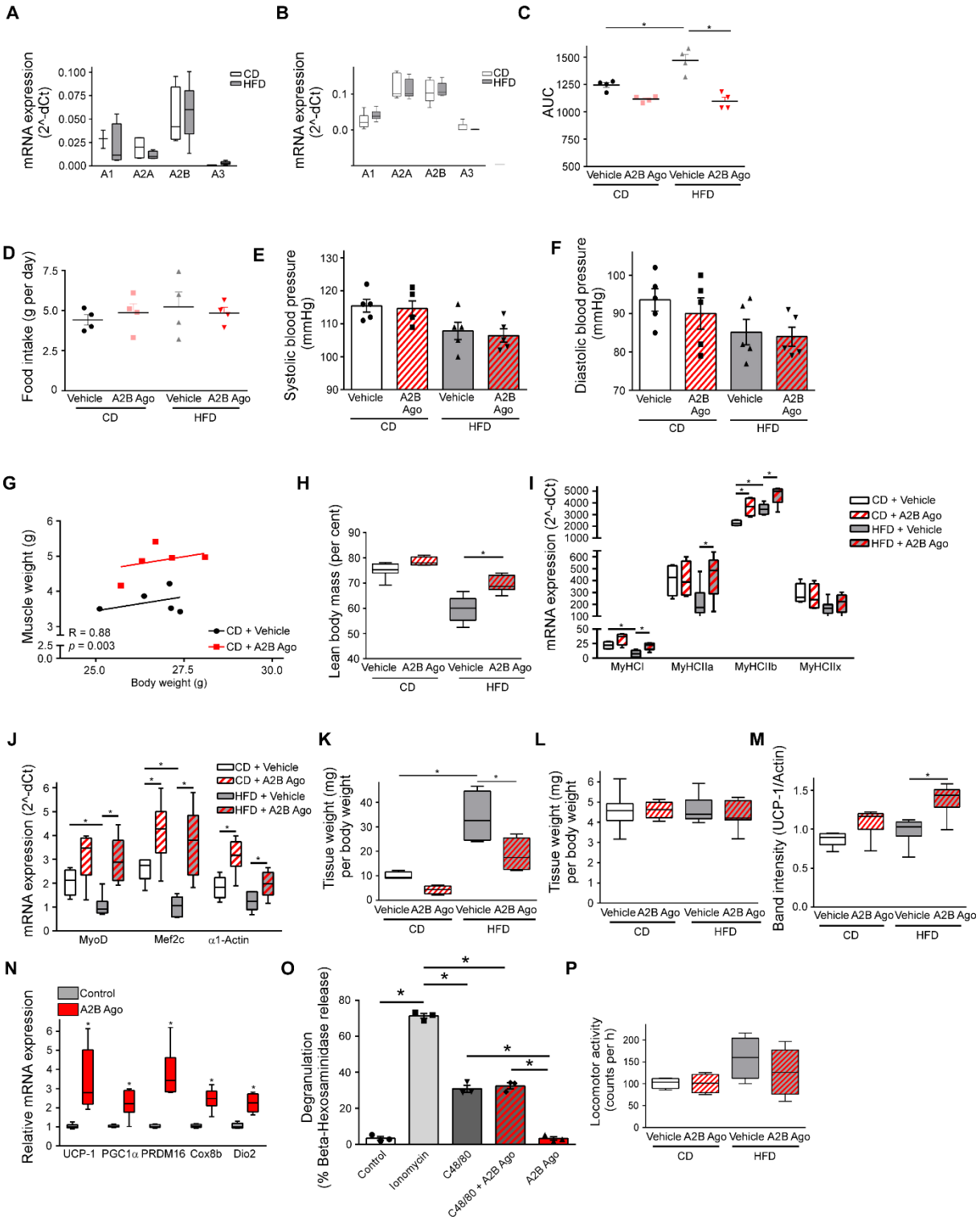

Figure S4. Related to Figure 4. A2B-treatment counteracts DIO.

(A,B) Adenosine receptor expression in BAT (**A**) and SKM (**B**) of mice fed control diet (CD) or HFD for 12 weeks. **(C,D)** Relative glucose tolerance (**C**) and food intake (**D**) of mice on HFD or CD and treated with and without A2B agonist (1 mg/kg). **(E-H)** Systolic (**E**) and diastolic (**F**) blood pressure, muscle mass (**G**), as well as lean body mass (**H**) of mice fed CD or HFD for 12 weeks and treated with A2B agonist (1 mg/kg). **(I,J)** Expression of MyHC isoforms (**H**) and myogenic marker genes (**I**) in *extensor digitorum longus*. **(K,L)** Tissue weight of gonadal WAT (**K**) and BAT (**L**). **(M)** Quantification of UCP-1 protein expression in inguinal WAT. **(N)** Thermogenic marker gene expression in primary murine white adipocytes treated with and without A2B agonist (300 nM)(n=4). (**O**) Degranulation of peritoneum-derived primary mast cells treated with ionomycin and C48/80 in the presence or absence of A2B agonist (300 nM) (n=3). **(P)** Locomotor activity of mice on HFD or CD and treated with and without A2B agonist. n=5. * $P < 0.05$. Data are shown as mean + SEM (C-F; O) or Boxplot (with median) and whiskers (1.5x interquartile range) (A,B,H-N,P). Data were analyzed with ANCOVA (G), ANOVA with Newman-Keuls post-hoc test (A-F,H-M,O,P) or two-tailed student's t-test (N).

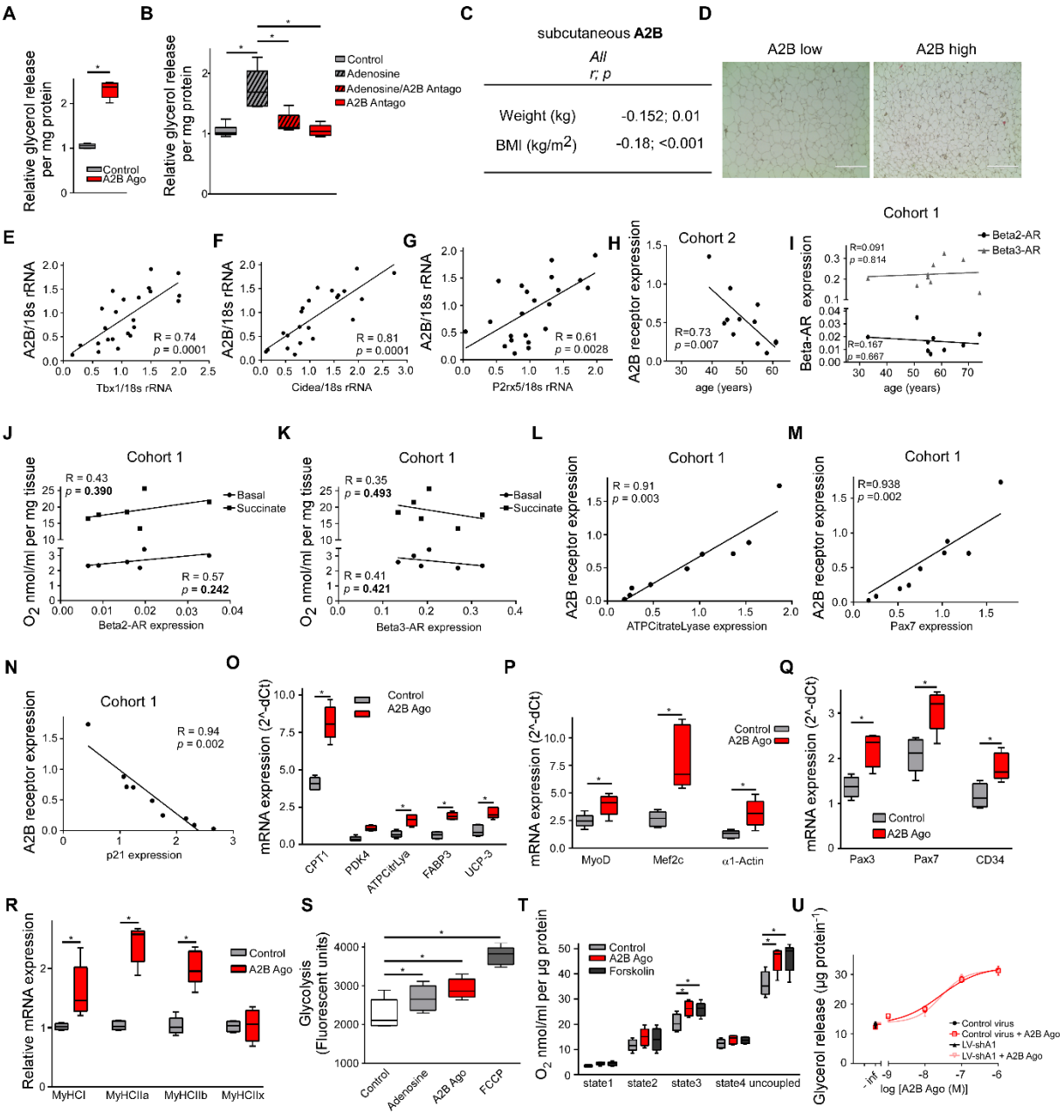

Figure S5. Related to Figure 5. A2B-signaling in human BAT and SKM.

(A,B) Lipolysis of primary human brown adipocytes treated with A2B agonist (300 nM) (**A**) or adenosine (1 µM) and A2B antagonist PSB603 (150 nM) (**B**) (n=4). (**C**) Correlation of A2B expression and BMI in inguinal WAT of 405 subjects. Data were analyzed with Spearman correlation coefficient. (**D**) Representative HE stain of inguinal WAT with high or low A2B expression. (**E-G**) Correlation of A2B expression with beige adipocyte marker genes Txb1 (**E**), Cidea (**F**) and P2rx5 (**G**) from 10 subjects with high or low A2B expression, respectively, in subcutaneous WAT. (**H**) Correlation of A2B expression with age in SKM of former master athletes (n=12). (**I**) Correlation of beta2- and beta3-AR expression with age in SKM of overweight/obese subjects (c.f. Figure 5J and Table S8) (n=9). (**J, K**) Correlation of beta2-AR (**J**) and beta3-AR (**K**) expression with oxygen consumption in human SKM explants (n=6). (**L-N**) Correlation of A2B with of ATP Citrate Lyase (**L**), Pax7 (**M**) and p21 (**N**) in SKM explants of overweight/obese subjects (c.f. Figure 5J and Table S8) (n=9). (**O-R**) Expression of oxidative metabolism (**O**), myogenic (**P**) and satellite markers (**Q**) as well as myosin heavy chain isoforms (**R**) in human primary myocytes treated with A2B agonist (300 nM) (n=5). (**S**) Glycolysis of primary human myocytes treated with adenosine (1 µM), A2B agonist (300 nM), or uncoupling reagent FCCP (1 µM) (n=5). (**T**) O_2 consumption of primary human myocytes treated with A2B agonist (300 nM) or Forskolin (1 µM) (n=4). **(U)** Lipolysis of murine brown adipocytes transduced with control or shA1-overexpressing lentivirus and stimulated with increasing concentrations of Bay 60-6583 (n=4). * $P < 0.05$. Data are depicted as mean + SEM (U) or Boxplot (with median) and whiskers (1.5x interquartile range) (A,B,O-T). Data were analyzed with two-tailed student's t-test (A; O-R), ANOVA with Newman-Keuls post-hoc test (B,S,T) or Pearson correlation coefficient (E-N).

Target	Sample	Expression (2^-dCt)
5-HT4	SM	0.0009
	BAT	not detectable
5-HT7	SM	0.0008
	BAT	0.0006
Adora2a	SM	0.0022
	BAT	0.5730
Adora2b	SM	0.4210
	BAT	0.3678
Adrb1	SM	0.0008
	BAT	0.0154
Adrb2	SM	0.0277
	BAT	0.0020
Adrb3	SM	0.2778
	BAT	0.4962
Avpr2	SM	0.0008
	BAT	0.0178
Calcr	SM	0.0205
	BAT	not detectable
Calcrl	SM	0.0103
	BAT	not detectable
Crhr1	SM	0.0011
	BAT	0.0117
Crhr2	SM	0.0012
	BAT	0.0177
Drd1	SM	0.0814
	BAT	0.0647
Drd5	SM	0.0370
	BAT	not detectable
Fshr	SM	0.0034
	BAT	not detectable
Gcgr	SM	0.0004
	BAT	0.0233
Ghrhr	SM	not detectable
	BAT	not detectable
Gipr	SM	0.0433
	BAT	0.0001
Hrh2	SM	0.0011
	BAT	0.0158
Lhcgr	SM	0.0580
	BAT	0.0004
Mcr1r	SM	0.0005
	BAT	0.0004
Mcr2r	SM	0.0003
	BAT	0.0029
Mcr3r	SM	0.0011
	BAT	not detectable
Mcr4r	SM	0.1088
	BAT	0.0530
Mcr5r	SM	0.1051
	BAT	0.0029
Ptgdr2	SM	not detectable
	BAT	0.0002
Ptgir	SM	0.0011
	BAT	not detectable
Pth1r	SM	0.0327
	BAT	0.0006
Sctr	SM	0.0001
	BAT	0.0182
Taar1	SM	not detectable
	BAT	not detectable
Tshr	SM	0.0837
	BAT	0.0583

Table S1. Related to **Figure 1**. Gs-coupled GPCR expression in murine BAT and SKM.

Parameter	Con-A2B	SKMA2B-KO
Body weight g	27.17 +/- 0.31	26.52 +/- 0.36
lenght (nose/anus)	8.55 +/- 0.06	8.61 +/- 0.04
food intake g/day/bw	0.172 +/- 0.007	0.182 +/- 0.009
Basal glucose mg/dl	126.2 +/- 2.01	136.5 +/- 2.54
Plasma insulin (pM)	60.1 +/- 8.5	63.92 +/- 9.9
Body temperature °C	36.52 +/- 0.210	36.01 +/- 0.184
BAT weight (g/bw)	0.0080 +/- 0.0012	0.0084 +/- 0.0007
Inguinal WAT (g/bw)	0.0126 +/- 0.0011	0.0131 +/- 0.0010
gonadal WAT (g/bw)	0.0328 +/- 0.0022	0.0364 +/- 0.0031

Table S2. Related to **Figure 1**. Basal metabolic parameters of Con-A2B and SKMA2B-KO mice.

Parameter	A2B-Con	ATA2B-KO
Food intake (g/day/body weight)	0.193 +/- 0.008	0.190 +/- 0.005
Body weight (g)	25.46 +/- 0.26	26.14 +/- 0.40
Gonadal WAT (g/body weight)	0.037 +/- 0.001	0.044 +/- 0.006
BAT (g/body weight)	0.008 +/- 0.00017	0.009 +/- 0.00013
Fasting glucose (mg/dl)	112.2 +/- 5.45	115.6 +/- 7.71

Table S3. Related to **Figure 2**. Basal metabolic parameters of A2B-Con and ATA2B-KO mice.

Parameter	Vehicle	A2B antagonist (PSB603)
Mean oxygen consumption (ml/h)	74.73 +/- 2.62	75.11 +/- 3.84
Motility (counts/h)	54.16 +/- 4.93	52.01 +/- 6.63
Heart rate (bpm)	595 +/- 7	589 +/- 5
Body temperature (° C)	36.74 +/- 0.276	36.53 +/- 0.117

Table S4. Related to **Figure 3**. Basal metabolic parameters of A2B antagonist (PSB603; 1mg/kg) treated mice.

Parameter	Vehicle	A2B agonist (Bay 60-6583)
Sodium (mmol/l)	145.2 +/- 2.33	147.0 +/- 2.12
Potassium (mmol/l)	4.16 +/- 0.13	4.16 +/- 0.18
Calcium (mmol/l)	2.22 +/- 0.08	2.25 +/- 0.07
Total protein (g/l)	48.4 +/- 2.80	46.6 +/- 2.29
Cholesterol (mg/dl)	68.4 +/- 1.89	76.4 +/- 3.11
ALT (U/l)	23.20 +/- 2.44	28.00 +/- 2.68
AST (U/l)	22.80 +/- 2.60	20.80 +/- 1.93
ALP (U/l)	111.4 +/- 6.22	129.6 +/- 8.28
Albumin (g/l)	16.80 +/- 2.01	17.60 +/- 1.63
Alpha-amylase (U/l)	1648 +/- 53.36	1756 +/- 49.98
Glucose (mg/dl)	226 +/- 17.10	237 +/- 22.10
Blood urea nitrogen (mg/dl)	18.10 +/- 1.20	18.10 +/- 1.39
Creatinine (mg/dl)	0.214 +/- 0.014	0.2440 +/- 0.022
Uric acid (mg/dl)	1.88 +/- 0.17	2.40 +/- 0.16
Total Bilirubin (mg/dl)	0.44 +/- 0.06	0.50 +/- 0.05
Gamma-GT (U/l)	5.94 +/- 0.26	6.40 +/- 0.47
Creatinine kinase (U/l)	336.0 +/- 19.05	347.0 +/- 25.33

Glutamate dehydrogenase (U/l)	6.84 +/- 0.41	7.16 +/- 0.28
HDL (mg/dl)	65.0 +/- 3.76	65.6 +/- 2.77
LDL (mg/dl)	97.4 +/- 5.34	103.8 +/- 4.36

Table S5. Related to **Figure 4**. Clinical chemistry parameters from mice chronically-treated with vehicle or A2B agonist (Bay 60-6583; 1 mg/kg).

Number	Weight group	Age	BMI	Fat%	Total fat mass	Waist (cm)
1	Lean	51	26,6	24,8	18,8	82
2	Lean	38	23,0	19,0	14,3	80
3	Lean	37	25,4	22,9	17,2	85
4	Lean	21	26,5	20,3	16,8	87
5	Lean	22	21,8	13,9	8,6	73
6	Lean	22	22,6	14,2	10,6	77
7	Lean	28	24,1	19,4	14,3	80
8	Lean	18	23,1	16,0	12,1	79
9	Overweight	25	33,3	27,7	29,4	110
10	Overweight	54	30,9	25,6	23,9	108
11	Overweight	46	33,8	31,5	30,4	116
12	Overweight	28	30,6	26,8	26,5	111

	BMI	Age
Lean average (n=8):	**24,1**	**29,6**
s.d.	1,7	10,7
Overweight average (n=4):	**32,1**	**38,3**
s.d.	1,7	14,0

Table S6. Related to **Figure 5A-G**. Characteristic parameters of analyzed subjects.

Number	A2B expression	Age	BMI	sex	diabetes
1	low	65	26	m	no
2	low	63	25	m	no
3	low	59	43	m	T2D
4	low	58	48	m	T2D
5	low	52	56	f	T2D
6	low	48	52	m	T2D
7	low	37	56	f	T2D
8	low	36	50	f	no
9	low	35	48	m	no
10	low	31	48	m	no
11	high	83	19	m	no
12	high	60	41	f	T2D
13	high	52	39	f	no
14	high	49	33	m	T2D
15	high	49	22	m	no
16	high	37	20	m	no
17	high	36	54	f	T2D
18	high	35	19	m	no
19	high	35	32	f	no
20	high	19	30	m	T2D

	BMI	Age
A2B low average	**45,0**	**48,4**
s.d.	11,0	12,6
A2B high average	**32,2**	**45,3**
s.d.	11,5	17,4

Table S7. Related to **Figure 5H,I** and **Figure S5D-G.** Characteristic parameters of analyzed subjects.

Number	Age	BMI	diabetes
1	33	26	T2D
2	51	25	T2D
3	54	43	T2D
4	55	48	T2D
5	55	56	T2D
6	56	52	T2D
7	57	56	T2D
8	61	50	T2D
9	68	48	T2D
10	74	48	T2D

Table S8 (Cohort 1). Related to Figure **5J-M** and Figure **S5I-N**. Characteristic parameters of analyzed subjects.

Number	Age	BMI	sex
1	39	23.04	m
2	55	21.72	m
3	61	23.24	m
4	61	22.49	m
5	45	24.07	m
6	46	21.72	f
7	49	31.40	m
8	58	27.10	f
9	44	22.49	f
10	54	24.93	f
11	47	20.91	m
12	55	17.09	f

Table S9. Related to Figure **S5H**. Characteristic parameters of analyzed subjects.

Acronym	Meaning
Adrb3	Beta3-adrenergic receptor
Drd1	Dopamine receptor d1
Mc4r	Melanocortin 4 receptor
Tshr	Thyroid stimulating hormone receptor
Pax3	Paired box 3
Pax7	Paired box 7
CD34	Cluster of differentiation 34
4-MU	4-methylumbelliferone
p16	Cyclin Dependent Kinase Inhibitor 2A
p21	Cyclin Dependent Kinase Inhibitor 1A
eMyHC	Embryonic myosin heavy chain
PGC1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
Nrf1	Nuclear Respiratory Factor 1
TFAM	Mitochondrial Transcription Factor 1
Cox-1	Cytochrome c oxidase subunit I
CPT1	Carnitine Palmitoyltransferase 1
PDK4	Pyruvate Dehydrogenase Kinase 4
ATP	ATP Citrate Lyase
FABP3	Fatty Acid Binding Protein 3
UCP-3	Uncoupling protein-3
Sirt1	Sirtuin 1
Sirt3	Sirtuin 3
FoxA3	Forkhead Box A3

Adipoq	Adiponectin
Cav1	Caveolin 1
VEGFA	Vascular Endothelial Growth Factor A
Ndufa	NADH:Ubiquinone Oxidoreductase Subunit A1
PRDM16	PR domain containing 16
ND5	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5
HE	Hematoxylin and eosin
A1	Adenosine receptor A1
A3	Adenosine receptor A3
MyoD	Myogenic Differentiation 1
Mef2c	Myocyte Enhancer Factor 2C
MDA	Malondialdeyde
Cox-8	Cytochrome C Oxidase Subunit 8
Dio2	Iodothyronine Deiodinase 2
Tbx1	T-Box Transcription Factor 1
Cidea	Cell Death Inducing DFFA Like Effector A
P2rx5	Purinergic Receptor P2X 5
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

Table S10. Related to **Figure 1, Figure 2, Figure 4 and Figure 5**. Definition of acronyms.