Mouse fertility is not dependent on the CREB coactivator Crtc1

To the Editor:

Cyclic AMP response element–binding protein (CREB)-regulated transcription coactivators (Crtcs), also known as transducers of regulated CREB activity (TORCs), are coactivators that function as calcium- and cyclic AMP–sensitive coincidence detectors[1,2]. Ubiquitously expressed Crtc2 is a key regulator of energy metabolism modulating gluconeogenesis and insulin signaling[3–5]. Crtc1 is primarily expressed in the brain, where its functions still need to be determined more clearly. Our previous work showed that Crtc1 is required for the maintenance of late-phase long-term potentiation in the mammalian hippocampus, suggesting that it may be involved in learning and memory processes[6].

Altarejos *et al.*[7] recently characterized a *Crtc1*-trapped mouse line and found that *Crtc1*$^{-/-}$ mice are hyperphagic, obese and infertile. They related this phenotype to an altered hypothalamic expression of the *Cartpt* and *Kiss1* genes, which encode neuropeptides that mediate leptin's effects on satiety and fertility. Furthermore, they provided evidence that *Crtc1*-mutant mice have a deficit in circulating luteinizing hormone levels, which is a characteristic of infertility. On the basis of their results, these authors concluded that the CREB-Crtc1 pathway mediates the central effects of hormones and nutrients on energy balance and fertility.

To follow up our evaluation of Crtc1's physiological role in the brain[6], and before the publication of the article by Altarejos *et al.*[7], we independently generated a *Crtc1*-trapped mouse line using the same strategy. However, unlike their *Crtc1*-mutant mice, our *Crtc1*$^{-/-}$ males and females are fertile, thus questioning the crucial role of Crtc1 for mouse fertility.

Like Altarejos *et al.*[7], we obtained the mouse embryonic stem cell line XK522 containing an insertional gene trap in the *Crtc1* gene from

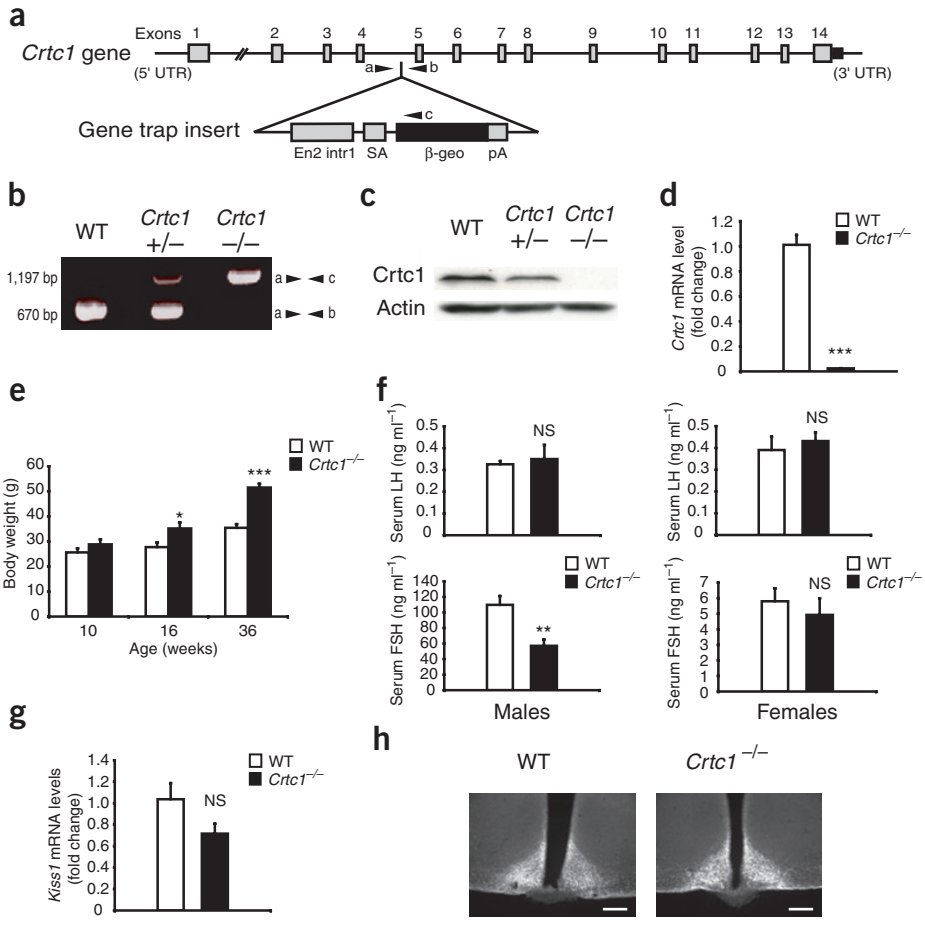

Figure 1 *Crtc1*$^{-/-}$ mice are obese but not infertile. (**a**) Schematic representation of the *Crtc1* gene with the insertion of the gene trap vector pGT0lxf containing engrailed-2 intron 1 sequences (En2 intr1), a splice acceptor (SA), a β-galactosidase-neomycin resistance (β-geo) cassette and a polyadenylation sequence (pA). Primers used for genotyping (a, b and c) are indicated by arrowheads. UTR, untranslated region. (**b**) PCR genotyping of wild-type (WT), *Crtc1*$^{+/-}$ and *Crtc1*$^{-/-}$ mice. The 670-bp and 1,197-bp bands correspond to the WT allele amplified with the primers a and b and the mutant allele amplified with the primers a and c, respectively. (**c**) Crtc1 protein amounts in brain extracts from WT, *Crtc1*$^{+/-}$ and *Crtc1*$^{-/-}$ mice. β-actin levels are shown as loading controls. (**d**) Quantitative PCR of *Crtc1* mRNA levels in hypothalami of WT and *Crtc1*$^{-/-}$ mice (***$P < 0.001$, $n = 5$ or 6; data are means ± s.e.m.). (**e**) Average weights of WT and *Crtc1*$^{-/-}$ mice at 10, 16 and 36 weeks of age (*$P < 0.05$, ***$P < 0.001$, $n = 6$ or 7; data are means ± s.e.m.). (**f**) Plasma luteinizing hormone (LH) and FSH levels in WT and *Crtc1*$^{-/-}$ males and proestrus females (**$P < 0.005$; NS, not significantly different, $P > 0.05$, $n = 5$ or 6; data are means ± s.e.m.). Vaginal smears were performed on female mice to determine their estrous cycle stage. (**g**) Q-PCR of *Kiss1* mRNA levels in hypothalami of WT and *Crtc1*$^{-/-}$ males ($P > 0.05$, $n = 5$ or 6; data are means ± s.e.m.). (**h**) Immunofluorescence detection of kisspeptin in the arcuate nucleus of WT and *Crtc1*$^{-/-}$ females in proestrus with an antiserum to kisspeptin-10. Scale bars, 200 μm. Mouse studies were approved by the Cantonal Veterinary Service of Lausanne in accordance with the Swiss Federal Veterinary Office's guidelines.

BayGenomics. We verified by PCR the presence of the gene trap vector pGT0lxf and determined that it was inserted within the fourth intron of the *Crtc1* gene (**Fig. 1a**). We then injected embryonic cells into C57BL/6N blastocysts to generate chimeric mice. We backcrossed heterozygous mice with C57BL/6N mice for six generations and then intercrossed them to obtain homozygous *Crtc1*$^{-/-}$ mice and wild-type littermates. We genotyped *Crtc1*-mutant mice by PCR amplification of wild-type and mutant alleles (**Fig. 1b**). *Crtc1* messenger RNA and protein were undetectable in the brains of *Crtc1*$^{-/-}$ mice, thus confirming the efficiency of the trapping vector (**Fig. 1c,d**). *Crtc1*$^{-/-}$ mice were born at the expected mendelian frequency and showed no obvious phenotype before 10 weeks of age. In agreement with the results of Altarejos *et al.*[7], our male and female *Crtc1*$^{-/-}$ mice then developed an obese phenotype on a normal chow diet (**Fig. 1e**). These results highlight the importance of Crtc1 for the control of energy balance in adult mice.

Contrary to the findings of Altarejos *et al.*[7], however, we did not find any major infertility problems in our *Crtc1*$^{-/-}$ mice (**Table 1**). To assess the role of Crtc1 in fertility, we mated 8- to 12-week-old homozygous mutant male or female mice with wild-type C57BL/6N mice of the same age. We also intercrossed homozygous mutants and allowed them to mate for up to 3 weeks. At the day of birth, we assessed the number of pups. Male and female *Crtc1*$^{-/-}$ mice gave rise to a normal number of productive matings, regardless of whether they were crossed with wild-type or mutant mice (**Table 1**). In all cases, the average number of pups per litter was comparable to the mean litter size of *Crtc1*$^{+/-}$ intercrosses, except for the *Crtc1*$^{-/-}$ intercrosses, which produced fewer pups (**Table 1**). Altarejos *et al.*[7] generated *Crtc1*$^{-/-}$ mice from heterozygotes that were backcrossed with C57BL/6 mice for three generations. However, the difference in fertility between our *Crtc1*$^{-/-}$ mice and their mutants cannot be attributed to the different numbers of backcrosses, because our *Crtc1*$^{-/-}$ mice were also fertile when they were produced after only three backcrosses (data not shown). In agreement with the fertility of our *Crtc1*$^{-/-}$ mice, we did not observe any significant differences in plasma luteinizing hormone levels between wild-type and mutant mice of both sexes (**Fig. 1f**). We collected female blood at the proestrus stage, which we determined daily by vaginal smears on wild-type and *Crtc1*$^{-/-}$ females over a period of 2 to 3 weeks. Mutant females showed normal estrous cycles, which further indicates that their fertility is not altered. In addition, we measured the plasma follicle-stimulating hormone (FSH) concentrations of both genotypes and sexes. Whereas the FSH concentrations were comparable in wild-type and mutant proestrus females, they were significantly lower in *Crtc1*$^{-/-}$ males (**Fig. 1f**). This latter observation is consistent with an earlier report demonstrating no impact of low FSH levels on male mouse fertility[8]. Moreover, this alteration of gonadotropin expression does not necessarily result from a dysregulation in the hypothalamus of *Crtc1*-deficient males, because FSH expression is regulated by CREB in pituitary gonadotropes[9] and may require Crtc1 coactivation. Finally, hypothalamic *Kiss1* mRNA levels were not significantly different between *Crtc1*$^{-/-}$ mice and wild-type littermates (**Fig. 1g**), and immunofluorescence detection of kisspeptin, a cleavage product of the Kiss1 precursor, revealed comparable amounts in the arcuate nucleus of wild-type and *Crtc1*$^{-/-}$ mice (**Fig. 1h**).

In conclusion, we used exactly the same gene trap strategy as Altarejos *et al.*[7] to generate *Crtc1*$^{-/-}$ mice that develop obesity but are clearly not infertile. Defects in leptin signaling lead to increased weight and infertility, but leptin controls body energy homeostasis and fertility via different neural pathways[10]. Therefore, our data suggest that Crtc1 may be required for the hypothalamic expression of genes that mediate the effects of leptin on energy balance but not on fertility. The latter observation is in contrast with the report of Altarejos *et al.*[7], because they observed no offspring from matings of either *Crtc1*$^{-/-}$ males or *Crtc1*$^{-/-}$ females with wild-type mice (0/6 matings). The genetic background of the two *Crtc1*-mutant mouse lines should be comparable, because they were both generated from the same embryonic stem cell line and were both backcrossed with C57BL/6 mice. However, we cannot exclude that subtle genetic differences between the two mouse lines allow the differential expression of modifier genes affecting the fertility of Crtc1-deficient mice. Although the C57BL/6 mouse is the most well-known inbred mouse strain, several substrains were derived from the founder line, and genetic variations have been recently reported among these substrains[11]. We used the C57BL/6N substrain to backcross heterozygous mice, whereas Altarejos *et al.*[7] did not clearly state which substrain they used for their backcrosses. We do not question the involvement of Crtc1 in the regulation of hypothalamic *Kiss1* gene expression. However, it has been shown that other transcription factors than CREB are implicated in this regulation[12], and, therefore, additional coactivators should be involved. Hypothetically, the genetic background of the *Crtc1*$^{-/-}$ mouse line used by Altarejos *et al.*[7] might impinge on the efficiency of these transcriptional regulators, which, in the absence of Crtc1, might have led to a lower expression of the *Kiss1* gene and infertility of their mutant mice. In any event, and regardless of the reason for the difference in fertility between the *Crtc1*$^{-/-}$ mouse lines, our data do not support the view that the CREB coactivator Crtc1 is indispensable for mouse fertility.

Table 1 Fertility of *Crtc1*-mutant mice

Genotype (male × female)	Number of productive matings	Average number of pups
Crtc1$^{-/-}$ × WT	5/6	7.2
WT × *Crtc1*$^{-/-}$	6/6	7.8
Crtc1$^{-/-}$ × *Crtc1*$^{-/-}$	4/5	4.3
Crtc1$^{+/-}$ × *Crtc1*$^{+/-}$	10/10	7.8

Note: Supplementary information is available on the Nature Medicine website.

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Altarejos *et al.* reply:

We find Cardinaux's correspondence[1] quite interesting. Both laboratories used a practically identical approach to knock out the *Crtc1* gene in similar genetic strains. In contrast to the infertility that we observed in our *Crtc1*$^{-/-}$ mice[2], Cardinaux found that *Crtc1*-mutant mice show only a mild reproductive phenotype[1]. The energy balance phenotypes appear quite consistent between the two groups, however.

Although we are not sure why there is a discrepancy in the fertility phenotype, it may reflect the different substrains used. The lack of a reproductive phenotype could also reflect compensatory upregulation of other CRTC family members, a consideration that will require further study. We should also note that disruption of the single *CRTC* homolog in *Drosophila melanogaster* disrupts energy balance and reduces fertility[3].

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Innovating for impact: The Affordable Medicines Facility-malaria (AMFm)

To the Editor:

Your story[1] on AMFm does not provide a balanced picture of the evidence pertaining to the proposed approach adopted by AMFm and the prevailing development approaches used to combat malaria.

AMFm is an innovative financing mechanism to expand access to affordable artemisinin-based combination therapies (ACTs) for malaria, thereby saving lives and reducing the use of inappropriate treatments. By increasing access to ACTs and displacing artemisinin monotherapies from the market, AMFm also seeks to delay resistance to artemisinin. AMFm aims to enable countries to increase the provision of affordable ACTs through the public, private and nongovernmental organization sectors. Contrary to your report, AMFm's key feature is not "heavily subsidizing the private market"[1]. In both the public and the private sector, it will subsidize the buyer, rather than the manufacturer, of ACTs through copayments.

You also wrote about "provisions in the scheme that allow subsidies for artemisinin monotherapy" and quoted claims that the pilot subsidies have not proven AMFm's effectiveness[1]. Your report would have been more balanced if it had specified how much has been spent globally on approaches other than the AMFm and discussed their effectiveness in improving access to ACTs and displacing ineffective drugs from the market.

We welcome critical comments, as they catalyze improvements in planning and implementation, and we especially welcome critiques based on evidence, such as those we have come to enjoy from *Nature Medicine*.

Olusoji Adeyi & Rifat Atun

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