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Consequences of *ATAD3* Hemizygosity in *C57Bl6j* Mice

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Abstract

ATAD3 is a mitochondrial ATPase involved in reticulum/mitochondria interactions and cholesterol transport and essential for mitochondrial biogenesis as vital at an early stage of development but still with unknown molecular functions.

To evaluate the role of *ATAD3* expression at the animal scale we used *ATAD3* hemizygote mice of *C57bl6j* genetic background to test the impact on mitochondrial biogenesis. For this goal, we studied two physiological capacities involving mitochondrial biogenesis: (i) training efficiency for running performance, with males, and (ii) reaction to high-fat/high-sucrose diets with females.

We found that *ATAD3* hemizygosity decreases male's heart and body weights, especially at the adipose tissue level, and almost suppress running training improvement, but surprisingly increases the gripping strength and modifies the circadian activity. These phenotypes are associated with a lower mitochondrial mass and a major increase of Acetyl-CoA Carboxylase (ACC) phosphorylation ratio status in muscles. *ATAD3* hemizygosity favors body weight increase under high-fat/high-sucrose diet in females, especially at the adipose tissue level, and induces a major increase of ACC phosphorylation status in the adipose tissue.

We show therefore that *ATAD3* gene does not present allelic compensation, or haplo-sufficiency, under training and high-calorie dieting phases in mice and that *ATAD3* hemizygosity is potentially involved in pathologies such as obesity and not severe myopathies.

Keywords: *ATAD3*; Mitochondria; Exercise; Diet

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Introduction

ATAD3 (ATPase family AAA Domain-containing protein 3) is a 68 KDa mitochondrial inner membrane ATPase which appeared phylogenetically in the pluricellular organism [1]. Despite a still undiscovered function, *ATAD3* is known as vital, as essential for mitochondrial biogenesis [2-4] and for early development of animals like nematodes [5,6] drosophila [7] and mouse [8]. As others AAA+ ATPases, *ATAD3* is expected to be active in multimeric states like forming a ring-shaped P-loop exerting its activity through energy-dependent remodeling and translocation of macromolecules [9,10]. Among the cellular phenotypes induced by *ATAD3* miss-of-function is the impact on the mitochondrial biogenesis [4,11] and network formation [4,12] as on mtDNA maintenance [2]. *ATAD3* is therefore a new and major actor of the mitochondrial physiology, becoming a candidate potentially involved in some unclassified mitochondrial-associated diseases. Either, *ATAD3* expression was also tightly linked to cancer appearance and progression [1,13].

Today, the strongest putative function for *ATAD3* is thought to be a direct contribution in cholesterol import and/or more likely in lipid rafts transport of nuclear-encoded mitochondrial proteins, from the reticulum to the mitochondria [2,4,14]. *ATAD3* is proposed therefore to act as a molecular motor inducing morphological modification of membranes and their translocation. Recent works showed indeed that *ATAD3* could interact in complex with VDAC (Voltage-Dependent Anion Channel) and Translocator

Protein (TSPO)[14] or also with *COX* assembly factor [15]. In the side, *ATAD3* interacts with Mitochondrial Associated Membranes (MAM) [16,17]. This would support *ATAD3* as a potential partner in sterol transport and especially in cholesterol transport. In addition, it is known from others and us that *ATAD3* functionally and structurally interacts with Mfn2/Drp1 fusion/fission machinery [12,16]. Then, both observations lead to think that *ATAD3* could be involved in contact sites between mitochondria and the endoplasmic reticulum and contributing there in lipid-protein exports to mitochondria. More recently, *ATAD3* was also shown to regulate mitochondrial iron homeostasis [18].

Again, and without knowing with its exact function, *ATAD3* dysfunction can be potentially involved in mitochondria-linked diseases, especially in yet unclassified ones, among which are myopathies, neuropathies, or metabolic diseases and some cancers. Indeed, three recent studies have shown in a set of family patients, that *ATAD3* mutation, mono-or bi-allelic, were linked to severe neuro-myopathies [19-21]. Also, the causality was clearly shown by the skeletal muscle-specific knockout of *ATAD3* in mice that induces muscle weakness and mitochondrial abnormalities [22].

Therefore, to address the question of knowing deeper about the direct impact of *ATAD3* loss of function at the body level, we went to *ATAD3*+/-mice model to screen for basic muscular behaviors and metabolic characteristics [8]. Because *ATAD3*+/-mice do not present any obvious phenotype (normal fertility and sex ratio), we enterprise to watch the mice along periods requiring significant mitochondrial biogenesis, as muscle training and adipose tissue fat uptake [4,23-25]. Mitochondrial biogenesis was investigated by training male mice for running 7 times a week for 6 weeks, as males react higher to physical training, and adipose tissue physiology was tested by submitting female cohorts to high-fat/high glucose diet for 4 weeks, as females react higher to high-calorie diets.

We found that *ATAD3* hemizygosity inhibits running training capacities and mitochondrial biogenesis and impacts body weight increase under high-calorie diet, both phenotypes involving a major increase of ACC phosphorylation in the concerned tissues.

ATAD3 gene does not therefore present allelic compensations, or haplo-sufficiency, under physical training and high-calorie dieting phases in mice and therefore *ATAD3* hemizygosity is potentially involved in pathologies such as obesity and not severe myopathies.

Materials and Methods

Mice-breeding

ATAD3+/-male *C57bl/6j* mice were obtained from Pr. Kolanus laboratory, at the Life and Medical Sciences Institute (LIMES) in Bonn [8] and crossed with *C57bl/6j* female mice. Progenies were genotyped by PCR (Figure 1A) using a distal finger tarsus cut performed for numbering newborn.

All experiments followed the European Union recommendations concerning the care and use of laboratory animals for experimental and scientific purposes. All animal work was approved by the local board of ethics for animal experimentation and notified to the research animal facility of our laboratory (authorization ComEth Grenoble n°244-04427.04). The performed research was in compliance with Animal Research: Reporting of *in vivo* Experiments (ARRIVE guidelines) on animal research.

Running training, limit length/speedrunning and gripping tests

Ten male mice of each genotype (WT and *ATAD3*+/-) were housed each together and trained daily for running on a treadmill (15 cm/sec) for 30 minutes, 5/7 days, after a week of habituation. For limit length/speedrunning tests, mice ran at increasing speeds: 5 min at 10 cm/sec, 5 min at 20 cm/sec, 5 min at 30 cm/sec and 35 cm/sec until the limit for abandon (five consecutive electrifications at 10 mV).

Gripping tests were performed by returning mice on a grid, sixty centimeters over a pillow, and measuring the time before falling.

All these experiments were performed according to animal care and under ethical watching (ComEth Grenoble n°244).

Activimetry

Ten male mice of each genotype (WT and *ATAD3*+/-) were video-recorded in individual cages over a 12 h night period and the spontaneous physical activity was calculated as previously described [26].

High glucose-high fat diet

Ten female mice of each genotype (WT and *ATAD3*+/-) were housed each together and fed with a high sucrose/high-fat regime (31.8% proteins, 50% lipids (46% milk fat-4% oil) and 23% sucrose) over a two-week period. Immediately, the same cohorts were fed with a high-fat diet (31.5% proteins, 54% lipids (50% lard, 4% soya-bean oil w/w), and 7% cellulose) over another two weeks period. The energy from fat in this diet typically represents more than 50% of total calories as in an average Western diet. All groups were fed ad libitum with free access to water and their body weight and food intake were measured regularly.

Genotyping PCR

Animal genotyping of gene-trapped and wild type (WT) *ATAD3* genes was performed on newborn mice the first tarsus, according to the two-steps PCR method of Goller et al. [8]. Biopsies were lysed overnight at 56°C with proteinase K in Viagen buffer™, inactivated at 85°C one hour, and used directly for PCR (Titanium™ PCR kit). PCR products were analyzed by electrophoresis on a 2% agarose gel and imaged by Sybergreen™ staining.

Western-blot

Proteins were extracted from tissues with potter by lysis in Laemmli buffer containing 10 mM DTT and 5% v/v β-mercapto-ethanol. Samples were dosed for protein concentration (Biorad) and the same protein amounts (100 µg to 600 µg) were separated by 10% SDS-PAGE and electro-blotted onto a nitrocellulose membrane. The nitrocellulose membranes were incubated for 1 h at room temperature with a blocking buffer containing TBST (Tris-Buffered Saline (25 mM Tris), 0.1% Tween 20) complemented with 3% non-

fat dry milk powder. They were further incubated at 4°C overnight with primary antibodies in TBST, washed three times for 10 min each with TBST at room temperature, and finally incubated at room temperature for 1 h with secondary antibodies in TBST. After washing three times for 10 min each in TBST, the detection was performed by enhanced chemo-luminescence (ecl plus, ge healthcare). Primary rabbit polyclonal antibodies (all from cell signaling technology and used at 1:1000 dilutions unless stated otherwise) were directed against Drp1, OPA1, 4EBP1, Ser333-phospho-ACC, ACC and β-actin. *ATAD3* was detected using rabbit polyclonal antibodies raised against a specific sequence of *ATAD3* (Anti-Nter: R40PAPKDKWSNFDPTG53 in *ATAD3As;* produced by Eurogenetec, Belgium) and used as purified immune-globulins. Secondary antibodies were anti-mouse or anti-rabbit IgG-horse radish peroxidase conjugates (GE Healthcare). The membranes were occasionally incubated with TBST again with sodium azide for irreversible inactivation of HRP. Imaged were obtain by chemoluminescence imager and signals were quantified using ImageJ.

Citrate synthase activity measurements

The reduction of 5, 5-dithiobis (2-nitrobenzoic acid) (DNTB) by Citrate Synthase (CS) at 412 nm (extinction coefficient 13.6 $mM^{-1}cm^{-1}$) was followed in a coupled reaction with coenzyme A and oxaloacetate. A reaction mixture of 150 mM Tris-HCl, pH 8.0, 5 mM acetyl-coenzyme A, 3 mM DTNB, and 20 µl of cells lysates were incubated at 37°C for 5 min. The reaction was initiated by the addition of 0.5 mM oxaloacetate and the absorbance changes were monitored for 5 min. All assays were normalized to protein amounts.

MtDNA/GH genomic PCR

DNA from soleus biopsies was extracted by overnight digestion at 56°C with proteinase K in Viagen Buffer™ and inactivated at 85°C for one hour. DNA purification was performed using Promega™ Wizard cartridges. After spectrophotometric dosage, 5 ng of each DNA samples were used directly for genomic-PCR amplification of the growth hormone gene (reverse primer: TCCCCTACCCCATAGTTTCC; forward primer: CTGGCTGCTGACACCTACAA). After a 400 times dilution of the DNA samples, 12 pg of DNA was used for the amplification of mtDNA (reverse primer: GGGCTAGGATTAGTTCAGAGTG; forward primer: CCGTCACCCTCCTCAAATTA). For both two-steps PCR, cycling parameters were 95°C 1 min at the first step and then 95°C for 30 sec/68°C for 1.5 min for 25 cycles. DNA samples were run on 2% agarose gel and imaged by SYBR green staining. Linear responses of the PCR were checked using 5 ng, 10 ng, 20 ng, and 50 ng, or 5 pg, 10 pg, 20 pg, or 50 pg of each DNA templates respectively (GH and mtDNA).

Statistics

Experiments were repeated at least three times and mostly analyzed in different parallels. Resulting data are given as means +/- standard deviation. Differences between data were analyzed by two-tailed, two-sample, unequal-variance student's T-test using XLSTAT software with P-levels indicated according to * for P<0,1; ** for P<0,5 and *** for P<0,05.

Results

ATAD3 +/-mice breeding

Because constitutive invalidation of *ATAD3* in mice is early lethal for embryo development, at gastrula and implantation stage 8, we used *ATAD3*+/-and+/+ mice to search for haplo-deficiency and differential phenotypes under physical training and high-calorie dieting. Breeding of *ATAD3*+/- and +/+ mice were therefore undergone as follows: *C57Bl6j* (*ATAD3*+/+) females were crossed with *ATAD3*+/-*C57Bl6j* males and the progenies were genotyped 5 days after birth (Figure 1A). Among the 84 animals obtained at F1 and F2 generations, 39 were WT and 45 were *ATAD3*+/-, showing no impact of *ATAD3* deletion on embryonic viability. Among WT mice were 19 females and 20 males, and among *ATAD3*+/-mice were 23 females and 22 males, showing no impact of *ATAD3*+/-genotype on the sex ratio (Figure 1B). No impact of *ATAD3* hemizygosity was observed on the female body weights at 2 and 5 months, under standard breeding (Figure 1B). However, at 5 months old, *ATAD3*+/-males were significantly lighter than the wild-type males (-10%). As a second characterization step, we checked by Western-blot for *ATAD3* expression levels in different organs. As shown in Figure 1C, *ATAD3* expression was significantly reduced, even more than twice, in *ATAD3*+/-animals. This is true in the skeletal muscle, the heart, the brain, the liver, and the adipose tissue. In adipose tissue, we observed that the expression of *ATAD3* is almost 3-5 times more in females than in males, for both genotypes (data not shown).

ATAD3+/-male mice have reduced running training performances, lower weights, higher gripping performances, and disturbed circadian activity

To reach our task in looking for *ATAD3*+/-phenotypes under a solicitation for mitochondrial biogenesis, we started to train males cohorts (5 months old, n=10) for daily running on a running platform, for a 6 weeks period (after one week of habituation). Running performances were regularly measured along the training period by performing limit length/speedrunning tests (see materials and methods). As can be seen in Figure 2A, and at the opposite of *ATAD3*+/+*C57Bl6j* males who improved normally their running performances (200% increase in limit length/speedrunning tests), *ATAD3*+/-males were unable to improve their running performances, all over the 6 weeks of training. Watching at mice body weights along this period was to observe that both males lost weight during the training period (5% for both), while *ATAD3*+/-males with a weight is always 10% less than the WT males (Figure 2B).

Our second approach to appreciate the muscle efficiency of *ATAD3*+/-and WT mice were to perform a gripping test (Figure 2C). As depicted in this figure, the gripping force was higher for *ATAD3*+/-animals.

Finally, at the term of training experiments, and before sacrifice, we measured the activity of the males along with a 12 h night period by movement video recording, in individual cages. As can be seen in Figure 3A and Figure 3B, we observed a significant difference in the distribution of activity periods, even if the

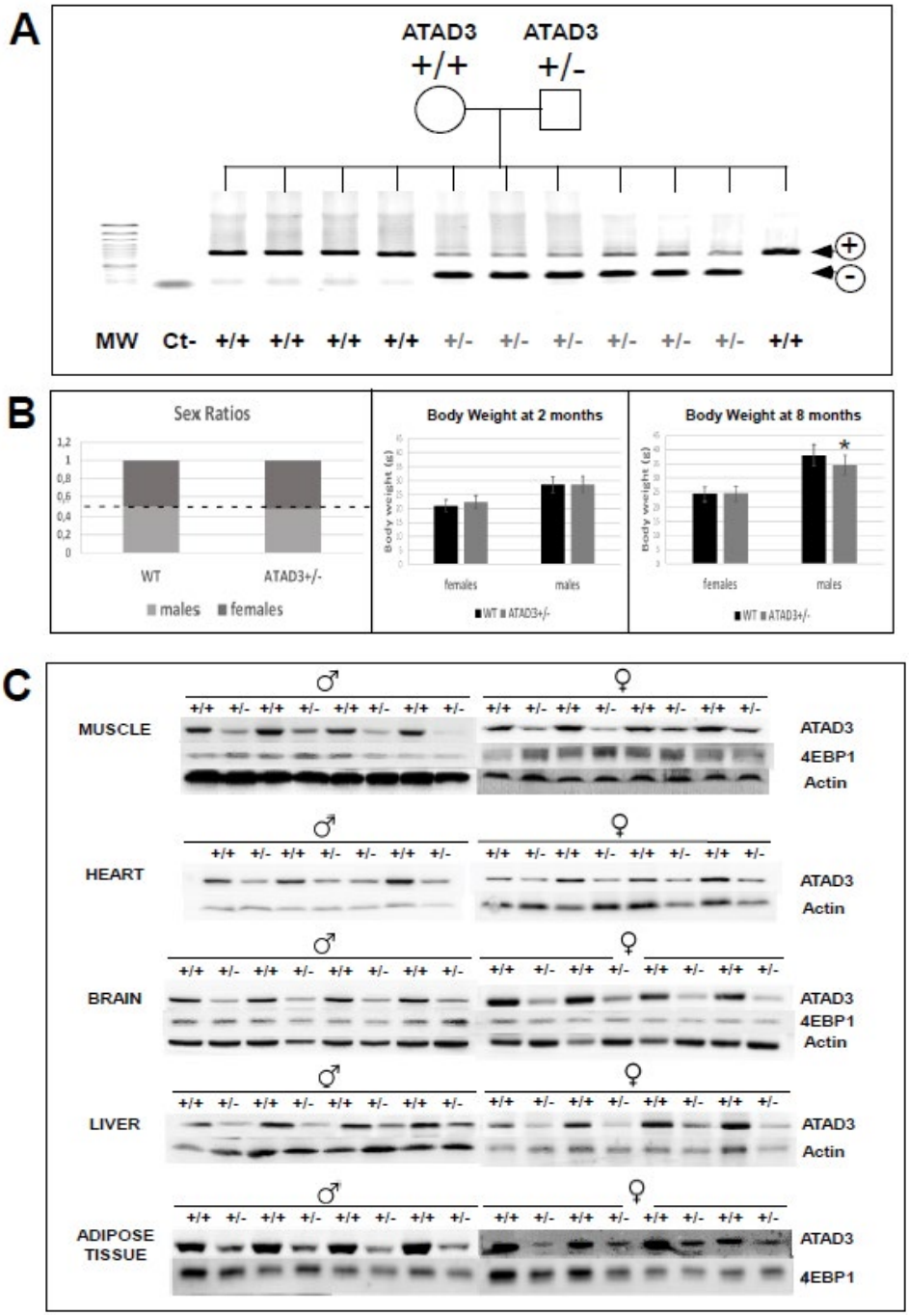

Figure 1 *ATAD3*+/-mice breeding. A: *ATAD3*+/-mice breeding plan. WT *ATAD3*+/+*C57Bl6j* females were crossed with *ATAD3*+/- males of identical genetic background. More than 84 animals were obtained from F1 and F2 generations and were PCR-genotyped for *ATAD3* allele's structures (+ for WT allele, -for gene-trapped mutated one); B: The sex ratio of progenies (left panel) and the body weights of cohorts (n=10) at 2 and 5 months (middle and right panel) are represented by histograms; C: Western-blot analysis of *ATAD3* expression levels was performed on muscles, hearts, brains, livers, and adipose tissues of *ATAD3* +/- and +/+ mice, males, and females (n=4 for each), 4E-BP1 and actin were used as a loading control.

overall activity remains the same (Figure 3B). While WT mice have a peak of activity during the night (22 h to 3 h), the *ATAD3*+/- mice did not present this peak and are more active in the evening and early morning. In addition, measured as a control, both mice were presenting almost the same overall inactivity even if *ATAD3*+/-mice were seen slightly more inactive (Figure 3C). This result shows that *ATAD3* hemi-deletion can affect the circadian system.

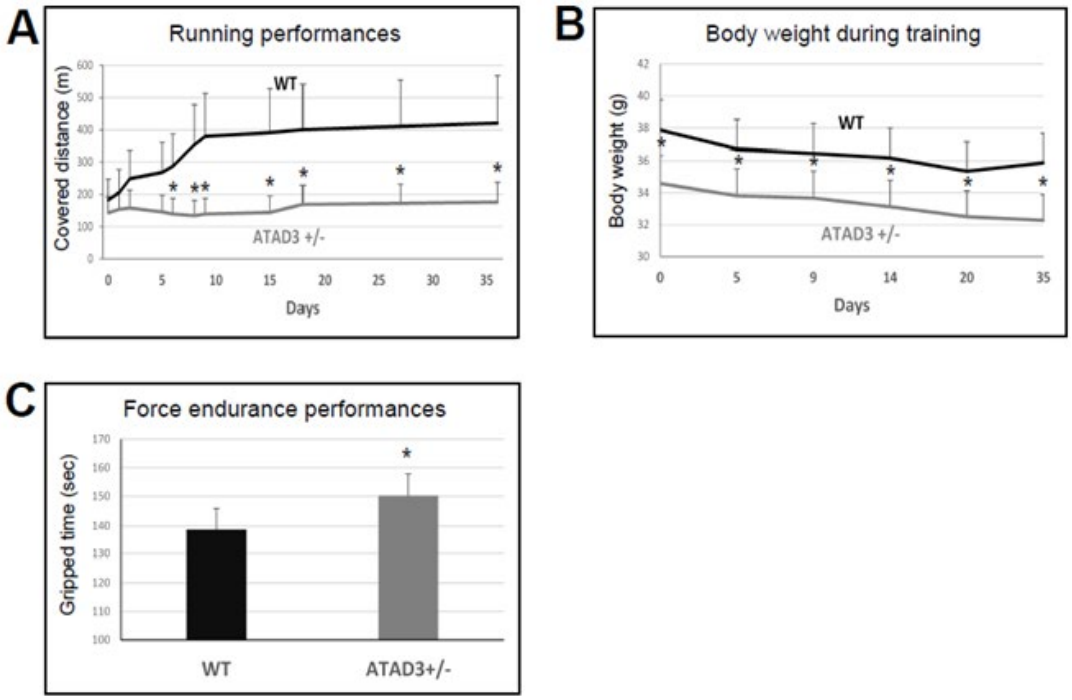

Figure 2 *ATAD3*+/-male mice in physical activity. A: Males (n=10) were tested regularly along the training period for time/length limit running. Meters ran are represented on the curve along the training period; B: The body weights of animals (n=10) over the training period are represented on the curve; C: Gripping tests were performed with males (n=10) after the training period (number of experiments=5).

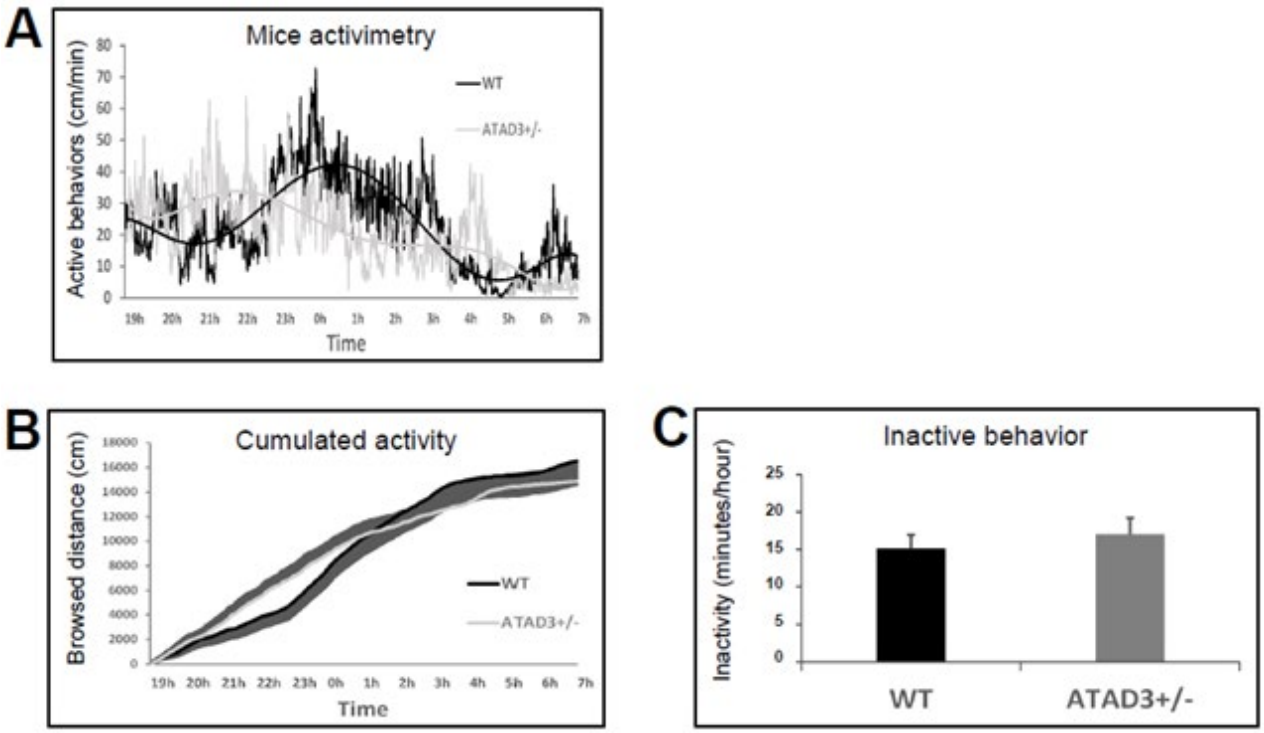

Figure 3 *ATAD3*+/-male mice circadian activity. A: Activimetry was measured individually by video recording of 9 WT and 9 *ATAD3*+/- males during a night period. Movements of each mouse were quantified by computer analysis and restituted as an activimetric curve; B: Movements of each mice restituted as accumulative activimetric curve; C: Cumulated inactivity (min/h) is represented as a histogram.

ATAD3+/-male mice leg muscles have lower mitochondrial mass and present ACC hyper-phosphorylation

To analyze the biological characteristics of *ATAD3*+/-males' muscles after the training period, mice were sacrificed and dissected for the following analysis. At first, calf muscles, hearts, and epididymal adipose tissues were weighted (Figure 4A). No major change was observed for muscle weights, excepted that the gastrocnemius is slightly smaller in *ATAD3*+/-, and the plantaris and soleus a little bigger. In addition, the hearts of *ATAD3*+/-mice were smaller, even very slightly. Concerning the adipose tissue (epididymal fat), a very significant difference was observed in *ATAD3*+/-males (-40%=-0.5 g/1.325 g), explaining, but only in part, the lower whole body mass of *ATAD3*+/-mice (-4 g). Finally, these differences were observed the same on untrained animals.

To look for a cellular *ATAD3*+/-phenotype, we evaluated the mitochondrial mass levels of the different calf muscles by the citrate synthase assay (Figure 4B), and mtDNA PCR-based measurements (Figure 4C). Citrate synthase activities were clearly lowered in trained *ATAD3*+/-mice muscles compared to WT, especially in the plantaris and soleus muscles, as expected for a mitochondrial miss-of-function. Also, the mtDNA content was found to decrease in *ATAD3*+/-soleus to 42% compared to WT. Checking for the levels of few mitochondrial-associated proteins (OPA1, Drp1, and NRF1) in the soleus of trained +/+ and +/-mice, we found no main difference in any of the proteins studied excepted a slight down-regulation of *NRF1*, indicating a possible genetic down-regulation of mitochondrial biogenesis.

We looked finally at ACC phosphorylation status, an enzyme-linked to mitochondrial-based lipogenesis. As can be seen in Figure 4D, we observed an increased level of pACC in *ATAD3* +/- animals, and a very strong down-expression of the whole ACC amount, both showing a very important increase of the phosphorylation ratio of ACC (pACC/ACC). This result indicates a very significant inhibition of lipogenesis, even if the sampling is too low for statistics.

All the results obtained here let us think that in males, *ATAD3* level can impact the circadian activity, the fat deposition level (most probably by ACC inactivation), and the muscle training capacity, by modulation of the muscle mitochondrial mass.

ATAD3 is then described as haplo-insufficient in males under training conditions, this emphasizing why *ATAD3* gene status has been studied as a potential cause of unclassified myo- and neuropathies cases.

ATAD3+/-female mice have a higher propensity for obesity under high-calorie diet

Along with the term of our project, we bred female cohorts in parallel (n=10) with high-energetic diets, typical hypercaloric diets requiring as inducing mitochondrial biogenesis [25,27]. A high-glucose/high-fat diet was applied at the start for 3 weeks, followed by a simple high-fat diet for 2 other weeks (Figure 5A). At the beginning of the experimentation, both *ATAD3*+/- and WT females (5 months old) were of similar weights. Under high-fat/high glucose regime, *ATAD3*+/-mice were with increasing weights along the 20 days period, over 15% (+3.6 g), while WT mice only increased their weights of 2% (+0.7 g). Switching then to a simple high-fat hyper-caloric diet (14 days), we found that *ATAD3*+/-mice started to decrease in weight (-4%), while WT mice were continuing with the weight increase. At the term of the experiment, we measured epididymal adipose tissue weights of both cohorts (Figure 5B) and we found that *ATAD3*+/-mice had a 50% weight increase of their epididymal fat tissue (0.75 g/0.5 g), this representing however only 10% of the whole body weight difference (Figure 5A). This phenotypic difference at the epididymal fat tissue level was not observed on female mice under standard diet. An important parameter of this diet experiment to mention is that both mice were exactly with the same food intake, given in excess, at the term of the experiment. Also, importantly, WT mice were looking anxious during the diet, more than *ATAD3*+/-mice.

To address better the question of this specific fat increase in *ATAD3*+/-mice, we analyzed the phosphorylation status of ACC in adipose tissue samples since ACC is mainly involved in lipogenesis/lipolysis regulation. As can be seen in Figure 5C, we observed that the phosphorylated ACC form increased in *ATAD3*+/-mice, while the whole ACC amount decreased very drastically, even if the sampling is too low for the statistic. Both phenomenon evoke therefore a strongly decreased in the lipogenesis potential.

Therefore, *ATAD3* hemizygosity induces a weight increase capacity upon a high-calorie diet by affecting the adipose tissue mass associated with the inactivation of ACC activity. All of our results obtained here to let us think that *ATAD3* is involved in whole-body metabolism and that *ATAD3* loss of function can participate in a tendency for obesity in females.

Discussion

From the earliest studies on *ATAD3*, up today, it has been shown a strong link between *ATAD3*, lipid, and mitochondrial mass and metabolism, as observed here at the animal level. Knowing that *ATAD3* is essential for mitochondrial biogenesis and for down-stream processes like lipogenesis, steroidogenesis as proliferation and differentiation, the results obtained in this study are not surprising [2-4,8,14,15,18,28,29]. In addition, many works have focused on the role of *ATAD3* in cancers [1,13]. Despite a still unknown precise function in mitochondrial biogenesis, all researches have converged today to the hypothesis that *ATAD3* is involved in the transit of MAM-derived cholesterol-proteins rafts to mitochondria. This may furnish mitochondria with both lipids (for the synthesis of mitochondrial membranes, growth, renewing, and other syntheses like for steroids) and imported/neo-synthetized nuclear-encoded mitochondrial proteins essential for these processes [2,14,15,29].

To approach *ATAD3* function better we addressed here the question of understanding its role at the animal level in mouse.

The knockout of *ATAD3* in mice is early lethal, as at day 4-5 of embryonic development [8], just at trophoblast growth and embryo implantation. This time corresponds to the first zygotic mitochondrial biogenesis [30]. Using a gene-trapped mouse

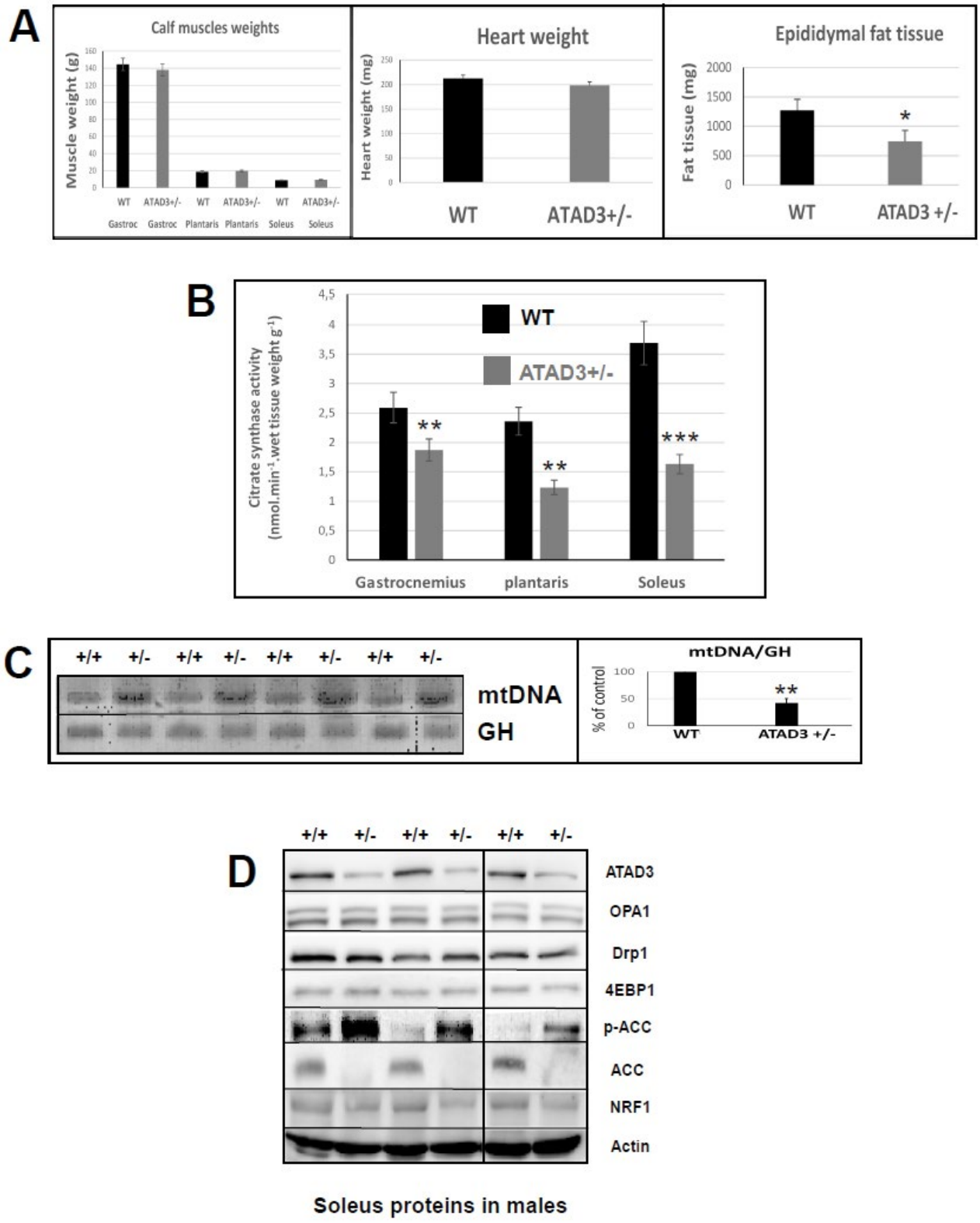

Figure 4 Biological characteristics of **ATAD3**+/-male mice muscles. A: Left panel: Weights of calf muscles (gastrocnemius, plantaris, and soleus) were measured after the sacrifice of males (n=10) and represented by a histogram. Middle panel: Weights of hearts were measured after the sacrifice of males (n=10) and represented by a histogram. Right panel: Weights of epididymal fat tissues of trained males were measured after sacrifice (n=10) and represented by a histogram; B: Citrate synthase activities were measured on the soleus of trained males (n=10) and are represented by a histogram; C: mtDNA PCR amplification was performed using Growth Hormone (GH) as a genomic gene control (see M and M). Quantified data are represented by a histogram; D: Western-blot analysis of mitochondrial proteins was performed on soleus protein extracts of trained males (n=6).

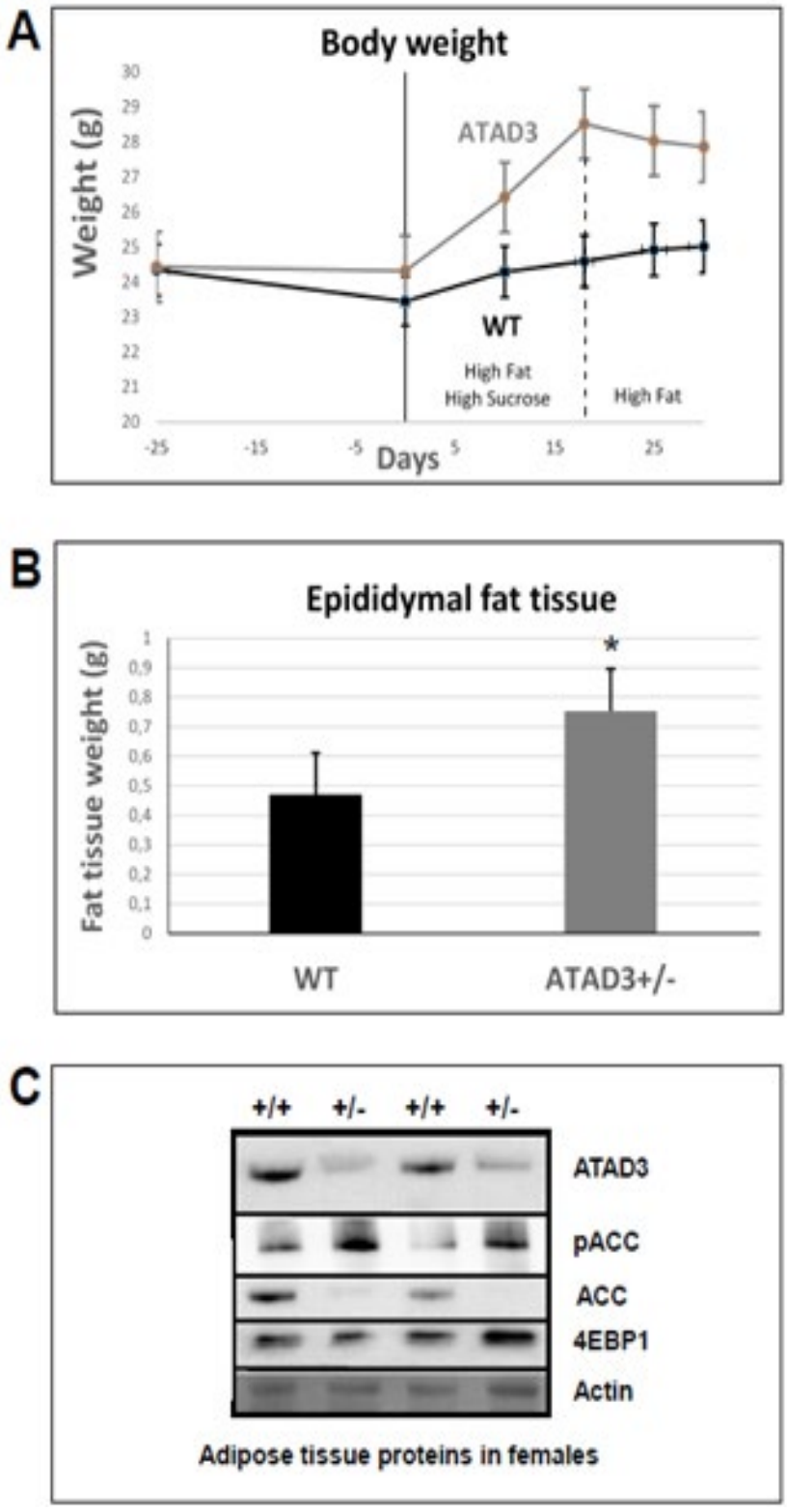

Figure 5 *ATAD3*+/-female mice have a higher propensity for obesity under a high-calorie diet. A: The weights of female cohorts (n=10) were measured before and along with the diets and represented on the curve. High sucrose/high fat was a two weeks diet and high fat was, immediately after, a two weeks diet; B: The weights of epididymal fat tissues were measured after the sacrifice of females (n=10) and represented by a histogram; C: Western-blot analysis of pACC/ACC was performed on adipose tissue extracts of trained females (n=4). Actin and 4EBP1 were used as loading controls.

model, we investigate the physiology of hemizygous *ATAD3*+/- mice, versus their WT counterparts.

Because *ATAD3*+/-mice do not present any obvious phenotype under regular breeding, the choice was to test the animals for a haplo-sufficiency during periods involving significant mitochondrial biogenesis, in muscle along with training and in adipose tissue under high-calorie diet. Even testing only males for muscular improvement and females for high-calorie diet we succeeded to observe strong phenotypic differences in both sexes.

For males, we observed that the standard weights of *ATAD3*+/- mice were 10% lower (not in females). We showed that *ATAD3*+/- males, trained for running, are unable to improve their running performances. This phenotype is associated with a lower citrate synthase activity in calf muscles after training as a lower mtDNA level, both indicating a lower mitochondrial mass. Nonetheless, *ATAD3*+/-mice have a higher gripping force and a modified circadian rhythm.

Such a result about the complete inability for *ATAD3*+/-mice to improve their performances was even not expected the so, but this is clearly understandable since *ATAD3* is firstly involved in mitochondrial biogenesis, even being a limiting factor [3,4,11]. Also, if it was also a surprise, the increased gripping force of *ATAD3*+/-mice could be understood as a miss of mitochondria, provoked by *ATAD3* depletion, would normally reduce aerobic muscle fibers which could, in turn, be compensated by the production of glycolytic fibers that will ameliorate gridding strength. The potential plasticity of mammalian muscles can explain this phenotype [31,32]. In addition, this phenotype was observed more significantly in aerobic fibers (soleus) than in glycolytic ones (gastrocnemius). For the few mitochondrial proteins studied, we did not observed the difference between *ATAD3*+/-and WT mice, excepted for *NRF1* which expression is decreased in *ATA3*+/+ mice. This can be considered as a feedback regulation to adapt the mitochondrial biogenesis at the genetic level.

Looking to Acetyl-CoA Carboxylase activity (pACC/ACC ratio) we found it inhibited in *ATAD3*+/-male soleus (increased inhibitory phosphorylation ratio), indicating a lower lipogenesis in this tissue. This phenomenon may contribute to the inability of *ATAD3*+/- males to improve their running performance by reducing their lipid storage and reserves.

The other phenotype observed in *ATAD3*+/-males is the loss of the night-active peak period, normally observed in WT mice [33]. In spite of a normal active period in the night (22 h-3 h), *ATAD3*+/- mice were active more homogeneously along the night, without a night activity peak but an evening and early morning ones. However, the overall night activity was the same compared to WT mice. One could think that hormone levels, steroids particularly, are deregulated in *ATAD3*+/-mice [14], especially considering the decrease of fertility that we have observed for *ATAD3*+/-males.

To explain why *ATAD3*+/-males are lighter, before and after training, is to underline that *ATAD3*+/-males have a smaller epididymal adipose tissue (30% less). This can be due to a modified ACC activity in the adipose tissue as the one observed in the muscle. It appears then that the level of *ATAD3* expression controls male body weight, probably because of lower steroid levels.

To conclude about the phenotypes observed in the males *ATAD3* hemi-deletion is not haplo-sufficient under training solicitation. This observation may not be only linked to muscle status since *ATAD3* is ubiquitously expressed and at a high level in the brain [4]. It is possible that brain activities of *ATAD3*+/-mice are altered like could be for the resistance to the pain, their motivation, or stress resistance. Steroids (cortisol/corticosterone, testosterone) levels can also be responsible for the observed phenotypes since *ATAD3* controls steroid production [2]. Again, the decreased fertility observed in males that would fit with the other observations and the hypothesis that *ATAD3* deletion is associated with a lower mitochondrial mass and steroid levels. Finally, the combination of both, like the change in ACC activity, can potentially have a strong impact on behaviors and running potential, in addition to a major role of the differential mitochondrial mass observed.

For females, we observed that the standard weights for *ATAD3*+/- mice are similar to WT counterpart, contrary to the males, indicating thus a clear impact of the sex on *ATAD3* hemi-deletion phenotypes. While in males we detected weight differences we find here in females that it exists a full compensation under normal breeding conditions. Concerning body weight, we can state therefore that it exists a male-linked *ATAD3* phenotype. However, we showed that *ATAD3*+/-female mice are much more prompt to increase their weight under a high-fat/high-glucose diet compare to WT. This phenotype (+3 g of weight increase after a 3 weeks diet) is due in part to an increase of epididymal adipose tissue (+0.3 g) but this plays only for 10% of the weight difference. Like for male's weight under training, we will have to find which of other organs' weight are impacted during the diet, like could be the liver or the other adipose tissues. Such a result about the propensity of *ATAD3*+/-mice for obesity was not expected, as in vitro *ATAD3* down-regulation reduces adipogenesis and lipogenesis [4]. Then, we expected that in vivo, *ATAD3*+/-mice would have limited lipogenesis and then a limited propensity for weight increase. However, we observed that ACC was inactivated in female adipose tissue-like observed in male muscles. Then, the increased weight under high diet might be linked somehow and finally to a reduced lipogenesis. In addition, at the animal level, it can be expected opposite responses, for compensation or other reasons. Then, we can hypothesize that *ATAD3*+/-mice can become more fat under high-calorie diet because all the other organs are not able to use so much of the calories (all mice on the same diet), because of less mitochondrial mass and energy use. We can hypothesize that ACC inactivation observed at sacrifice is the end-consequence of increased lipogenesis during the diet, as observed at bodyweight level. Calorimetric studies are the main clue to be explored, like to study mitochondrial mass in adipose tissue. As for males, this phenotype could also be due to different behaviors, the different activities of the mice, as *ATAD3* hemi-deletion could affect brain activity. Therefore it will be interesting to study activimetry of females too, like training potential and other behaviors like cognitive studies. While performing the two-steps diet, with an only high-fat regime in the second period, we observed that *ATAD3*+/-mice were starting

to lose weight, while WT mice continued to increase in weight. This observation allows us to think that *ATAD3*+/- obesity is not only due to fat consumption but could be linked to either high-sugar consumption or its combination with fat. This will have to be discriminated against by further experiments. At this point is precise that *ATAD3* is 4-5 times more expressed in adipose tissues of females versus males (data not shown). This would mean that females have either more mitochondria in adipose cells than males or that females contain *ATAD3*-enriched mitochondria. Deeper studies are needed to address this question. Then, the differences in *ATAD3*+/-phenotypes between sexes may be also due to hormonal differences.

To conclude about the phenotypes observed in females, it is clear that **A***TAD3* hemi-deletion in females induces a phenotype of propensity for obesity under a high-fat/high-glucose regime. This phenomenon, like in male muscles, is linked to a diminished ACC activity.

Conclusion

The search for *ATAD3*+/-phenotypes, even not observed in standard breeding conditions, was with good chances to be found because *ATAD3* is playing a major role in mitochondrial biogenesis. Considering all the data on *ATAD3*, *ATAD3* may likely contribute to the transport of lipid rafts of cholesterol and neo-synthetized nuclear-encoded mitochondrial proteins, linked to Drp1-driven fission processes, from the MAM (Mitochondrial Associated Membranes), part of the endoplasmic reticulum, to the mitochondria. This process may drive mitochondrial biogenesis, which occurs during cellular growth and differentiation steps, as for simple multiplication of mitochondria, or for differentiation-linked proteomic specification, and mitochondrial renewal.

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