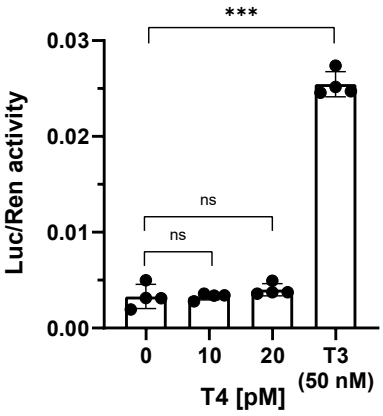
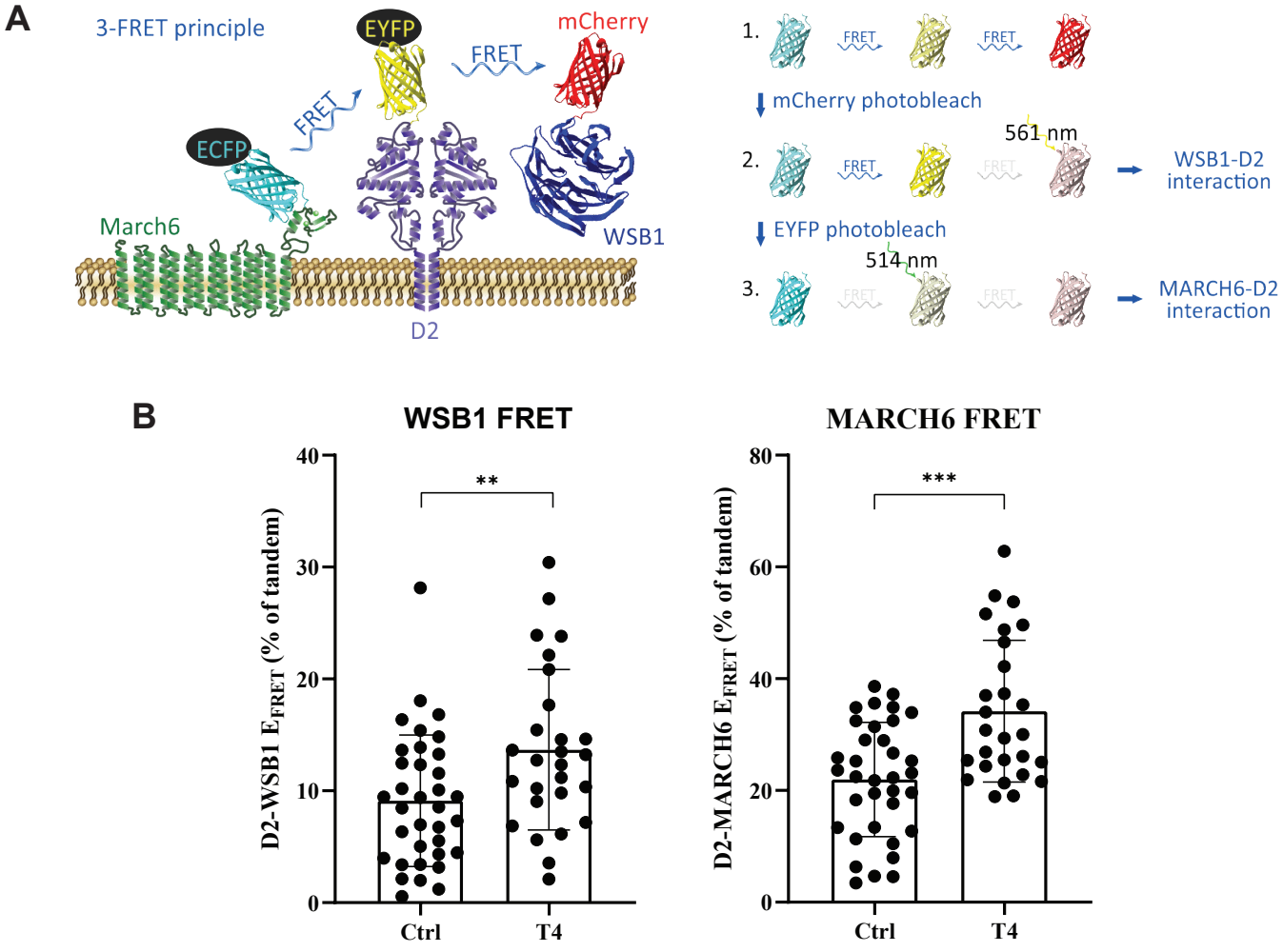
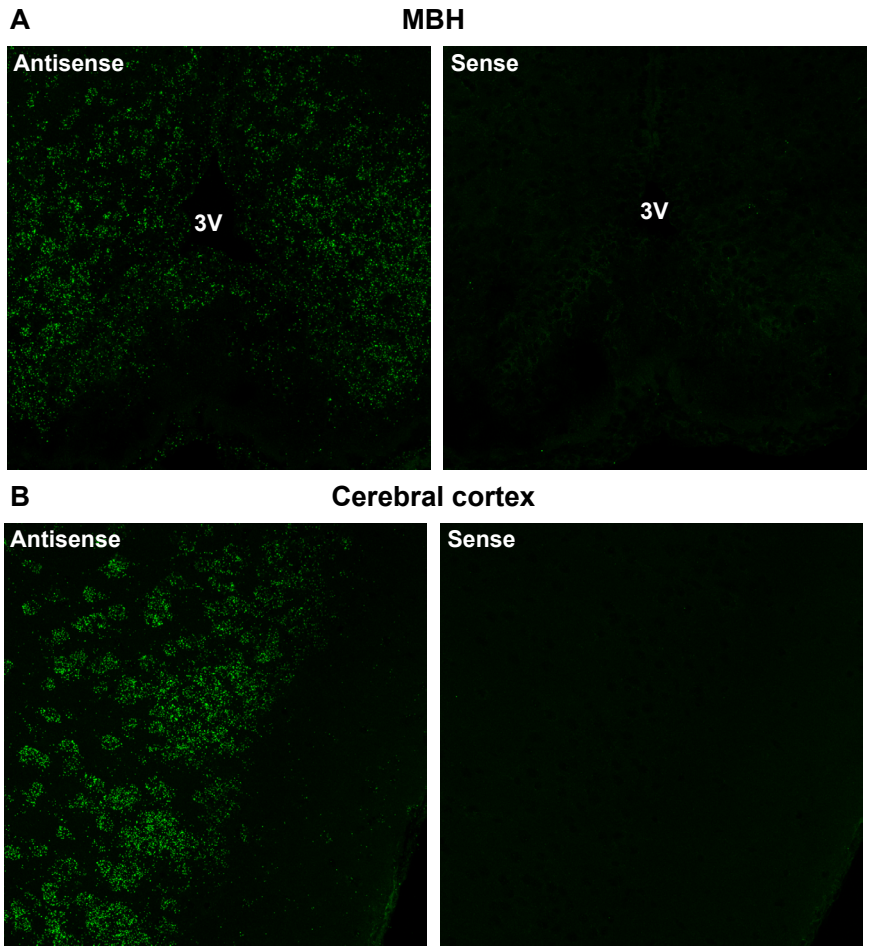
Suppl. fig. 1



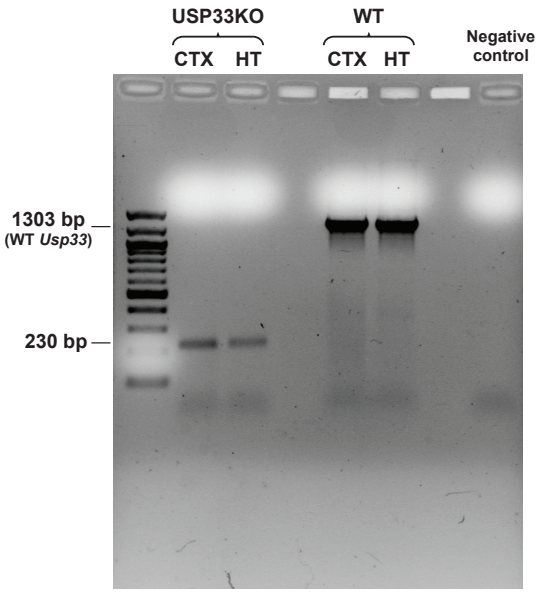
Suppl. fig. 2


A
3-FRET principle
EYFP
mCherry
FRET
FRET
ECFP
March6
WSB1
D2
1.
FRET
FRET
mCherry photobleach
561 nm
2.
FRET
FRET
WSB1-D2 interaction
EYFP photobleach
514 nm
3.
FRET
FRET
MARCH6-D2 interaction
B
WSB1 FRET
D2-WSB1 E_FRET (% of tandem)
**
Ctrl
T4
MARCH6 FRET
D2-MARCH6 E_FRET (% of tandem)
***
Ctrl
T4

Suppl. fig. 3


A
MBH
Antisense
3V
Sense
3V
B
Cerebral cortex
Antisense
Sense

Suppl. fig. 4


USP33KO
WT
CTX
HT
CTX
HT
Negative control
1303 bp
(WT Usp33)
230 bp

Supplementary Table 1.

Statistics of regression models

Fig. 3 D,F	***Luc* vs T4**		***Dio3* vs T4**	
	MBH	**Cerebral cortex**	**MBH**	**Cerebral cortex**
Slope	-1.1857	-0.2778	-1.3842	-0.2968
r^2	0.8547	0.5974	0.7271	0.1802
p	<0.0000	0.00004	<0.0000	0.0551
Homogeneity of slopes	F= 57.2441 p< 0.0000		F= 17.4428 p= 0.00018	
Fig. 3 G,H	**MBH** ***Luciferase* vs *Dio3***		**Cerebral Cortex** ***Luciferase* vs *Dio3***	
Slope	1.0779		1.3399	
r^2	0.7879		0.4998	
p	p< 0.0000		p= 0.0002	

Parameters and statistics of regression models in **Fig. 3. D,F,G,H**. Homogeneity of slopes test was used to compare linear correlation of tissue tT4 and tissue TH action between regions (slope[MBH] vs slope[cerebral cortex]; **Fig. 3. D,F**). Linear regression and Pearson correlation coefficient was used to characterize connection between *Luciferase* and *Dio3* expression in regions (**Fig. 3. G,H**).

Supplementary Table 2.

List of Taqman gene expression assays

Gene symbol	Gene name	Assay ID
dCpG Luc (Luc)	modified luciferase	AIY9ZTZ
Dio3	type 3 deiodinase	Mm00548953_s1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Hprt1	hypoxanthine phosphoribosyltransferase 1	Mm01545399_m1
Msi1	musashi RNA binding protein 1	Mm01203522_m1

Supplementary legends

Supplementary figure 1. The THAI luciferase reporter responds to T3 but not to T4. *Luc* mRNA levels in HEK293T cells transfected with the THAI luciferase construct (Mohacsik et al. Endocrinology 2018) in the indicated treatments. Values are mean ± SD of 4 independent experiments; One-way ANOVA and Tukey post-hoc test were used for multiple comparisons; ***$p < 0.001$; ns: non-significant; Luc: luciferase activity, Ren: renilla luciferase activity, T4: thyroxine, T3: triiodothyronine.

Supplementary figure 2. T4 increases the interaction of D2 with MARCH6 and with WSB1. (A) Scheme of the 3-FRET principle. D2-WSB1 and D2-MARCH6 interaction results in emission fluorescence at 561 nm and 514 nm, respectively. (B) Changes in emission fluorescence at 561 nm (left panel) and 514 nm (right panel) in the indicated conditions (10 µM T4) in HEK-293T cells. Values are mean ± SD of 27-35 independent experiments. Two-tailed Student's test was used for comparing between conditions. **: $p< 0.01$; ***: $p< 0.001$; FRET: Förster resonance energy transfer, ECFP: enhanced cyan fluorescent protein, EYFP: enhanced yellow fluorescent protein, WSB-1: WD repeat and SOCS box containing protein 1, MARCH6: membrane associated ring-CH-type finger 6, E_{FRET}: FRET energy, D2: type 2 deiodinase, T4: thyroxine.

Supplementary figure 3. *Usp33* mRNA hybridization shows high selectivity. (A) Hybridization shows high signal with antisense probe while sense probe barely resulted in any signal in the MBH. (B) Hybridization shows high signal with antisense probe while sense probe barely resulted in any signal in the cerebral cortex. MBH: mediobasal hypothalamus.

Supplementary figure 4. No wild-type *Usp33* mRNA is expressed in the cortex and hypothalamus of USP33KO mice. Isolated RNA was reverse transcribed and subjected to PCR with the following primers: sense on exon 3: GCGCTTGTCAGGACTGTAAAGTCA; antisense on exon 12: CCTATCGCACGTCAGACACTGTA. Wild type *Usp33* mRNA is not detectable either in the cortex or the hypothalamus of USP33KO mice, the 230 bp amplicon corresponds to the truncated *Usp*33 mRNA lacking exons 5 to 11, while the intact mRNA was readily detectable (1303 bp amplicon) in wild type mouse; USP33KO: ubiquitin specific peptidase 33 knock out animal, WT: wild type animal, *Usp33*: ubiquitin specific peptidase 33 mRNA, CTX: cerebral cortex, HT: hypothalamus.

Supplementary Table 1. Statistics of Fig. 3D,F,G,H. r^2: square of Pearson correlation coefficient.

Supplementary Table 2. List of Taqman probes

Supplementary Methods

Animals. Mice were housed in temperature-controlled (21 ± 1°C) facilities, with automated light and dark cycles of 12 h, fed ad libitum with chow diet, and studied at the indicated ages. THAI and Rax-CreErt2 strains were bred in-house, ZsGreen strain was obtained from The Jackson Laboratory (strain number: 007906), C57Bl6/J mice were acquired from Charles River Laboratories. Unless otherwise specified, 8-10-week-old male THAI mice were used throughout experiments. Hypothyroidism was induced by adding methimazole (MMI) (0.05%) to the drinking water and a low iodine diet (1). Some animals received an L-T4 treatment given at 1.7 and 1.9 µg/100g BW/day via gavage for 2 weeks (Fig. 1A). The other group received one *i.p* injection of 2.5 µg L-T4 and was sacrificed 8 hours later (Fig. 2A). For experiments involving stereotaxic surgery, we administered L-T4 in the lateral brain ventricle (Fig. 3A) at a concentration of 3 mg/ml in a volume of 2 µl over 2 minutes using our established method (2), and mice were sacrificed 6 hours later. The USP33KO C57Bl/6 mice lack the exons 5 to 11 of the USP33 gene and were obtained from GemPharmatech Co., Ltd. (Nanjing, China).

TaqMan Real-Time quantitative PCR. Total RNA was isolated from tissue samples with Qiagen RNEasy Mini kit. DNA contaminants were digested with DNase as previously described (3). 1 µg RNA was reverse transcribed with High Capacity Reverse Transcription kit (Applied Biosystems). cDNA concentration was determined with Qubit ssDNA assay (Invitrogen), and 10 ng cDNA was used in all Taqman qPCR reactions. The expression of *Luciferase*, *Dio3 and MsiI* was detected using a specific TaqMan probe as previously described (4-6) and compared with Glyceraldehyde 3-phosphate Dehydrogenase (*Gapdh*) or hypoxantine phosphoribosyl transferase 1 (*Hprt1*) reference genes featuring low variability within groups and brain regions (probe details are provided in Suppl. Table 2). Reactions were assayed on a Viia7 instrument (Applied Biosystems).

Determination of TSH, T3, and T4 concentrations. Under isoflurane anesthesia, blood samples (~1 mL) were taken from the heart ventricle using a heparinized syringe at the end of the treatment period (3-4 mice/group). Blood was centrifuged, and plasma was kept at -20 ºC. After extraction and purification of the plasma samples, TSH was measured by ELISA using a MILLIPLEXTM Rat thyroid panel (Millipore Corporation) and read on a Magpix (Millipore Sigma). T4 and T3 plasma concentrations were obtained by radioimmunoassay as previously described (7). To determine total tissue T4 concentrations in the hypothalamus and the cerebral cortex, samples were homogenized by sonication in 200 µl PBS and measured with an AccuLite CLIA Microwells kit (Monobind Inc.) on a Luminoskan Ascent instrument (Thermo Fisher Electron Corporation) according to the manufacturer's instructions. Tissue T4 levels were normalized with the tissue sample weight.

Determination of D2-mediated deiodination. D2 deiodination was assessed in cerebral cortex, striatum, hippocampus, hypothalamus, and cerebellum sonicates in the presence of 10 mM dithiothreitol, 0.25 M sucrose, and 1 nM ^{125}I-T4 (PerkinElmer); 1 mM propylthiouracil was added to inhibit potential D1 activity and 10 nM T3 to saturate D3 (8). For assays in primary hypothalamic tanycytes and cortical astrocytes, cells (grown as described below) were washed twice with PBS, harvested, sonicated in 0.25 M sucrose in PE buffer (0.1 M phosphate-buffered saline (PBS), 1 mM ethylenediaminetetraacetic acid), and processed for D2 assay. Essentially, 100 µg protein sonicate was incubated at 37°C in the presence of 10 mM dithiothreitol, 0.25 M sucrose,

1 mM propylthiouracil, 100 nM T3 and 1 nM T4 (approximately 100.000-250,000 cpm ^{125}I-T4) for 3 to 6 hours (for tissue or cell pellets, respectively). The reaction was stopped by moving the tubes to ice-cold water and adding a 1:1 volume of ice-cold methanol (tissue samples) or to normal horse serum: 50% trichloroacetic acid (cells) (1). The amount of radioactive iodine (from ^{125}I-T4 deiodination) was measured in a 2470 Automatic Counter Wizard2 (Perkin-Elmer).

Primary cultures of tanycytes and astrocytes. Tanycytes were cultured as described with modifications (9). Cells were isolated from postnatal day 10 (P10) mice as indicated on the figures; pups were decapitated followed by brain dissection. The mediobasal hypothalamus (MBH) was isolated and enzymatically digested using trypsin-DNase. Cells obtained from the MBH of 3-4 pups were seeded on poly-L-lysine coated 3.5 cm tissue culture plate in thrombin-free tanycyte culture medium (DMEM/F12 1:1 without Phenol red, 0.18 UI/ml insulin, bovine serum albumin 50ug/ml, transferrin 12.5 µg/ml 40 µg/ml gentamicin sulfate salt). 3 days after seeding, the medium was changed to 1.25 UI/ml thrombin-containing culture medium. Experiments were performed after 7 days in culture of (~80 % confluency) after treatment with 10 uM forskolin (Merck) in serum-free media for 4 hours. Thyroid hormones, MG132 and 2,6-Diamino-3,5-dithiocyanopyridine (PR-619) were obtained from Merck.
Astrocytes were isolated from P1-3 mice. The pups were decapitated, the brain dissected, and meninges and blood vessels were removed. The cerebral cortices were collected in ice cold 2.5 µg/ml amphotericin containing 1XPBS. Tissue pieces were washed with 37 ^{o}C 1X PBS 3-times with brief centrifugation at 240 RCF after each wash and were enzymatically digested using trypsin-DNAse (10 minutes at 37 ^{o}C). To obtain a single-cell suspension, 10 ml MEM was added to stop digestion followed by vigorous trituration with a 5 ml serological pipette. Cells were pelleted by centrifugation for 10 minutes at 160 RCF at room temperature, resuspended in fresh culture medium (MEM, 10 % FBS, 0.2M L-glutamine, 2.5 µg/ml amphotericin B, 40 µg/ml gentamicin) and seeded at poly-L-lysine coated 6 cm diameter tissue culture dishes. Tissue samples of two pups were seeded on one plate. Cells were incubated for 7 days with medium changes every 72 hours. Experiments were done when the cells reached a confluency of 80% (after ~7 days in culture) after treatment with 10 uM forskolin (Merck) in serum-free media for 4 hours.

Fluorescent *in situ* hybridization combined with immunofluorescence. Adult male C57Bl6/J (15-week-old) mice were anesthetized with ketamine-xylazine and then decapitated. The brains were rapidly removed, frozen in powdered dry ice, and stored at -80°C until sectioning. Serial 16 µm thick whole brain coronal sections at the level of the MBH were obtained on cryostat (Leica Microsystems, Austria), thaw-mounted on Superfrost Plus glass slides (Fisher Scientific Co.), air-dried and stored at -80°C until used. Sections were hybridized with a digoxigenin-labeled antisense Usp33 riboprobe, corresponding to 414-1306 bases of the mouse *Usp33* mRNA (GenBank #NM_133247) as previously described (10). Post-hybridization, sections were treated with 0.5% Triton X-100/0.5 % H2O2 in PBS for 15 min, rinsed in PBS, immersed in maleate buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5; 10 min), and in 1 % blocking reagent for nucleic acid hybridization (Roche Applied Sciences). The sections were incubated overnight in peroxidase-conjugated sheep anti-digoxigenin antibody Fab fragments (Roche, diluted 1:100 in 1% blocking reagent) using CoverWell incubation chambers (Grace Bio-Labs Inc.). The hybridization signal was amplified with the TSA Plus Biotin Kit (Akoya Biosciences) for 30 min, using the reagent at 1:500 dilution in 0.05M Tris containing 0.01% H2O2. The biotin deposits were detected with Alexa Fluor 488-conjugated Streptavidin (ThermoFisher, 1:500). Sections were then incubated

overnight with a rabbit monoclonal antibody against vimentin (ThermoFisher, 1:4000) or with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (Millipore, 1:4000). The primary antibodies were detected with Alexa Fluor 555-conjugated anti-rabbit or anti-mouse IgG, respectively (ThermoFisher; 1:500). Sections were coverslipped with SlowFade Diamond mounting medium containing DAPI. Images were obtained with a Zeiss LSM 780 confocal microscope and analyzed with Zen 2012 and Adobe Photoshop software (Adobe System Inc., USA).

Förster Resonance Energy Transfer (FRET). To examine the interaction between the proteins D2, WSB1 and MARCH6, we modified previously established protocols (11, 12). HEK-293T cells were plated into 35-mm glass-bottom dishes and transfected with equal amounts of plasmids for D2, WSB1, and MARCH6 using X-tremeGENE HP reagent (Roche) following the manufacturer's instructions. The next day, the media was changed to complete media containing charcoal-stripped FBS. Two days after transfection, FRET imaging was performed using a Nikon A1R laser scanning microscope equipped with a Tokai Hit stage top incubator (37 °C and 5 % CO2). The interactions between D2 WSB1 and MARCH6 were calculated using the sequential acceptor photobleaching method (12).

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