SUPPLEMENTARY INFORMATION

miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit

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Supplementary Figures S1-8
Supplementary Table S1

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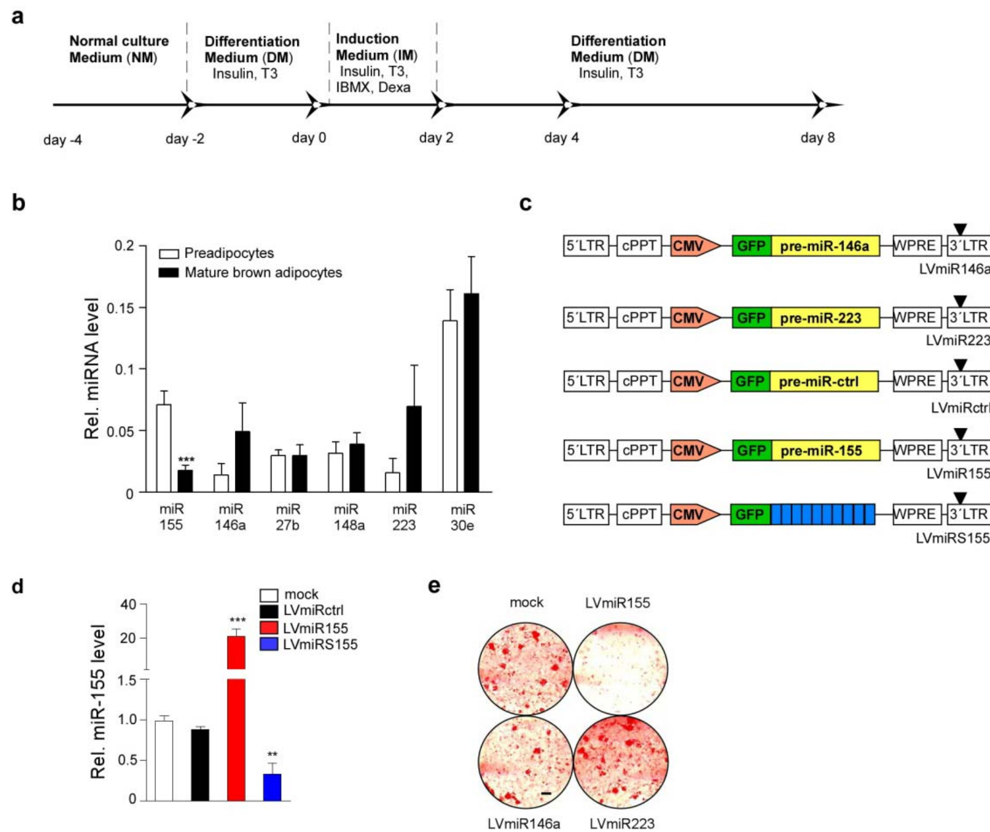
Supplementary Figure S1

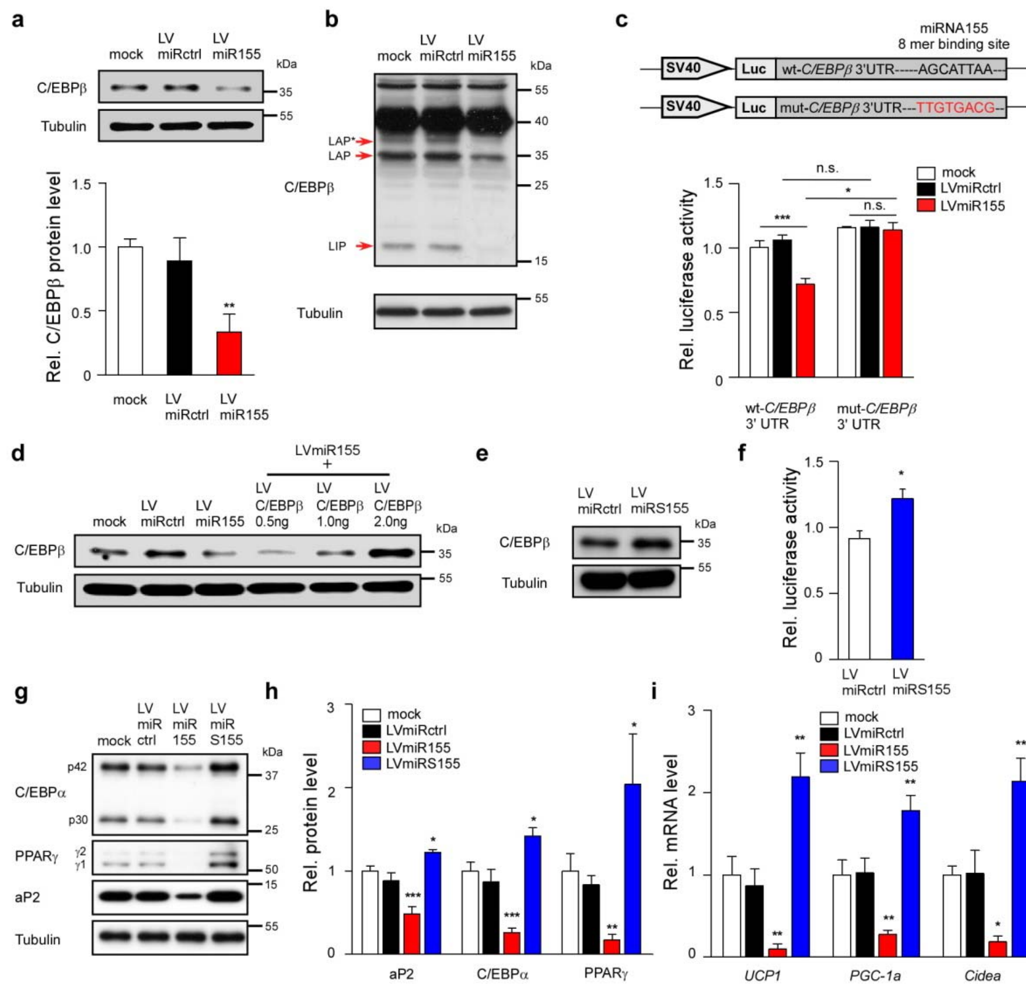
Validation of identified miRNA candidates, lentiviral over-expression of miRNAs in brown adipocytes.



(**a**) Protocol for *in vitro* differentiation of brown adipocytes (day -4 to day 8). NM, normal growth medium; DM, differentiation medium; IM, induction medium. (**b**) qRT-PCR analysis of miRNA expression in preadipocytes isolated from brown fat tissue (SVF) versus *in vitro* differentiated mature adipocytes. Data are normalized to sno202RNA expression and are represented as means ± SEM. (***$P < 0.001$ student´s *t*-test, n=6). (**c**) Lentiviral expression vectors; LVmiRs express miR-146a, miR-223, or miR-155 precursors (yellow) coupled to GFP mRNA (green), LVmiRctrl contains a scrambled, non-targeting sequence. LVmiRS155 contains a miR-155 \`sponge´ - an octameric decoy target (blue), complementary to miR155. LTR, long terminal repeat; cPPT, central polypurine tract; CMV, cytomegalovirus promoter; GFP, green fluorescent protein; pre-miR-155, precursor miR-155; WPRE, woodchuck hepatitis responsive element; triangle, self-inactivating mutation. (**d**) qRT-PCR analysis of miR-155 levels in LVmiR155 and LVmiRS155 transduced preadipocytes (normalized to snoRNA202). Untreated cells (mock) were set as one; data are presented as mean±SEM (***$P < 0.001$; **$P < 0.01$ one way ANOVA; n=3). (**e**) Oil Red O staining of differentiated brown fat cells transduced with LVmiR155, LVmiR146a and LVmiR223 as compared to uninfected wild-type cells (mock); scale bar = 3mm.

Supplementary Figure S2

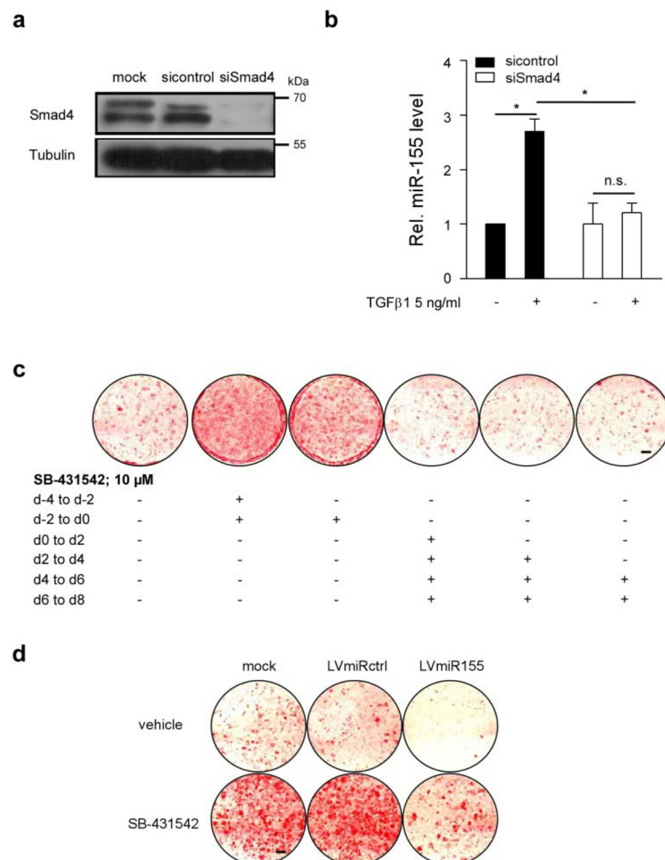
C/EBPβ is targeted by miR-155 and is able to rescue the miR-155 phenotype in BAT differentiation.



(**a**) Western Blot analysis (top) of C/EBPβ expression in cells transduced with LVmiRctrl or LVmiR155; mock, uninfected cells. Quantification of Western blot analyses (bottom), mock was set as one. Data are represented as means ± SEM. (**P* < 0.05 one way ANOVA; n=3). **(b)** Western blot analysis of the three C/EBPβ isoforms (LAP*/38kDa, LAP/35kDa, and LIP/20kDa) in mock, LVmiRctrl or LVmiR155 transduced brown adipocytes. (**c**) Luciferase assay to examine interaction between miR-155 and its predicted target site in the C/EBPβ 3´UTR. Reporter plasmids carrying the wt or "seed" mutated C/EBPβ 3´UTR (top) were transfected into HIB-1B cells transduced with LVmiRctrl or LVmiR155. Data are presented as means ± SEM. (**P* < 0.05; ****P* < 0.001 one way ANOVA; n.s., not significant, n=3). (**d**) Western blot analysis of C/EBPβ expression in the LVmiR155 cells transduced with increasing amounts of LVC/EBPβ to rescue the effect of miR-155. (**e**) Western blot analysis of C/EBPβ protein levels in LVmiRctrl or LVmiRS155 transduced preadipocytes. (**f**) Luciferase assay to analyse miR-155 interaction with the C/EBPβ 3´UTR. Reporter plasmids containing the C/EBPβ 3´UTR were transfected into LVmiRctrl or LVmiRS155 transduced HIB-1B cells. LVmiRctrl transduced controls were set as one. Data are presented as means ± SEM. (**P* < 0.05 student´s *t*-test; n=3). (**g**) Representative Western blot of C/EBPβ, PPARγ, and aP2 in differentiated brown adipocytes transduced with LVmiRctrl, LVmiR155, and LVmiRS155, respectively; mock, uninfected control. (**h**) Quantification of Western blot analyses of fat cell markers. Uninfected controls (mock) were set as one. Data were normalized to Tubulin and are represented as means ± SEM. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001 one way ANOVA; n=4). (**i**) qRT-PCR analyses of brown fat cell markers *PGC-1α*, *UCP1*, and *Cidea* in differentiated brown adipocytes transduced with the indicated lentiviral vectors. Untreated cells (mock) were set as one. Data were normalized to HPRT expression and are represented as means ± SEM. (**P* < 0.05; ***P* < 0.01 one way ANOVA; n=4). Tubulin served as loading control to all western blot analysis.

Supplementary Figure S3

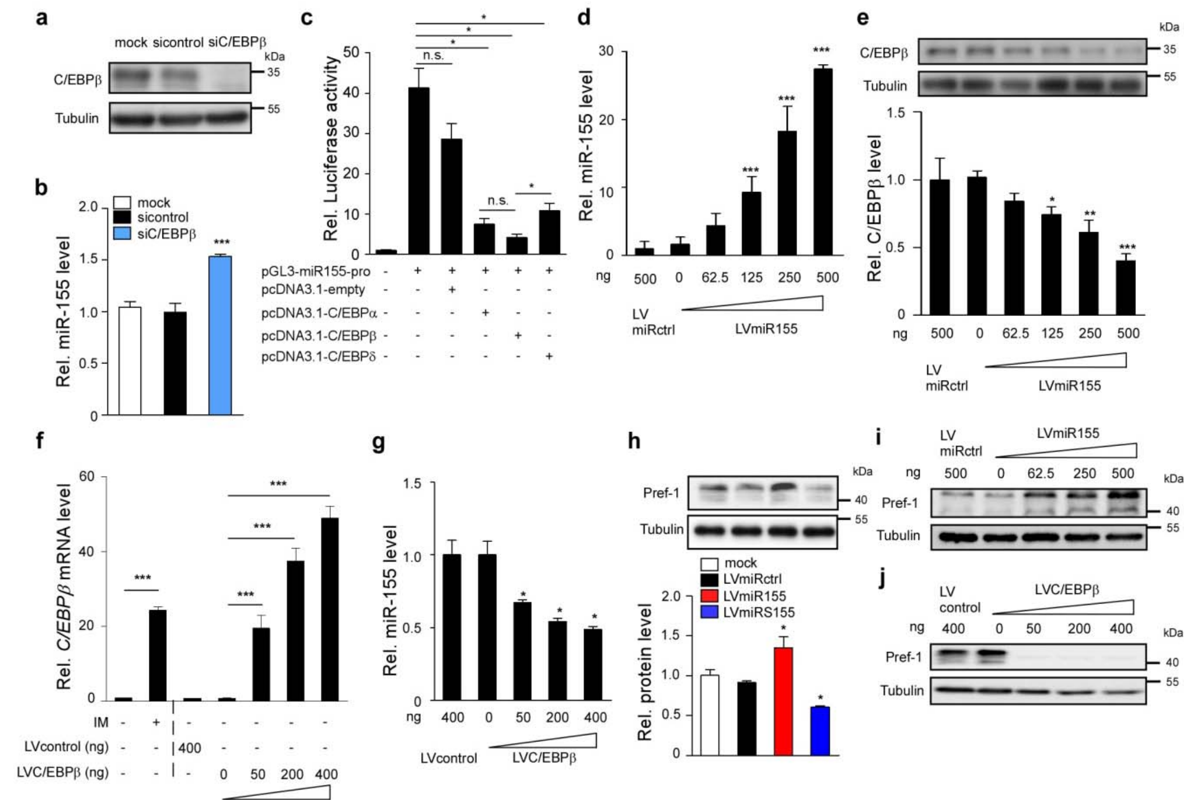
TGFβ regulates miR-155 expression via the TGFβ/Smad4 pathway in brown adipocytes.



(**a**) Western blot analysis of Smad4 protein levels in untransduced (mock), control siRNA (RVsictrl) and Smad4 siRNA (RVsiSmad4) transduced preadipocytes. (**b**) qRT-PCR analysis of miR-155 expression in RVsictrl or RVsiSmad4 transduced preadipocytes with or without TGFβ1 treatment (5 ng/ml; 24 h). Untreated, RVsictrl transduced cells served as control. Data are presented as means ± SEM. (*$P < 0.05$ student´s *t*-test; n.s., not significant; n=3). (**c**) Oil Red O staining (day 8) of brown fat cells treated with 10μM SB-431542 (TGFβ1 receptor inhibitor) at indicated time points during *in vitro* differentiation, scale bar = 3mm. **(d)** Oil Red O staining of fully differentiated brown adipocytes infected with LVmiRctrl or LVmiR155 and treated with vehicle or SB-431542 (10μM, day -2 to day 0); mock, uninfected cells; vehicle, distilled water, scale bar = 3mm.

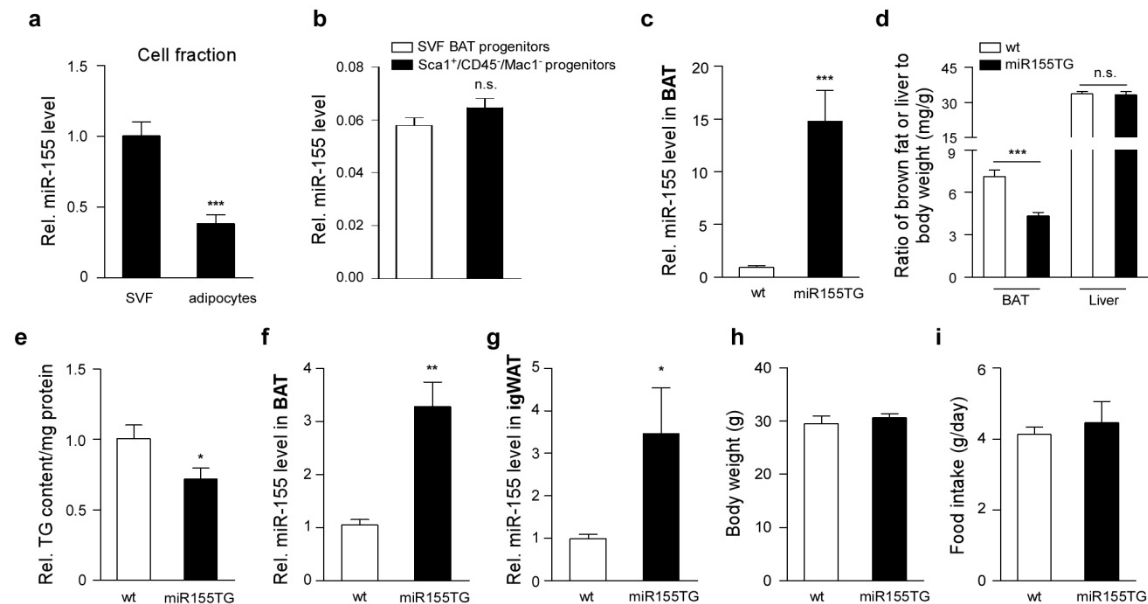
Supplementary Figure S4

C/EBPβ and miR-155 constitute a negative feedback loop in brown adipocytes.



(**a**) Western blot analysis of C/EBPβ expression in preadipocytes 48h post transduction with sicontrol (control-siRNA) or siC/EBPβ (C/EBPβ-siRNA). (**b**) qRT-PCR analysis of miR-155 levels in preadipocytes 48h after transduction with sicontrol or siC/EBPβ. miR-155 levels were normalized to snoRNA202; untransduced cells were set as one. Data are presented as means ± SEM (***P < 0.001 one way ANOVA; n=3). (**c**) Luciferase reporter assay to analyze regulation of the *BIC/miR-155* promoter by C/EBPs. HIB-1B cells were co-transfected with C/EBPα, C/EBPβ, or C/EBPδ expression vectors and the *BIC/ miR-155* promoter luciferase construct. Untransfected cells were set as one; data are represented as means ± SEM (*P < 0.05 one way ANOVA; n.s., not significant; n=3). (**d**) qRT-PCR analysis of miR-155 expression levels in preadipocytes transduced with LVmiRctrl or different amounts of LVmiR155. Data are normalized to sno202 expression, untreated cells were set as one; data are presented as means ± SEM (***P < 0.001 one way ANOVA; n=3). (**e**) Western-blot analysis of C/EBPβ protein levels in preadipocytes 48h post transduction with LCmiRctrl or LVmiR155 (top). Quantification of C/EBPβ protein levels (bottom). Data were normalized to Tubulin; untreated cells were set as one; data are presented as means ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001 one way ANOVA; n=3). (**f**) qRT-PCR analysis of C/EBPβ mRNA levels in preadipocytes transduced with LVcontrol or LVC/EBPβ as indicated, or stimulated with induction medium (IM) for 8 hours. Expression was normalized to HPRT; untransduced cells were set as one; data are presented as means ± SEM (***P < 0.001 one way ANOVA; n=3). (**g**) qRT-PCR analysis of miR-155 expression levels in preadipocytes transduced with LVcontrol or LVC/EBPβ. miR-155 levels were normalized to snoRNA202; untransduced cells were set as one. Data are presented as means ± SEM (*P < 0.05 one way ANOVA; n=3). (**h**) Western-blot analysis of Pref-1 protein levels in differentiated brown adipocytes transduced with LVmiRctrl, LVmiR155 or LVSmiR155. One representative blot is shown (top). Quantification of Pref-1 protein levels (bottom). Data were normalized to Tubulin; untreated cells (mock) were set as one; data are presented as means ± SEM (*P < 0.05 one way ANOVA; n=3). (**i,j**) Western-blot analysis of Pref-1 expression in preadipocytes transduced with (**i**) LVmiRctrl and LVmiR-155 or (**j**) LVcontrol and LVC/EBPβ at different dosages. Tubulin served as loading control.

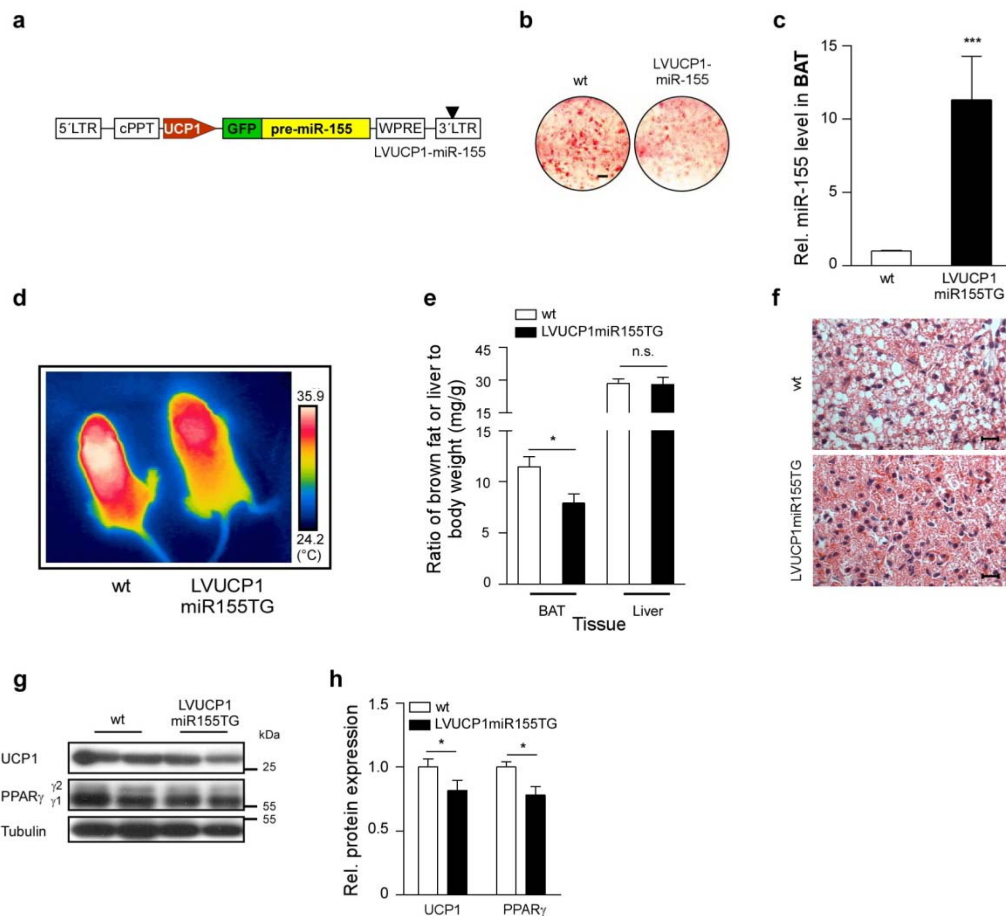
Supplementary Figure S5
miR-155 regulates brown fat differentiation *in vivo*.



(**a**) qRT-PCR analysis of miR-155 expression in cells of the SVF and the mature adipocyte fraction isolated from BAT of new-born mice. SVF data were set as one; data are represented as means ± SEM (***$P < 0.001$ student´s *t*-test; n=3). (**b**) qRT-PCR analysis comparing miR-155 expression levels in the total SVF versus the sub-fraction of FACS-sorted Sca-1$^+$, CD-45$^-$ and Mac-1$^-$ SVF-derived cells. Data are normalized to sno202RNA, and represented as means ± SEM (n.s., not significant student´s *t*-test; n=3). (**c**) qRT-PCR analysis of miR-155 levels in interscapular BAT from one week old wt or miR-155 transgenic (miR155TG) mice. miR-155 levels were normalized to snoRNA202. Data of wt were set as one; data are represented as means ± SEM (***$P < 0.001$ student´s *t*-test, n=3). (**d**) Ratios of interscapular BAT and liver weight to total body weight of 1 week old wt and miR155TG mice. Data are presented as means ± SEM (***$P < 0.001$; ns: not significant student´s *t*-test; wt group n=6, miR155TG group n=6). (**e**) TG content of interscapular BAT isolated from 1 week old wt or miR155TG mice. TG content was normalized to total protein concentration of lysates. wt was set as one, and data are represented as means ± SEM (*$P < 0.05$;**$P < 0.01$ student´s *t*-test; n=3). (**f,g**) qRT-PCR analysis of miR-155 expression levels in (**f**) interscapular BAT, or in (**g**) igWAT from ten week old wt or miR155TG mice. miR-155 levels were normalized to snoRNA202. wt was set as one; data are represented as means ± SEM (*$P < 0.05$;** $P < 0.01$ student´s *t*-test; n=3). (**h**) Body weight and (**i**) daily food intake of wt and miR-155TG littermates at 10-13 weeks of age (wt group n=5, miR155TG group n=9). Data are represented as means ± SEM.

Supplementary Figure S6

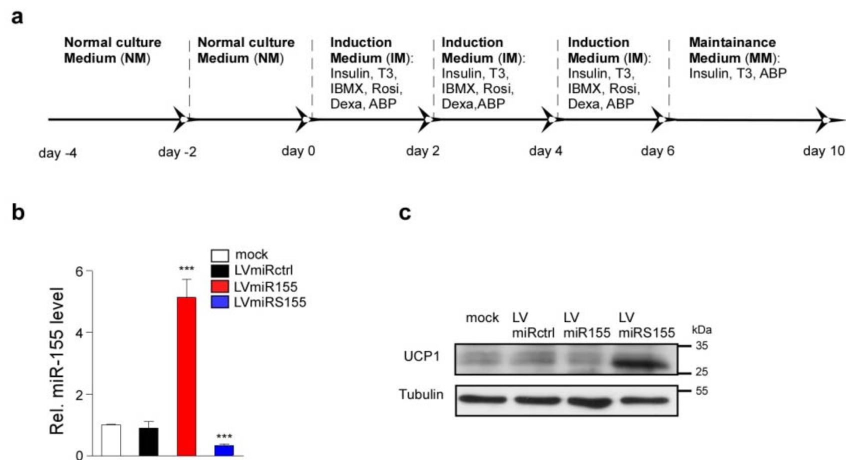
miR-155 impairs brown adipogenesis in an *in vivo* model with BAT specific miR-155 overexpression.



(**a**) Schematic representation of the lentiviral construct (LVUCP1-miR-155) for brown fat-specific expression of miR-155. LTR, long terminal repeat; cPPT, central polypurine tract; UCP1, uncoupling protein 1 promoter; GFP, green fluorescent protein; pre-miR-155, precursor miR-155; WPRE, woodchuck hepatits responsive element; triangle, self-inactivating mutation. (**b**) Oil Red O staining of differentiated brown adipocytes transduced with LVUCP1-miR-155 as compared to wt cells, scale bar = 3mm. (**c**) qRT-PCR analysis of miR-155 expression levels in 1 week old wt and miR-155 transgenic (LVUCP1miR155TG) mice generated by transduction of preimplantation embryos with LVUCP1-miR-155. miR-155 levels were normalized to snoRNA202. Data are represented as means ± SEM. (***$P < 0.001$ student´s *t*-test; wt group n=7, LVUCP1miR155TG group n=7). (**d**) Body surface temperature of wt and LVUCP1miR155TG mice was measured by infrared thermography. (**e**) Ratios of BAT and liver weight versus total body weight. Data are represented as means ± SEM. (*$P < 0.05$ student´s *t*-test; n.s., not significant; wt group n=7, LVUCP1miR155TG group n=7). (**f**) Hematoxylin and eosin staining of BAT sections from one week old wt and LVUCP1miR155TG littermates; (scale bar = 15 µm). (**g**) Western Blot analysis of UCP1 and PPARγ protein levels in BAT isolated from wt and LVUCP1miR155TG littermates. Tubulin served as loading control. 2 representative pairs are shown. (**h**) Densitometric quantification of Western blot analyses (right). Proteins expression was normalized to Tubulin. Data are presented as means ± SEM. (*$P < 0.05$ student´s *t*-test; wt group n=7, LVUCP1miR155TG group n=7).

Supplementary Figure S7

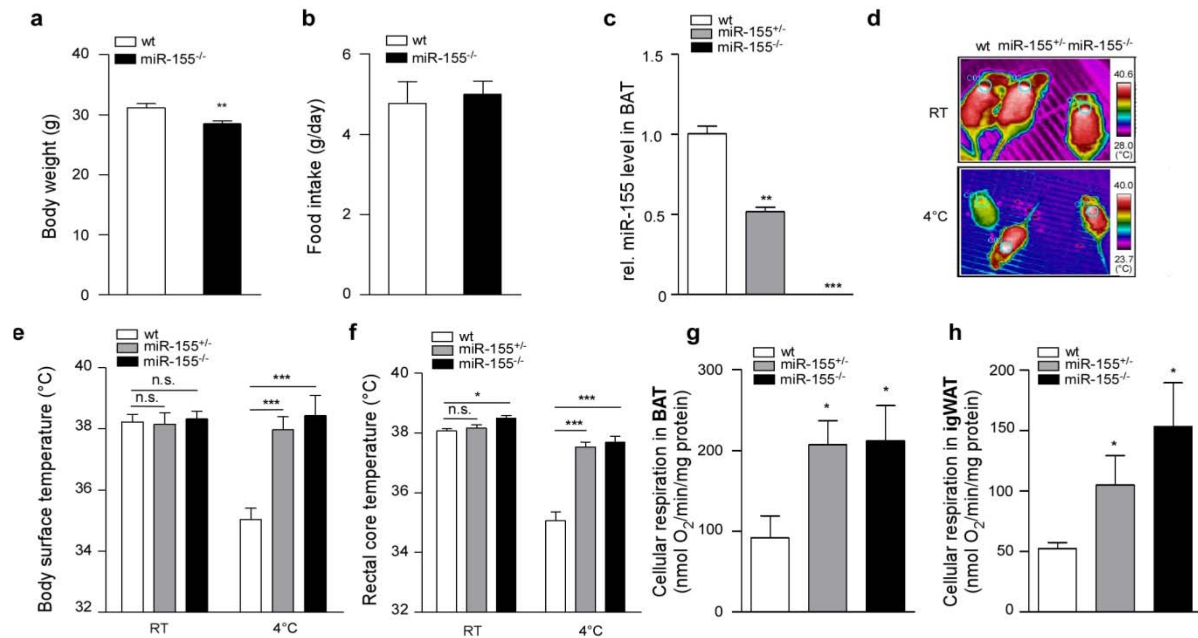
miR-155 regulates white fat differentiation and browning of WAT *in vitro*.



(**a**) Differentiation protocol used for *in vitro* differentiation of white preadipocytes (day -4 to day 10). NM, normal growth medium; MM, maintenance medium; IM, induction medium. (**b**) qRT-PCR analysis of miR-155 expression levels in LVmiRctrl, LVmiR155 or LVmiRS155 transduced white preadipocytes (normalized to snoRNA202). Untransduced cells (mock) were set as one. Data are represented as means ± SEM (***P < 0.001 one way ANOVA; n=3). (**c**) Western Blot analysis of UCP1 protein levels in LVmiRctrl, LVmiR155 or LVmiRS155 transduced fully differentiated white adipocytes at day 9. Tubulin served as loading control.

Supplementary Figure S8

Thermogenesis and respiration are increased in miR-155$^{+/-}$ mice.



(**a**) Body weight and (**b**) daily food intake of wt and miR-155$^{-/-}$ littermates at 12-16 weeks of age; Data are represented as means ± SEM (**$P < 0.01$ student´s *t*-test; wt group n=12, miR-155$^{-/-}$ group n=16). (**c**) RT-PCR analysis of miR-155 expression levels in interscapular brown adipose tissue (BAT) from sixteen week old mice. Data are normalized to sno202RNA expression; data of wt group were set as onedata are represented as means ± SEM (**$P < 0.01$; ***$P < 0.001$ one way ANOVA; wt group n=3, miR-155$^{+/-}$ group n=3, and miR-155$^{-/-}$ group n=3). (**d**) Infrared thermography analysis of body surface temperature of 12-16 weeks old wt, miR-155$^{+/-}$ or miR-155$^{-/-}$ littermates exposed to 4°C. (**e**) Statistical analysis of body surface temperature. Data are represented as means ± SEM (*$P < 0.05$ one way ANOVA; n.s., not significant) (wt group n=7, miR-155$^{+/-}$ group n=5, and miR-155$^{-/-}$ group n=5). (**f**) Statistical analysis of rectal body core temperature measurement of 12-16 weeks old wt, miR-155$^{+/-}$ or miR-155$^{-/-}$ littermates exposed to either RT or 4°C. Data are represented as means ± SEM (*$P < 0.05$; ***$P < 0.001$ one way ANOVA; n.s., not significant) (wt group n=14, miR-155$^{+/-}$ group n=14, and miR-155$^{-/-}$ group n=12). (**g,h**) *Ex vivo* Oxygraph measurement of cellular mitochondrial respiration in (**g**) interscapular BAT (BAT), or in (**h**) igWAT of wt, miR-155$^{+/-}$ and miR-155$^{-/-}$ littermates at 12-16 weeks of age. Data are represented as means ± SEM (*$P < 0.05$; one way ANOVA; n.s., not significant; wt group n=6, miR-155$^{+/-}$ group n=6, and miR-155$^{-/-}$ group n=5).

Supplementary Table S1

Supplementary Table S1: qRT-PCR primer sequences

Primer name/ cDNA	Primer sequence
UCP1 forward	5' - GGT GAA CCC GAC AAC TTC CGA AGT G - 3'
UCP1 reverse	5' - GGG TCG TCC CTT TCC AAA GTG TTG A - 3'
PGC-1α forward	5' - GCA CAC ACC GCA ATT CTC CCT TGT A - 3'
PGC-1α reverse	5' - ACG CTG TCC CAT GAG GTA TTG ACC A - 3'
Cidea forward	5' - ATT TAA GAG ACG CGG CTT TGG GAC A - 3'
Cidea reverse	5' - TTT GGT TGC TTG CAG ACT GGG ACA T - 3'
HPRT forward	5' - ACA TTG TGG CCC TCT GTG TGC TCA - 3'
HPRT reverse	5' - CTG GCA ACA TCA ACA GGA CTC CTC GT - 3'
C/EBPα forward	5' - CGA GGA GGA CGA GGC GAA GCA - 3'
C/EBPα reverse	5' - TGC GCA GGC GGT CAT TGT CAC - 3'
C/EBPβ forward	5' - CAC CAC GAC TTC CTC TCC GAC CTC T - 3'
C/EBPβ reverse	5' - GTA CTC GTC GCT CAG CTT GTC CAC C - 3'
aP2 forward	5' - TGA AAG AAG TGG GAG TGG GCT TTG C - 3'
aP2 reverse	5' - CAC CAC CAG CTT GTC ACC ATC TCG T - 3'
PPARγ2 forward	5' - TCC GTA GAA GCC GTG CAA GAG ATC A - 3'
PPARγ2 reverse	5' - CAG CAG GTT GTC TTG GAT GTC CTC G - 3'
ChIP C/EBPβ A fwd	5' – TGT GTG GTA GGA AGA AAA CCT TCA - 3'
ChIP C/EBPβ A rev	5' – TGT CAG ATT CGT GGA CAC AGA AA - 3'
ChIP C/EBPβ B/C/D fwd	5' – CCA GAT ACT ACA GGG ATC TAA AGA GGT T -3'
ChIP C/EBPβ B/C/D rev	5' – TCA GGC CTT CCC CTA AAA CTC - 3'
ChIP C/EBPβ E fwd	5' – CAG AGT GAC CTG CCT TAT CTT AAA AA - 3'
ChIP C/EBPβ E rev	5' – GAC CCT TTA ATA CAG TTC CTT CTG TTG - 3'