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SUPPLEMENTARY ONLINE DATA

Brd2 disruption in mice causes severe obesity without Type 2 diabetes

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EXPERIMENTAL

ES cell lines and mice

The RRT234 line of ES cells (BayGenomics) provided the starting point for derivation of homozygous *brd2*$^{(-/-)}$ ES cells by culture in G418. Single colonies were expanded. Both ES cells and mice were genotyped by PCR with a primer set: P1, 5′-GCGTCTGTCTTCCTTGTTTCAG-3′; P2, 5′-CAAT-CCTCCAGCTCGTCCTAG-3′; and P3, 5′-AAGGTTCATAT-GGTGCCGTGC-3′. P1+P2 generate a 660 bp wild-type band and P1+P3 generate a 1 kb mutant band. Unless otherwise specified, all primers reported in the present study were homologous to murine genes or transcripts.

Metabolic measurements and IGTT

Mice were housed individually in metabolic cages. We used a comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments) that was also equipped with photocells to quantify habitual physical activity. For IGTT, 1.5 g of D-glucose/kg of body weight was injected intraperitoneally after starvation overnight. Blood glucose and insulin were measured at the time points described.

Plasmid construction

A Brd2 siRNA expression vector, pSiBrd2, was constructed using pRNA-U6.1/Neo (GenScript), according to the manufacturer's protocol. The sense sequence is 5′-AGATGGGGCAGGAAGG-CTCCG-3′. The annealed synthesized double-stranded fragment was cloned into the BamHI and HindIII sites of the vector. pRNA-U6.1/Neo/CTL (GeneScript) was used as a negative control (pSiC) for Brd2 shRNA. To construct pmBrd2, the long-form Brd2 expression vector, mouse Brd2 cDNA was obtained from R1 murine ES cells (American Type Culture Collection) that express a high level of Brd2 by RT–PCR using the primers 5′-GTGGTC GGTACCATGGTGCAAAACGTGACTCCCCACA-3′ and 5′-GGCTAGGAATTCAATCGTATTTTGTCCATG-3′. The cDNA was then ligated into KpnI and EcoRI sites of pcDNA3.1 (+). The pcDNA3.1 empty vector was used as a negative control for Brd2 overexpression.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated, then incubated with rabbit polyclonal antibody against Brd2, as described in main text, and a guinea-pig antibody against insulin (Invitrogen). Alexa Fluor®-conjugated secondary antibodies were Alexa Fluor® 594 anti-rabbit and Alexa Fluor® 488 anti-guinea-pig (Invitrogen). DNA counterstain was with DAPI (4′,6-diamidino-2-phenylindole).

Flow cytometry

BrdU incorporation

β-TC-6 cells in six-well plates were pulsed with 10 μM BrdU for 30 min. Then the cells were fixed and stained using a BrdU Flow Kit (BD Biosciences) with a FITC-conjugated anti-BrdU antibody, and DNA content was determined with 7-aminoactinomycin D. All flow cytometry antibodies were from eBioscience unless otherwise specified.

Macrophages

Primary adipose tissues were isolated immediately after killing of the mice and cells of the SVF were isolated by collagenase-1 digestion (1 mg of enzyme/g of tissue; ICN Biomedicals) at 37 °C for 1 h and centrifugation in the cold. Erythrocytes were removed (1× RBC lysis buffer), then nucleated cells were stained with a FITC-conjugated anti-F4/80 antibody ($IgG_{2a,\kappa}$ clone BM8) in the presence of an F_c-blocking antibody (CD16/32 clone 93). The isotype control was a FITC-conjugated rat $IgG_{2a,\kappa}$ antibody. The HFD (Figures 3E and 4D in the main paper) was D12492 (Research Diets), with 60 % kcal % from fat (where 1 kcal≡1 kJ).

Splenic lymphocytes

Spleens were harvested from 3 month-old *w*/*lo* female mice or age-matched *w*/*w* controls and stained with an APC (allophycocyanin)-conjugated anti-B220 antibody as a pan-B-cell marker and a PE (phycoerythrin)-Cy5 conjugated anti-MHC class II antibody in the presence of an F_c-blocking antibody. Lymphocytes were gated by forward- and side-scatter properties, and appropriate isotype controls were used to set the colour gates. Cell cycle parameters and surface markers of stained cells were measured at the BUSM Flow Cytometry Core Facility using an LSR II multi-laser bench top flow cytometer (BD Biosciences). Fluorochrome compensation and population analysis was performed with FlowJo software.

3T3-L1 pre-adipocyte differentiation and insulin resistance

Culture and induction of differentiation of 3T3-L1 cells were as follows: confluent 3T3-L1 pre-adipocytes in 12-well plates were differentiated for 8 days with initiation mixture containing 10 μg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma). Neutral lipid content relative to mock transfection was measured by Oil Red O stain. Induction of insulin resistance with TNF-α and glucose uptake assay were performed as follows: confluent 3T3-L1 pre-adipocytes were differentiated for 10 days with 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 1.5 μM pioglitazone (Alexis Biochemicals). Mature adipocytes were treated with 4 ng/ml TNF-α (Cell Sciences) for 4 days to

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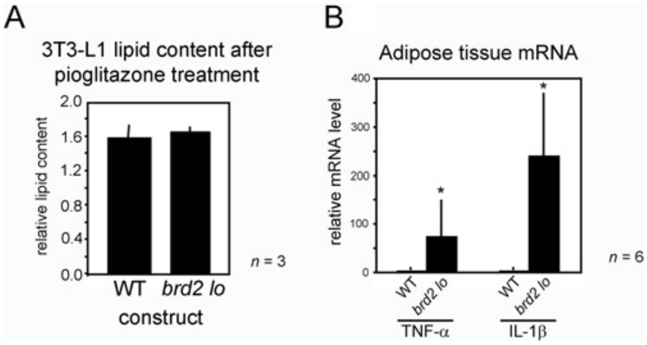

Figure S1 Knockdown of Brd2 affects 3T3-L1 adipogenesis and subsequent insulin sensitivity.

See Figures 4H, 4I, 6B and 6C in the main text. In control experiments, we established that differentiation of 3T3-L1 pre-adipocytes is not blocked by knockdown of Brd2. The enhanced adipogenesis in 3T3-L1 pre-adipocytes observed in the context of Brd2 knockdown was due to increased PPAR-γ activity when no agonist was added to the medium. When a PPAR-γ agonist (pioglitazone) was used, 3T3-L1 pre-adipocytes differentiated to a similar extent, whether Brd2 was knocked down or not, as measured by lipid content (**A**) ($n = 3$). Thus knockdown of Brd2 in 3T3-L1 pre-adipocytes does not interfere with their ability to undergo differentiation. We found that not only were pro-inflammatory cytokines elevated in the serum of obese *w/lo* mice at 9 months of age (Table 1 in the main paper), but the mRNA levels of pro-inflammatory cytokines were also elevated in adipose tissue. We assayed *Tnf* (TNF-α) and *Il1b* (IL-1β) transcripts by real-time PCR of visceral fat depots in *w/lo* mice and *w/w* mice ($n = 6$; $P < 0.05$) (**B**). The transcript level of *Il1b* in WAT (white adipose tissue) from *w/lo* mice, for example, was elevated to a mean value of 250-fold in excess of the transcript level in WAT from *w/w* mice. These results suggest that WAT contributes at least part to the elevated serum levels of pro-inflammatory cytokines observed in obese *w/lo* mice.

Table S1 Numbers of observed compared with expected pups of litters descended from RRT234 chimaeras

Values are the observed numbers (with expected values in parentheses). The expected Mendelian frequency of *w/w*:*w/lo*:*lo/lo* is 1:2:1, supplying two degrees of freedom (ν) for a χ^2 analysis of the observed frequencies. In each case, a significance level (α) of 0.05 was chosen. For 62 neonates, $\chi^2 = 14.52$, and for 108 weaned mice, $\chi^2 = 33.04$, both of which exceed the critical value 5.991 for $\alpha = 0.05$ and ν=2. Thus Mendelian frequencies were not observed in litters because homozygous mice were highly under-represented among survivors. For 27 E14.5 embryos, $\chi^2 = 1.20$; for 26 E18.5 embryos, $\chi^2 = 4.38$; and for 19 E20.5 embryos, $\chi^2 = 4.26$. Although these embryos are few in number, the sample size is large enough to calculate an unbiased χ^2. The ratios for embryos are Mendelian, leading us to speculate that some homozygous embryos are capable of surviving until birth, but the pups are generally not viable. This interpretation is consistent with the results by Gyuris et al. [34], who report that lethality increased towards the end of embryonic development of homozygous mice derived from RRE050 ES cells (25 out of 28 homozygous embryos died before birth), which is more severe than the phenotype reported in the present study for homozygous mice derived from RRT234 ES cells.

	Age				
Litter	E14.5	E18.5	E20.5	Neonate	Weaned (3 weeks)
Total	27	26	19	62	108
w/w	9 (6.75)*	7 (6.5)	5 (4.75)	23 (15.5)	32 (27)
w/lo	13 (13)	17 (13)	13 (9.5)	36 (31)	76 (54)
lo/lo	5 (6.75)	2 (6.5)	1 (4.75)	3 (15.5)	2 (27)
χ^2	1.20	4.38	4.26	14.52	33.04

induce insulin resistance. For the glucose uptake assay, cells in 12-well plates were treated with or without 170 nM insulin for 30 min at 37 °C, then with 1 μCi/ml [^{3}H]-2DG (2-deoxyglucose) for 5 min. Uptake of [^{3}H]-2DG was determined with a scintillation counter.

Real-time PCR

Total RNA from cultured cells or tissues was extracted with TRIzol® (Invitrogen) and cDNA was prepared with the ImProm-II reverse transcription system (Promega). Real-time PCR was performed using SYBRGreen PCR master mix (Applied Biosystems). Relative mRNA levels were determined by $\Delta\Delta Ct = \Delta C_{t,\ sample} - \Delta C_{t,\ reference}$ and β-actin was the reference. Real-time PCR primers for assay of transcripts from pro-inflammatory cytokine genes were as follows (F, forward; R, reverse). For *Tnf*: F, 5′-CTCCAGGCGGTGCCTATG-3′ and R, 5′-GGGCCATAGAACTGATGAGAGG-3′; and for *Il1b*: F, 5′-GCACACCCACCTGCA-3′ and R, 5′-ACCGCTTTTCCATCTTCTTCTT-3′. Other primers were as follows. For *Ins* (insulin): F, 5′-GGGGAGCGTGGCTTCTTCTA-3′ and R, 5′-GGGGACAGAATTCAGTGGCA-3′; for *Brd2*: F, 5′-TACTGGGCTGCCTCAGAATG-3′ and R, 5′-CCAGTGTCTGTGCCATTAGGA-3′; and for *Actb* (β-actin): F, 5′-CCCAGATCATGTTTGAGACCTTC-3′ and R, 5′-AGTCCATCACAATGCCTGTGGTA-3′. Primers for assay of transcripts from Ucp genes were as follows. For *Ucp1*: F, 5′-CCCGACAACTTCCGAAGTGCA-3′ and R, 5′-GGAAGCCTGGCCTTCACCTTG-3′; and *Ucp2*: F, 5′-CTACAAGACCATTGCACGAGAGGA-3′; and R, 5′-GAGGTTGGCTTTCAGGAGAGTATC-3′; for *Ucp3*: F, 5′-CATCGCCAGGGAGGAAGGAG-3′ and R, 5′-TCCAAAGGCAGAGACAAAGTGAC-3′; and for control transcripts, *Tbp*: F, 5′-ACCCTTCACCAATGACTCCTATG-3′ and R, 5′-TGACTGCAGCAAATCGCTTGG-3′.

Protein immunoblot and co-immunoprecipitation

For co-immunoprecipitation, antibodies were cross-linked to Protein A/G beads using dimethylpimelimidate, according to the manufacturer's instructions (Pierce). The cross-linked antibodies were washed extensively before binding to cell lysates. Reagents used were rabbit polyclonal Brd2 antibody [14], monoclonal and polyclonal PPAR antibodies and Protein A/G Plus agarose (Santa Cruz Biotechnology), monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Millipore) and β-actin polyclonal antibody (Sigma).

Statistical analysis

Histological images in Figures 2 and 5 of the main paper were analysed with NIH Image J software, version 1.41o. Adipocyte area was calculated from best polygon fit to individual adipocytes measured in three separate fields for each genotype and each tissue type. The area distribution for wild-type adipocytes was calculated in SigmaPlot 11.0 using non-linear regression to a five parameter Weibull curve. Pancreatic β-cells were counted in Image J by converting RGB micrographs into eight-bit greyscale, then defining a threshold value such that each stained nucleus was counted once using the 'analyse particles' function; the number of nuclei were then taken as a proxy for the number of β-cells, which was expressed as a box plot. The boundary of the box closest to zero indicates the 25th percentile, a line within the box indicates the median and the boundary of the box farthest from zero indicates the 75th percentile. Error bars indicate the 90 and 10th percentiles, and outliers were identified. Islet area was calculated as for adipocytes. Linear regression and statistical tests for significance were performed in SigmaStat 3.11.

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