Ablation of Arginylation in the Mouse N-End Rule Pathway: Loss of Fat, Higher Metabolic Rate, Damaged Spermatogenesis, and Neurological Perturbations

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Abstract

In the N-end rule pathway of protein degradation, the destabilizing activity of N-terminal Asp, Glu or (oxidized) Cys residues requires their conjugation to Arg, which is recognized directly by pathway’s ubiquitin ligases. N-terminal arginylation is mediated by the Ate1 arginyltransferase, whose physiological substrates include the Rgs4, Rgs5 and Rgs16 regulators of G proteins. Here, we employed the Cre-lox technique to uncover new physiological functions of N-terminal arginylation in adult mice. We show that postnatal deletion of mouse Ate1 (its unconditional deletion is embryonic lethal) causes a rapid decrease of body weight and results in early death of ~15% of Ate1-deficient mice. Despite being hyperphagic, the surviving Ate1-deficient mice contain little visceral fat. They also exhibit an increased metabolic rate, ectopic induction of the Ucp1 uncoupling protein in white fat, and are resistant to diet-induced obesity. In addition, Ate1-deficient mice have enlarged brains, an enhanced startle response, are strikingly hyperkinetic, and are prone to seizures and kyphosis. Ate1-deficient males are also infertile, owing to defects in Ate1+/− spermatocytes. The remarkably broad range of specific biological processes that are shown here to be perturbed by the loss of N-terminal arginylation will make possible the dissection of regulatory circuits that involve Ate1 and either its known substrates, such as Rgs4, Rgs5 and Rgs16, or those currently unknown.

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Introduction

N-terminal arginylation of intracellular proteins by Arg-tRNA-protein transferase (R-transferase) is a part of the N-end rule pathway of protein degradation (Fig. 1A). In eukaryotes, this pathway is a part of the ubiquitin (Ub)-proteasome system. The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue (reviewed in [1,2,3,4]). Degradation signals (degrons) that can be targeted by the N-end rule pathway are of two distinct kinds: N-terminal degrons, called N-degrons, and internal (non-N-terminal) degrons [1,5]. The main determinant of an N-degron is a destabilizing N-terminal residue of a substrate protein (Fig. 1A). The other determinants of N-degron are a substrate’s internal Lys residue (the site of formation of a poly-Ub chain) and a nearby unstructured region [6,7]. An N-degron is produced from a Precursor, called a pre-N-degron, through a protease-mediated cleavage of a substrate that exposes a destabilizing N-terminal residue.

The N-end rule has a hierarchic structure (Fig. 1A). N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu [8]. Destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by the Ate1-encoded R-transferase [9,10,11,12]. In eukaryotes that produce nitric oxide (NO), R-transferase arginylates not only N-terminal Asp and Glu but also Cys, after its conversion to Cys-sulfinate or Cys-sulfonate, in reactions that require NO and oxygen (Fig. 1A) [11,13]. Alternative splicing of the mammalian Ate1 pre-mRNA produces isoforms of R-transferase, a metabolically unstable protein whose enzymatic activity and the in vivo half-life are down-regulated by heme [10,12,14]. E3 Ub ligases of the N-end rule pathway are called N-recognins. An N-recognin is an E3 that can recognize (target for polyubiquitylation) at least a subset of N-degrons (Fig. 1A) [1,4]. Some of substrate-binding sites of an N-recognin target N-degrons, while other sites of the same N-recognin are specific for structurally unrelated internal (non-N-terminal) degrons [15,16]. At least four N-recognins, Ubr1, Ubr2, Ubr4 and Ubr5, mediate the mammalian N-end rule pathway (Fig. 1A) [4,17].

The functions of the N-end rule pathway in eukaryotes include selective degradation of misfolded proteins; the sensing of heme, oxygen, nitric oxide (NO), and short peptides; the regulation of DNA repair and peptide import; the signaling by transmembrane receptors, through the NO/O2-connected degradation of G-protein regulators Rgs4, Rgs5 and Rgs16; the fidelity of chromosome segregation; regulation of apoptosis, meiosis, spermatogenesis, neurogenesis, and cardiovascular development; the functioning of specific organs, in particular the brain and the pancreas; and regulation of leaf senescence, seed germination, and other processes in plants [2,12,16,18,19,20], and refs. therein). A partial N-terminal arginylation of the apparently long-lived...
mammalian α-actin [21] suggests that arginylation of some proteins may not alter their in vivo half-lives. Although there are many putative intracellular substrates of the Ate1 R-transferase, for example, among C-terminal fragments of proteins that are cleaved in vivo by proteases such as MetAPs, caspasas, calpains or secretases, the set of definitively identified Ate1 substrates is still small. It includes the Drosophila antiapoptotic Ub ligase DIAP1 [22]; the mammalian G-protein regulators Rgs4, Rgs5 and Rgs16 [11,13]; and the separase-produced fragment of the mammalian Rad21/Scc1 cohesin subunit that bears N-terminal Glu, a secondary destabilizing residue (Fig. 1A) [J. Zhou, D. Pati and A.V., unpublished data].

Figure 1. Postnatal ablation of the mouse Ate1 R-transferase, a component of the N-end rule pathway. (A) The mammalian N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. “Primary”, “secondary” and “tertiary” denote mechanistically distinct subsets of destabilizing N-terminal residues (see Introduction). C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate. MetAPs, Met-aminopeptidases. (B) Bidirectional promoter between the mouse Ate1 exons 1A and 1B [14]. Green arrows indicate transcriptional units, including a previously uncharacterized gene, termed Dfa (“divergent of Ate1”), that is transcribed from the bidirectional promoter. (C) Immunoblotting-based comparisons of Ate1 levels in the indicated mouse tissues from Ate1+/+ and Ate1flox/2; CaggCreER mice 76 days after the tamoxifen (TM)-induced, Cre-mediated Ate1flox→Ate1 conversion that yielded Ate1-deficient mice. The band of 60-kDa Ate1, detected by antibody to mouse Ate1, is indicated on the right. Total (Ponceau-stained) protein patterns are shown below, with positions of molecular-mass markers on the left. (D) IB assays for the levels of Ate1 and Rgs4 (25 kDa) in brain extracts from Ate1+/+ and Ate1-deficient mice (Ate1flox/2; CaggCreER mice 30 days after TM treatment). doi:10.1371/journal.pone.0007757.g001
Heterozygous Ate1<sup>+/−</sup> mice appear indistinguishable from their wild-type counterparts, whereas Ate1<sup>−/−</sup> mice die around embryonic day 15 (E15) with abnormalities that include cardiovascular defects [10].

To bypass the embryonic lethality of nonconditional Ate1<sup>−/−</sup> mice, we employed the Cre-lox technique [24]. As shown below, a systemic postnatal deletion of the sole active Ate1<sup>lox<sub>1A</sub></sup> allele in juvenile Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup> mice causes a rapid decrease of body weight and results in early death of ∼15% of Ate1-deficient mice, with surviving mice retaining only ∼70% of normal weight. This failure to thrive occurs despite higher than normal food intake by Ate1-deficient mice. These mice contain little or no visceral fat, exhibit an increased metabolic rate, a decreased fasting blood glucose level, and an increased intestinal import and retention of amino acids and/or peptides. Ate1-deficient mice are also resistant to diet-induced obesity and exhibit ectopic induction of the Ucp1 uncoupling protein in white adipose tissue (WAT). In addition, Ate1-deficient mice have enlarged brains, an enhanced startle response, and are strikingly hyperkinetic. They often suffer from kyphosis, i.e., an excessive curvature of the upper back, and from frequent seizures as well. Ate1-deficient males are also infertile, owing to defects in meiotic Ate1<sup>−/−</sup> spermatocytes. The remarkably broad range of specific biological processes that are shown here to be perturbed by the loss of N-terminal arginylation will facilitate the dissection of regulatory circuits that involve Ate1 and either its known substrates, such as Rgs4, Rgs5 and Rgs16 [11,13], or those currently unknown.

**Results**

**Ate1<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub> Mouse Strains and Production of Ate1<sup>−/−</sup> Mice**

Standard methods were employed to produce, initially, ATE<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub> mouse strains in which a specific segment of Ate1 was “floxed”, i.e., flanked by 34-bp loxP repeats (Fig. 2C–E). The targeting vector contained ∼14 kb of Ate1, including the exon 1A-exon 4 segment that encodes an essential part of R-transferase [9] (Fig. 2A). Our previous work has shown that the Ate1 promoter (P<sub>Ate1</sub>) is bidirectional, expressing both Ate1 and an oppositely oriented gene termed Dfa (divergent from Ate1), which overlaps with exon 1A of Ate1 (Fig. 1B) ([14]; C.S.B. and A.V., unpublished data). To minimize the possibility of perturbing the expression of Dfa, the “floxed” region of Ate1 encompassed exons 2–4, away from exon 1A (Fig. 1B). Our aim was to produce ATE<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub> mouse strains that were “poised” to lose their remaining active ATE<sup>lox<sub>1A</sub></sup> allele through the expression of Cre recombinase. To do so, heterozygous matings were carried out among the above ATE<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub> mice, the previously constructed ATE<sup>−/−</sup>/lox<sub>1A</sub> mice [10], and a mouse strain that contained the CaggCreER gene, expressed from the ubiquitously active chimeric Cagg promoter [25]. CaggCreER encoded CreER, a fusion between Cre and a derivative of the mouse estrogen receptor ligand binding domain. CreER was functionally inactive (sequestered in the cytosol) but could be activated by intraperitoneal (IP) injections of tamoxifen (TM) [25]. Depending on configurations of their Ate1 alleles, the resulting mice, poised for the loss of Ate1, were termed Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CreER or Ate1<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub>CaggCreER.

Using standard methods, we could demonstrate the presence of Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CreER mice, at expected (Mendelian) frequencies, in the progeny of above matings. These mice expressed TM-inducible CreER recombinase and contained a single copy of Ate1<sup>lox<sub>1A</sub></sup>, the active Ate1 allele (Figs. 2 and 3A). The functional intactness of Ate1<sup>lox<sub>1A</sub></sup> was inferred from the fact that Ate1<sup>lox<sub>1A</sub></sup>/lox<sub>1A</sub>CaggCreER mice survived embryogenesis (in contrast to Ate1<sup>−/−</sup>/lox<sub>1A</sub> mice [10]) and were phenotypically similar (in the absence of TM treatment) to Ate1<sup>−/−</sup>/lox<sub>1A</sub> and Ate1<sup>−/−</sup> mice. To induce the Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>→Ate1<sup>−/−</sup> conversion, ∼1 month old Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice and their Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER littermates, used as controls, were treated with TM (see Materials and Methods for details, including the ages of TM-treated mice). Southern hybridization and PCR-based analyses of DNA from tissues of the resulting mice (sampled 1 month after TM treatment) confirmed the TM-induced, Cre-mediated excision of the Ate1<sup>lox<sub>1A</sub></sup> allele in Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice. The frequency of Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>→Ate1<sup>−/−</sup> conversion was nearly 100% in the brain and kidney of these mice, but significantly lower in several other tissues (Figs. 2G, H and 3B).

We also used an affinity-purified antibody to mouse Ate1 [11] to carry out immunoblotting (IB) with extracts from brain, heart, kidney, liver, muscle, brown adipose tissue (BAT) and white adipose tissue (WAT) that were harvested up to 8 months after TM treatment of Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice, versus identically TM-treated control littermates. No Ate1 could be detected by IB in several tissues of TM-treated Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice, in contrast to readily detectable Ate1 in TM-treated control mice (Fig. 1C). The only significant exception was liver (Fig. 1C, lanes 7, 8; cf. lanes 5, 6 or lanes 11–14; see also below). One effect of Ate1 deletion in the mouse brain was a striking increase of Rgs4, a physiological Ate1 substrate (see Introduction) that down-regulates specific G proteins by acting as a GTPase-activating protein (GAP) (Fig. 1D). Whereas no Rgs4 could be detected in the Ate1-containing brain (owing to degradation of Rgs4 by the N-end rule pathway [11]), an intense band of Rgs4 was present in the Ate1-deficient brain, illustrating high penetrance of Ate1 deletion in the brain (Fig. 1D).

We also performed in vitro arginylation assays with extracts from several tissues of Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice 21 days after TM treatment, versus extracts from identically treated Ate1<sup>+/+</sup> or ATE<sup>+/−</sup> mice. The TM-induced decrease of arginylation activity in specific organs of ATE<sup>+/−</sup>/CaggCreER mice ranged from ∼90% in the brain and kidney to ∼60% in the liver (Fig. 3C–E). Although heterozygous Ate1<sup>+/−</sup> mice were phenotypically similar to their wild-type (Ate1<sup>+/+</sup>) counterparts, we found that Ate1<sup>−/−</sup> mice grew slightly but consistently slower than Ate1<sup>+/+</sup> mice, and reached a lower average weight (Fig. 4B). In agreement with this mild but detectable haploinsufficiency of Ate1, the arginylation activity in extracts from, e.g., brains or hearts of Ate1<sup>−/−</sup> mice was significantly below its wild-type (Ate1<sup>+/+</sup>) levels (Fig. 3C), implying the absence of a compensatory (e.g., autoregulated) increase of Ate1 expression upon a decrease of Ate1 gene dosage.

**Retarded Growth, Kyphosis, and a Transient Increase in Lethality of Ate1-Deficient Mice**

TM treatment produced abnormal phenotypes within 1 week in Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice, in comparison to identically TM-treated controls. Specifically, ∼1 month old and previously growing Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice failed to thrive (in comparison to control mice) after their TM-induced conversion to Ate1<sup>−/−</sup>/CaggCreER mice (Figs. 4A–C and 5A). During the first ∼3 weeks after becoming Ate1<sup>−/−</sup>, these mice experienced a rapid loss of weight and decreased growth (Fig. 4A, B), despite no decrease in their consumption of food (see below). The average body length (measured from tip-of-nose to base-of-tail) of Ate1<sup>−/−</sup>/CaggCreER mice was 5% smaller (p<0.08) than that of their Ate1-containing, identically TM-treated counterparts (Ate1<sup>+/−</sup>/CaggCreER, Ate1<sup>−/−</sup>/CaggCreER, or Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice) (Figs. 4D and 5C).

In the entire cohort of TM-treated post-natal Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER mice, 15% of them (18 of 119 mice) died over 42 days after TM treatment. Crucially, none of identically TM-treated control mice (Ate1<sup>−/−</sup>/CaggCreER, Ate1<sup>+/−</sup>/CaggCreER, or Ate1<sup>lox<sub>1A</sub>/lox<sub>1A</sub></sup>CaggCreER) died in the same time interval. The frequency of Ate1<sup>−/−</sup>/CaggCreER mice died within 42 days, was 15% (18 of 119 mice). This difference was statistically significant (p<0.05).

"Ablation of R-Transferase"
Figure 2. Genomic configurations at the Ate1 locus of Cre-lox-based mouse strains constructed in the present work. (A) The 5′ end of the previously produced unconditional Ate1 knock-out allele [10], in which the Ate1 exons 1b through 3 were replaced by a cassette encoding a promoter-lacking, NLS-containing LacZ (NLS-bgal) (it was expressed from the endogenous P_{Ate1} promoter) and the Neo selection marker expressed from the phosphoglycerate kinase P_{PGK} promoter (green rectangles). (B) A diagram of the 5′ end of wild-type (wt) mouse Ate1, indicating approximate locations of exons 1a through 5. (C) The −22.5 kb targeting construct containing a −6 kb long-arm region of Ate1 homology (shown as a shaded rectangle on the left); a single loxP site (red triangle) upstream of Ate1 exon 2, a “floxed” hygromycin-resistance (hph) cassette, expressed from the P_{PGK} promoter (blue arrow between two red triangles) downstream of Ate1 exon 4; a −2 kb short-arm region of homology (an inclined shaded rectangle), and the HSV thymidine kinase (tk) negative-selection cassette expressed from the P_{HSV} promoter (yellow arrow). Wavy line indicates an abutting sequence of the pBR322 plasmid DNA. (D) The tri-lox Ate1 allele obtained after a correctly targeted double crossover event. (E) In the notations here and elsewhere in the paper, “flox-on” indicates a configuration depicted in this panel (the functionally active Ate1^{flox} allele), whereas “flox-off” indicates a configuration depicted in panel F (the null Ate1^{2} allele). The functionally active, “flox-on” (Ate1^{flox}) allele, obtained by the removal of the hph cassette, using the in vivo expression of Cre-recombinase driven by the P_{Stre} promotor, which is active only in pre-implantation blastocysts. The null “flox-off” (Ate1^{2}) allele obtained by the inducible expression of CreER recombinase from the P_{Cagg} promoter and posttranslationally induced by tamoxifen (TM) treatment (see the main text and Materials and Methods). H, approximate locations of HindIII sites used in Southern analyses with DNA probe A (see panel G); E, approximate locations of EcoRI sites used in Southern analyses with DNA probe D (see panel H); black boxes marked “A” and “D” indicate the regions specific for DNA probes A and D, respectively. (G) Southern hybridization analysis using DNA probe A and HindIII-digested genomic DNA. The wt Ate1 allele (panel B) yields the 11.8 kb HindIII fragment. The previously constructed [10] unconditionally null Ate1^{2} allele (panel A), denoted as “null” on this panel, yields the 9.8 kb HindIII fragment. The functionally active flox-on (Ate1^{flox}) allele (panel E) yields the 6.3 kb HindIII fragment. Lane 1, Ate1^{+/+}; lane 2, Ate1^{+/2}; lane 3, Ate1^{flox}; lane 4, Ate1^{flox}. (H) Southern hybridization analysis using DNA probe D and EcoRI-digested genomic DNA. The previously constructed [10] unconditionally null Ate1^{2} allele (denoted as “null”) yields the 5.8 kb fragment. Both the wild-type Ate1 allele and the flox-on (Ate1^{flox}) allele yield the 9.7 kb fragment, whereas the null flox-off (Ate1^{2}) allele yields the characteristic 3.6 kb fragment. The use of DNA probe D and EcoRI-digested DNA from specific tissues of tamoxifen (TM)-treated Ate1^{flox}/CaggCreER mice allowed approximate estimates of the levels of Cre-mediated recombination that produced the flox-off (Ate1^{2}) allele. For example, whereas no flox-on (Ate1^{flox}) allele could be detected in the kidney and brain of Ate1^{flox}/CaggCreER mice after TM treatment (lanes 5, 6), approximately equal amounts of flox-on (Ate1^{flox}) and flox-off (Ate1^{2}) alleles were present in the heart of TM-treated Ate1^{flox}/CaggCreER mice. Lanes 1–3, 1,000, 250, and 25 ng of EcoRI-digested wt mouse genomic DNA (from a tail biopsy), respectively. Lane 4, EcoRI-digested genomic DNA from the tail of a previously constructed [10] Ate1^{flox} mouse. Lanes 5–7, EcoRI-digested genomic DNA from the indicated tissues of TM-treated Ate1^{flox}/CaggCreER mice. Lane 8, same as lane 7, but from a TM-treated Ate1^{flox} mouse (lacking the CaggCreER transgene).

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succumbing upon the acquisition of Ate1−/− genotype was age-dependent. Specifically, 46% of Ate1−/−;CaggCreER mice younger than 30 days at the beginning of TM treatment died within 42 days after TM treatment. In contrast, only 12% of Ate1−/−;CaggCreER mice died if they were older than 30 days (by up to 56 days) at the beginning of TM treatment. Those among Ate1−/−;CaggCreER mice that survived for at least 42 days after TM treatment eventually resumed growth, but the rate of growth and their maximum weight were significantly below those parameters for identically TM-treated control mice (Fig. 4A–C).

In addition to their retarded growth (despite a higher than normal food intake; see below), 53% of Ate1−/−;CaggCreER mice (95 of 180 mice) appeared “scruffy”, and 66% of them (109 of 180) had a kyphotic posture, i.e., an excessive curvature of the upper back (Fig. 5B). In contrast, only 3% of Ate1-containing mice (8 of 244) were scruffy, and only 2% (5 of 244) exhibited kyphosis. Among surviving Ate1−/−;CaggCreER mice, 10% (8 of 80) developed patches of red hair among their normally black hair, in contrast to identically TM-treated Ate1-containing mice (data not shown), suggesting a misregulation of melanocytes in Ate1-deficient mice. The liver, spleen, intrascapular brown adipose tissue (BAT), pancreas, and testis of Ate1−/−;CaggCreER mice appeared normal and were of appropriate sizes (if the smaller size of these mice (Fig. 4C) was taken into account), whereas the brains, hearts and kidneys of these Ate1-deficient mice were disproportionately large, in comparison to those of Ate1-containing siblings (Fig. 5D). Intact brains of Ate1-deficient mice appeared swollen, in comparison to brains harvested, in parallel, from identically treated Ate1-containing siblings (Fig. 6A). In addition, Ate1-deficient males were infertile, in agreement with defects in their testes (Fig. 6F–I). Yet another abnormality of Ate1-deficient mice

Figure 3. Cre-mediated conversion to Ate1-null genotype in different mouse tissues. (A) PCR-based genotyping of tail DNA to detect the Cre-mediated Ate1flox→Ate1− conversion of the functionally active flox-on (Ate1flox) allele to the null Ate1− allele in a 27-day old Ate1flox−/−;CaggCreER mouse immediately after the fourth (daily) intraperitoneal (IP) injection of tamoxifen (TM+), or in the absence of TM treatment (TM−). Upper panel: the 512 bp DNA fragment characteristic of the flox-on (Ate1flox) allele and the 472 bp DNA fragment characteristic of either wild-type or the previously constructed [10] unconditionally null Ate1− allele, with primers CB110 and CB157 (Table 4); and the 324 bp DNA fragment (control), amplified from the IL-2 gene using primers IMR42 and IMR43, in the same PCR reaction. (B) The Cre-mediated Ate1flox→Ate1− conversion, detected by PCR (as described in panel A) in genomic DNA isolated from the indicated tissues immediately after the fourth (daily) IP injection of tamoxifen in a 24-day old Ate1flox−/−;CaggCreER mouse. (C) Relative in vitro arginylation activity (cpm/reaction) in extracts of the indicated tissues from a wild type mouse (Ate1+/+ (black bar), a heterozygous mouse (Ate1+/−) (blue bar), and an Ate1−/− mouse (the latter mouse was initially Ate1flox−/−;CaggCreER (red bar) from the same litter 76 days after TM treatment. A white bar on the right indicates the relative arginylation activity obtained with purified recombinant mouse Ate1 (denoted as “rAte1”) that had been expressed in S. cerevisiae. Shown here are “cpm/reaction” after subtracting “cpm/reaction” in the null-control (“buffer alone”) sample. The control incorporation was approximately equal to that observed in extracts from spleen and thymus. In other words, the assay configured as described in this panel and in Materials and Methods was not sensitive enough to robustly detect the arginylation activity in extracts from spleen and thymus. (D) Relative in vitro arginylation activity (cpm/reaction) in the whole brain, cerebellum, and hippocampus harvested from wild type mice (Ate1+/+; n = 3), heterozygous mice (Ate1+/−; n = 3), and Ate1−/− mice (specifically, Ate1flox−/−;CaggCreER; n = 3) mice 40 days after TM treatment. Standard deviations are indicated. (E) Relative in vitro arginylation activity (cpm/reaction) in tests extracts from Ate1+/+ mice (n = 3) and Ate1−/− mice (specifically, Ate1flox−/−;CaggCreER; n = 3) −130 days after TM treatment. Standard deviations are indicated.

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was their strikingly lower content of the peritoneal white adipose tissue (WAT), on average only 16% of WAT in Ate1-containing mice (Figs. 5D and 7A–C). These phenotypes are discussed below.

Spermatogenesis Defects and Infertility of Ate1-Deficient Male Mice

The marking of Ate1−/− allele with NLS-β-galactosidase (βgal) expressed from the P_{Ate1} promoter revealed high levels of Ate1 expression in the neural tube and other specific, often sharply delineated, regions of Ate1+/− embryos [10]. An earlier study detected high levels of Ate1 expression in spermatogonia (stem cells, located at the periphery of testis’ seminiferous tubules), and possibly also in early meiotic spermatocytes of adult mice [26]. Male Ate1+/−;CaggCreER mice that were produced by TM treatment (Fig. 4C) were found to be infertile in matings with Ate1-containing females, in contrast to identically TM-treated Ate1+/+ males [27]. XGal staining of testis sections of NLS-βgal-marked Ate1+/− mice in the present work (Fig. 6F–I) confirmed and extended the earlier evidence [26] for the pattern of Ate1 expression in testis. Whereas the lumens of seminiferous tubules in Ate1-containing testis were filled with inward-pointing sperm tails, the lumens of tubules in Ate1-deficient testis contained few sperm cells, in a disorganized arrangement (Fig. 6F–I), in agreement with the observed infertility of Ate1-deficient males.

To address the timing of requirement for Ate1 during spermatogenesis, we mated wild-type females with Ate1flox/− males that contained (instead of the CaggCreER gene) the PrpCreER gene (line 28.8) [27] or the PrmCre gene [28]. PrpCreER expresses TM-inducible CreER from the Prp promoter, whose activity in testis is confined to spermatogonia and meiotic spermatocytes [27]. In contrast, PrmCre expresses the (unconditionally active) Cre recombinase from the protamine promoter, which is active at later stages of spermatogenesis, in haploid round and elongating spermatids. PmrCreER mice were infertile [28,29]. 

Figure 4. Growth rate consequences of postnatal ablation of Ate1. (A) Weights of Ate1-containing (n = 4; black curve) and Ate1-deficient (n = 2; red curve) mice from the same litter as a function of time after tamoxifen (TM) treatment. Weights were measured at weekly intervals. Vertical bars indicate the ranges of measured weights. (B) Averaged growth curves for the indicated numbers of mice after TM treatment, plotted as a percentage of their weight immediately before TM treatment. Red, black and blue curves: Ate1−/− (n = 87), Ate1+/− (n = 55), and Ate1+/+ (n = 66) mice. Red arrow indicates the time (~21 days) after TM treatment by which ~15% of Ate1-deficient mice have died while the rest of them began to gain weight. Note a slightly but clearly decreased weight of heterozygous (Ate1+/−) mice (blue curve), in comparison to Ate1+/− mice (black curve) ~1 year after TM treatment. Error bars indicate standard deviations (SD). (C) Typical appearance of Ate1+/− versus wt mice (a smaller, leaner Ate1+/− mouse) ~1 year after TM-mediated ablation of Ate1. (D) Mean body lengths (± SD) (from tip of nose to base of tail) between pairs of Ate1+/− (red bar) and Ate1+/+ (black bar) mice. This comparison was derived from the data in Fig. 5C. Statistical analysis was performed using an unpaired t-test (p<0.08). doi:10.1371/journal.pone.0007757.g004
spermatids [28]. Three breeding pairs for each of two kinds of Ate1flox/2 males (PrpCreER-based and PrmCre-based) and wild-type females were set up. 33% fewer litters and 50% fewer pups were produced with Ate1flox/2; PrpCreER males, in comparison to Ate1flox/2; PrmCre males (Table 1). (This substantial difference is expected to be even larger in a setting where an expressed Cre does not require a second, TM-mediated step for activation, as is the case with TM-independent Cre expressed from the Prm promoter, but not with TM-inducible CreER, expressed from the Ptp promoter.) Nearly equal numbers of the Ate1flox (active) and Ate1+ (inactive) alleles were present in the heterozygous progeny of matings that involved Ate1flox/2; PrmCre males (13 versus 14 pups containing Ate1flox versus Ate1+ alleles, respectively). In contrast and most revealingly, only one Ate1+ (inactive) allele but 12 Ate1flox (active) alleles were present in the progeny of matings that involved Ate1flox/2; PrpCreER males (Table 1). These findings suggest that the PrmCre-mediated inactivation of the
Figure 6. Brain, behavioral, and testis abnormalities of Ate1-deficient mice. (A) Enlarged brains of Ate1-deficient mice. Upper panel: comparison of representative brains harvested from an Ate1<sup>+/+</sup> and an Ate1<sup>−/−</sup> mouse, respectively, 134 days after tamoxifen (TM) treatment. Lower panel: brain weights expressed as percentages of total body weights in Ate1<sup>+/+</sup> (n = 41) and Ate1<sup>−/−</sup> (n = 40) mice. Horizontal bars and numbers indicate mean values. (B) Wet (0.4053 g versus 0.4608 g) and dry (0.1022 g versus 0.1119 g) weight components of the total mean brain weights (±SD) in Ate1<sup>+/+</sup> and Ate1<sup>−/−</sup> mice. (C) Total distance traveled (in meters), over 15 min, in an open field test among mice of different genotypes belonging to the same litter, 44 days after TM-treatment. Bar 1, Ate1<sup>flox/+;CaggCreER</sup> mouse. Bar 2, Ate1<sup>+/+</sup>;CaggCreER mouse. Bar 3, Ate1<sup>-/-</sup> mouse. Bar 4, Ate1<sup>flox/-;CaggCreER</sup> mouse that was converted to Ate1<sup>-/-</sup> by TM treatment. Blue and red bars denote Ate1-containing and Ate1-deficient mice, respectively. (D) Same as in C but maximum lengths of single movements (in centimeters). (E) Same as in C but mean velocities (in cm/second) over 15 min. (F) Paraffin sections (4 μm) of testis showing cross-sections of seminiferous tubules in Ate1<sup>+/+</sup> testis stained with hematoxylin and eosin (150× magnification). (G) Same as in F but Ate1<sup>-/-</sup> testis. Note that sperm tails in the lumens of Ate1<sup>-/-</sup> tubules are sparse in comparison to those in Ate1<sup>+/+</sup> testis. (H) Same as in F but at 600× magnification. (I) Same as in G but at 600× magnification. (J) XGal staining for β-gal activity in a 10-μm section of Ate1<sup>+/+</sup> testis in which one copy of Ate1 was replaced by an ORF encoding NLS-β-galactosidase and expressed from the P<sub>Ate1</sub> promoter (100× magnification). (K) Immunoblotting analysis, using antibody to poly (ADP-ribose) polymerase (PARP), of testis extracts from an Ate1-containing (Ate1<sup>flox/+</sup> (−/+)) and an Ate1-deficient (Ate1<sup>flox/-;CaggCreER</sup> (−/−)) mouse 16 days after TM treatment. Note the loss of the full-length length 116 kDa PARP and the presence of the 85 kDa PARP fragment (lane2). An asterisk denotes a protein crossreacting with anti-PARP antibody.

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Ate\textsuperscript{flox} allele, which occurs at a post-meiotic stage of spermatogenesis [28], takes place at a time when Ate1 is no longer essential for production of viable sperm cells, thus accounting for high frequency of the Ate1\textsuperscript{2} allele in the progeny of matings that involve Ate1\textsuperscript{flox/2};Prm\textsuperscript{Cre} males. In contrast, the Prp\textsuperscript{CreER}-mediated inactivation of the Ate1\textsuperscript{flox} allele, which takes place in meiotic spermatocytes [27], clearly discriminated against the transmission of the Ate1\textsuperscript{flox} allele, most likely because spermatocytes that become Ate1-deficient before they became haploid were sufficiently perturbed by the absence of arginylation to either

Figure 7. Loss of white adipose tissue (WAT), resistance to high fat diet-induced obesity, and ectopic Ucp1 in WAT of Ate1-deficient mice. (A–C) Visceral fat content of Ate1-containing mice. Shown here are representative examples of Ate1-containing (Ate1\textsuperscript{flox/+};Cagg\textsuperscript{CreER}) (A) and Ate1\textsuperscript{flox/2} (B) and Ate1-deficient (Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}) (C) mice 37 days after TM-treatment. Note the loss of both visceral fat (large white arrow in A and B) and fat surrounding the kidney (small white arrows in A and B) in an Ate1-deficient mouse (C). (D) Hematoxylin/eosin staining of a 10-μm section of white adipose tissue (WAT) harvested from an Ate1-containing mouse (TM-treated Ate1\textsuperscript{flox/+};Cagg\textsuperscript{CreER}). The bar denotes 100 μm. (E) Same as in D except that WAT was from an Ate1-deficient mouse (TM-treated Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}). (F) Average weights of TM-treated Ate1-containing (n = 12; black curve) and Ate1-deficient (n = 11; red curve) mice as a function of time after the beginning of ad libitum high-fat diet. Weights were measured at weekly intervals for 10 weeks. Error bars indicate ±SD. (G) Comparisons, by immunoblotting, of Ucp1 protein levels in extracts from brown adipose tissue (BAT) (lanes 1 and 2) and WAT (lanes 3 through 6) from Ate1\textsuperscript{+/+} and Ate1\textsuperscript{−/−} mice 46 days (lanes 1 and 2) or −1 year (lanes 3–6) after TM treatment. Specific genotypes were as follows (genotypes after TM treatment are indicated in parentheses here, and also on top of the gel): lane 1, Ate1\textsuperscript{flox/+/+}; lane 2, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}; lane 3, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}; lane 4, Ate1\textsuperscript{flox/+};Cagg\textsuperscript{CreER}; lane 5, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}; lane 6, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}. Note abnormally high expression of Ucp1 in WAT of Ate1-deficient mice (lanes 3 and 5). An asterisk denotes a protein in WAT that cross-reacts with anti-Ucp1 antibody. (H) RT-PCR analyses of leptin and Ucp1 mRNA levels in BAT (lanes 1–4) and WAT (lanes 5–8) of Ate1-containing (denoted as “+/+”; lanes 2, 4, 6, and 8) and Ate1-deficient (denoted as “−/−”; lanes 1, 3, 5, and 7) mice −1 year after TM treatment. Specific genotypes: lanes 1 and 5, Ate1\textsuperscript{flox/+/+};Cagg\textsuperscript{CreER}; lanes 2 and 6, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}; lanes 3 and 5, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}; lanes 4 and 8, Ate1\textsuperscript{flox/2};Cagg\textsuperscript{CreER}. (I) RT-PCR analyses of Ucp1 and Ucp2 mRNA levels in BAT, liver, muscle, and WAT of an Ate1\textsuperscript{flox} mouse (denoted as “+/+”) and an Ate1\textsuperscript{flox};Cagg\textsuperscript{CreER} mouse (denoted as “−/−”) −1 year after TM treatment. doi:10.1371/journal.pone.0007757.g007
undergo apoptosis or differentiate abnormally, yielding defective sperm cells.

Previous work demonstrated a defective assembly of synaptosomal complexes and massive apoptosis of spermatocytes in Ub2−/−/− mice [26]. The Ate1 R-transferase acts upstream of Ub2 and other Ub ligases of the N-end rule pathway (Fig. 1A). Given a role of Ate1 in spermatogenesis demonstrated in the present study, it is possible that the currently unknown N-end rule substrate(s) whose degradation is in down-regulated in Ub2−/−/− spermatocytes is an Ate1 substrate. To assess the extent of apoptosis in Ate1-deficient spermatocytes, we employed immunohistochemistry with antibody to poly(ADP-ribose)polymerase (PARP), which is cleaved by caspases late in apoptosis. Anti-PARP antibody detected the (expected) 116 kDa full-length PARP in extracts from Ate1-containing mouse testis, but no 85-kDa PARP fragment, a marker of apoptosis (Fig. 6K, lane 1) [29]. In contrast, Ate1-deficient testis contained the 85-kDa fragment of PARP but virtually no full-length PARP, indicating extensive apoptosis in the absence of Ate1 (Fig. 6K, lane 2; cf. lane 1), in agreement with cytological and Ate1−/− male-infertility data (Fig. 6F–I). The 85-kDa PARP fragment is expected to bear N-terminal Gly [29], which is not a substrate of the Ate1 R-transferase (Fig. 1A). Thus the absence of the 85-kDa PARP fragment in Ate1-containing testis (Fig. 6K, lane 1) signifies the lack of production of this fragment by caspases, rather than its degradation by the Ate1 enzyme. The virtually null Ate1 state of the brain in Ate1−/−/−;CaggCreER mice was also indicated by a strong accumulation of R484, a physiological substrate of Ate1 (see Introduction) (Fig. 1D).

We carried out cell proliferation assays with Ate1−/−/−;CaggCreER mice (and controls), using 5-ethynyl-2′-deoxyuridine (EdU). In examinations of EdU-labeled brain sections, we paid particular attention to regions such as the hippocampus and the periven- tricular zone of the lateral ventricles, where neurogenesis is known to occur. However, no differences in EdU incorporation between Ate1-deficient and Ate1-containing brains were observed (data not shown), consistent with a brain edema (fluid accumulation) being a significant cause of brain enlargement in Ate1-deficient mice. We also determined the water content of freshly isolated brains, by subtracting their “dry weights” (after freeze-drying) from their total weights. The average water content and dry weight of control (Ate1-containing) brains was 79.9% and 20.1%, respectively, versus 80.5% and 19.5%, respectively (p<0.03), for Ate1-deficient brains (Fig. 6B). Thus cerebral edema at least contributes to the observed differences in brain weight between Ate1-deficient and Ate1-containing mice. It remains to be determined whether an edema (owing, e.g., to an osmotic imbalance or inflammation) suffices to account for consistently observed Ate1-dependent differences in brain weights (Fig. 6A, B).

There was also a 10-fold higher propensity for seizures among Ate1-deficient mice. For example, during routine cage changes and handling of mice, ~3.1% of Ate1-deficient mice (38 of 1,232) versus ~0.3% of identically TM-treated Ate1-containing mice had tonic-clonic seizures. The skulls of Ate1-deficient mice appeared to be thinner, “softer” than the skulls of Ate1-containing mice. Although MRI analyses did not reveal statistically significant abnormalities in the shape or size of skulls in Ate1-deficient mice (Fig. 8B, C), the MRI data did not preclude the possibility that bone structure may be perturbed in the absence of Ate1. These issues remain to be addressed.

The neurological/behavioral abnormalities of Ate1-deficient mice included an enhanced startle response, a marker for increased anxiety in rodents. Specifically, the latency between stimulus and response (Tmax) for Ate1-deficient mice was between 54% and 76% of the average latency for Ate1-containing controls, i.e., Ate1-deficient mice reacted significantly faster (Fig. 8D), thus exhibiting an enhanced startle response. The open field test is used to assess locomotor, exploratory and anxiety-like behavior in rodents. This test revealed a remarkably hyperkinetic behavior of Ate1-deficient mice (Figs. 6C–E and 8A), consistent with their increased startle response (Fig. 8D). The initial test involved a 15-min comparison of movements of Ate1-deficient mice versus Ate1-containing siblings of the same litter. An Ate1-deficient mouse traveled, during the test, a 3-fold greater distance than their (identically TM-treated) Ate1-containing counterpart (175.71 m versus 55.63 m, respectively) (Fig. 6C). The mean velocity of an Ate1−/−/−;CaggCreER mouse was 19.5 cm/sec, in comparison to 7.0 cm/sec for a wild-type (Ate1+/+; PrmCre) mouse, 6.2 cm/sec for an Ate1−/−/−;CaggCreER mouse, and 5.5 cm/sec for an Ate1+/−/−;CaggCreER mouse (Fig. 6E).

## Table 1. Genotypes of mice from matings of Ate1+/+ females with Ate1floxed− males containing testis-specific Cre transgenes.

| Genotypes | Ate1floxed−;PmrCre ∩ | Ate1floxed−;Ppr28.8Cre ∩ |
|-----------|----------------------|--------------------------|
| # breeding pairs | 3 | 3 |
| # litters | 6 | 4 |
| Average litter size | 7 5.25 |
| total pups | 42 21 |
| % floxOFF | 14 1 |
| % floxON | 33 4.7 |
| % floxON | 13 12 |
| % floxON | 31 5.7 |

Matings involving the Ppr28.8 Cre strain occurred ~1 month following TM treatment.

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controls. In particular, the brains of some Ate1-deficient mice reached 5% of TBW (Fig. 6A). Histological patterns of NLS-Bgal [10] expressed from the Pnol promoter in the brains of Ate1−/−/− mice (data not shown) were in agreement with in situ hybridization data in the Allen Brain Atlas (http://www.brain-map.org/), in that Ate1 was expressed at varying but significant levels throughout the mouse brain, particularly in the hippocampus, dorsal thalamus, and cerebellum. No Ate1 protein could be detected in brain extracts of Ate1−/−/−;CaggCreER mice, in contrast to extracts from wild-type or Ate1−/−/− brains (Fig. 1C, D). The virtually null Ate1 state of the brain in Ate1−/−/−;CaggCreER mice was also indicated by a strong accumulation of R484, a physiological substrate of Ate1 (see Introduction) (Fig. 1D).

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To assess generality of this striking phenotype, we repeated the open field test with three Ate1-deficient mice at 10, 26, 38, and 82 days after TM treatment, in parallel with TM-treated Ate1-containing (control) mice. At 10 days after TM treatment, i.e., soon after the acquisition of the Ate1<sup>−/−</sup> genotype, the differences between distances travelled by Ate1-deficient versus Ate1-containing mice were small (Fig. 8A). However, by 26 days after TM treatment, there was a statistically significant difference between Ate1-deficient and Ate1-containing mice in regard to their locomotor activity (Fig. 8A). By 82 days after TM treatment, the locomotor activity of Ate1-deficient mice, in conjunction with their elevated overall anxiety, increased so much that the device in which the open field tests were performed became nearly impractical, as Ate1-deficient mice (in contrast to Ate-containing ones) kept jumping out of the testing box.

Figure 8. Brain abnormalities and behavioral phenotypes of Ate1-deficient mice. (A) Ate1-deficient mice become hyperactive as a function of time after TM-mediated ablation of Ate1. Total distance (in cm) traveled over 15 min in the open field test box (2500 cm²). This test was repeated every ~2 weeks after the end of TM treatment. The data for Ate1-containing mice (n = 5; their genotypes were Ate1<sup>flx</sup>/+, Ate1<sup>flx</sup>/2, and Ate1<sup>flx</sup>/2:CaggCreER) and Ate1-deficient mice (n = 3; Ate1<sup>flx</sup>−/−:CaggCreER) are indicated by black diamonds and red circles, respectively. The horizontal bars indicate mean values. The average total distance traveled over 15 min for all Ate1-containing mice (n = 37) was 4,870 cm. (B) Representative magnetic resonance images showing equivalent horizontal planes of Ate1-containing (Ate1<sup>flx</sup>/+:CaggCreER on the left, Ate1<sup>flx</sup>/2: on the right) brains ~3 months after TM treatment. The indicated average width of the skull (measured at the widest point from left to right in the same plane) of four Ate1-containing mice was 10.6 mm (±0.89 mm). (C) Same as in B except with brains from Ate1-deficient (Ate1<sup>flx</sup>−/−:CaggCreER) mice ~3 months after TM treatment. The average width of the skull (measured as in B) of four Ate1-deficient mice was 10.8 mm (±0.38 mm). (D) Comparison of the response latency (T<sub>max</sub>) recorded in msec between Ate1-containing (n = 3; black bars) and Ate1-deficient mice (n = 3; red bars) to a 40-msec pulse of 120 dB (p120; p < 0.3), a 40-msec pulse of 120 dB preceded by a pre-pulse of 5 dB (pp5; p < 0.09), or a 40-msec pulse of 120 dB preceded by a pre-pulse of 15 dB (pp15; p < 0.01). Statistical analysis was performed using an unpaired t-test. doi:10.1371/journal.pone.0007757.g008
Depletion of White Adipose Tissue in Ate1-Deficient Mice, and Their Resistance to Diet-Induced Obesity

To address the cause of a strikingly lower content of the peritoneal white adipose tissue (WAT) in Ate1-deficient mice, on average only 16% of WAT in Ate1-containing mice (Figs. 5D and 7A–C), we examined sections of intraperitoneal WAT. The average diameter of WAT adipocytes from Ate1-deficient mice was ~30% of the average diameter of such cells in identically TM-treated Ate1-containing mice (25.5 ± 7.4 μm versus 76.2 ± 16.2 μm, respectively) (Fig. 7D, E). Thus, at least the bulk of WAT decrease in Ate1-deficient mice resulted from a decreased lipid content of individual adipocytes, rather from an extensive loss of adipocytes. Similar results were obtained with intrascapular brown adipose tissue (BAT) (Fig. 9A, B). The leanness of Ate1-deficient mice was particularly striking in view of their hyperphagy (see below).

We also asked whether the consistent difference in weight between Ate1-deficient and Ate1-containing mice on a standard ad libitum diet (Fig. 4A–C) could be reduced by an energy-rich, high-fat diet (HFD). At the end of the resulting 10-week test, the average weight of HFD-treated Ate1-containing mice was 152% of their starting weight (40.0 g versus 26.3 g). In contrast, the average weight of identically HFD-treated Ate1-deficient mice was only 122% (24.0 g versus 19.7 g) of their starting weight (Fig. 7F), indicating their relative resistance to diet-induced obesity. Yet
another phenotype of Ate1-deficient mice, observed during their initial loss of weight after TM treatment (Fig. 4A, B), was their lower core body temperature, on average 35.1°C, in comparison to identically TM-treated Ate1-containing control mice, whose average core body temperature was 36.0°C during the same time, in the absence of weight loss (Fig. 9E). After the early deaths of ~15% of Ate1fl/fl-CaggCreER mice (Fig. 4A, B), the average temperature of surviving mice (36.6°C) was not significantly different from that of Ate1-containing control mice (36.7°C) (Fig. 9E). As one would expect from their depletion of WAT (Fig. 7A–C), Ate1-deficient mice were strongly hypersensitive to cold (Fig. 9F).

Ucp1 is a proton carrier in the mitochondrial inner membrane that mediates a partial uncoupling of oxidative phosphorylation from ATP synthesis, an alteration that can increase heat production and thereby regulate body temperature and energy homeostasis. Although Ucp1 is normally expressed in BAT but not in WAT, several mouse mutants other than Ate1-/- are resistant to diet-induced obesity have been shown to ectopically express Ucp1 in WAT [32,33]. Using RT-PCR and immunoblotting with anti-Ucp1 antibody, we found that the levels of Ucp1 mRNA and Ucp1 protein in BAT did not change significantly between Ate1-deficient and Ate1-containing mice (Fig. 7G–I). Remarkably, however, the levels of both Ucp1 mRNA and Ucp1 protein were strongly increased in WAT of Ate1-deficient mice (Fig. 7G–I). A Ucp1-Ate1 connection revealed by these findings adds a new dimension to the understanding of Ucp1 regulation ([32] and refs. therein), and may also provide an experimental route to identifying a relevant circuit that involves Ate1.

Increased Metabolic Rate in Ate1-Deficient Mice

During the week prior to TM treatment, Ate1fl/fl-CaggCreER and control (Ate1fl/fl-CaggCreER) mice (at that point, both strains contained Ate1) consumed 0.63 and 0.62 kcal of standard chow per gram of body weight per day, respectively (Fig. 10C). Within a week after TM treatment the now Ate1-deficient Ate1fl/fl-CaggCreER mice increased their food consumption on average to 125% of identically TM-treated Ate1-containing mice (Fig. 10C). This pattern of significant hyperphagia of Ate1-deficient mice continued for the duration of this study, i.e., up to ~8 months, with regular measurements for 6 weeks following TM treatment and intermittent comparisons afterwards (Fig. 10C). Thus, despite their initial decline of weight shortly after TM treatment and the early death of ~15% of Ate1-deficient mice, and despite their subsequent failure to gain, on average, more than ~63% and ~69% of the weights of Ate1-/- and Ate1+/+ mice, respectively, the Ate1-deficient mice consumed significantly more food than their Ate1-containing counterparts (Fig. 10C). To address their patterns of glucose utilization, we fasted these mice for 16 hr and measured blood glucose before after administering a 50-ml (0.2 ml) bolus of glucose by gavage. The kinetics of rise and fall of blood glucose levels under these conditions was similar for Ate1-deficient and Ate1-containing mice (Fig. 10A). Ate1-deficient mice had lower fasting glucose levels than Ate1-containing mice (88.6 mg/dl versus 125.3 mg/dl, respectively; p<0.04), and also lower glucose levels 6 hr after administration of glucose (80.9 mg/dl versus 109.7 mg/dl, respectively; p<0.04), consistent with the (expected) higher energy expenditure of Ate1-deficient mice, and suggesting normal sensitivity of these mice to insulin (Fig. 10B). There were no other significant differences in blood composition (well as urine composition) between Ate1-containing and Ate1-deficient mice (Tables 2 and 3).

To measure metabolic rate, we employed indirect calorimetry (see Materials and Methods), determining CO2 consumption and
through deamidation, arginylation, polyubiquitylation and pro-
cessive degradation, into an output of proteolysis-derived short
peptides (Fig. 1A). The rate and selectivity of the proteasome-
mediated protein degradation by the N-end rule pathway are
modulated by physiological effectors, including specific phospho-
kinases, short peptides, redox, heme and nitric oxide (see
Introduction). Some of N-end rule substrates are produced by
proteases that include MetaPs, separases, caspas and calpains.
These and other nonprocessive proteases, which function as
upstream components of the N-end rule pathway, have in
common their ability to convert, through a cleavage, a pro-N-
degeon into an N-degron.
The present study expanded the earlier understanding of the Ate1 R-transferase (Fig. 1A) by making possible a postnatal inactivation of mouse Ate1. (Unconditional deletion of Ate1 results in embryonic lethality [10].) Described and discussed in Results is a large set of defects, some of them quite striking, in juvenile and adult mice upon the postnatal inactivation of Ate1 and the resulting loss of N-terminal arginylation (Figs. 1C, D). The initial abnormality is a rapid decrease of body weight and early death of ~15% of Ate1-deficient mice, with surviving mice attaining, gradually, only ~70% of the weight of wild-type mice identically treated with tamoxifen (TM) (Fig. 4A–C). Both “partial” lethality and the transiency of acute crisis, over ~3 weeks after TM treatment (red arrow in Fig. 4B), remain to be understood in molecular terms. This crisis and subsequent failure to thrive occur despite higher than normal food intake by Ate1-deficient mice (Fig. 10C). These mice contain little or no visceral fat (Figs. 5D and 7A–E), and exhibit an increased metabolic rate (Fig. 10E), resistance to diet-induced obesity (Fig. 7F), enlarged brains (Figs. 5D and 6A), kyphosis (Fig. 5B), a striking hyperkinesia (Figs. 6C–E and 8A), and male sterility (Fig. 6F–K).

Owing to current constraints of the Cre-lox technology ([25] and refs. therein), the extent of Ate1 inactivation, while nearly 100% in some mouse tissues, was variable in others. The TM-induced Ate1<sup>2/2</sup>;Cre<sup>ERT2</sup> mouse strains are thus mosaics of Ate1<sup>flox/2</sup> and Ate1<sup>2/2</sup> cells, where Ate1<sup>2/2</sup> cells are a great majority in most organs, such as the brain, but even there do not reach 100% of all cells (see Results). The initial weight loss upon the TM-induced conversion of Ate1<sup>flox/2</sup> mice to Ate1<sup>2/2</sup> mice was accompanied by death of ~15% of Ate1<sup>2/2</sup> mice (Fig. 4A, B). Such a “partially” lethal phenotype suggests that an adult-onset Ate1<sup>2/2</sup> genotype in all cells (as distinguished from most cells) of a mouse might be incompatible with viability, similarly to the

| Table 2. Serum analyses. |
|--------------------------|
|                         | Ate1-deficient | Ate1-containing |
| Glucose                 | Mean           | StdDev | Mean          | StdDev | Units | normal range |
|                         | 153.3          | 41.9   | 182.6         | 73.3   | mg/dl | 62–175       |
| BUN                     | 26.0           | 3.8    | 22.0          | 8.0    | mg/dl | 12–28        |
| Creatinine              | 0.4            | 0.1    | 0.4           | 0.1    | mg/dl | 0.3–1.0      |
| Sodium                  | 158.0          | 2.8    | 129.8         | 62.3   | mg/dl |             |
| Potassium               | 8.5            | 1.2    | 6.7           | 2.8    | mg/dl |             |
| NA/K ratio              | 18.8           | 2.4    | 17.1          | 7.2    | mg/dl |             |
| Chloride                | 112.8          | 3.4    | 91.9          | 43.4   | mg/dl |             |
| CO2                     | 21.5           | 2.8    | 20.8          | 9.1    | mg/dl |             |
| Anion gap               | 32.3           | 1.9    | 24.3          | 11.6   | mg/dl |             |
| Calcium                 | 9.3            | 0.4    | 8.1           | 3.4    | mg/dl | 3.2–8.5      |
| Phosphorus              | 10.5           | 2.8    | 8.6           | 3.8    | mg/dl | 2.3–9.2      |
| Osm, Calc               | 328.0          | 6.6    | 270.9         | 129.6  | mg/dl |             |
| TP                      | 5.2            | 0.4    | 4.5           | 1.8    | g/dl  | 3.5–7.2      |
| Albumin                 | 3.1            | 0.3    | 2.7           | 1.1    | g/dl  | 2.5–4.8      |
| Globulin                | 2.0            | 0.1    | 1.8           | 0.7    | g/dl  | 0.6          |
| Albumin/Globulin        | 1.5            | 0.1    | 1.3           | 0.5    | mg/dl | 4.1–8        |
| Bilirubin               | 0.2            | 0.1    | 0.1           | 0.0    | mg/dl | 0.1–0.9      |
| AP                      | 102.3          | 41.6   | 113.7         | 48.0   | U/L   | ~70          |
| gamma gt                | 0.0            | 0.0    | 0.0           | 0.0    | U/L   | ~60          |
| ALT                     | 57.0           | 14.4   | 39.5          | 11.5   | U/L   | ~100         |
| AST                     | 409*           | 286.5  | 199.2         | 83.9   | U/L   | ~100         |
| Cholesterol             | 70.1           | 4.0    | 71.5          | 31.8   | mg/dl | 26–82 (~1.5 mmol/L) |
| T4                      | 2.7            | 0.9    | 2.4           | 0.8    | mg/dl |             |
| T3                      | 54.3           | 7.4    | 50.9          | 19.7   | µg/dl |             |

*Ate1-deficient mice with more severe phenotypes tended to have higher AST levels.

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| Table 3. Urinalysis. |
|----------------------|
|                      | Ate1-deficient | Ate1-containing |
| Glucose              | neg            | neg             | mg/dL   |
| Bilirubin            | neg/small      | neg/small       |         |
| Ketone               | neg            | neg             | mg/dL   |
| Specific Gravity     | 1.03           | 1.02            |         |
| Blood                | neg            | neg             |         |
| pH                   | 6.2*           | 7.1             |         |
| Protein              | 100            | 100             | mg/dL   |
| Urobilinogen         | 0.2            | 0.2             | mg/dL   |
| Nitrite              | neg            | neg             |         |
| Leukocytes           | neg            | neg             |         |

*Ate1-deficient mice had a significantly lower urine pH.

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embryonic lethality of unconditional Ate1⁻/- mice. Thus, paradoxically, the discovery, in the present study, of specific Ate1-linked defects in adult mice might have been made possible by incomplete penetration of the Cre-induced conversion of Ate1flo to the Ate1 allele.

The set of definitively identified mammalian N-end rule substrates that involve N-terminal arginylation consists, at present, of fewer than 10 proteins. They subserve different functions, from chromosome segregation to control of apoptosis and regulation of G proteins (see Introduction). This set is the tip of the iceberg, as several considerations [2], in addition to our findings above, strongly suggest a larger number of physiological Ate1 substrates. Given this complexity, the specific and often striking phenotypes of Ate1-deficient mice that were discovered in the present work will be of major assistance in deciphering the underlying Ate1 circuits.

Methods

Animal Care and Treatments

All animal care and procedures in the present study were conducted according to the relevant NIH guidelines, and were approved (Protocol #1328) by the Institutional Animal Care and Use Committee (OLAR) at the California Institute of Technology, where the entire present study was carried out. Mice were housed at ~22°C, in a pathogen-free (barrier) facility, using a 12 hr light/12 hr dark cycle, with Laboratory Rodent Diet 5001 (PMI International, Richmond, IN) ad libitum. Mice aged between 3 and 8 weeks were treated with tamoxifen (TM) (Sigma) (2 mg in 0.2 ml sesame oil) by daily intraperitoneal (IP) injections over 5 days. Mice were weighed weekly, starting 3 days before the first TM treatment. For a high-fat diet (HFD) study, mice were fed ad libitum a diet containing 35.5% fat (BioServe, Frenchtown, NJ), and were weighed weekly.

Construction of Ate1flo/CaggCreER Mouse Strains

Mouse genomic DNA encoding Ate1 was isolated from a BAC library [10]. Two pBluescript-based plasmids were used to construct the targeting vector. In one insert, a ~12 kb HindIII fragment contained Ate1 exons 1a, 1b, 2, and 3 as well as ~1.9 kb of DNA 3' of exon 3 (ending just before exon 4). In the other insert, a ~2.9 kb fragment contained Ate1 exons 4 and 5. The entire ~12 kb HindIII fragment and a part of the ~2.9 kb fragment were modified as described below and assembled into a final ~22.5 kb targeting vector consisting of the following parts (Fig. 2C): (i) pBR322 backbone (New England Biolabs, Ipswich, MA); (ii) a ~6.3 kb “long arm” of Ate1 homology containing the Ate1 exon 1a, the bidirectional Pα promoter [14], and exon 1b; (iii) A single lOX P site ~300 bp upstream of Ate1 exon 2; (iv) a ~2 kb fragment that contains, 50 bp downstream of Ate1 exon 4, a “floxed” Hph (hygromycin) antibiotic-resistance marker, expressed from the Pα promoter [36]; (v) a ~1.2 kb “short arm” of Ate1 homology that spans most of the intron between exons 4 and 5; (vi) a gene encoding HSV-TK (herpes simplex virus thymidine kinase), expressed from the Pα promoter. The targeting vector was linearized with BamHI and electroporated into C57 embryos by DRNT electroporation into ES cells (a gift from Dr. Thomas Gridley, formerly of Jackson Laboratories, Bar Harbor, ME). ES cells were grown in DMEM supplemented with 15% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM pyruvate, and 1,000 U/ml leukemia inhibitory factor (LIF) [37], using a feeder layer of hygromycin-resistant mouse primary fibroblasts that had been treated with 10 μg/ml mitomycin C for 3 hr at 37°C. Selection with hygromycin (at 0.2 mg/ml) and 1-(2′-deoxy, 2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (HIAU; at 0.4 μM) was started 24 hr after electroporation. Correctly targeted ES cell clones that contained “tri-loxed” Ate1 allele (Figs. 2C, D) were identified using Southern hybridization and PCR. Southern DNA probes and positions of primers for PCR are indicated in Fig. 2.

Two correctly targeted, independently produced ES cell lines that had apparently normal karyotypes were injected into 3.5-days postcoitum C57BL/6j blastocysts and implanted into pseudopregnant females. The resulting male chimeric offspring were mated with C57BL/6j females. In some of the progeny, “floxed” ES cells became a part of germline line. Standard mating techniques [36,38] were then used to produce, initially, mouse strains that contained a “tri-lox” Ate1 configuration, in that they also contained the floxed positive-selection Pα-ek/ek cassette (Fig. 2D). This DNA segment was removed by mating Ate1-tri-lox heterozygotes with EIIα-Cre mice that expressed Cre recombinase only in early, pre-implantation blastocysts [39,40,41]. Owing to the presence of three loxP sites at the initial floxed Ate1 locus, F1 progeny from this cross were mosaic, i.e., their tissues, including germ line, contained varying configurations of retained loxP sites, depending on specific patterns of Cre-mediated recombination (Fig. 2A–F). To isolate a mouse strain with the desired configuration of (retained) loxP sites (Fig. 2E), the above F1 mosaic mice were mated to wild-type C57BL/6j mice. This produced, among other progeny, a strain that lacked the Pα-ek cassette and had the desired Ate1flo/+ genotype, in the (mixed) C57BL/6j-129SvEv background.

Through the use of appropriate mating pairs, with genotyping of resulting progeny, we produced Ate1flo/+;CaggCreER mice as well as Ate1floflo/+;CaggCreER mice (Fig. 2). The former strain harbored one unconditionally null Ate1 allele (derived, through matings, from the previously constructed unconditional heterozygous Ate1+/– mice [10]) and one “floxed”, conditionally active Ate1flo/+ allele that could be made null in the presence of active Cre recombinase. In the latter strain Ate1floflo/+;CaggCreER, both copies of Ate1 were Ate1flo. These mouse strains also contained the CaggCreER gene, expressed from the ubiquitously active chimeric Cagg promoter (Fig. 2F) [25]. CaggCreER encoded CreER, a fusion between the phage P1 Cre recombinase and a derivative of the mouse estrogen receptor ligand binding domain. CreER was functionally inactive (sequestered in the cytosol) but could be activated by intraperitoneal (IP) injection of tamoxifen (TM) [25]. To produce Ate1floflo/+;CaggCreER mice, we mated Ate1flo/+;CaggCreER (the latter were generated by mating Ate1+/–;CaggCreER mice) with Ate1+/–;CaggCreER mice (Fig. 2E). To produce Ate1floflo/+;CaggCreER mice, we mated Ate1flo+/+;CaggCreER mice (the latter were generated by mating Ate1+/+;CaggCreER mice). In the notations here and elsewhere in the paper, “floxo-off” indicates a configuration depicted in Fig. 2E (the functionally active Ate1flo/+ allele), whereas “floxo-off” indicates a configuration depicted in Fig. 2F (the null Ate1 allele).

Southern Hybridization and PCR

Total genomic DNA was isolated from ES cells by washing them twice with phosphate-buffered saline PBS, followed by an overnight incubation at 50°C in 10 mM EDTA, 10 mM NaCl, 0.5% Sarcosyl,10 mM Tris-HCl (pH 7.5) containing Proteinase K at 0.2 mg/ml. Thereafter an equal volume of 75 mM NaCl in 100% ethanol was added. Precipitated genomic DNA was then gently washed twice with 70% ethanol and resuspended in T1,lF buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA). Total genomic DNA was isolated from mouse tails or other tissues by overnight incubation at 55°C, with constant rotation, in 5 mM EDTA, 0.2 M NaCl, 0.3% SDS, 0.1 M Tris [pH 8.5] containing Proteinase K at 0.4 mg/ml.
Thereafter an equal volume of isopropanol was added, and the mixture was gently inverted several times. Genomic DNA was then precipitated, and washed twice, with 70% ethanol, followed by a gentle resuspension in T10E0.1 buffer.

Southern hybridization was performed as described [42], with a 32P-labeled mouse DNA probes that was produced by PCR using the following primers: CB108F and CB107R (Table 2) to amplify a 219 bp genomic fragment containing Ate1 exon 5 (probe D, external probe); CB23 and CB24 (Table 4) to amplify a 929 bp genomic fragment that was a part of the long arm of the targeting vector (Probe A, internal probe) (Fig. 2). DNA probes were labeled with 32P-labeled using the Rediprime-II Random Prime Labeling System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s protocol. Hybridization with Probe A was carried out overnight at 57°C in ExpressHyb solution (Clontech). The membrane was then washed once for 10 min at room temperature (RT) in 2xSSC/0.1% SDS, once for 30 min at 55°C in 2xSSC/0.1% SDS, once for 30 minutes at 58°C in 0.5xSSC/0.1% SDS, and once for 30 minutes at 65°C in 0.1xSSC/0.1% SDS, followed by autoradiography. (1xSSC is 0.15 M NaCl, 15 mM Na-citrate, pH 7.4.) Hybridization with Probe D was carried out overnight at 55°C in ExpressHyb solution (Clontech). The membrane was then washed once for 10 min at RT in 2xSSC/0.1% SDS, once for 30 min at 55°C in 2xSSC/0.1% SDS, and once for 30 min at 58°C in 1xSSC/0.1% SDS, followed by autoradiography.

Genotyping of Mouse Strains

PCR-based genotyping was carried out with total genomic DNA isolated from various mouse tissues. Routine genotyping was performed using DNA from mouse tails. Specific Ate1 alleles and transgenes encoding specific derivatives of Cre were identified as

| Name   | Nucleotide sequence (5’ to 3’)                  | Use                                      |
|--------|------------------------------------------------|------------------------------------------|
| CB23   | ACTTTACGTTGCTAGATAAGC                         | for PCR of Southern Probe A              |
| CB24   | AGGAGGTACCAGTCATCCAGCTC                       | for PCR of Southern Probe A              |
| CB107R | AATTCTTTAGACCCCTTCTTTTTGT                    | for PCR of Southern Probe D              |
| CB108F | TGCTACTAAATGACGTCATGGAGGGCCTCTCTTCTTCTTTAGATGGCCCATGGATCTAC | for PCR of Southern Probe D              |
| CB156F | CAGGCAGGAGAAGGACGC                           | PCR detection ATE1-floxON                |
| CB157R | TTCAGGTAGCTTCCTGCTGG CTG GTG GT              | PCR detection ATE1-floxON and ATE1-floxOFF |
| AK49   | GGGATTGCTGCTGCGAGTGTG                        | PCR detection of ATE1-null               |
| YT641  | CTGCTTCCATATACTATCTCTACGA                     | PCR detection of ATE1-null               |
| AK83-Clfix | CTGGAGACGAAAGCCCGGAGCTTCT                   | PCR detection of Cre gene A             |
| Cre-1  | GTGCGAACGGACATGACGAC                        | PCR detection of Cre gene C             |
| Cre-2  | GGGAGGTACCAAGCATGGGCTCTGT                    | PCR detection wild type ATE1            |
| CB159R | ACATGAGAGGACATGACGAGCT                      | PCR detection wild type ATE1            |
| CB160F | ACAGAAGATCAGATGACGAC                      | PCR detection wild type ATE1            |
| CB110F | GTGCTTCTTCAATCAGGCTCACC                      | PCR detection ATE1-floxOFF              |
| oIMR0042 | GTAAGGAAATGATCGACAGATCTG                    | PCR detection IL-2 control              |
| oIMR0043 | GGGAGGTACCAAGCATGGGCTCTGT                    | PCR detection IL-2 control              |
| AgRP-for | GGGAGGTACCAAGCATGGGCTCTGT                    | RT-PCR                                   |
| AgRP-rev | GGGAGGTACCAAGCATGGGCTCTGT                    | RT-PCR                                   |
| NPY-for | TCCGCGCTCGCACTCAGACCT                     | RT-PCR                                   |
| NPY-rev | GGGCGCTCGCACTCAGACCT                     | RT-PCR                                   |
| POMC-for | ACCTCACACGGACGCCAAGCA                     | RT-PCR                                   |
| POMC-rev | GGGCGCGCTCGCACTCAGACCA                    | RT-PCR                                   |
| MCH-for | ATTCAAGGCAACAGCAGGCTCACAC                    | RT-PCR                                   |
| MCH-rev | CAGATCCCTTCCAGCCATGCAAGAGTA                | RT-PCR                                   |
| cyclophilin-for | GGGAGGTACCAAGCATGGGCTCTGT                    | RT-PCR                                   |
| Cyclophilin-rev | GGGAGGTACCAAGCATGGGCTCTGT                    | RT-PCR                                   |
| Ucp2-5’ | GGGGGGGCGGCGCATGTTGCGGAGACCCCTGTG          | RT-PCR                                   |
| Ucp2-3’ | GGGGGGGCGGCGCATGTTGCGGAGACCCCTGTG          | RT-PCR                                   |
| Aco2-5’ | ATGGAGGTCATGATGTCGCTGTG                    | RT-PCR                                   |
| Aco2-3’ | GAAGCTGCTAGCAGCCATGGGCTCTGT                | RT-PCR                                   |
| Leptin-5’ | GGGGGCGGCGGCGCATGTTGCGGAGACCCCTGTG        | RT-PCR                                   |
| Leptin-3’ | GGGGGGGCGGCGCATGTTGCGGAGACCCCTGTG        | RT-PCR                                   |
| Ucp1-5’ | GGGGGGGCGGCGCATGTTGCGGAGACCCCTGTG        | RT-PCR                                   |
| Ucp1-3’ | GGGGGGGCGGCGCATGTTGCGGAGACCCCTGTG        | RT-PCR                                   |

Table 4. PCR primers used in the present study.

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follows. A two-primer PCR using the CB156F and CB157R primers (Table 4) was employed to produce and detect a 512 bp fragment of the Ate1\textsuperscript{+} ("floxOFF", active) allele as well as a 472 bp fragment of the wild-type Ate1\textsuperscript{+} allele. A four-primer PCR using the CB101F, CB157R, OMR0042, and OMR0045 primers \(Z\) (Table 4) was employed to produce and detect a 470 bp fragment of the Ate1\textsuperscript{flox} derived Ate1\textsuperscript{−} allele ("floxOFF") as well as a (control) 324 bp fragment of the B2-gene. A three-primer PCR using the AK49, YT641, and AK38-CBSee (Table 4) was employed to detect both a 300 bp fragment of the unconditional Ate1\textsuperscript{−} allele \(10\) and a 560-bp fragment of the wild-type Ate1\textsuperscript{+} allele. A four-primer PCR using the Cre-1, Cre-2, CB159R, and CB160F primers (Table 4) was employed to detect both a 320 bp fragment of the CaggCreER transgene as well as a 1,060 bp fragment of the wild-type Ate1\textsuperscript{+} allele. All PCR reactions except for those to detect the CaggCreER transgene were carried out using HotStar Taq DNA polymerase, standard buffer conditions (Qiagen, Valencia, CA), 35 cycles of template denaturation for 30 seconds at 95°C, followed by primer annealing for 30 seconds at 60°C and primer extension for 1 minute at 72°C. PCR reactions for detecting CaggCreER were carried out using 30 cycles of template denaturation for 30 seconds at 95°C, followed by primer annealing for 30 seconds at 58°C and primer extension for 45 seconds at 72°C.

**Northern and RT-PCR Analyses of RNA**

Total RNA was isolated from various mouse tissues using the RNaseasy Protect Mini Kit (Qiagen). Tissue disruption and homogenization were done in Buffer RLT and the MP FastPrep-24 instrument with Lysing Matrix D (MP Biomedicals, Solon, OH) for 2 runs at 6.5 m/s. First-strand cDNA was primed with oligo-dT using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and PCR was carried out using primers cited in legends to the corresponding figures and in Table 4.

**Tissue Extracts and Immunoblotting**

Various mouse tissues were harvested and lysed in “Tissue Lysis Buffer” (10% glycerol, 0.05% NP40, 0.15 M NaCl, 2 mM EDTA, 1 mM dithiothreitol (DTT) 1 mM phenylmethylsulfonyl fluoride PMSF 50 mM HEPES, pH 7.5) plus freshly dissolved “Complete EDTA-Free Protease Inhibitors” (Roche), using the MP FastPrep-24 instrument and Lysing Matrix D (MP Biomedicals, with 2 or 3 runs at 6.5 m/s for 25 sec each, and with 5-min incubations on ice between the runs. The lysates were centrifuged at 10,000g for 20 min at 4°C. The supernatants were fractionated by SDS-12.5% PAGE, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA), and analyzed by immunoblotting (IB) with antibodies indicated in specific figures. Immunoblots were visualized using SuperSignal West Pico or SuperSignal West Dura reagents (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions.

**Other Analyses of Mouse Tissues**

Specific mouse tissues were dissected immediately after euthanasia by CO2 inhalation. The tissues washed with PBS, blotted dry on Kimwipes, and weighed (wet). For dry-weight measurements, mouse brains were dissected intact, washed in PBS, blotted dry on Kimwipes, weighed wet, then incubated overnight in acetone. After acetone incubation, individual brains were lyophilized until their (dry) weight no longer decreased.

For routine histological examinations, tissues or organs were fixed in Bouin’s solution or in 4% formaldehyde, using standard procedures [57]. Fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. To stain for LacZ (NLS-\(\beta\text{-gal}\)), dissected tissues or organs were fixed in LacZfix (0.2% glutaraldehyde, 5 mM EGTA, 0.1 M MgCl\(_2\) in PBS (pH 7.3) for 4 h, rinsed twice with PBS, dehydrated overnight at 4°C in 50%

**Blood and Urine Analyses**

Blood (~0.6 ml per mouse) was withdrawn by cardiac puncture and transferred into BD Microtainer SST tubes (BD, Franklin Lakes, NJ). The serum fraction was prepared by centrifugation in a microcentrifuge after clotting occurred, immediately frozen in liquid N\(_2\) and stored at ~80°C. The levels of glucose, cholesterol, sodium, potassium, chloride, calcium, phosphorus, blood urea nitrogen, creatine, total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, \(\gamma\)-glutamyltransferase (gamma gt), as well as the T3 and T4 hormones were determined by Phoenix Central Laboratories (Everett, WA).

Urine was obtained by placing the external urethra over a test tube. Urine samples collected from Ate1-deficient mice were pooled and compared with pooled urine from Ate1-containing mice. The levels of glucose, bilirubin, ketones, blood, protein, urobiligen, nitrite, leukocytes, as well as the pH and specific gravity were determined using the Multistix 10 SG Reagent Strips (Bayer, Tarry Town, NY).

**Measurements of Body Temperature and Cold Sensitivity**

Mice (housed at one mouse per cage, without bedding but with food and water) were exposed to a 4°C environment for up to 6 hr. Their core body temperature was monitored every 30 min via a rectal probe digital thermometer (Thermalert TH-8; Physitemp Instruments, Clifton, NJ).

**Measurements of 14C-Protein Uptake from Gastrointestinal Tract**

E. coli DH5\(_{\alpha}\) cells were grown in minimal M9 media supplemented with 0.5% glucose and 50 \(\mu\)g of 14C-amino acids

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(derived from $^{13}$C-protein hydrolysate (Amersham)) until the incorporation of ~70% of the added $^{13}$C amino acids. Cells were lysed by one freeze/thaw cycle in PBS containing 1 mg/ml lysozyme and incubated with RNase H and DNase I for 45 min at 37°C. A crude protein fraction was isolated by precipitation with cold 10% TCA (C$_2$H$_5$COOH), and the pellet was washed with ice-cold acetone. The pellet was redissolved in PBS, with a brief sonication to facilitate solubilization. 0.2 ml of the resulting cold acetone. The pellet was redissolved in PBS, with a brief sonication to facilitate solubilization. 0.2 ml of the resulting sample, containing 260,000 cpm of $^{13}$C-labeled E. coli proteins, was fed to a mouse by oral gavage. Urine and feces was collected at various times post-gavage. Total $^{13}$C was measured, using a scintillation spectrometer, in feces, urine, and (eventually) in mouse tissue samples that were collected either 48 hr or 15 days post-gavage.

Measurements of Glucose Uptake

For glucose analyses, mice were fasted for 24 hr, then gavaged with 50 mg glucose in 0.2 ml of water. Blood was collected through the lateral tail vein at 15, 30, 60, 90, 120, and 360 min post-gavage. Blood glucose levels were determined using the OneTouch UltraMini Blood Glucose Monitoring System (LifeScan, Johnson and Johnson, Milpitas, CA).

Metabolic Analyses

The resting metabolic rate was determined at the Mouse Physiology Laboratory in the Department of Physiology at the Geffen School of Medicine, UCLA using indirect calorimetry as previously described [43,44]. Single mice were placed into a custom-made enclosed plexiglas chamber (25 cm x 12 cm x 7.5 cm, with 4 room air intake vents and one outflow port) and allowed to come to rest over a period of 30 min to 2 hr. Outflow of expired gases was sampled by the gas analyzer and recorded using a computerized acquisition system during a 30-min resting interval.

MRI Analyses of Mouse Brains

The procedures used were essentially the same as previously described [45]. Mice were given an IP injection of 40 mg/kg of Na-pentobarbital (Nembutal, Hospira, Inc., Lake Forest, IL). Once fully anesthetized, mice were transcardially perfused with 4% formaldehyde in PBS. MRI analysis was performed by the Caltech Brain Imaging Center. Briefly, mouse heads were excised and postfixed in 4% formaldehyde/PBS overnight. Hair and skin were removed from fixed heads, which were then soaked in 5 mM Gdodium-based MR contrast agent (Prohance Bracco Diagnostics, Durham, NC) for 10 days, to decrease the intrinsic tissue relaxation rates and improve the MR acquisition efficiency. A gradient echo sequence (TE/TR = 8 msec/50 msec, 16 averages) was used to acquire 3D data sets of the mice heads, using a Bruker 7T Biospec animal magnet system. Images were reconstructed with an isotropic resolution of ~90 µm and analyzed using BrainSuite 2 software [46].

Open-Field and Startle Response Tests

For the open-field activity measurements, individual mice were placed into a square chamber (30 by 50 cm). Movements along the x and y axes were tracked and analyzed using Ethovision software (Noldus, Leesburg, VA) over 15-min intervals. Startle response tests were carried out essentially as described previously [47].

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Author Contributions

Conceived and designed the experiments: CSB AV. Performed the experiments: CSB. Analyzed the data: CSB AV. Contributed reagents/materials/analysis tools: CSB AV. Wrote the paper: CSB AV.

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