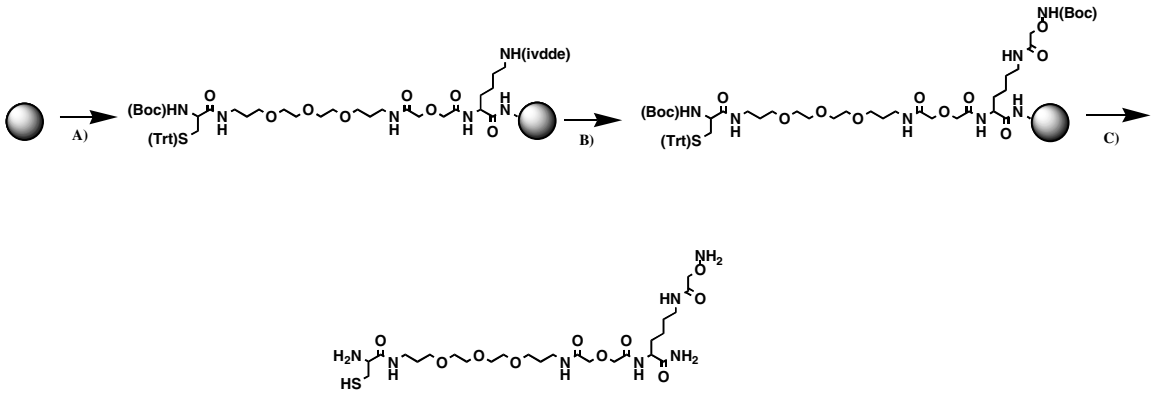
Supporting information for publication



Scheme S1. Solid phase synthesis of peptide **2**. Reaction conditions: A) Standard Fmoc-SPPS. B) 2% Hydrazine in DMF followed by 8 equivalents of Boc-aminooxyacetic acid activated with diisopropylcarbodiimide. C) 1% triisopropyl silane, 2.5% ethanedithiol, 2.5% water in trifluoroacetic acid, 1 h.

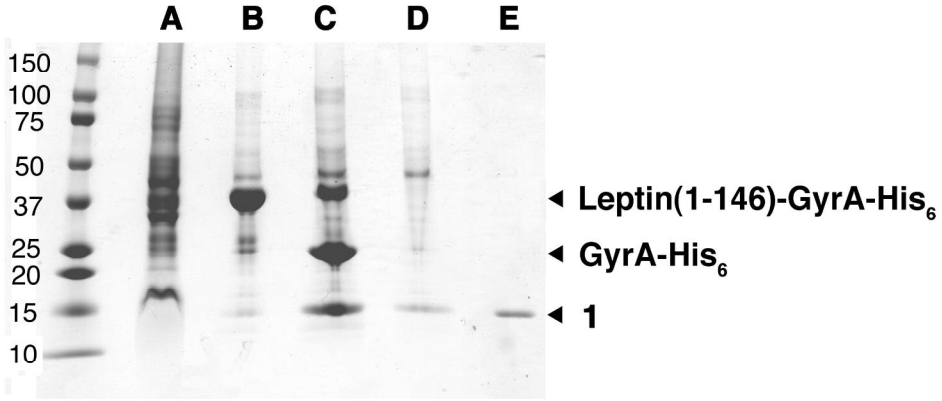


Figure S1. Expression and purification of leptin α-thioester **1**. Coomassie stained 4-20% gradient SDS-PAGE analysis of leptin purification fractions. A: Crude insoluble fraction from cell lysate. B: Leptin-GyrA-His_6 purified on a Ni-NTA column. C: Cleavage of leptin-GyrA-His_6 using 100 mM MESNa. D: Eluate of leptin α-thioester **1** from the Ni-NTA column, E: Purified leptin α-thioester **1**.

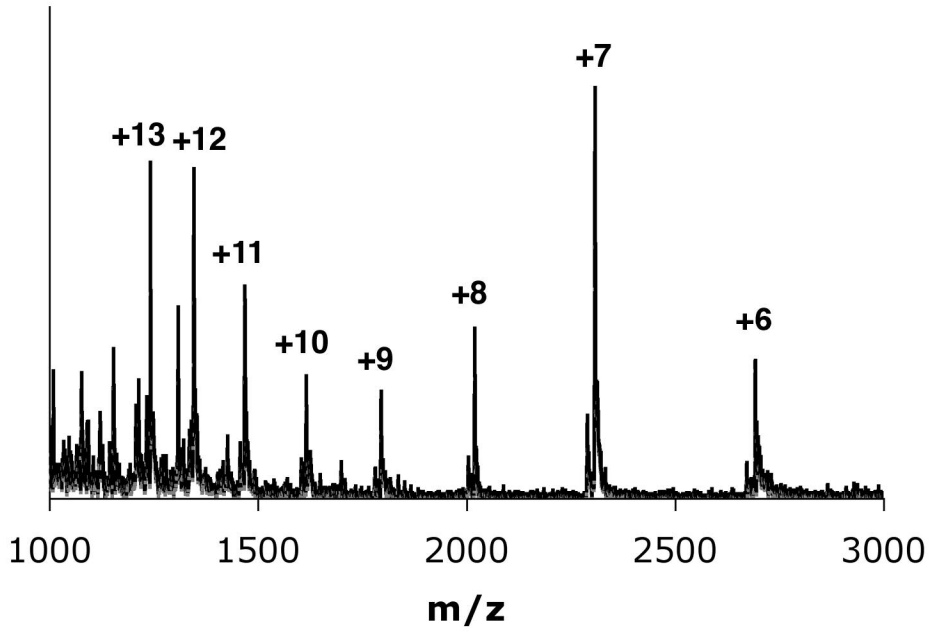


***Figure S2*.** ESI-MS of leptin α-thioester **1** (calculated mass 16156.2 Da, obtained 16157.2 ± 3.0 Da). Charge states are indicated.

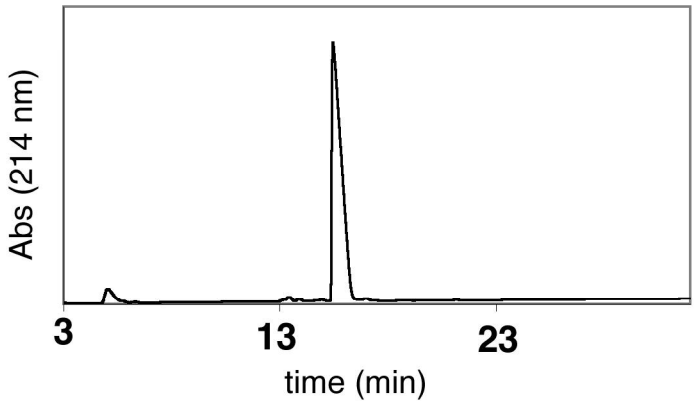


Figure S3 C18 analytical RP-HPLC chromatogram of purified peptide **2** on a 5-20% B gradient.

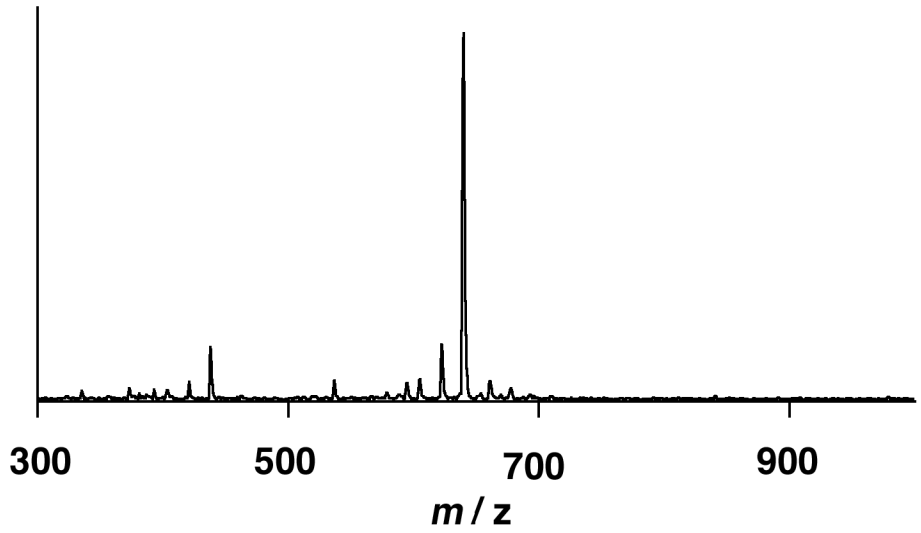


Figure S4 ESI-MS of peptide **2** (calculated mass 639.8 Da, obtained [M+H] = 641.0 Da).

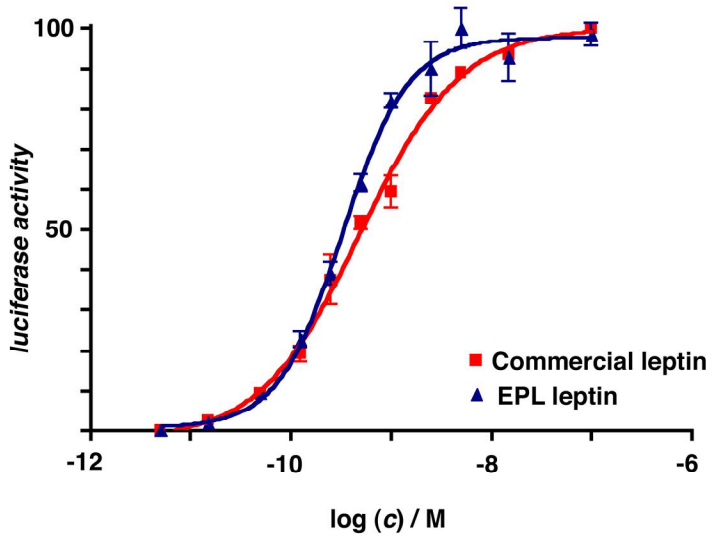


Figure S5. Leptin receptor-STAT3 dependant luciferase assay comparing the activity of recombinant leptin produced by an EPL protocol and recombinant leptin obtained from a commercial source. EC_{50} for recombinant leptin = 0.49 nM (95% confidence intervals 0.39-0.61 nM). EC_{50} for semisynthetic leptin = 0.31 nM (95% confidence intervals 0.25-0.38 nM)

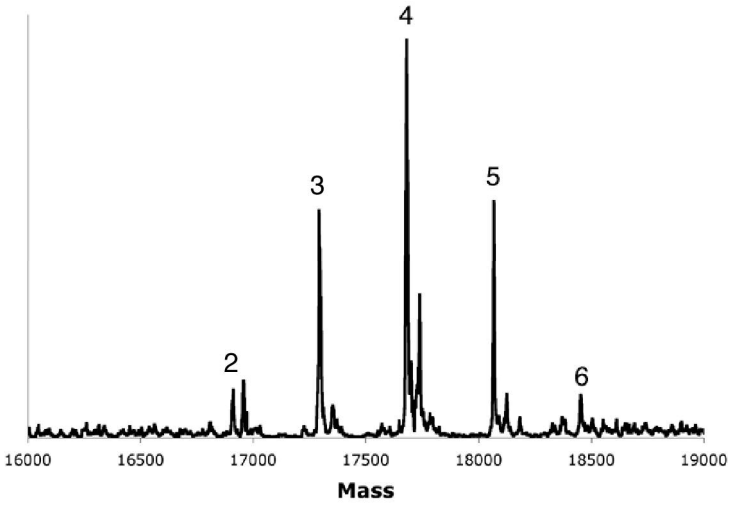


Figure S6. ESI-MS reconstruct of DOTA-Leptin prepared using DOTA-NHS ester. The number of DOTA chelators per leptin molecule is indicated. Expected and obtained masses: +2 DOTAs, expected 16908.5, obtained 16909.1 ± 0.5, +3 DOTAs, expected 17294.9, obtained 17295.0 ± 1.9, +4 DOTAs, expected 17681.3, obtained 17679.6 ± 2.6, +5 DOTAs expected: 18067.7, obtained 18067.5 ± 3.0, +6 DOTAs expected: 18454.1, obtained 18452.8 ±3.6.

General Methods

Amino acid derivatives, resin and coupling reagents were purchased from Novabiochem (San Diego, CA) unless otherwise indicated. Boc-aminooxyacetic acid was purchased from Sigma-Aldrich (Milwaukee, WI). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). Recombinant murine leptin was a gift of Amgen (Thousand Oaks, CA). Analytical gradient reversed phase HPLC was performed on a Hewlett Packard 1100 series instrument and a Vydac C18 or C4 column (5 micron, 4 X 150 mm). Flow rate was 1 mL/min and detection was at 214 and 280 nm. Preparative scale HPLC was performed on a Waters Delta Prep 4000 fitted with a Waters 486 tunable absorbance detector and using either a Vydac C4 process scale column (5 x 25 cm) at 30 mL/min, a Vydac C18 process scale column (5 x 25 cm) at 30 mL/min, or a Vydac C4 semiprep scale column (1 x 25 cm) at 5 mL/min. Radiochemical HPLC was performed on a Varian ProStar series instrument fitted with a model 320 UV detector and a Bioscan flowcount radiodetector (Bioscan Inc., Washington D.C.), and either a Vydac analytical C4 column (5 micron, 4 X 150 mm) at 1 mL/minute or a Waters μBondapak C18 (7.8 x 300 mm) column at 3 mL/min as indicated. HPLC mobile phase buffers A and B were 0.1% trifluoroacetic acid in water, and 90% acetonitrile, 0.1% trifluoroacetic acid in water, respectively. Electrospray mass spectrometry was routinely used on all peptides and proteins, using a Sciex API-100 single quadrapole spectrometer. Thin layer chromatographic (TLC) analysis was conducted using normal phase silica 60, F254, 250 μm, 2.5 x 7.5 cm plates (Spectrum Chemical, Gardena, CA). Quantification of radiochemical thin layer chromatography plates was performed on a Bioscan Mini-Scan TLC scanner (Bioscan, Washington, D.C.).

All enzymes involved in the construction of the plasmids were obtained from New England Biolabs (Ipswich, MA). All molecular biology kits were obtained from Qiagen unless otherwise specified.

Cloning of Leptin(1-146)-GyrA-His_6

The sequence encoding the chitin binding domain in the pTXB vector (New England Biolabs, Ipswich, MA) was replaced with a His_6 tag sequence to facilitate protein purification under denaturing conditions. Synthetic oligonucleotides encoding a His_6 tag flanked by AgeI and PstI restriction sites (sense strand; 5'-CCGGTGGAGCGCATCATCATCATCATCATGGAGCGTAACTGCA and antisense strand; 3'-GTTACGCTCCATGATGATGATGATGATGCGCTCCA) were phosphorylated using T4 DNA kinase and annealed. The pTXB vector was digested with AgeI and PstI, and the annealed His-oligos inserted using T4 ligase to give plasmid pGyrAHis, the identity of which was verified by sequencing. cDNA encoding leptin(1-146) was amplified by PCR from a leptin-EGFP plasmid (obtained from A. Zeigerer, Friedman lab) using forward and reverse primers (GGAATTCCATATGGTGCCTATCCAGAAAGTCCAGG and GGACTAGTGCATCTCCCGTGATGCATTCAGGGCTAACATCCAACTG, respectively) encoding the NdeI and SpeI restriction sites. The PCR product was purified by gel extraction and ligated into the pre-digested pGyrAHis plasmid using T4 ligase to give the plasmid pLeptin-GyrA, the identity of which was verified by sequencing.

Overexpression and purification of Leptin(1-146)-GyrA-His_6

E. coli BL21(DE3) cells (Novagen, Darmstadt, Germany) were transformed using the plasmid pLeptin-GyrA. Cells were grown to an OD_{600} of 15-25 in a Bioflo 3000 Batch/Continuous bioreactor (New Brunswick Scientific, Edison, NJ) in superbroth media (Qbiogene, Irvine, CA) with 50 μg/mL ampicillin. The culture medium was supplemented with oxygen to keep oxygen gas levels constant at 30% and agitated at 350 RPM. The pH was maintained at 7.0 automatically using 5N sodium hydroxide. The culture medium was supplemented with 2 mL/min 50% glucose and 2 mL/min 3X superbroth solution during growth. Overexpression was induced with 1 mM IPTG, and the flow of glucose and superbroth was increased up to 10 mL/min. After four hours of induction at 37 °C, the cells were harvested by centrifugation. Using this protocol 200-250g of biomass was typically obtained per culture prep.

The cells were resuspended in lysis buffer consisting of 1% triton, 50 mM sodium phosphate buffer, pH 7.0, 200 mM sodium chloride supplemented with one complete protease inhibitor tablet, EDTA free (Roche, Basel, Switzerland) per 50 mL of buffer. The cells were lysed by passage through a French Press (Emulsiflex C5 homogenizer, Avestin, Ottawa, Canada.). The inclusion bodies were separated by centrifugation at 16,000 rpm for 30 minutes, and subsequently resuspended overnight in one volume (relative to the cell pellet) of resuspension buffer consisting of 8 M urea, 20 mM imidazole, 150 mM sodium chloride, 100 mM sodium phosphate, pH 7.5. This suspension was centrifuged again at 16,000 RPM for 30 minutes to remove insoluble debris, and applied to 100 mL of Ni-NTA beads which were pre-equilibrated with

resuspension buffer supplemented with 20 mM imidazole. The beads were then washed with resuspension buffer supplemented with 20 mM imidazole, and the fusion protein was eluted from the column with 10 x 50 mL fractions of resuspension buffer supplemented with 500 mM imidazole. The fractions were analyzed by SDS-PAGE and those containing the 39 kDa fusion protein were pooled.

Generation of Leptin(1-146) α-Thioester, 1

The combined Leptin(1-146)-GyrA-His_6 fractions were refolded by extensive dialysis against 4 M urea, 100 mM sodium phosphate, 150 mM sodium chloride pH 7.0 at 4 °C. The first dialysis buffer was further supplemented with 10 mM EDTA to remove trace amounts of contaminating nickel. Intein cleavage to form the leptin (1-146) thioester **1** was initiated by adding sodium 2-mercaptoethane sulfonate to a final concentration of 100 mM. Cleavage was allowed to proceed for 2 days at 4 °C. The crude cleavage reaction was reapplied to 100 mL of nickel-NTA beads, which were pre-equilibrated with dialysis buffer supplemented with 20 mM imidazole. The beads were washed with dialysis buffer supplemented with 20 mM imidazole and 100 mM sodium 2-mercaptoethane sulfonate. The pooled flowthrough and washes from the Ni-NTA column containing principally the leptin(1-146) thioester **1** were then purified by process scale C4 RP-HPLC using a gradient of 50-65% buffer B. Fractions were analyzed by analytical RP-HPLC and ESI-MS, and those containing pure leptin thioester **1** were pooled and lyophilized. Residual uncleaved fusion protein on the Ni-NTA column could be recovered from the column by elution with 8 M urea, 500 mM imidazole, 500 mM sodium chloride, 100 mM sodium phosphate pH 7.0, and refolded and recleaved as

above. Using this procedure, ~60 mg of pure leptin(1-146) α-thioester **1** could be obtained from an initial 5L culture.

Synthesis of Peptide 2

The peptide was prepared using manual Fmoc-based solid phase peptide synthesis as indicated on Scheme S1. Agitation was achieved using a flow of nitrogen gas. Fmoc deprotection was achieved using 5 mL of 20% piperidine in DMF for 3 minutes followed by another treatment for 10 minutes. The resin was acetylated after every coupling using 5 mL of 10% acetic anhydride, 10% diisopropylethylamine (DIEA) in DMF for 10 minutes. Washes with DMF were performed in between all coupling, acetylation, and deprotection steps.

Fmoc-Lys(ivDde) (0.379 g, 0.66 mmoles) was coupled to Rink amide AM resin (420 mg, 0.71 mmol/g) using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 0.228 g, 0.6 mmoles), 0.5 mL DIEA, in 4 mL DMF for two hours. After capping and deprotection, Fmoc-NH-PEG_2(20 atoms)-COOH linker (0.2 g, 0.36 mmoles) was coupled to the resin using HBTU (0.125 g, 0.33 mmoles), 0.5 mL DIEA in 4 mL DMF for two hours. The coupling reaction was repeated. Following capping and deprotection, Boc-Cys(Trt)-OH (1.02 g, 2.2 mmoles) was coupled using diisopropylcarbodiimide (310 μL, 2 mmoles), N-hydroxybenzotriazole (0.270 g, 2 mmoles) for 20 minutes. The coupling was then repeated. The ivDde group was deprotected using 2% hydrazine in DMF three times for one hour. Boc-aminooxyacetic acid (0.460 g, 2.4 mmoles) was then coupled using DIC (370 μL, 2.4 mmoles) for 10 minutes as described.[1] The resin was washed with DCM and dried. Cleavage from the

resin and deprotection was achieved using 5 mL of 1% triisopropyl silane, 2.5% ethanedithiol, 2.5% water in trifluoroacetic acid (TFA) for 2.5 hours. The peptide was precipitated using 50 mL of 5% 2-mercaptoethanol in diethyl ether, centrifuged at 4800 rpm for 10 minutes, resuspended in 5 mL of HPLC buffer A, and lyophilized. The crude residue was then purified by process scale RP-HPLC on a C18 column using a gradient of 5-20% B. Fractions containing the purified peptide **2** were identified by HPLC/MS, pooled, and lyophilized. Yield from 0.3 mmol scale: 120 mg, (0.19 mmol), 63%.

Synthesis of native murine leptin by EPL

Leptin-GyrA-His_6 was expressed, purified, and refolded as indicated above. EPL with cysteine was initiated using 100 mM sodium 2-mercaptoethanesulfonate and 10 mM cysteine, and the reaction was allowed to proceed for 3 days at 4 °C. The crude ligation was then refolded and purified using the same method as aminooxy-Leptin **4**.

Generation of a Stable Cell Line Overexpressing Leptin Receptor Long Isoform (Ob-Rb) and a Stat3-Responsive Element-Luciferase Reporter

A stable Flp-In 293 cell line[2] expressing the long isoform of the leptin receptor (Ob-Rb) that is capable of full signaling (kind donation of Dr. Cai Li, University of Texas Southwestern) was stably transfected with a p4xm67 luciferase plasmid, containing a STAT3 responsive element and luciferase DNA (kindly provided by Dr Jim Darnell, The Rockefeller University). Cells were transfected with p4xm67 luciferase and a puromicin resistance plasmid following the Lipofectamine 2000 method (Invitrogen). Stable integrants were selected in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and puromicin (1.5 μg/ml) for about 4 weeks. Single cell clones were then

selected for leptin induced luciferase production and the clone with the best response to leptin used for further experiments.

Animal Maintenance

Male C57BL6/J ob/ob mice and wild type were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in individual cages in temperature controlled rooms. All animals were subjected to a 12 h light/dark cycle. All procedures and measurements were performed between 14:00 and 17:00 hours. Animal care and experiments were conducted in accordance with approved guidelines of The Rockefeller University.

Synthesis and Reformulation of [18F]-FBA

The radiosynthesis of [18F]-FBA was accomplished essentially as previously described, however in order to facilitate labeling of leptin, which precipitates in solutions containing a high proportion of organic solvents, it was reformulated into 5 μL of DMF. The precursor (4-formylphenyl)trimethylammonium trifluoromethanesulfonate was synthesized as previously described.[3] This precursor was dried under vacuum and stored under argon atmosphere. Cyclotron produced ^{18}F in ^{18}O water was loaded onto a QMA light cartridge (Waters), which had been preconditioned with 5 mL 0.5M potassium carbonate and 10 mL water. The cartridge was eluted with a solution of 20 mg kryptofix 222 (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane), 2 mg potassium carbonate in 1:1 acetonitrile:water into a 3 mL reactivial (Pierce, Rockford, IL) equipped with a stir bar. The resulting solution was evaporated at 95 °C with a gentle nitrogen

stream, and dried azeotropically using 3 x 1 mL dry acetonitrile. The resulting solid was resuspended in 400 μL of dry DMSO and transferred to a second 3 mL reactivial equipped with a stir bar and containing 1-2 mg of (4-formylphenyl)trimethylammonium trifluoromethanesulfonate. The vial was incubated for 8 minutes at 120 °C, and then cooled to room temperature. The reaction was then diluted with 1.5 mL of water, and filtered through a GHP filter (Pall, East Hill, NY) and loaded on a Waters μBondapak C18 (7.8 x 300 mm) semiprep column, and eluted isocratically using 13:87 acetonitrile:water with 0.1% TFA at a flow rate of 3 mL/min. The peak eluting at 14 minutes was diluted with 15 mL water, and loaded onto a C18 sep-pak plus cartridge (Waters). The cartridge was then eluted with 1 mL of diethyl ether. A small amount of DMF (5 μL) was added during this step to minimize loss of the FBA during evaporation. The diethyl ether was then evaporated at 45° C, leaving a small organic residue (about 5-10 μL in a typical reaction). The product had the same R_f in 3:1 hexanes:ethyl acetate as authentic 4-fluorobenzaldehyde (0.65) and the same retention time on C18 HPLC as the authentic sample. Starting from 9.25-13 GBq, typically 1.3-1.85 GBq of reformulated ^{18}FBA could be recovered with a synthesis time of 90 minutes.

Preparation of [68Ga]-DOTA-Leptin

A lysine-directed labeling approach using DOTA-NHS ester (Macrocyclics) was employed. Briefly, to 100 μL leptin solution at 13.1 mg/mL (Amgen) was added 162 μL metal free (by chelex resin treatment, Biorad) 100 mM sodium phosphate pH 7.5 (to dilute to 5 mg/mL). 100 equivalents of DOTA-NHS (8.2 μmol, 4.1 mg) were added, and the pH brought to 7.5 using 1N NaOH. The reaction proceeded for 3 hours at which point

it was purified over C4 analytical HPLC on a 45-60%B gradient. The product was characterized by ESI-MS (figure S6), which indicated the incorporation of 2-6 DOTA moieties per leptin molecule. The biological activity of this compound was found to be unaltered when compared against unmodified leptin *in vitro* and *in vivo* as above. DOTA-Leptin was labeled using ^{68}Ga eluted from a ^{68}Ge generator (Isotope Products Laboratories) using a previously described procedure with slight modifications.[4] The specific activity of the conjugate at the end of synthesis was typically 200 mCi/μmol at greater than 90% purity as determined by radiochemical HPLC and iTLC. The full details of the synthesis and characterization of this compound will be reported elsewhere.

1. Jimenez-Castells, C.; de la Torre, B. G.; Gutierrez Gallego, R.; Andreu, D., Optimized synthesis of aminooxy-peptides as glycoprobe precursors for surface-based sugar-protein interaction studies. *Bioorg Med Chem Lett* **2007,** 17, (18), 5155-8.
2. Ge, H.; Huang, L.; Pourbahrami, T.; Li, C., Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors in vitro and in vivo. *J Biol Chem* **2002,** 277, (48), 45898-903.
3. Michael S. Haka, M. R. K., G. Leonard Watkins and Steven A Toorongian, Aryltrimethylammonium trifluoromethanesulfonates as precursors to aryl [18F]Fluorides: improved synthesis of [18F]GBR-13119. *Journal of Labeled Compounds and Radiopharmaceuticals* **1988,** XXVII, (7), 823-833.
4. Hoffend, J.; Mier, W.; Schuhmacher, J.; Schmidt, K.; Dimitrakopoulou-Strauss, A.; Strauss, L. G.; Eisenhut, M.; Kinscherf, R.; Haberkorn, U., Gallium-68-DOTA-albumin as a PET blood-pool marker: experimental evaluation in vivo. *Nucl Med Biol* **2005,** 32, (3), 287-92.