

A UCP1-IRES-Cre knock-in mouse enables specific brown adipocyte targeting without CNS off-target expression

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Abstract

Uncoupling protein 1 (UCP1) is predominantly expressed in brown adipocytes and plays a central role in thermogenesis and energy metabolism. BAC-transgenic UCP1-Cre mouse lines have been widely used to target thermogenic adipocytes, but ectopic Cre activity in the hypothalamus and peripheral organs has raised concerns regarding specificity. Here, we characterize a novel knock-in UCP1-IRES-Cre mouse model that drives Cre expression from the endogenous UCP1 promoter. UCP1-IRES-Cre mice were crossed with Ai14 reporter mice to visualize Cre-dependent tdTomato expression across adipose depots, brain regions, and peripheral tissues and compared with UCP1 protein localization. Reporter expression in male and female UCP1-IRES-Cre mice closely mirrored endogenous UCP1 expression in brown and white adipose tissue depots, with substantially reduced ectopic activity compared with previously reported BAC-transgenic models. Very limited reporter signal was observed in the hypothalamus and peripheral organs, although robust reporter expression was detected in the choroid plexus despite absent UCP1 protein staining. Surface image analysis in various adipose depots demonstrated generally broader reporter labeling than UCP1 immunoreactivity, consistent with transient or prior UCP1 promoter activation during development or adult life. Together, these findings demonstrate that the UCP1-IRES-Cre model faithfully recapitulates endogenous UCP1 expression while minimizing off-target Cre activity and provides a knock-in alternative for genetic targeting of thermogenic adipocytes.

Keywords UCP1 · Adipose tissue · IRES-Cre · BAC-transgenics · Hypothalamus

Introduction

Brown adipose tissue (BAT) is an important thermogenic and energy-expending tissue that is present in all mammals, including humans. Brown adipocytes are distinguished from energy-storing white adipocytes by their expression of uncoupling protein-1 (UCP1), a mitochondrial protein that bypasses ATP synthesis and instead releases energy as heat. UCP1 serves as a defining molecular marker for brown and beige adipocytes and their role in thermogenesis^{1–3}.

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Joshua Meyer and Jaclyn Williams contributed equally to this work.



Recent validation studies of the BAC Tg(UCP1-Cre)Evdr/J mouse line showed robust ectopic Cre activity in the central nervous system, including the hypothalamus, even though endogenous Ucp1 expression is at or near the limit of detection in the adult brain⁸⁻¹¹. Cre activity was also reported in peripheral tissues, including kidneys and adrenal gland, indicating that recombination driven by the transgene is not restricted to thermogenic adipose tissue^{8,9}. Other genetic studies suggested that the Tg(UCP1-Cre)Evdr/J line may harbor transgene-associated chromosomal rearrangements that could also contribute to off-target recombination⁸. Together, these findings highlight the potential limitation of transgenic mouse models, particularly BAC-based constructs, which may be more susceptible to off-target recombination than knock-in strategies¹².

Results

We assessed the accurate expression of Cre activity from UCP1-IRES-Cre mice by crossing them with Ai14-tdTomato reporter mice (Fig. 1). Both males and females were analyzed for UCP1 protein and Cre-dependent tdTomato reporter expression. In core iBAT (cBAT), UCP1-expressing brown adipocytes are easily distinguishable from the surrounding white adipocytes due to their increased cytoplasmic content in H&E images (Fig. 2, A1-D1) and aligns with UCP1 protein signals (Fig. 2, A2-D2). Cre-induced tdTomato labeling overlapped UCP1 labeled areas in both male and female mice but was absent in controls (Fig. 2, A3-D3). This data overall demonstrates that Cre-induced tdTomato reporter expression generally corresponds with UCP1 expression in cBAT.

A.  UCP1-IRES-Cre mice

UCP1 promoter

E1 E2 E3 E4 E5 E6

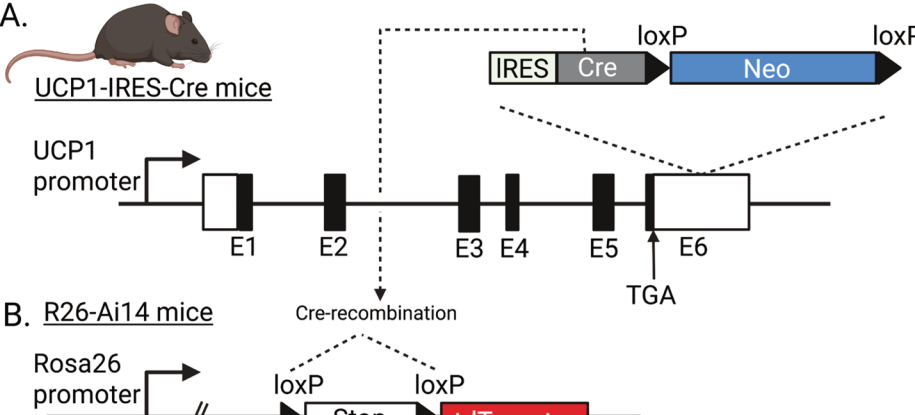
IRES Cre loxP Neo loxP

TGA

B. R26-Ai14 mice

Rosa26 promoter

loxP Stop loxP tdTomato



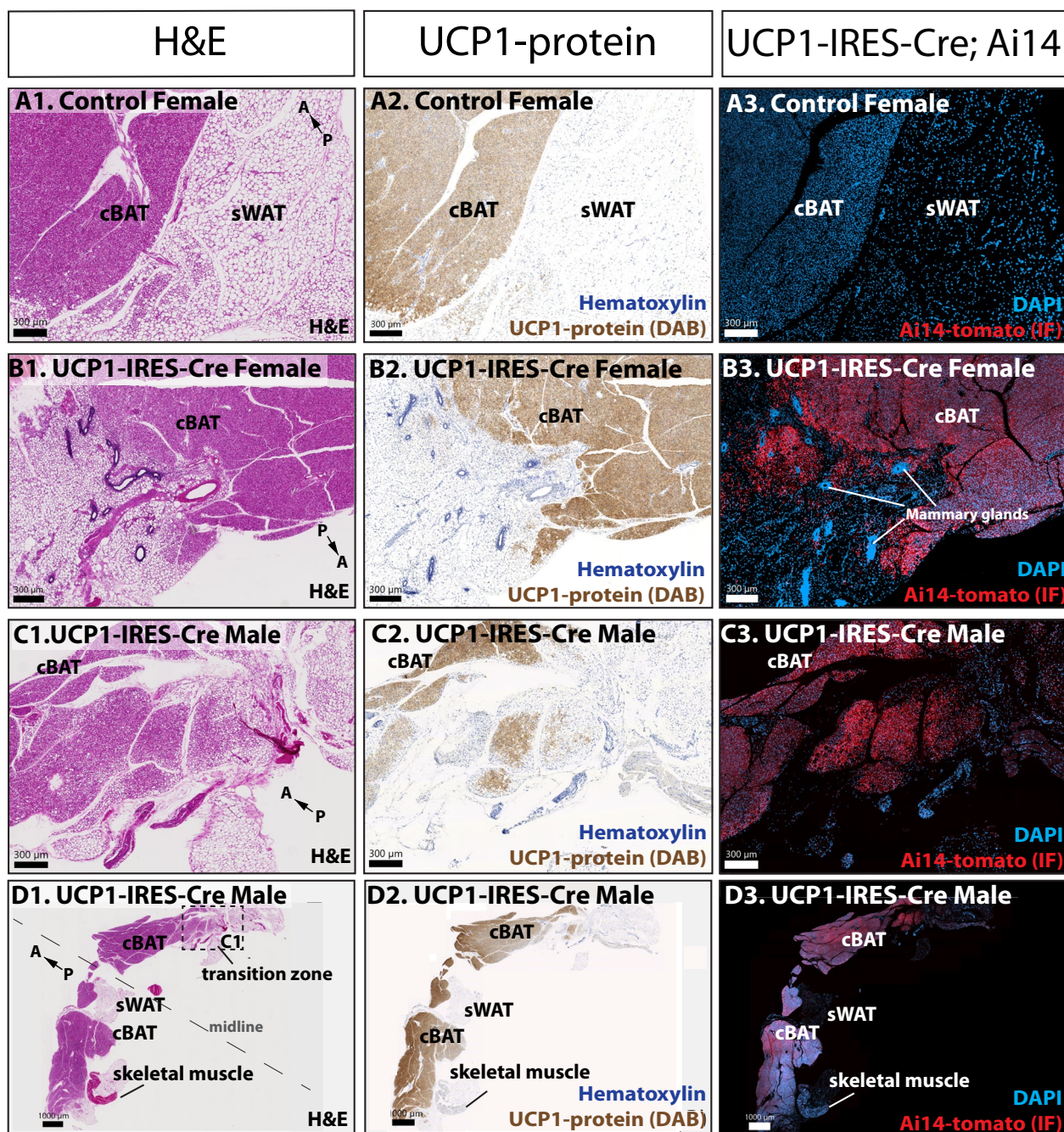


Fig. 2 UCP1-reporter expression reflects UCP1-protein expression in iBAT. Paraffin-embedded 5 μ m cross-sections spanning the full bilateral extent of the interscapular brown adipose tissue (iBAT) depot were stained with hematoxylin/eosin (H&E) (**A1-D1**), UCP1 peptide (DAB brown) with hematoxylin counterstain (blue) (**A2-D2**), or anti-RFP detection of Ai14-tdTomato reporter (red) with DAPI (blue) (**A3-D3**). **A1-C1**: Higher-magnification images from core BAT (cBAT) and surrounding WAT (sWAT) from a control female, a UCP1-IRES-cre female (**B1-B3**) and a UCP1-IRES-cre male (**C1-C3**). **D1-D3**: Low-magnification overview of the iBAT depot from a male UCP1-IRES-cre mouse showing midline, adjacent skeletal muscle, and anterior (A) and posterior (P) to show orientation. **A1-D1**: H&E staining distinguishes dense, multilocular brown adipocytes in cBAT from unilocular white adipocytes in sWAT. **A2-D2**: UCP1 protein labeling is prominent in cBAT but absent in sWAT. **A3-D3**: Ai14-tdTomato is absent in control mice (**A3**) but robustly labels cBAT in UCP1-IRES-Cre mice in females (**B3**) and males (**C3, D3**). Note the higher DAPI signal density in cBAT compared to sWAT, reflecting differences in adipocyte size and density. Scale bars are 300 μ m (**A-C**) or 1000 μ m (**D1-D3**). Anterior (A) and posterior (P) tissue orientations are indicated by arrows.

Upon closer inspection of the transition zone from cBAT to surrounding white adipocytes, we found that many adipocytes without a UCP1 protein signal were positive for the tdTomato reporter signal. This discrepancy is consistent with prior UCP1 expression, indicating the presence of extensive “pre-beige” adipocytes, e.g., during development or postnatal/early adult life cold-stimuli^{13,14}, which would induce irreversible tdTomato expression.

A quantitative surface image analysis across the full extent of the unilateral iBAT depot, including cBAT, transition zone and surrounding WAT (Supplementary Fig. 1), confirmed that the tdTomato positive area was significantly greater than the UCP1-positive area across the iBAT of UCP1-IRES-Cre mice (Supplementary Fig. 1B4; paired two-tailed t-test, Cre $n=5$; $P=0.0085$). Importantly, the UCP1-positive area across the iBAT depot, as well as Ucp1 mRNA quantification in cBAT in a separate experiment, were comparable between genotypes (Supplemental Fig. 1E, 1G, unpaired two-tailed t-test, E: WT $n=4$; Cre $n=5$; $P=0.7999$, G: WT $n=5$; Cre $n=4$; $P=0.4638$), suggesting that the IRES-Cre knock-in does not substantially alter endogenous Ucp1 expression. Further, we confirmed that Cre mRNA expression was, as expected, several-fold higher in cBAT of UCP1-IRES Cre mice compared to WT mice Supplementary Fig. 1H; unpaired two-tailed t-test, WT $n=5$; Cre $n=4$; $P=0.0394$).

We also compared the total adipose tissue area analyzed and found no significant effects of labeling, genotype or labeling x genotype interaction in iBAT (Supplemental Fig. 1F; WT $n=4$, Cre $n=5$; repeated measure two-way ANOVA: labeling effect, $P=0.8851$; genotype effect, $P=0.8449$; labeling x genotype interaction, $P=0.2569$), supporting that differences in positive area were not attributable to unequal tissue sampling.

Cre expression is more widespread than adult UCP1 expression in iWAT

In iWAT (Fig. 3) the existence of anatomically defined regions with beige islands, where clusters of UCP1-expressing, multilocular beige adipocytes and dense sympathetic innervation is found are well documented^{13,15–18}. Interestingly, we found only a few sites with UCP1 protein expression in both wildtype and UCP1-IRES-Cre mice (Fig. 3, A2-D2, Supplemental Fig. 2), which was in striking contrast to the widespread tdTomato reporter signal (Fig. 3, A3-D3) in a pattern that mirrored the expected location of beige islands proximal to the iWAT lymph node and further down the inguinal extension of iWAT^{13,15–18}. We observed more tdTomato-positive adipocytes than UCP1 immunopositive cells (Fig. 3, C2-C3). The tdTomato signal in adipocytes extended well beyond the region with detectable UCP1 protein, similar to our observation in the iBAT transition zone (Supplemental Fig. 1). As expected, no tdTomato signal was detected in control iWAT ($n=4$) (Fig. 3, A3, B3), supporting the interpretation that tdTomato-positive adipocytes represent adipocytes with prior UCP1 expression.

We further quantified the full extent of unilateral iWAT cross-sections for UCP1 positive areas and show no difference between WT and UCP1-IRES-Cre mice, and similarly the total adipose tissue area analyzed was comparable between groups (Supplemental Fig. 2C, D, unpaired t-test, WT=4, Cre=5; C: $P=2667$; D: $P=0.9915$).

Very limited ectopic reporter expression in the brain

Because the Tg(UCP1-Cre)Evdr/J mouse model showed strong ectopic reporter expression in the hypothalamus and choroid plexus, we investigated the expression levels in control and UCP1-IRES-Cre mice (Fig. 4). Overall, tdTomato expression in the brain was limited to a prominent signal in the choroid plexus and a small number of discrete neuronal populations in a subset of UCP1-IRES-Cre mice (Fig. 4A2, B2, E2) compared to no signal in control mice (Fig. 4, A1, B1 E1). We further verified that endogenous UCP1 protein is not expressed in the adult choroid plexus (Fig. 4, A3), so the Cre-induced reporter signal may be due to UCP1 expression during development or prior to tissue harvest, which would maintain reporter expression once Cre recombination occurs. There was no reporter signal in the ventromedial hypothalamus (VMH) (Fig. 3, C1,C2), although in two female mice, we found a cluster of reporter-positive neurons in the dorsomedial hypothalamus (DMH) (Fig. 4E), while two other UCP1-IRES-Cre brains (one male and one female) exhibited minimal reporter-positive neurons. A more caudal section (Fig. 4D) within the hypothalamus of the same brains shows minimal tdTomato reporter expression

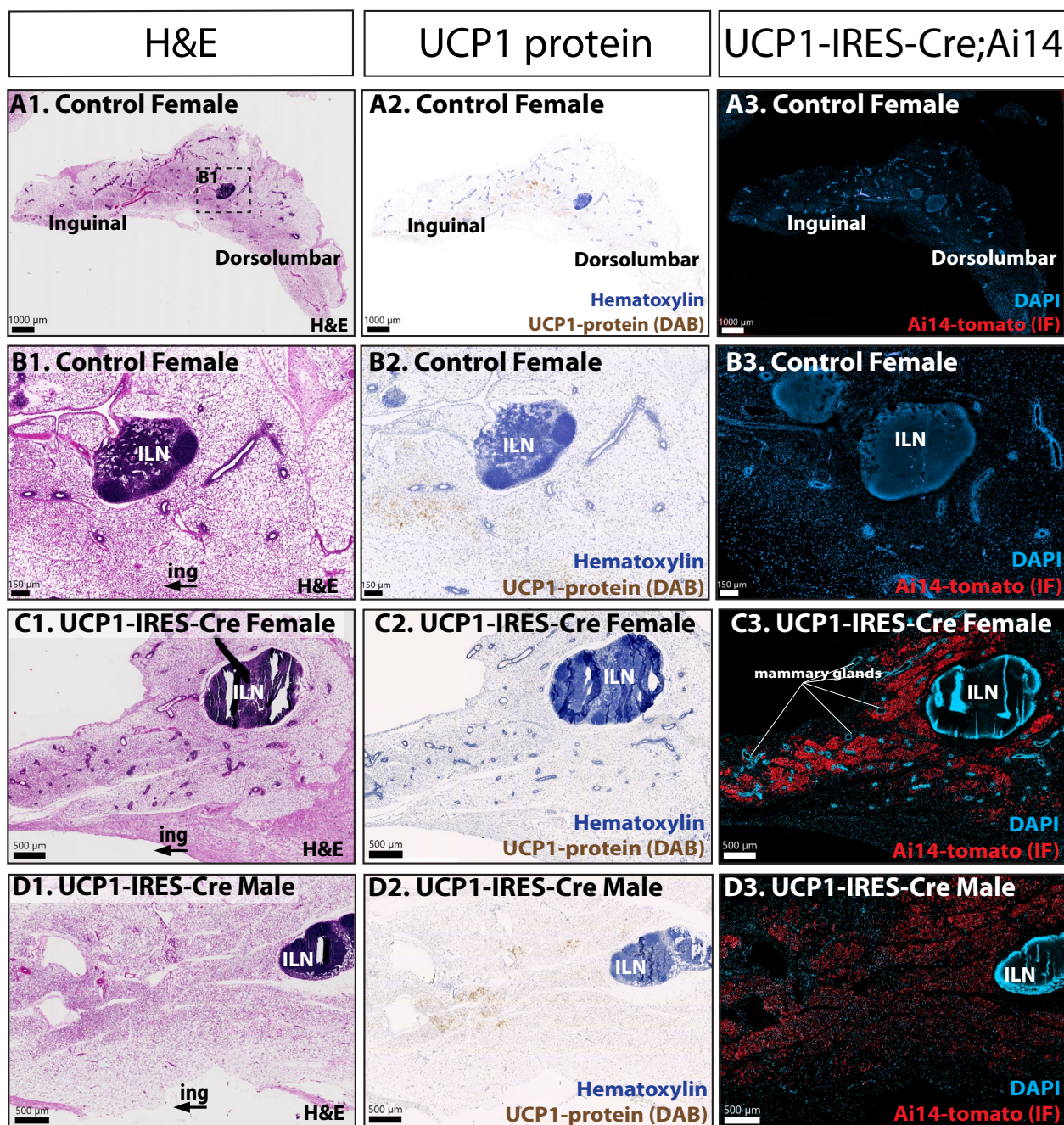
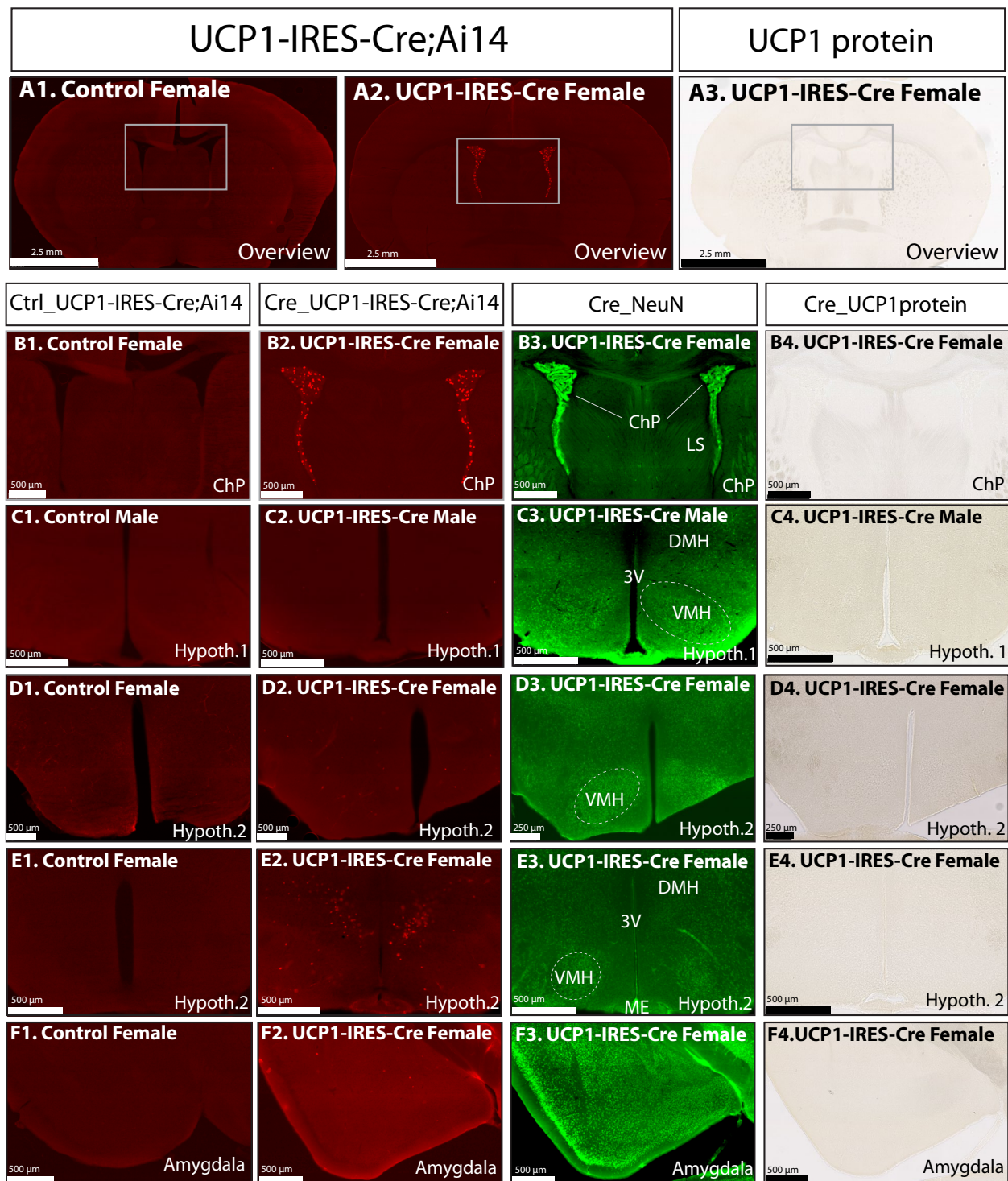


Fig. 3 UCP1-reporter expression is more expansive than UCP1-protein expression in iWAT. Paraffin-embedded 5 μ m cross-sections spanning the full unilateral extent of the inguinal white adipose tissue (iWAT) (A, B, C, D) mice was processed for H&E staining (A1-D1), UCP1 peptide (DAB brown) with hematoxylin (blue)(A2-D2) or anti-RFP detection of Ai14-tdTomato (red) with DAPI (blue) (A3-D3). A-D: Images sampled near the inguinal lymph node (ILN) as an anatomical landmark (A, B, D), arrows indicate the direction of the inguinal (ing) portion of the depot for tissue orientation. A2-D2: UCP1 protein expression was low or undetectable in iWAT across mice, including regions with increased cellular density and suggestive of beige islands. A3-D3: Ai14-tdTomato expression was absent in control mice but robustly detected in all UCP1-IRES-cre mice. Reporter-positive labeling in some cases overlapped with denser eosin-stained regions observed by H&E, despite minimal UCP1 peptide detection at the time of analysis, consistent with prior or transient UCP1 expression in these adipocytes. Scale bars are 700 μ m (Panel A) 500 μ m (Panel C, and D)) or 100 μ m (D1-D3).



and confirms the anatomical restriction of the positive cluster. Finally, neither controls nor UCP1-IRES-Cre mice exhibited tdTomato reporter signals or UCP1 protein expression in the amygdala (Fig. 4F). However, the few sites with reporter signals were observed in only UCP1-IRES-Cre mice and indicated some expression in choroid plexus cells at some point during the life of the mice. We additionally evaluated tdTomato reporter expression in the parafascicular thalamic nucleus (PaF) as a direct comparison with previous reports in Tg(UCP1-Cre)Evdr/J mice (Supplemental Fig. 3)⁹. We observed tdTomato expression in half ($n = 2$) of the UCP1-IRES-Cre brains (n

Fig. 4 Very limited ectopic reporter expression in the brain of UCP1-IRES-Cre mice. Coronal 30 μ m brain sections show endogenous reporter expression (Ai14-tdTomato, red) in control mice (**A1-F1**) or UCP1-IRES-cre mice (**A2-F2**). Adjacent sections were stained for the neuronal marker NeuN (green, **B3-F3**) or UCP1-peptide (**A3, B4-F4**). **A1-A2**. Low-magnification overview ($\sim$ Bregma +0.1 mm) depicting the choroid plexus (ChP) shows no reporter signal in control mice (**A1**), while UCP1-ires-cre mice (**A2**) exhibit prominent tdTomato labeling in the ChP despite the absence of detectable UCP1 protein. **B1-B2** Higher-magnification images of the ChP confirming tdTomato labeling. **C1-C2**: Control and UCP1-IRES-Cre sections of a anterior hypothalamus ($\sim$ Bregma – 1.5 mm) showed no tdTomato reporter or UCP1 protein expression. **D2-D4**: A posterior hypothalamic level ($\sim$ Bregma – 1.9 mm) from a UCP1-IRES-Cre mouse showed minimal tdTomato labeling and no detectable UCP1 protein. **E2-E4**: An adjacent posterior hypothalamic level ($\sim$ Bregma – 2.1 mm) from the same animal similarly showed minimal reporter expression, but with scattered tdTomato-positive neurons observed bilaterally in the dorsomedial hypothalamus (DMH). UCP1 protein was not detected in this region. **F1-F2**: The amygdala showed no reporter expression in controls or UCP1-IRES-Cre mice. Scale bars are 2.5 mm (**A1-A3**) or 500 μ m (**B1-F4**). *3rd ventricle (3 V), median eminence (ME), choroid plexus (ChP), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH)*.

= 4) (Supplemental Fig. 3). Interestingly, the same two animals with more localized tdTomato expression in the DMH (Fig. 4E) also showed expression in the PaF.

Minimal or no ectopic expression in heart, liver, pancreas, kidney/adrenal tissue

We analyzed heart, liver, pancreas, and kidney tissue for UCP1 and reporter expression. UCP1 was found in adipocytes located around those organs, specifically heart, kidney and adrenal glands (Fig. 5). In the myocardium, we did not observe UCP1 reporter expression; however, in the surrounding heart-associated white adipose tissue (hWAT) tdTomato reporter expression (Fig. 5, A3) corresponds with UCP1 protein expression (Fig. 5, A4). Across all liver (Fig. 5, B1-B4), pancreas (Fig. 5, C1-C4) and adrenal tissues (Fig. 5, D1-D4), neither tdTomato nor UCP1 protein expression was observed in Cre-positive or control mice, supporting minimal off target reporter activity. In the kidney (Fig. 5, D1-D4), a noticeable but scattered tdTomato signal was observed within the renal medulla and cortex. The surrounding renal adipose tissue showed tdTomato signals (Fig. 5, D3) as a truthful representation of UCP1 protein-positive adipocytes (Fig. 5, D4), and “pre-beiged” adipocytes with tdTomato signal but lacking UCP1 protein expression were also identified. Again, we found large portions of pre-beiged tdTomato-positive adipocytes that lacked endogenous UCP1-protein labeling, similar to the pattern found in iBAT and iWAT. Importantly, all tissues analyzed in this group (experimental and control) showed minimal leakiness of IRES-UCP1 reporter expression.

A quantitative surface image analysis across perirenal adipose tissue (Supplementary Fig. 1C1-D4, I, L) confirmed that the tdTomato positive area was significantly greater than UCP1-positive area labeling (Supplemental Fig. 1D4; paired two-tailed t-test, Cre $n=5$; $P=0.0085$) in UCP1-IRES-Cre mice. In contrast, UCP1-positive areas were comparable across genotypes at room temperature (Supplemental Fig. 1I; unpaired two-tailed t-test, WT $n=4$, Cre $n=5$; $P=0.2473$), indicating that IRES-Cre knock-in does not substantially change endogenous UCP1 protein labeling.

Because these were corresponding serial sections, we further compared the total adipose tissue area analyzed and found no significant effects of labeling, genotype or labeling x genotype interaction in perirenal adipose tissue (Supplemental Fig. 1J; WT $n=4$, Cre $n=5$; repeated measure two-way ANOVA: labeling effect, $P=0.1677$; genotype effect, $P=0.1387$; labeling x genotype interaction, $P=0.2280$), confirming the difference in positive area was not attributed to uneven tissue sampling.

UCP1 reporter expression is also found in the ovary and testis

UCP1 reporter expression was examined in female and male reproductive tissues to assess Cre activity (Supplementary Fig. 4). In the female control mice, the ovary, oviduct and surrounding white adipose tissue did not show UCP1 protein or tdTomato signal (Supplementary Fig. 4, A1-3). In contrast, the UCP1-IRES-Cre females showed tdTomato labeling within the ovarian tissues (Supplementary Fig. 4, B3), concentrated within the ovarian

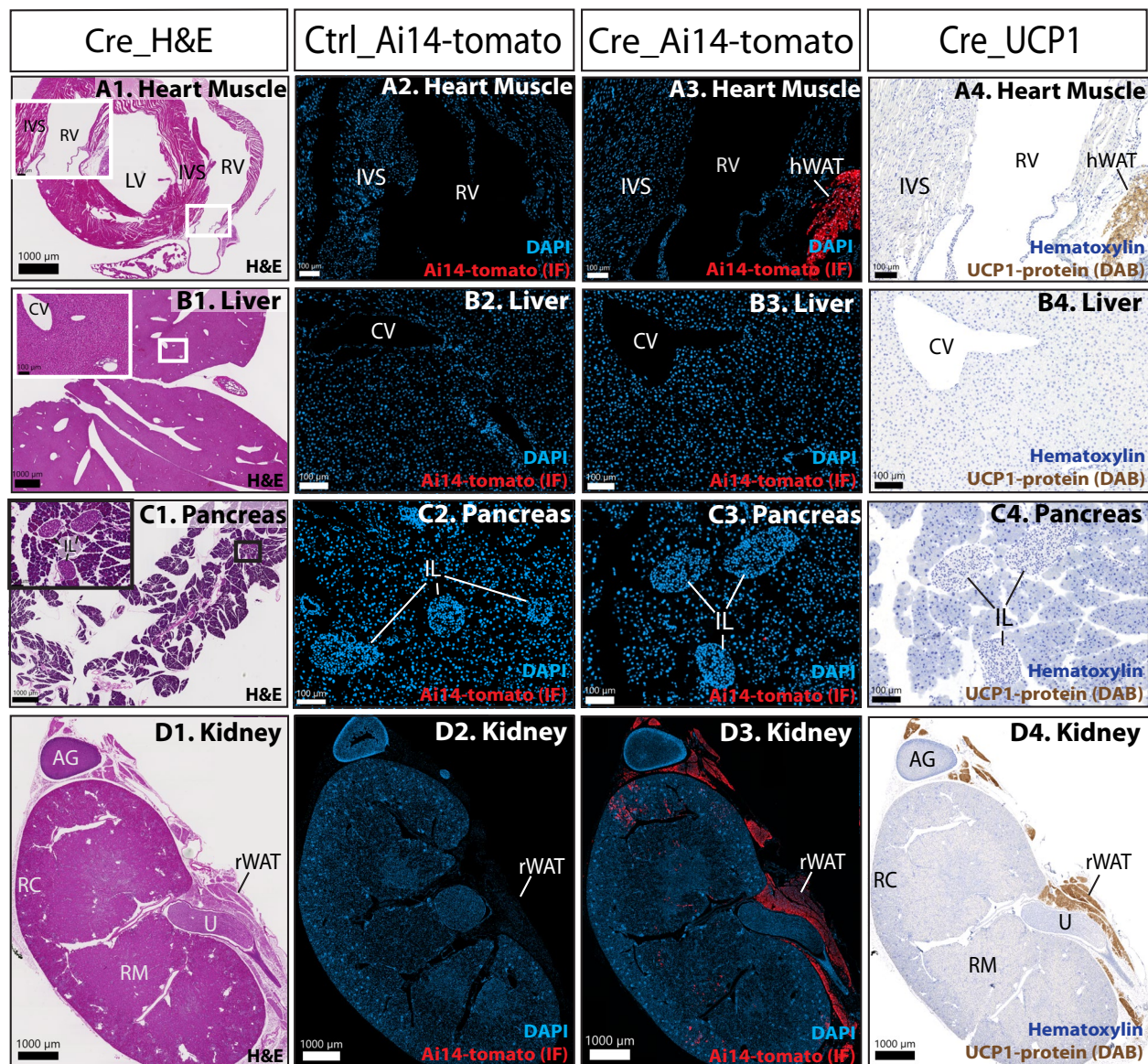


Fig. 5 Lack of UCP1-reporter expression in muscle, liver, kidney, and pancreas. Paraffin-embedded 5 μ m sections of heart (A1-A4), liver (B1-B4), pancreas (C1-C4), and kidney (D1-D4) were processed for H&E stain (A1-D1), anti-RFP detection of Ai-tdTomato (red) with DAPI (A2-D2) or UCP1-DAB protein stain with hematoxylin (A3-D3). Low magnification overviews show characteristic anatomical landmarks for each tissue (heart: IVS, LV, RV; liver: CVs; pancreas: ILs; kidney RC, RM, U, AG) with boxed regions shown at higher magnification insets. Ai14-tdTomato reporter expression was absent in cardiac muscle, liver, pancreas and kidney parenchyma of both controls and UCP1-IRES-cre mice, consistent with the lack of detectable UCP1 protein in these tissues. In contrast, adjacent adipose tissue depots exhibited strong reporter expression such as heart-associated WAT (hWAT, A2) and renal WAT (rWAT, D3) showed prominent Ai-tdTomato labeling together with detectable UCP1 protein (D4), consistent with their classification as beige fat depots. Rare scattered tdTomato-positive cells were consistently observed in the kidney of all UCP1-IRES-cre mice (D3), whereas no signals were observed in the adrenal gland or ureter. *interventricular septum (IVS), left and right ventricles (LV, RV), central veins (CV), islets of Langerhans (IL), renal medulla (RM) and renal cortex (RC), ureter (U), adrenal gland (AG).*

follicles including cells in the secondary and pre-antral follicles. However, there was no indication of Cre-activity in oocytes, consistent with limited germline recombination (Supplementary Fig. 4E).

In male mice, UCP1 protein was not detected in the testis or caput epididymis in either control or UCP1-IRES-Cre mice (Supplementary Fig. 4, C2-D2). However, UCP1-IRES-Cre males showed robust tdTomato reporter

expression throughout the testis and caput epididymis (Supplementary Fig. 4, D3). Reporter expression in the testis corresponds to cells within the seminiferous tubules and suggested stage-restricted labeling, with no labeling in the basal spermatogonia but strong labeling in elongating spermatids (Supplementary Fig. 4F). Accordingly, use of this model requires careful breeding strategies, and UCP1-IRES-Cre males are not recommended for breeding to avoid unintended germline recombination. Unlike other adipose tissues, the gWAT showed no signs of “pre-beiged” adipocytes and lacked UCP1 protein or tdTomato reporter labeling (Supplementary Fig. 4, A2,B2), which aligns with the limited potential for beiging in this adipose tissue depot^{19–22}.

Discussion

Cre-driver lines generated by BAC transgenesis have been widely used to understand the biological roles of adipose tissue and thermogenic adipocytes^{1,2}. The utility of these models has been called into question by findings of Cre activity in non-adipose tissues, including the brain and other peripheral tissues^{8,9}. Because hypothalamic circuits may impact thermogenesis and energy balance, any ectopic recombination in the CNS complicates the interpretation of BAT-specific genetic manipulations. These findings underscore the need for improved genetic tools that faithfully reflect endogenous UCP1 expression while minimizing ectopic recombination. In this study, we demonstrate that the UCP1-IRES-Cre knock-in mouse model faithfully labels brown and beige adipocytes, with greatly reduced off-target Cre activity, providing an alternative tool for studying thermogenic adipocyte biology.

We examined the distribution of UCP1 and Cre activity throughout the body of UCP1-IRES-Cre; tdTomato-Ai14 reporter mice. tdTomato reporter distribution and UCP1 protein localization closely corresponded across adipose tissues. Interestingly, tdTomato expression was more widespread than endogenous UCP1 protein expression in both iBAT transition zones, perirenal WAT and iWAT. The adipocytes without a detectable UCP1 signal, but Cre-induced reporter expression is consistent with prior UCP1 expression during development, postnatally or at some point in their adult life. Lineage tracing of adipocytes have established that beige adipocytes derive *de novo* from differentiation of progenitors or by conversion from mature white to brown adipocytes^{2,23}. Transient UCP1 expression during development, that is avoided by adult onset Cre-activation, has been reported in non-adipose tissue¹⁰. Also, spatially restricted regions with increased beiging potential have been identified in the iBAT transition zone, the peri-lymph-node region and the inguinal portion of the iWAT depot. These regions show dynamic, cold-induced changes with multilocular adipocytes, increased UCP1 expression and sympathetic innervation^{13,15–18}. Together, these findings suggest the existence of genetically predisposed, pre-beiged adipocytes in anatomically defined regions. Indeed, UCP1 expression and adipose tissue innervation peaks during postnatal development of iWAT¹³ and beiging can be reversed through “whitening”²⁴, consistent with early developmental beiging that may be lost in adulthood dependent on environmental stimuli. Thus, Cre expression throughout the transition zone between core BAT and surrounding WAT, perirenal WAT and iWAT depots likely reflects “legacy” activation during postnatal development rather than ongoing thermogenic identity. Quantitative histological analysis demonstrated that areas with tdTomato-positive labeling were significantly greater than areas with UCP1-positive labeling in both interscapular BAT and perirenal adipose tissue. Nevertheless, we note the existence of many “never-beiged” adipocytes that lack UCP1 and tdTomato signals. Similarly, gWAT exhibited minimal UCP1 protein and tdTomato reporter expression, suggesting that gWAT lacks prior beiging episode in contrast to iWAT and is in line with many reports highlighting limited beiging capacity when compared to subcutaneous adipose depots^{19–22,25}.

Importantly, we observed substantially less ectopic reporter expression in the brain compared to the Tg(UCP1-Cre)Evdr/J line. We confirmed a robust tdTomato signal in the choroid plexus, while only sparse tdTomato labeling was found in scattered neurons of the DMH and PaF, and no UCP1 protein expression was detected in those regions, suggesting potential UCP1 expression during development. This contrasts with Tg(UCP1-Cre)Evdr/J mice, in which strong Cre activity has been observed across multiple brain regions, including the DMH

and VMH⁹, both key CNS areas involved in regulating energy homeostasis and BAT thermogenesis^{20–22,25,26}. While Ucp1 mRNA has been detected at low levels in single-cell RNA seq datasets and histological analysis of the choroid plexus⁹, the Tg(UCP1-Cre)Evdr/J and Ucp1-CreERT2;NuTRAP strains have demonstrated that Ucp1 promoter activity is not strictly confined to thermogenic adipose tissues^{9,10}.

In contrast to the recent characterization of the Tg(UCP1-Cre)Evdr/J line⁹, which reported off-target recombination in peripheral tissues including the kidney and adrenal glands, tdTomato reporter expression of our UCP1-IRES-Cre line was largely restricted to adipose tissues. There was sparse reporter expression in the renal medulla and cortex, while other peripheral tissues, including pancreas, liver, heart, and adrenal glands, lacked tdTomato reporter signal. In contrast with Ucp1-CreERT2 mice¹⁰, tdTomato reporter expression was not observed in the iWAT mammary glands UCP1-IRES-Cre mice. The absence of tdTomato reporter expression across metabolically active peripheral tissues supports the conclusion that recombination driven by the UCP1-IRES-Cre line reduces potential confounding effects in future metabolic studies.

Because UCP1-IRES-Cre is a constitutively active Cre line, the tdTomato reporter expression reflects historical Ucp1 promoter activity and cannot be completely distinguished between developmental and active expression, particularly in iWAT and reproductive tissues. Consistent with the recent characterization of Ucp1-iCre mice¹¹, tdTomato reporter expression was identified in both male and female reproductive tissues. These findings likely reflect low level transient endogenous Ucp1 promoter activity in male germ cells during spermatogenesis, resulting in permanent lineage labeling of elongating spermatids and sperm rather than thermogenic identity^{9,11,27}.

This limitation should be considered when designing experiments. However, ectopic recombination is markedly reduced compared to BAC transgenic lines. Off-target recombination in earlier models may have confounded interpretations of BAT-specific genetic manipulations such as food intake, energy expenditure and thermoregulation which are strongly influenced by central neuronal pathways regulating sympathetic communication with adipose tissue^{8,9,28}.

A limitation of this current study is that quantification was performed only under room temperature conditions, which represents a mild cold exposure for mice. At room temperature endogenous Ucp1 protein labeling or mRNA expression in UCP1-IRES-cre mice remained comparable to wildtype mice in iBAT, perirenal WAT and iWAT. Our work cannot rule out the possibility that Cre recombination under more extreme challenges, such as cold exposure, may not maintain normal UCP1 expression. Further studies would need to confirm that the knock-in allele maintains normal UCP1 induction and beige adipocyte recruitment in adipose tissue also during select metabolic challenges.

The knock-in design of the UCP1-IRES-Cre places Cre recombinase under the control of the endogenous Ucp1 locus and contributes to the improved tissue specificity observed relative to BAC transgenic models. In conclusion, the UCP1-IRES-Cre line described here supports consistent and specific genetic manipulation of thermogenic adipocytes with minimal confounding effects from ectopic recombination and represents an improved genetic tool for studying thermogenic tissue biology.

Materials and methods

Mouse generation and husbandry. Mice were bred and housed at Pennington Biomedical Research Center (PBRC) under standard conditions. The UCP1-IRES-Cre mouse line was originally generated in the laboratory of the late Dr. Randall Mynatt at PBRC but has not been previously described in the literature. This mouse line is a non-commercial, investigator-generated mouse line that was originally developed at PBRC. Cryopreserved sperm from the original colony was preserved and later rederived by the PBRC Transgenic Core, after which the line has been maintained through in-house breeding at PBRC and was not obtained from any commercial vendor. No externally owned or client-owned animals were used in this study.

Due to recent findings of widespread ectopic recombination in the hypothalamus of the BAC Tg(UCP1-Cre)Evdr/J model, we recognized the value of this knock-in mouse line for the research community. *Mouse*

ethics declaration: All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Pennington Biomedical Research Center (PBRC). All methods were carried out in accordance with relevant institutional guidelines and regulations for the care and use of laboratory animals. Animals were deeply anesthetized with inhaled isoflurane prior to euthanasia by transcardial perfusion. Animal anesthesia and euthanasia procedures were performed in accordance with American Veterinary Medical Association (AVMA) guidelines. ARRIVE guidelines were followed in all animal experiments.

Mouse genotyping

To validate the integrity of the UCP1-IRES-Cre construct, we performed targeted sequencing (Quintara Bioscience, Houston, TX) of the UCP1 locus from a PCR-amplified fragment using the following primers: (FWD: 5'-TGGGCATCTGAAGAGGTAGA-3'; REV: 5'-GCTTGAGGAGAGCCATTGA-3'). Sequencing results confirmed that the IRES-Cre cassette was inserted immediately downstream of UCP1 exon 6, following the stop codon at the targeted Ucp1 locus (Fig. 1A). Heterozygous UCP1-IRES-Cre (C+; C57BL/6J background) female mice were crossed with homozygous male Rosa-Ai14-tomato^{ff} (RRID: IMSR_JAX:007914; C57BL/6J background) reporter mice to generate double heterozygous male and female UCP1-reporter (c+) and control (++) littermate mice. Genotyping was performed with PCR on DNA extracted from tail biopsies using the following primers: *UCP1-Cre*: (Cre FWD: 5'-CGTACTGACGGTGGGAGAAT-3'; Cre REV: 5'-CCC GGCAAAACAGG TAGTTA-3' U-Cre FWD: 5'-CCTCGGTACACATGCTTTACAT-3'; U-Cre REV: 5'-CTGATGGACATGTTTCAG GGATC-3') *Ai14 reporter*: (Ai14 WT FWD: 5'-AAGGGAGCTGCAGTGGAGTA-3'; Ai14 WT REV: 5'-CCGA AAATCTGTGGGAAGTC-3'; Ai14 Mut FWD: 5'-CTGTTCTGTACGGCATGG-3'; Ai14 Mut REV: 5'-GGC ATT AAA GCA GCG TAT CC-3').

Histology, Immunostaining and Imaging: UCP1-reporter (c+: $n=5$; 2 males, 3 females) and control mice (++: $n=5$, 3 males, 2 females) were deeply anesthetized with inhaled isoflurane and euthanized by transcardial perfusion with heparinized PBS followed by 4% paraformaldehyde. Peripheral tissues: BAT, iWAT, gonadal WAT (gWAT), heart, liver, pancreas, testes, ovaries and kidney/adrenals) were post fixed, paraffin embedded and sectioned (5 μ m). Brains (c+: $n=4$; 1 male, 3 females; ++: $n=4$, 2 males, 2 females) were cryoprotected in 30% sucrose and sectioned in 4 series of 30 μ m sections spanning rostro-caudally from approximate Bregma level +1.3 mm to -8.5 mm. Immunostaining was performed on Leica Bond RXM automated system (Leica Biosystems) using standard protocols. For primary antibodies we used rabbit anti-RFP antibody (1:300; Rockland, Cat#600-401-379, RRID: AB_2209751) or rabbit anti-UCP1 primary antibody (1:4000 dilution; Abcam Cat# ab209483, RRID: AB_2722676), for detection we used either Alexa Fluor 647 (1:200; Invitrogen, Cat#A32733) or HRP/DAB (ImmPRESS/PACT, Cat#MP-7451, SK-4105, Vector Laboratories), respectively. In peripheral tissues, Hematoxylin Normal (Cat#812, Anatech, Cancer Diagnostics) was used for nuclear stain. In brain, NeuN was stained to visualize anatomical landmarks (mouse anti-NeuN; Millipore Sigma Cat# MAB377; RRID: AB_2298772, 1:1000) and Hoechst (15 μ g/ml) was used as a nuclear counterstain in RFP-stained peripheral tissues. Tissues were imaged with the Axioscan 7 slide scanner (ZEISS Research Microscopy Solutions, Carl Zeiss AG, Oberkochen, Germany) and the NanoZoomer Digital Pathology System (Hamamatsu Photonics, Hamamatsu City, Japan). Data were qualitatively assessed for UCP1 and reporter expression from all animals, and representative overview and high-magnification images are shown. For peripheral tissues one consecutive section was used and analyzed each for H&E, UCP1/Hematoxylin and RFA/DAPI. For brains, one series was used for endogenous tdTomato, one series was used for UCP1 stain and one series was used for NeuN stain.

Histological quantitative analysis of UCP1 peptide and tdTomato labeled areas

Slide scanner images of UCP1 peptide and tdTomato immunohistochemistry were further used to quantify and compare the extent of labeling in perirenal adipose tissue and interscapular BAT. Whole-slide images of interscapular brown adipose tissue and perirenal adipose tissue were analyzed using QuPath²⁹ (v0.7.0)), Fiji/ImageJ³⁰,

v1.54t), and Imaris (v11.0, Oxford Instruments, Abingdon, UK). Brightfield DAB images were processed with QuPath to separate the hematoxylin and DAB signals by color deconvolution. The isolated UCP1/Hematoxylin channels were converted to an 8-bit grayscale image, inverted and stacked to a single image for further analysis with Imaris software. The target surface areas for quantification were manually outlined (unilateral iBAT spanning the core BAT and surrounding WAT, unilateral perirenal fat from the level of the adrenal gland but excluding the ureter) and non-adipose tissue areas (e.g. iBAT muscle) were manually excluded. Within these areas UCP1- or tdTomato-positive signals were detected and segmented using the intensity-based surface generation tool in Imaris. Surfaces were generated to define the total adipose tissue area and the positive immunostained area with non-adipose tissue regions excluded manually. tdTomato and UCP1 staining were quantified on adjacent tissue sections. Total surface and positive immunostained areas were measured in mm² and as relative portion of positive immunostained area of total surface area (% Positive Area). Statistical analysis was performed using GraphPad Prism (v.10.0.0 GraphPad Software, Boston, MA, USA). All statistical tests defined significance as $\alpha = 0.05$. For quantification, paired, two-tailed t-tests were used to compare two labels within Cre mice. Unpaired, two-tailed t-tests with Welch's correction were used for comparison between genotypes. Repeated measures two-way ANOVA was used to assess the effects of labeling and genotypes and their interaction, with labeling treated as the within-subject factor. Individual data points are shown as mean $\pm$ SEM. For paired comparisons, individual data from the same animal are connected by lines. Open symbols represent females and filled symbols represent males.

Quantification of UCP1 and Cre mRNA

Tissue was dissected from wildtype ($n=5$; 4 males, 1 females) and UCP1-Ires-cre ($n=4$; 4 males) mice housed at room temperature. Interscapular BAT was dissected and surrounding adipose tissue was removed. Total RNA was isolated from adipose tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen) and RNA concentrations were assessed using a Nanodrop spectrophotometer. RNA was reverse transcribed into cDNA and diluted to a final concentration of 4 ng/ μ l. qPCR was performed for Cre and Ucp1 and normalized to the housekeeping gene cyclophilin. Relative gene expression was calculated using the Δ Ct method and normalized to the mean of the wildtype control group. Unpaired, two-tailed t-tests with Welch's correction were used for comparison between genotypes ($\alpha=0.05$). Individual data points are shown as mean $\pm$ SEM. Open symbols represent females and filled symbols represent males.

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Data availability Representative data supporting the findings of this study are included within the article and supplementary information files. Additional raw imaging data are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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