TIGAR deficiency enhances skeletal muscle thermogenesis by increasing
neuromuscular junction cholinergic signaling

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Summary

Cholinergic and sympathetic counter-regulatory networks control numerous physiologic functions including learning/memory/cognition, stress responsiveness, blood pressure, heart rate and energy balance. As neurons primarily utilize glucose as their primary metabolic energy source, we generated mice with increased glycolysis in cholinergic neurons by specific deletion of the fructose-2,6-phosphatase protein TIGAR. Steady-state and stable isotope flux analyses demonstrated increased rates of glycolysis, acetyl-CoA production, acetylcholine levels and density of neuromuscular synaptic junction clusters with enhanced acetylcholine release. The increase in cholinergic signaling reduced blood pressure and heart rate with a remarkable resistance to cold-induced hypothermia. These data directly demonstrate that increased cholinergic signaling through the modulation of glycolysis has several metabolic benefits particularly to increase energy expenditure and heat production upon cold exposure.

Highlights:

1. Deficiency of a negative regulator of glycolysis (TIGAR) in cholinergic neurons increases the biosynthesis and content of the neurotransmitter acetylcholine.
2. Increased cholinergic tone reduces blood pressure and heart rate while enhancing signaling at neuromuscular junction.
3. Upregulation of neuromuscular junction activation provides protection against the paralytic curare and cold-induced hypothermia.

4. Modulation of cholinergic neuron glycolysis may provide a novel therapeutic approach for treatment of diseases stemming from reduced acetylcholine signaling such as myasthenia gravis and sarcopenic pre-synaptic dysfunction.

Keywords

TIGAR, cholinergic neurons, acetylcholine, neuromuscular junction, skeletal muscle thermogenesis

Introduction

TIGAR (Tp53-induced Glycolysis and Apoptosis Regulator) was originally identified as a p53-inducible protein that functions as a fructose-2,6-bisphosphatase (F2,6P) but subsequently shown to have phosphatase activities for a variety of phosphorylated metabolic intermediates and allosteric regulators including 2,3-bisphosphoglycerate (2,3BPG), 2-phosphoglycerate, phosphoglycolate and phosphoenolpyruvate (Bensaad, Tsuruta et al. 2006, Rigden 2008, Bolanos 2014, Tang, Chen et al. 2021). Due to its F2,6P dephosphorylation activity, TIGAR is generally considered as a suppressor of glycolysis as F2,6P is a potent allosteric activator for 6-phosphofructo-1-kinase (PFK1), the rate-limiting step in glycolysis. However, the dephosphorylation of 2,3BPG to generate 3-phosphoglycerate would also be expected to increase glycolysis (Bolanos 2014). In addition, the role of TIGAR in regulating carbohydrate metabolism is also complicated by the presence of the related phosphofructokinase bis-phosphatase (PFKBP) family that has both F6P 2-kinase as well as F2,6P bisphosphatase activities (Mor, Cheung et al. 2011). Thus, the biologic

readout of TIGAR function is likely to be highly cell context dependent. In this regard, TIGAR has been reported to differentially modulate multiple different pathophysiologic outcomes. For example, in the brain TIGAR expression was reported to protect against ischemic/reperfusion injury (Li, Li et al. 2021), whereas in the heart TIGAR deficiency protected myocardial infarction (Hoshino, Matoba et al. 2012) and in pressure overloaded hearts (Okawa, Hoshino et al. 2019). In cancer models, TIGAR plays a complex role on the formation and progression of different types of cancer via suppressing aerobic glycolysis and controlling of reactive oxygen species (ROS) production (Tang, Chen et al. 2021). Recently, it has been reported that TIGAR can both enhance the development of premalignancies and suppress the metastasis of cancer invasion by the way of inhibition of ROS production (Cheung, DeNicola et al. 2020).

As carbohydrate metabolism and glycolysis are essential normal physiologic processes in all cells and tissues, we have examined the metabolic phenotype of whole body and tissue specific TIGAR knockout mice. Surprisingly, we have observed that TIGAR deficiency in cholinergic neurons of mice results in a marked protection against hypothermia following an acute cold challenge. At a molecular level, this results from enhanced acetylcholine levels and increased cholinergic signaling at the neuromuscular junction driving skeletal muscle shivering induced thermogenesis.

Results

Previously we reported that the TIGAR can modulate NF- κ B signaling through a direct binding interaction and inhibition of the E3 ligase activity of the linear ubiquitin

binding assembly complex, LUBAC in cultured cells (Tang, Kwon et al. 2018). To examine the role of TIGAR in vivo, we initially generated whole body *Tigar* knockout mice (TKO). Surprisingly however, during our phenotypic characterization we observed that the TKO male and female mice both display a remarkable resistance to cold induced-hypothermia. As shown in Figure 1, both male (panel A) and female (panel B) control mice when shifted from room temperature to a 4°C environment display the typical 4-5°C decline in core body temperature during the 1 h acute exposure period. In contrast, the TKO male and female mice only display less than a 1°C decline in core body temperature under the identical conditions. The level of *Ucp1* mRNA (Figure 1-figure supplement 1A) and protein (Figure 1-figure supplement 1B) were not significantly different in interscapular brown adipose tissue (iBAT) at room temperature. *Ucp1* mRNA did increase approximately 1.5-fold following 1 h cold exposure but this was not significantly different between the control and TKO mice. Although a very small amount of *Ucp1* mRNA was detected in inguinal adipose tissue (iWAT) of control mice at room temperature, it was lower in the TKO mice (Figure 1-figure supplement 1C). Moreover, following 4°C exposure for 2 h there was an increase in *Ucp1* mRNA indicative of beige adipocyte induction. However, the induction of *Ucp1* mRNA was significantly reduced in the TKO mice compared to the control mice, probably due to decrease sensitivity of cold-induced sympathetic activation (see Figure 7).

Changes in tissue distribution of muscle and fat mass as well as modulation of basal energy expenditure have important roles in the control of thermogenesis. However, there was no significant difference in the relative fat mass or lean mass (Figure 1-figure supplement 1D) between the control and TKO mice. Hematoxylin and

Eosin staining of oxidative soleus and glycolytic extensor digitorum longus, EDL (Figure 1-figure supplement 1E) skeletal muscle also indicated the absence of any gross morphological differences between the control and TKO mice. Similarly, food intake (Figure 1-figure supplement 1F), spontaneous locomotor activity in the AMB+Z dimensions (Figure 1-figure supplement 1G) and energy expenditure (Figure 1-figure supplement 1H) were essentially identical.

As the TKO mice are whole body knockouts, the temperature phenotype could result from an effect on a single or combinatorial number of cell types. We first generated *Tigar^{fl/fl}* mice that were crossed with *Adipoq^{Cre}* mice to generate adipocyte-specific *Tigar* knockout mice (Figure 1C). These mice had the identical temperature sensitivity as the control *Tigar^{fl/fl}* mice both displaying an approximate 4°C decline in core body temperature following exposure to 4°C for 1 h (Figure 1D). To further rule out BAT as a contributor to the enhanced cold protection in the TKO mice, we crossed the cold sensitive *Ucp1* deficient (Enerbäck, Jacobsson et al. 1997) with the TKO mice (both strains with C57BL/6J background) to produce wild type (WT), TKO, UKO, and *Ucp1^{-/-}/Tigar^{-/-}* double knockout (UTKO) mice (Figure 1E). As expected, UKO mice were highly cold sensitive compared to their wild type littermates following a 1 h acute cold exposure (Figure 1F). Interestingly, the UTKO mice displayed a strong cold resistance, that was not significantly different from the TKO littermates. These data strongly suggest that thermogenic adipose tissue is not responsible for the resistance to hypothermia in the TKO mice.

Since skeletal muscle plays a major role in heat production, we next generated skeletal muscle-specific *Tigar* knockout mice by crossing the *Tigar^{fl/fl}* mice with the

skeletal muscle-specific myosin light polypeptide 1-Cre (*Myh1^{Cre}*) mice (Figure 1G). TIGAR is highly expressed in skeletal muscle, with the highest protein levels in depots containing glycolytic fibers (white gastrocnemius and EDL) skeletal muscle). The *Myh1^{Cre}*-driven TIGAR deletion resulted in efficient loss of TIGAR protein in all skeletal muscles examined with no effect on the heart (Figure 1G). Similar to the adipocyte-specific *Tigar* knockout mice, skeletal muscle *Tigar* deficient mice had no significant resistance to acute cold exposure (Figure 1H). Moreover, we did not find any significant change in skeletal muscle mRNA expression of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA1, *Atp2a1*; SERCA2, *Atp2a2*) in control or TKO mice maintained at room temperature or shifted to 4°C for 1 h (Figure 1-figure supplement 2A and B). Similarly, there was no change in myoregulin (*Mrln*) (Figure 1-figure supplement 2C). Although there was a small apparent increase in sarcolipin (*Slrn*) mRNA in the control wild type mice shifted to 4°C there was no statistical difference with the TKO mice (Figure 1-figure supplement 2D). Together these data are consistent with the absence of a cell autonomous effect in either thermogenic adipocytes or in skeletal muscle.

To unravel these unexpected findings, we next undertook a pharmacological approach to address the ability of the TKO mice to resist hypothermia. Treatment of control and TKO mice with the selective SERCA inhibitor cyclopiazonic acid (CPA) (Wang, Myles et al. 2021) reduced core body temperature at room temperature (Figure 2A). As expected, CPA treatment resulted in a faster rate of decline and to a lower extent when the mice were exposure to 4°C (Figure 2B). However, there was no significant difference in the response to CPA between the control and TKO mice. The CPA induced reduction in body temperature directly correlated with observable skeletal

muscle paralysis, consistent with skeletal muscle contraction activity as an important component in thermal regulation (Blondin and Haman 2018) and further indicates that SERCA functions downstream of TIGAR function.

Since *Tigar* deficiency in neither adipocytes nor skeletal muscle recapitulated the protection of acute hypothermia but that paralysis results in a rapid reduction in core body temperature, we next assessed whether the cold resistance of the TKO mice was due to shivering thermogenesis. To address this, we next treated mice with the highly specific skeletal muscle nicotinic acetylcholine receptor competitive antagonist tubocurarine (Bowman 2006). Control male mice treated with 0.4 mg/kg tubocurarine became relatively inactive with essentially no detectable locomotor activity at room temperature 10 min post injection that lasted for another 20-30 min (see Figure 2- video 1). Over this time period there was a concomitant decline in core body temperature that began to recover as the effects of tubocurarine began to wear off (Figure 2C). Tubocurarine treatment of the TKO male mice at room temperature also displayed a similar decline and recovery of body temperature but that remained somewhat higher than the control mice (Figure 2C). However, shifting the male mice to 4°C following tubocurarine injection, resulted in a rapid decline in body temperature such that by 30 min the control mice body temperature was reduced to less than 25°C (Figure 2D). In contrast, the tubocurarine injected TKO male mice only display a reduction in body temperature to approximately 34°C under the identical conditions. Moreover, while the tubocurarine treated control mice placed at 4°C remained completely immobile, while the TKO mice display apparent normal locomotor activity (Figure 2- video 1). Female mice are more sensitive to tubocurarine than male mice (Maurya, Periasamy et al. 2013). The body

temperature in WT female mice kept declining in one-hour period after a lower dose (0.15 mg/kg) tubocurarine injection at ambient temperature. Nevertheless, the decline of body temperature in TKO female mice was resistant to the effects of tubocurarine (Figure 2E).

The tubocurarine dose-response for male mice is shown in Figure 2F, with the control wild type mice have an LD50 of 0.44 mg/kg whereas the TKO mice have an LD50 of 0.55 mg/kg (Figure 2F). Injection of a lethal tubocurarine dose (0.60 mg/kg) initially resulted in 50% if the control mice displaying a loss of voluntary locomotor activity by 150 sec but this required 240 sec in the TKO mice (Figure 2G). At ten min 100% of the control mice were dead whereas it took nearly 25 min for 100% of the TKO mice to die (Figure 2H). The tubocurarine resistance of the TKO mice was not due to changes in expression of the nicotinic acetylcholine receptor subunits with no significant differences in the skeletal muscle expression of the $\alpha 1$ (*Chrna1*), $\beta 1$ (*Chrn1*) or γ (*Chrng*) subunits (Figure 2-figure supplement 1A, B, and C) and with a small reduction in the δ (*Chrnd*) and ϵ (*Chrne*) subunits (Figure 2-figure supplement 1D and E) in the TKO mice. These data suggest that the protection against hypothermia was due to cholinergic signaling to the skeletal muscle.

Consistent with increased skeletal muscle energetic demand, ATP levels were significantly decreased with an apparent increase in ADP and AMP in the TKO mice after 1 h at 4°C (Figure 3A). Stable isotope metabolic flux assessment with H₂O¹⁸ demonstrated increased incorporation of orthophosphate into ATP, ADP and AMP in the TKO mice at 4°C, that was not significantly different at room temperature (Figure 3B). Plasma creatine was also increased 2-fold in the TKO at 4°C (Figure 3C), reflecting the

increased phosphocreatine turnover (CP M4 phosphate fragment m/z 83, Figure 3D). The appearance of creatine in the plasma is likely due, in part, to the increase rate of shivering that is known to induce skeletal muscle leakage (Baird, Graham et al. 2012).

The mechanics of energetic supply at 4°C was further examined by using a widely targeted LC/MS/MS protocol (Hoshino, Matoba et al. 2012). Key pathways found to be significant were pentose phosphate pathway, purine nucleotide cycle, and amino acid utilization pathways (Figure 3-figure supplement 1A). The pentose phosphate pathway cycle fills the purine nucleotide cycle that acts in concert with the malate-aspartate shuttle and the TCA cycle to preserve cellular energetics (Hatazawa, Senoo et al. 2015). In particular glycine/serine, histidine and methionine metabolism also drives the input of the tricarboxylic acid cycle intermediates serving as anapleurotic inputs (Figure 3-figure supplement 1B). Together, these interactions serve to recycle AMP generated by increased skeletal muscle energy demand.

Mitochondria are a primary source of both ATP production and heat generation during muscle contraction. To determine whether mitochondrial oxidative phosphorylation (OXPHOS) efficiency and/or capacity are altered in TKO mice, permeabilized skeletal muscle fiber bundles were prepared from red portions of the gastrocnemius muscle from control and TKO mice immediately after 1 h cold exposure. ADP-stimulated respiratory kinetics (i.e., K_m and V_{max}) were virtually identical between wild-type and TKO muscle during respiration supported by carbohydrate (Figure 3-figure supplement 2A and B) and/or lipid-based substrates. Rates of ATP production (J_{ATP}) and oxygen consumption (JO_2) measured simultaneously under clamped submaximal and maximal ADP-demand states were also similar in muscle from control and TKO

mice (Figure 3-figure supplement 2C and D), yielding similar ATP/O efficiency ratios (Figure 3-figure supplement 2E). Finally, in vitro studies of the extensor digitorum longus muscle showed no differences in force-frequency, peak specific tension, or time to one-half relaxation between control and TKO mice (Figure 3-figure supplement 2F and G). Together these data indicate that the skeletal muscle contraction induced thermogenesis in TKO mice is not due to any intrinsic change in skeletal muscle mitochondrial efficiency of contractile function and therefore must result from a change in signaling that in turn drives an enhanced skeletal muscle response.

Since the TKO mice are resistant to tubocurarine and the major driver of skeletal muscle contraction is cholinergic signaling, we next generated cholinergic neuron specific *Tigar* knockout (chTKO) mice using the choline acetyl-CoA transferase *Chat*^{Cre} mice (Rossi, Balthasar et al. 2011). As the superior cervical ganglion (SCG) is composed of approximately 80% cholinergic neurons (Wang, Nelson et al. 1990, Morales, Holmberg et al. 1995, Juranek and Wojtkiewicz 2015), we collected the fresh SCGs (Figure 4-figure supplement1) and confirmed that *Chat*^{Cre} resulted in the loss of *Tigar* in cholinergic neurons by immunofluorescence co-localization of TIGAR with the vesicular choline transporter (VChAT) (Figure 4A) and by immunoblotting analyses of TIGAR protein in the SCGs (Figure 4B). Similar to the TKO mice, the chTKO mice were also resistant to acute cold exposure compared to the control *Chat*^{Cre} mice (Figure 4C). Treatment with the SERCA inhibitor CPA, at room temperature (Figure 4D) or at 4°C (Figure 4E), also resulted in paralysis and a decline in core body temperature that was not significantly different between the *Chat*^{Cre} and chTKO mice, although the reduction in core body temperature was much greater at 4°C than at room temperature. The

chTKO mice were also resistant to tubocurare at room temperature with both less of a core body temperature drop and a faster recovery (Figure 4F). In agreement with the TKO mice (Figure 2D), the resistance to cold induced hypothermia by acute 4°C challenge was very dramatic in the chTKO mice compared to control mice (Figure 4G). Similarly, the chTKO mice were highly resistance to the paralytic actions of tubocurare (Figure 4-video 1). Moreover, immunoblotting of the SCG for the neuron activation marker c-Fos, further demonstrated that control mice displayed a small increase in c-Fos protein levels when cold challenged that was further increased in the chTKO mice (Figure 4H). As controls, there was no significant differences in the protein levels of the vesicular acetylcholine transporter (VAChT) or the plasma membrane choline transporter (ChT) proteins. These data demonstrate the cholinergic neuron *Tigar* knockout mice recapitulate the cold resistance and pharmacological characteristics of the whole body *Tigar* knockout mice, consistent with these mice displaying increased cholinergic tone.

If increased cholinergic input is responsible for the increased energy demand and heat production by skeletal muscle, then the skeletal muscle metabolic characteristics of the TKO mice should be recapitulated in the chTKO mice. As shown in Figure 5, widely targeted LC/MS/MS in the gastrocnemius muscle of the chTKO mice identified the same three key pathways: pentose phosphate pathway, purine nucleotide cycle, and amino acid utilization pathways that display a greater induction at 4°C. Together, these data are consistent with increased cholinergic signaling to skeletal muscle as responsible for increased skeletal muscle thermogenesis following cold exposure.

To address the basis of the apparent increase in cholinergic signaling, we first asked whether there was a change in synaptic density in the neuromuscular junction. The acetylcholine receptor aggregates in the synaptic cleft and can be specifically labeled with α -bungarotoxin (Pratt, Iyer et al. 2018), as shown for whole extensor digitorum longus (EDL) muscle (Figure 6A). Quantification revealed a large degree of heterogeneity in the number of clustered acetylcholine receptors per muscle (Figure 6B). Nevertheless, there was a clear statistical increase in the *Tigar* knockout mice compared to control mice. Consistent with the increase in synaptic junctions, the total amount of acetylcholine was also increased in skeletal muscle extracts from the TKO and chTKO mice compared to their respective control mice at both room temperature and after cold exposure (Figure 6C and 6D).

As it is not possible to determine the rate of acetylcholine synthesis in vivo, we took advantage of SH-SY5Y cells that have characteristics of cholinergic neurons (Kovalevich and Langford 2013). Using sgRNA and Cas9, we effectively knocked out *Tigar* in several clonal SH-SY5Y cells, two representative control and *sgRNA* knockout cells are shown in Figure 6E. The amount of acetylcholine secreted into the medium over a 24 h period was increased in the *Tigar* knockout SH-SY5Y cells compared to controls (Figure 6F). Labeling with [1,2]- ^{13}C glucose demonstrate an increase in the m2-labeled acetylcholine levels in the *Tigar* knockout SH-SY5Y cells (Figure 6G). Similarly, labeling with U- ^{13}C glucose also demonstrated an increase in m2-labeled acetylcholine in the *Tigar* knockout SH-SY5Y cells (Figure 6H). Moreover, there was a 3-fold increase in the enrichment of the C2-C4 fragment, m1-glutamate m/z 129, indicating that the acetyl group in acetylcholine originated from glycolysis via pyruvate dehydrogenase,

302 PDH (Figure 6I, Figure 6-figure supplement 1A and B). This is because the m2 acetyl
303 group formed via PDH generates ^{13}C atoms at positions 4 and 5 of glutamate, whereas
304 the m2 ^{13}C glutamate (m/z 130) at position 2 and 3 is derived from pyruvate carboxylase
305 (PC) generated oxaloacetate (Madhu, Boneski et al. 2020) schematically shown in
306 Figure 6-figure supplement 1A. Analyses of the m/z 128-130 C2-C4 glutamate fragment
307 distinguishes these isotopomers with the enrichment of m1 glutamate (129 fragment)
308 approximately 5 times more abundant than the m2 glutamate (130 fragment) in the
309 *Tigar* knockout cells (Figure 6-figure supplement 1B).

310 If the changes in glucose flux occurred through increased flux through PDH, then
311 we should also observe a concomitant increase in the levels of acetyl-CoA that is
312 directly generated by PDH from pyruvate. As shown in Figure 6-figure supplement 1C,
313 acetyl-CoA levels were increased approximately 1.5-fold and acetyl-carnitine levels
314 approximately 2.5-fold (Figure 6-figure supplement 1D). Since acetyl-CoA is converted
315 to acetyl-carnitine in the mitochondria, these demonstrate that *Tigar* deficiency not only
316 increases glycolysis but also results in increased mitochondria uptake of acetyl-CoA (via
317 PDH). The increase in mitochondria PDH flux would in turn drive an increase in oxygen
318 consumption. Seahorse flux analyses of oxygen consumption rate (OCR) clearly
319 demonstrated that the *Tigar* knockout SH-SY5Y cells had higher rates of OCR
320 compared to the control SH-SY5Y cells (Figure 6-figure supplement 1E). Together,
321 these data demonstrate that *Tigar* deficiency in SH-SY5Y neuroblastoma cells results
322 from an increase in intracellular acetyl-CoA levels consistent with increased glycolysis
323 and direct conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase, as

opposed to pyruvate carboxylase. It is the increase in PDH flux into the tricarboxylic acid cycle that drives increased mitochondria oxidation.

A generalized increased cholinergic tone would be expected to reduce blood pressure and heart rate but with increased skeletal muscle shivering. To assess these physiologic parameters, we used implanted telemetry probes to simultaneously determine body temperature, mean arterial pressure (MAP), heart rate (BPM), and skeletal muscle electromyography in cold exposed *Chat^{Cre}* and chTKO mice (Figure 7). Consistent with the rectal temperature measurements previously observed, the telemetry probe also demonstrated that the chTKO are more resistant to cold induced hypothermia than *Chat^{Cre}* mice (Figure 7A). At room temperature, there was no significant difference in blood pressure, but following cold exposure the blood pressure of the control mice increased whereas the blood pressure of the chTKO mice decreased such that by 60 min the chTKO mice had a blood pressure 25 mmHg less than the control mice (Figure 7B). Similar, the heart rate was not statistically different at room temperature but increased substantially more in the control mice than in the chTKO mice following cold exposure (Figure 7C). As these data strongly suggest increase cholinergic signaling to enhance skeletal muscle contraction, we utilized electromyography (EMG) to directly determine skeletal muscle activity. A representative image of EMG tracers and root mean square (RMS) derivation are shown for *Chat^{Cre}* and chTKO mice when shifted to 4°C for 30 min (Figure 7D). Quantification of these types of data as the integral of RMS during the time course of cold exposure, demonstrated that at room temperature and following 10 min of cold exposure there is no significant difference in skeletal muscle contraction activity between *Chat^{Cre}* and

chTKO mice (Figure 7E). However, the contraction activity of the control *Chat^{Cre}* mice plateau at this level, whereas the chTKO mice shivering activity further increases to nearly twice the level of the control mice.

The increased skeletal muscle shivering associated with increased ATP turnover and energy expenditure would be expected to result in a compensatory increase in AMP-dependent protein kinase (AMPK) activity. As shown in Figure 7F, there was no significant difference in the activation site phosphorylation (T172) of the AMPK α subunit in the EDL muscle at room temperature between the control *Chat^{Cre}* and chTKO mice. However, after 1 h at 4°C, the control mice display the expected increase in T172-AMPK α phosphorylation that was further increased in the chTKO mice.

Together, the relative changes in cold induced blood pressure, heart rate, skeletal muscle shivering and increase in AMPK activation are fully consistent with the chTKO mice displaying a generalized increase in cholinergic signaling following cold exposure.

Discussion

Currently, there is a substantial effort to understand and develop approaches to increase energy expenditure through the development/activation of brown/beige adipose tissue thermogenesis. A fraction of adult humans can respond to cold stress by inducing the expression of thermogenic adipose tissue under relatively long-term cold acclimation in adult humans (McNeill, Suchacki et al. 2021). However, it remains somewhat controversial if this inducible thermogenic human brown adipose tissue is the rodent equivalent of beige or brown adipocytes (Cannon, de Jong et al. 2020, Samuelson and Vidal-Puig 2020, Virtanen and Nuutila 2021). In addition, whether the

370 mass of the human inducible thermogenic adipocytes is sufficient to significantly
371 contribute to cold responsive thermogenesis for core temperature maintenance or
372 weight loss has also been questioned (Blondin, Tingelstad et al. 2014, Virtanen and
373 Nuutila 2021). In contrast, skeletal muscle accounts for approximately 40% of healthy
374 body mass, is the dominant factor for basal metabolic rate and the major driver of
375 energy expenditure during physical exercise (Haman, Blondin et al. 2010, Blondin,
376 Tingelstad et al. 2014). In rodents, non-shivering thermogenesis (NST) is driven by
377 UCP1 dependent mitochondrial uncoupling in both brown and beige adipocytes, by the
378 creatine phosphorylation/dephosphorylation cycle in beige adipocytes (Cohen and
379 Spiegelman 2015, Kazak, Chouchani et al. 2015, Jung, Sanchez-Gurmaches et al.
380 2019). There are also reports of a skeletal muscle based NST via a sarcolipin-
381 dependent futile cycle of SERCA-dependent calcium transport in the sarcoplasmic
382 reticulum (Bal, Maurya et al. 2012, Maurya, Bal et al. 2015). In humans, maximal NST
383 can increase energy production equivalent to about 2 times above the resting metabolic
384 rate (RMR), while moderate shivering thermogenesis (ST) is 2.5 to 3.5 times RMR and
385 maximal shivering has been measured at about 5 times RMR (Haman, Blondin et al.
386 2010, Blondin, Tingelstad et al. 2014). In contrast, exercise at ~50% VO_2 max
387 generates approximately 15 times RMR (Weber and Haman 2005, Blondin, Tingelstad
388 et al. 2014). While a substantial effort has focused on NST, shivering and physical
389 activity are the dominant forms of involuntary and voluntary heat production during
390 acute cold exposure. Thus, it is essential to develop a deeper understanding of the
391 physiologic integrated role of skeletal muscle with thermogenic adipose tissue to have a

complete and detailed molecular understanding of energy balance and its implications for potential interventions to promote and maintain weight loss.

The data presented in this manuscript demonstrate that cholinergic neuron deficiency of *Tigar* results in an enhancement of cold-induced skeletal muscle contraction/shivering that is responsible for an increase in heat generation and protection of acute cold induced hypothermia. This response is likely mediated by an increase acetylcholine signaling at the neuromuscular junction, consistent with the parallel induction of tubocurare resistance and increased cholinergic neuron activation. Interestingly, despite the increase in synaptic density and acetylcholine levels mice at room temperature that are mildly cold stress (thermoneutrality for mice is approximately 29-31°C (Ganeshan and Chawla 2017), *Tigar* deficiency does not appear to significantly affect body temperature, skeletal muscle contraction activity or the overall skeletal muscle metabolite profile at room temperature. However, following acute cold challenge there are marked changes in these parameters between wild type and cholinergic *Tigar* knockout mice including a metabolic profile indicative of increased ATP turnover. These findings suggest that there is an increase in cholinergic signaling reserve that only becomes apparent when cholinergic signaling demand is high.

In addition to the well established role of sympathetic activation to increase brown and beige adipocytes thermogenesis (Zhu, Glazier et al. 2019), both cholinergic and sympathetic activation can affect vascular blood flow and behavioral activities that also contribute to body temperature regulation (Nakamura, Nakamura et al. 2022). Although we can not rule out the contribution of vasoconstriction to the conservation of core body temperature under cold exposure, heat loss from the mouse tail only

accounts for 5-8% of whole body heat loss (Škop, Liu et al. 2020) and is therefore unlikely to play a significant role in the thermoregulation observed by cholinergic *Tigar* deficiency.

In addition to TIGAR as a small carbohydrate phosphatase, previous studies have shown that TIGAR can directly interact with hexokinase 2 and the HOIP subunit of the linear ubiquitin assembly complex (Cheung, Ludwig et al. 2012, Tang, Kwon et al. 2018). Thus, although it is formally possible that TIGAR increases cholinergic signaling due to alterations in protein-protein interactions, we believe it is more likely a result of increased glycolysis due to its phosphatase function, that is occurring in the cholinergic neurons as this would result in an increase in acetyl-CoA levels. The steady-state acetyl-CoA concentrations in neuronal mitochondrial and cytosol are several folds lower than acetyl-CoA K_m values for choline acetyltransferase. More specifically, following cholinergic differentiation there is a further decrease in acetyl-CoA levels that results in further a substrate-dependence of acetylcholine biosynthesis (Ronowska, Szutowicz et al. 2018). In contrast, several studies have also shown that choline uptake via the choline transporter is rate limiting for acetylcholine synthesis (Lockman and Allen 2002). Although we have not measured choline uptake activity, *Tigar* deficiency had no significant effect on the amount of choline transporter protein. However, metabolic analyses clearly demonstrated an increased glucose derived acetyl moiety into acetylcholine, increased steady state acetylcholine levels with increased acetyl-CoA and acetyl-carnitine levels.

Thus, the simplest and most direct mechanism for the increase in acetylcholine levels induced by *Tigar* deficiency is the increased formation of acetyl-CoA through

enhanced glycolysis, which in turn leads to increased production of acetylcholine in cholinergic neurons. At present, it is not possible to distinguish the amount of synaptic acetyl-CoA from skeletal muscle acetyl-CoA at the neuromuscular junction, but future studies using spatial mass spectroscopy (MALDI) may be able to resolve this issue.

Finally, it is also important to recognize that myasthenia gravis is a neuromuscular disease caused by autoantibodies against components of the neuromuscular junction and in particular the nicotinic acetylcholine receptor (Gilhus, Tzartos et al. 2019). Current therapeutic strategies are focused on the use of steroids or immune suppressants to block the immune system and drugs to increase the transmission of acetylcholine signaling such as acetylcholine esterase inhibitors (Gilhus, Tzartos et al. 2019). Similarly, central cholinergic neurons undergo severe neurodegeneration in Alzheimer's disease and agents that enhance cholinergic signaling, particularly cholinesterase inhibitor therapies, provide significant symptomatic improvement in patients with Alzheimer's disease (Ferreira-Vieira, Guimaraes et al. 2016, Ahmed, Knowles et al. 2019). Interestingly, recent reports show that the neuromuscular junctions are stable in human age-related sarcopenia and in patients with cancer cachexia, which are different with the rodent models in that the sarcopenia is linked with the endplate fragmentation in NMJ (Jones, Harrison et al. 2017, Boehm, Miller et al. 2020). As such, inhibition of TIGAR function may also provide a novel approach to improve skeletal muscle function, memory and dementia through increased cholinergic signaling while limiting the deleterious metabolic consequences secondary to currently available therapies.

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Declaration of interests

The authors declare no competing interests.

Figure legends:

Figure 1: *TIGAR* deficiency mice are resistant to hypothermia induced by an acute cold challenge.

A, Male mice (WT n=10, TKO n=6) and B, Female mice (WT n=6, TKO n=7) core body temperatures were measured every fifteen-minutes starting at ambient laboratory temperature (0 min) and following placement at 4°C. C, Representative *TIGAR* immunoblot of gastrocnemius muscle (Gastroc), epididymal white adipose tissue (EWAT), subcutaneous inguinal white adipose tissue (iWAT) and interscapular brown adipose tissue (BAT) from control *Tigar*^{fl/fl} and adipocyte specific knockout

Tigar^{fl/fl}/Adipoq^{Cre} mice. D, *Tigar^{fl/fl}* and *Tigar^{fl/fl}/Adipoq^{Cre}* male mice (n=6) core body temperatures were measured every fifteen-minute starting at ambient laboratory temperature (0 min) and following placement at 4°C. E, Representative UCP1 and TIGAR immunoblots of brown adipose tissue from wild type (WT); whole body *Tigar* knockout (TKO), *Ucp1* knockout (UKO), and *Ucp1* and *Tigar* double knockout (UTKO) mice from two independent genotypes each. F, WT (n=6), TKO (n=6), UKO (n=7), and UTKO (n=8) mice core body temperatures were measured every fifteen-minute starting at ambient laboratory temperature (0 min) and following placement at 4°C. G, Representative TIGAR immunoblot of heart, gastrocnemius muscle (Gastroc), extensor digitorum digitorum longus (EDL) and soleus muscle from control (*Myf1^{Cre}*) and skeletal muscle specific *Tigar* knockout (*Myf1^{Cre}/Tigar^{fl/fl}*) mice. H, *Myf1^{Cre}* and *Myf1^{Cre}/Tigar^{fl/fl}* male mice (n=6) core body temperatures were measured every fifteen-minute starting at ambient laboratory temperature (0 min) and following placement at 4°C. Statistical analyses was described in the Method details and data presented as the mean ± S.D.. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****; $p < 0.001$, §.

Figure 2: TIGAR knockout mice are resistant to tubocurarine but not cyclopiazonic acid.

A, Wildtype (WT) and whole body *Tigar* knockout (TKO) male mice (n=6) were intraperitoneal injected with cyclopiazonic acid (CPA, 10 mg/kg body weight) and then core body temperature was measured every fifteen minutes at room temperature. B, WT and TKO male mice (n=6) were intraperitoneal injected with cyclopiazonic acid (CPA, 10 mg/kg body weight) at room temperature and then shifted to 4°C for 30

minutes, with core body temperature measured every fifteen minutes. C, WT and TKO male mice (n=6) were intraperitoneal injected with tubocurarine (0.4 mg/kg body weight) and then core body temperature was measured every ten minutes at room temperature. D, WT and TKO male mice (n=6) were intraperitoneal injected with tubocurarine (0.4 mg/kg body weight at room temperature and then ten minutes later shifted to 4°C for 30 minutes, with core body temperature measured every ten minutes. E, WT and TKO female mice (n=6) were intraperitoneal injected with tubocurarine (0.15 mg/kg body weight) and then core body temperature was measured every fifteen minutes at room temperature. F, WT and TKO male mice (n=10) were intraperitoneal injected with different tubocurarine doses and the LD₅₀ of curare was calculated using an online software LD₅₀ Calculator (AAT Bioquest, Inc., Sunnyvale, CA). G, WT and TKO male mice (n=10) were intraperitoneal injected with a lethal tubocurarine dose (0.6 mg/kg body weight) and the number of mice undergoing complete paralysis was plotted as a function of time in seconds. H, The time to death (absence of respiration) of the same mice was plotted as a function of time in minutes. Statistical analyses was described in the Method details and data presented as the mean ± S.D.. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****.

Figure 3: TIGAR deficient mice display increased ATP turnover in skeletal muscle.

A, Wildtype (WT) and *Tigar* knockout (TKO) male mice (n=6) were maintained at room temperature (RT) or shifted to 4°C for 1 hour. Quadriceps white skeletal muscle was isolated and extracts assayed for ATP, ADP and AMP levels as described in Method and Materials. B, Wildtype (WT) and *Tigar* knockout (TKO) male mice (n= 3-4 per

condition) were at room temperature or shifted to 4°C for 1 h and then given an oral gavage of 0.3 ml pure H₂O¹⁸ for 10 min at 4°C and an IP injection of 1 ml pure H₂O¹⁸ for another 10 min at 4°C. The quadricep muscles were freeze-clamped with liquid N₂ and extracts prepared for mass spectroscopic analyses. The heatmap shows the O¹⁸ enrichment fraction of ATP, ADP, AMP, and creatine-phosphate. C, Wildtype (WT) and *Tigar* knockout (TKO) male mice (n=6) were maintained at room temperature or shifted to 4°C for 1 hour and plasma creatine levels were determined by metabolomics analyses. D, The CP_M4_83 data was generated from the same experimental setting as B, indicating the increase of phosphocreatine turnover in TKO compared to that in WT at 4°C. Statistical analyses was described in the Method details and data presented as the mean ± S.D.. $p < 0.05$, *; $p < 0.01$, **

Figure 4: Cholinergic neuron specific TIGAR knockout mice recapitulate the protection against hypothermia of the whole-body TIGAR deficient mice.

A, Representative immunofluorescent images showing TIGAR, vesicular acetylcholine transporter (VACHT), and nuclei (DAPI) in the superior cervical ganglions (SCG) from control *Chat*^{Cre} and chTKO male mice. B, Representative TIGAR, choline acetyltransferase (ChAT) and actin protein immunoblots of the SCG from two independent control *Chat*^{Cre} and chTKO male mice. C, Male *Chat*^{Cre} and chTKO mice (n=6) core body temperatures were measured every fifteen-minutes starting at ambient laboratory temperature (0 min) and following placement at 4°C. D, Male *Chat*^{Cre} and chTKO mice (n=6) were intraperitoneal injected with cyclopiazonic acid (CPA, 10 mg/kg body weight) at room temperature and core body temperature measured every fifteen minutes. E, *Chat*^{Cre} and chTKO male mice (n=6) were intraperitoneal injected with

cyclopiazonic acid (CPA, 10 mg/kg body weight) at room temperature, shifted to 4°C and core body temperature measured every fifteen-minute. F, Male *Chat^{Cre}* and chTKO mice (n=6) were intraperitoneal injected with tubocurarine (0.4 mg/kg body weight) and then core body temperature was measured every fifteen minutes at room temperature. G, *Chat^{Cre}* and chTKO mice (n=6) were intraperitoneal injected with tubocurarine (0.4 mg/kg body weight) at room temperature and ten minutes later shifted to 4°C for 30 minutes. Core body temperature was measured every ten minutes. H, Representative TIGAR, c-Fos, VACHT, ChT and actin protein immunoblots of the SCG from two independent control *Chat^{Cre}* and chTKO male mice at room temperature or shifted to 4°C for 30 min. Statistical analyses was described in the Method details and data presented as the mean ± S.D.. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****.

Figure 5: Skeletal muscle of chTKO mice at 4°C display increased pentose phosphate pathway, purine nucleotide cycle, and amino acid utilization pathways.

Control *Chat^{Cre}* and chTKO male mice (n=4-6) were maintained at room temperature (RT) or shifted to 4°C for 1 hour. Quadriceps white skeletal muscle was isolated and extracts were subjected to widely targeted (MRM) small metabolite profiling using an ABSciex 6500+ QTRAP with ACE PFP and MERCK Zic-pHILIC columns as described in Method and Materials. The heatmap shows the metabolites/pathways differentially identified with corresponding p values.

Figure 6: TIGAR deficiency increases acetylcholine biosynthesis and acetylcholine receptor clustering at the neuromuscular junction.

A, Representative images of a-bungarotoxin immunofluorescent labeling of nicotinic acetylcholine receptor clusters in EDL muscle from wildtype (WT) and TKO mice. B, Quantification of the number of nicotinic acetylcholine receptor clusters following fifteen minutes exposure at 4°C (WT n=17, TKO n=13). These data represent the average of over six mice in each group of mean $\pm$ S.D. (Unpaired t test, two-tailed, $p=0.0035$, **). C, Acetylcholine levels in gastrocnemius muscle of WT and TKO male (n=7) mice at room temperature or following 1 hour at 4°C. D, Acetylcholine levels in gastrocnemius muscle of *Chat*^{Cre} and chTKO male (n=6) mice at room temperature or following 1 hour at 4°C. E, Representative immunoblots of TIGAR and actin proteins from two scrambled sgRNA and two *Tigar* sgRNA knockout Sh-SY5Y cell lines. F, Acetylcholine concentrations in the medium of scrambled and TKO SH-SH5Y neuroblastoma cells. G, m2 Acetylcholine enrichments in cells labeled with D-glucose-1,2-¹³C₂. H, m2 Acetylcholine enrichments in cells labeled with U-¹³C₆ D-glucose. I, m1 Glutamate (m/z 128, C2-C4 fragment) enrichment in the medium of scrambled and TKO SH-SY5Y human neuroblastoma cells labeled with D-glucose-[1,2]-¹³C₂. Statistical analyses was described in the Method details and data presented as the mean $\pm$ S.D.. $p < 0.05$, *; $p < 0.001$, ***; $p < 0.0001$, ****.

Figure 7: ChTKO mice display enhanced cold stimulated skeletal muscle shivering activity and increased cholinergic tone.

A, Male mice core body temperatures (*Chat*^{Cre} n=7, TKO n=10), B, blood pressure (mmHg) (*Chat*^{Cre} n=7, chTKO n=10), C, heart rate (beats per minute, BPM) (*Chat*^{Cre}

n=7, chTKO n=6), D, Representative electromyography traces (EMG) and root mean squares (RMS) for *Chat^{Cre}* and chTKO male mice when shifted to 4°C for 30 min, and E, neck electromyogram (EMG, (*Chat^{Cre}* n=5, chTKO n=6)) were measured continuously starting at ambient laboratory temperature (0 min) and for 60 min following placement at 4°C. F) EDL muscle from two independent control *Chat^{Cre}* and chTKO male mice at room temperature (RT) or shifted to 4°C for 1 hour were isolated and tissue extracts were immunoblotted with an antibody for the phospho-threonine 172 AMPK α subunit (pT172-AMPK α) or total α subunit (AMPK α). Statistical analyses was described in the Method details and data presented as the mean $\pm$ S.D.. $p < 0.01$, **

Supplemental figures legends

Figure 1-figure supplement 1: The TKO protection against hypothermia is independent of UCP1 expression with no change in basal metabolic rate.

A, Relative *Ucp1* mRNA levels in interscapular brown adipose tissue from wildtype (WT) and TKO male (n=6) mice maintained at ambient temperature and after 1 hour 4°C exposure. B, Representative TIGAR, UCP1 and actin protein immunoblot of interscapular brown adipose tissue from two independent wildtype (WT) and TKO male mice maintained at ambient temperature or after 1 hour 4°C exposure. C, Relative *Ucp1* mRNA levels in inguinal adipose tissue from WT and TKO male mice (n= 4-10) at ambient temperature or after 2-hour 4°C exposure. Please note that Ct values for brown adipose tissue was approximately 18, whereas the Ct values for the inguinal white adipose tissue was in the range of 33. D, The ratio of lean mass/body weight and fat mass/body weight were determined by EchoMRI at five months of age for male WT and

TKO mice. E, Representative images of Hematoxylin & Eosin staining of soleus and extensor digitorum longus (EDL) skeletal muscle from WT and TKO mice. F, Quantification of daily food intake/body weight in the light and dark cycles of WT and TKO mice of male. G, Indirect calorimetry determination of spontaneous locomotor activity in the AMB+Z dimensions from WT and TKO mice in the light and dark cycles. H, Indirect calorimetry determination of energy expenditure in the light and dark cycles of the WT and TKO mice. Statistical analyses were described in the Method details and data presented as the mean $\pm$ S.D.. $p < 0.01$, **; $p < 0.0001$, ****.

Figure 1-figure supplement 2: Changes in the expression of skeletal muscle SERCA, myoregulin, and sarcolipin does not account for the differential sensitivity to tubocurarine.

Relative mRNA levels of A, *Atp2a1*; B, *Atp2a2*; C, myoregulin (*Mrln*); and D, sarcolipin (*Slrn*) in the gastrocnemius muscle from male (n=3-10) wildtype (WT) and whole-body *Tigar* knockout (TKO) mice at ambient temperature or after one-hour 4°C exposure. Statistical analyses were described in the Method details and data presented as the mean $\pm$ S.D.

Figure 2-figure supplement 1: Changes in the expression of skeletal muscle nicotinic acetylcholine receptor subunits does not account for the differential sensitivity to tubocurarine.

Relative mRNA levels of the nicotinic acetylcholine receptor subunits A, $\alpha 1$ (*Chrna1*); B, $\beta 1$ (*Chrn1*); C, γ (*Chrng*); D δ (*Chrnd*); and E, ϵ (*Chrne*) in the gastrocnemius muscle from male (n=3-10) wildtype (WT) and whole-body *TIGAR* knockout (TKO) mice at ambient temperature or after one-hour 4°C exposure. Statistical analyses was described in the Method details and data presented as the mean $\pm$ S.D.

Figure 3-figure supplement 1: Skeletal muscle of TKO mice at 4°C display increased pentose phosphate pathway, purine nucleotide cycle, and amino acid utilization pathways.

Wildtype (WT) and whole-body *TIGAR* knockout (TKO) male mice (n=6) were maintained at room temperature (RT) or shifted to 4°C for 1 hour. a, Quadriceps white skeletal muscle was isolated and extracts were subjected to widely targeted (MRM) small metabolite profiling using an ABSciex 6500+ QTRAP with ACE PFP and MERCK Zic-pHILIC columns as described in Method and Materials. The heatmap shows the metabolites/pathways differentially identified with corresponding p values. B, Matched pathways, determined from MetaboAnalyst 5.0, which integrates enrichment analysis (metabolite over-representation more than expected by chance) and pathway impact (reflecting centrality of the metabolite in a topological network).

Figure 3-figure supplement 2: *TIGAR* deficiency has no significant effect on intrinsic skeletal muscle mitochondrial oxidative phosphorylation activity or ATP production.

Permeabilized skeletal muscle fiber bundles were prepared from red portions of the gastrocnemius muscle from wildtype (WT) and TKO mice immediately after 1 h cold

exposure. A, Oxygen consumption rate (JO_2) as a function of ADP concentration. B, The K_m and V_{max} for ADP-stimulatory respiratory kinetics. C, Rates of ATP production ($JATP$) and D, oxygen consumption (JO_2) measured simultaneously under clamped submaximal and maximal ADP-demand states. E, ATP/O efficiency ratios. F, Force-frequency of the extensor digitorum longus muscle isolated from WT and TKO mice. G, Peak specific tension and time to one-half relaxation of the extensor digitorum longus muscle from WT and TKO mice.

Figure 4-figure supplement1: Mouse superior cervical ganglion (SCG) dissection

A, The mouse ventral midline of the neck up to the mandible was incised and the salivary glands were exposed. B, The sternocephalicus and posterior belly digastric muscles were retracted after the removal of salivary glands. C, The carotid bifurcation and carotid sheath were identified. D, The SCG behind the carotid sheath was isolated and the fresh SCGs in two sides were subjected to immunofluorescence imaging and Western Blotting experiments for Figure 4. E, A representative image of SCG from ChAT^{<Cre>} and chTKO, respectively, was shown.

Figure 6-figure supplement1: Summation of flux results through glycolysis and the TCA cycle using [1,2]-¹³C-glucose by assessing glutamate isotopomers.

A, [1,2]-¹³C-glucose becomes [2,3]-¹³C pyruvate if it is directly metabolized through the glycolytic pathway. [2,3]-¹³C-pyruvate can enter the TCA cycle through pyruvate dehydrogenase (PDH) or pyruvate carboxylase (PC). Blue dots indicate ¹³C carbons from glucose entered TCA cycle via PDH. Red dots indicate ¹³C carbons from glucose entered TCA cycle via pyruvate carboxylase (PC). B, Representative spectra of the

glutamate C2-C4 fragments from TIGAR knockout SH-SY5Y cells labeled with D-glucose-[1,2]- $^{13}\text{C}_2$. M0 of the C2-C4 glutamate fragment is m/z 128, m1 of glutamate is m/z 129 (blue) that indicates the presence of ^{13}C at the fourth carbon position and m2 glutamate indicates the presence of ^{13}C at the second and third carbon position (red). C, The cellular acetyl-CoA and D, acetyl-carnitine levels in SH-SY5Y cell pellets were measured as described in the Method details and data presented as the mean $\pm$ S.D. E, Seahorse flux analyses of oxygen consumption rate (OCR) were performed as described in the Method details and data presented as the mean $\pm$ SEM. Statistical analyses was described in the Method details. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****

Figure 2-video 1: TKO mice are resistant to the paralytic effects of tubocurare.

Representative video clip of wildtype (WT) and whole-body TIGAR knockout (TKO) male mice behavior under ambient temperature and at 4°C following intraperitoneal injection of tubocurare (0.4mg/kg body weight). Rectal temperature recording of WT and TKO mice with curare injection was performed on Feb 12, 2019. The date of birth of both WT and TKO mice were Oct 15, 2018. The body weights of WT and TKO are 25.3 g and 27.6 g, respectively. The volume of curare saline solution (0.1 mg/ml in saline) for IP injection was 101.2 μl for WT mouse and 110.4 μl for TKO mouse. The mice were IP injected with curare, then caged at ambient temperature (21-23°C) for 10 minutes, and subsequently transferred into a pre-chilled cage in cold chamber at 4°C for 20 minutes. The mice were taken back to cages at ambient temperature (see the sketch below). Rectal temperature was recorded at the time of curare injection and every 10-

minute thereafter (see the table below). The mouse in left cage was WT and the mouse in right cage was TKO.

		RT	Curare 10 min	4 °C 10 min	4 °C 20 min
Rectal T. (°C)	WT	38.3	35	27	22.1
Rectal T. (°C)	TKO	38.6	37.6	37	36.5

Figure 4-video 1: Cholinergic neuron specific TIGAR knockout (chTKO) mice are resistant to the paralytic effects of tubocurare.

Representative video clip of control *Chat^{Cre}* and chTKO male mice behavior under ambient temperature and at 4°C following intraperitoneal injection of tubocurare (0.4mg/kg body weight). Rectal temperature recording of *Chat^{Cre}* and chTKO mice with curare injection was performed on Feb 7, 2019. The date of birth of both *Chat^{Cre}* and chTKO mice were Oct 6, 2018. The body weights of *Chat^{Cre}* and chTKO are 25.4 g and 27.7 g, respectively. The volume of curare saline solution (0.1 mg/ml in saline) for IP injection was 101.6 µl for *Chat^{Cre}* mouse and 110.8 µl for chTKO mouse. The mice were IP injected with curare, then caged at ambient temperature (21-23°C) for 10 minutes, and subsequently transferred into a pre-chilled cage in cold chamber at 4°C for 20 minutes. The mice were taken back to cages at ambient temperature (see the sketch below). Rectal temperature was recorded at the time of curare injection and every 10-

minute thereafter (see the table below). The mouse in left cage was *Chat*^{Cre} and the mouse in right cage was chTKO.

		RT	Curare 10 min	4 °C 10 min	4 °C 20 min
Rectal T. (°C)	<i>Chat</i> ^{Cre}	38.4	35.8	26.4	21.4
Rectal T. (°C)	chTKO	38.4	36.7	36	35.2

Legends for source data of raw immunoblotting images

Figure 1-source data 1

Fresh gastrocnemius muscle (Gastr), epididymal adipose tissue (EWAT), inguinal white adipose tissue, and interscapular brown adipose tissue from *Tigar*^{fl/fl} and *Tigar*^{fl/fl}/*Adipoq*^{Cre} mice were collected and 30 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1C. Detailed description of the raw images was shown in Source data 1.

Figure 1-source data 2

Interscapular brown adipose tissues were collected from WT, TKO, UKO, and UTKO mice and 30 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1E. Detailed description of the raw images was shown in Source data 1.

Figure 1-source data 3

Interscapular brown adipose tissues were collected from WT, TKO, UKO, and UTKO mice and 30 µg of the tissue lysate were used for UCP1 (33 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1E. The raw image was used for Figure 1E. Detailed description of the raw images was shown in Source data 1.

Figure 1-source data 4

Interscapular brown adipose tissues were collected from WT, TKO, UKO, and UTKO mice and 30 µg of the tissue lysate were used for vinculin (117 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1E. Detailed description of the raw images was shown in Source data 1.

Figure 1-source data 5

Fresh heart, gastrocnemius muscle (Gastr), extensor digitorum longus (EDL muscle), and soleus muscle were collected from *Myl1^{Cre}* and *Myl1^{Cre}/Tigar^{fl/fl}* mice and 30 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1 G. Detailed description of the raw images was shown in Source data 1.

Figure 1-source data 6

Fresh heart, gastrocnemius muscle (Gastr), extensor digitorum longus (EDL muscle), and soleus muscle were collected from *Myl1^{Cre}* and *Myl1^{Cre}/Tigar^{fl/fl}* mice and 30 µg of the tissue lysate were used for vinculin (117 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1 G. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 1

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The left four lanes of the raw image (lysate from the mice at ambient temperature) were used for Figure 4 B to confirm the efficiency of TIGAR protein loss in SCG of the chTKO mice. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 2

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for choline acetyltransferase (ChAT, 70 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The left four lanes of the raw image (lysate from the mice at ambient temperature) were used for Figure 4 B. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 3

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for Actin β (42 kDa) immunoblotting analysis as described in the

Immunoblotting, Method details. The left four lanes of the raw image (lysate from the mice at ambient temperature) were used for Figure 4 B. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 4

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from both ambient temperature housed and 4°C 30 minutes exposed *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The right eight lanes of the raw image (lysate from SCG tissues) was used for Figure 4 H to confirm the efficiency of TIGAR protein loss in SCG of the chTKO mice. The left two lanes of the raw immunoblotting image represent the lysates of soluble fraction (RIPA lysis buffer extracted) of whole brain tissues from the *Chat^{Cre}* and chTKO mice, respectively. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 5

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from both ambient temperature housed and 4°C 30 minutes exposed *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for c-Fos (62 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The right eight lanes of the raw image (lysate from SCG tissues) was used for Figure 4 H to show the increase of c-Fos protein in SCG of chTKO mice under cold exposed. The left two lanes of the raw immunoblotting image represent the lysates of soluble fraction (RIPA lysis buffer extracted) of whole brain tissues from *Chat^{Cre}* and chTKO mice, respectively. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 6

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from both ambient temperature housed and 4°C 30 minutes exposed *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for vesicular acetylcholine transporter (VACHT, 55 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The right eight lanes of the raw image (lysate from SCG tissues) were used for Figure 4 H to show VACHT protein in the SCGs. The left two lanes of the raw immunoblotting image represent the lysates of soluble fraction (RIPA lysis buffer extracted) of whole brain tissues from *Chat^{Cre}* and chTKO mice, respectively. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 7

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from both ambient temperature housed and 4°C 30 minutes exposed *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for choline transporter (ChT, 55 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The right eight lanes of the raw image (lysate from SCG tissues) were used for Figure 4 H to show ChT protein in that 63 and 70 kDa bands were observed in the SCGs. The left two lanes of the raw immunoblotting image represent the lysates of soluble fraction (RIPA lysis buffer extracted) of whole brain tissues from *Chat^{Cre}* and chTKO mice, respectively. Detailed description of the raw images was shown in Source data 1.

Figure 4-source data 8

The superior cervical ganglion (SCG) tissues were collected and snap frozen in liquid nitrogen from both ambient temperature housed and 4°C 30 minutes exposed *Chat^{Cre}* and chTKO mice, as shown in the Figure 4-figure supplement1 for SCG dissection. The SCGs from three mice of the same strain were pooled and processed by beads homogenization to acquire tissue lysates. 15 µg of the tissue lysate were used for actin β (42 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The right eight lanes of the raw image (lysate from SCG tissues) were used for Figure 4 H to show actin β protein in the SCGs. The left two lanes of the raw immunoblotting image represent the lysates of soluble fraction (RIPA lysis buffer extracted) of whole brain tissues from *Chat^{Cre}* and chTKO mice, respectively. Detailed description of the raw images was shown in Source data 1.

Figure 6-source data 1

The culture SH-SY5Y cells were collected from both scrambled and Tigar knockout (TKO) cells and 30 µg of the cell lysates were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The left four lanes of the raw image (lysate from neuroblastoma cells) were used for Figure 6 E to confirm the efficiency of TIGAR protein loss in the SH-SY5Y neuroblastoma TKO cells. The right four lanes of the raw image represent the TIGAR immunoblotting of the cell lysates from seven day differentiated neuroblastoma cells. Detailed description of the raw images was shown in Source data 1.

Figure 6-source data 2

The culture SH-SY5Y cells were collected from both scrambled and Tigar knockout (TKO) cells and 30 µg of the cell lysates were used for actin β (42 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The left four lanes of the raw image (lysate from neuroblastoma cells) were used for Figure 6 E to show actin β protein in the in the SH-SY5Y neuroblastoma cells. The right four lanes of the raw image represent the actin β immunoblotting of the cell lysates from seven day differentiated neuroblastoma cells. Detailed description of the raw images was shown in Source data 1.

Figure 7-source data 1

The extensor digitorum longus (EDL) muscles were collected from both ambient temperature housed and 4°C 1 hour exposed *Chat^{Cre}* and chTKO mice and 30 µg of the tissue lysate were used for AMPKα (62 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 7F. Detailed description of the raw images was shown in Source data 1.

Figure 7-source data 2

The extensor digitorum longus (EDL) muscles were collected from both ambient temperature housed and 4°C 1 hour exposed *Chat^{Cre}* and chTKO mice and 30 µg of the tissue lysate were used for phospho-^{Thr172}AMPKα (62 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 7F. Detailed description of the raw images was shown in Source data 1.

Figure 1-figure supplement 1-source data 1

Interscapular brown adipose tissues were collected from both ambient temperature housed and 4°C 1 hour exposed WT and TKO mice and 30 µg of the tissue lysate were used for TIGAR (30 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1-figure supplement 1 B. Detailed description of the raw images was shown in Source data 1.

Figure 1-figure supplement 1-source data 2

Interscapular brown adipose tissues were collected from both ambient temperature housed and 4°C 1 hour exposed WT and TKO mice and 30 µg of the tissue lysate were used for UCP1 (33 kDa) immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1-figure supplement 1 B. Detailed description of the raw images was shown in Source data 1.

Figure 1-figure supplement 1-source data 3

Interscapular brown adipose tissues were collected from both ambient temperature housed and 4°C 1 hour exposed WT and TKO mice and 30 µg of the tissue lysate were used for actin β immunoblotting analysis as described in the Immunoblotting, Method details. The raw image was used for Figure 1-figure supplement 1 B. Detailed description of the raw images was shown in Source data 1.

Legends for source data of metabolomics analyses

Figure 3-source data 1

Raw ¹⁸O enrichment data of stable isotope metabolic flux assessment with H₂O¹⁸

was collected in quadricep white muscle of both ambient temperature housed and 4°C 1 hour exposed WT and TKO mice, as described in Method details. The data was used in Figure 3.

Figure 3-figure supplement 1-source data 1

Raw metabolites data were collected in gastrocnemius muscle of both ambient temperature housed and 4°C 1 hour exposed WT and TKO mice as described in Method details. The data was used in Figure 3-figure supplement 1.

Figure 5-source data 1

Raw metabolites data were collected in gastrocnemius muscle of both ambient temperature housed and 4°C 1 hour exposed *Chat^{Cre}* and chTKO mice as described in Method details. The data was used in Figure 5.

Figure 6-figure supplement1-source data 1

Raw metabolites data including cellular acetyl-CoA and acetyl-carnitine were collected from SH-SY5Y control and TKO cell pellet as described in Method details. The data was used in Figure 6-figure supplement 1.

Materials and Methods

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeffrey E Pessin (Jeffrey.pessin@einsteinmed.edu)

Materials availability

This study did not generate new unique reagents. SH-SY5Y neuroblastoma both scrambled and TKO cell lines are available upon request from the lead contact. Mouse lines generated in this study are available from the lead contact, although there are timeline restrictions to the availability of the mice due to the labor and space limitations

of the laboratory staff and the facility of Institution for Animal Studies at Albert Einstein College of Medicine.

Data and code availability

The authors are willing to deposit all the data in this study before the manuscript being accepted for publication.

Experimental models and subject details

Mice models

All studies were performed in accordance with protocols approved by the Einstein Institutional Animal Care and Use Committee. *Tigar* whole body knockout (TKO) mice were made as described in our previous paper (Tang, Kwon et al. 2018). To establish conditional knockout mice of *Tigar* gene, we purchased C57BL/6N-TIGAR^{tm1b(EUCOMM)Wtsi}/Wtsi mice from Welcome Trust Sanger Institute (Hinxton Cambridge, U.K.). Briefly, loxP sites were generated to delete exon 3 of *Tigar*, chimeric mice were mated with C57BL/6 mice and germ-line transmission confirmed by PCR and Southern blotting. The progeny crossed sequentially with *FLPe* transgenic mice to remove *lacZ* and *neo* genes. The primer pairs TIGARCKO-5FRT-FW: TTGGGATCCCCTAGTTTGTG and TIGARCKO-5FRT-RV: AACTCAGCCTTGAGCCTCTG were used to confirm deletion of the *lacZ* and neomycin resistance genes. The primer pairs TIGARCKO-3loxP-FW: GAGAAGAGACCCCCTGGAAC and TIGARCKO-3loxP-RV: TTCCGGCCAAACAGACTTAC were used to confirm the *Tigar*^{fl/fl} mice. Mice were

backcrossed more than ten generations to C57BL/6J mice. The primers for subsequent genotyping for *Tigar*^{fl/fl} were listed in the Key Resources Table (KRT) and the mouse tail PCR (touchdown cycling protocol, Jackson Laboratory) results in 538 bp for mutant and 346 bp for wild type mice.

Adipocyte-specific *Tigar* knockout (*Tigar*^{fl/fl}/*Adipoq*^{Cre}) mice were produced by mating *Tigar*^{fl/fl} mice with *Adiponectin-Cre* (*Adipoq*^{Cre}) mice, as previously described (Feng, Amgalan et al. 2018). Skeletal muscle-specific *Tigar* knockout mice were produced by mating the *Tigar*^{fl/fl} mice with skeletal muscle-specific myosin light polypeptide 1 Cre (*Myl1*^{Cre}) mice purchased from Jackson Laboratory (stock number 024713). Cholinergic neuron-specific *Tigar* knockout (chTKO) mice were generated by mating *Tigar*^{fl/fl} mice with *Chat*^{Cre} mice purchased from Jackson Laboratory (stock number 028861). *Ucp1* knockout (UKO) mice, which were C57BL/6J background, were a gift from Dr. Victor Schuster (Albert Einstein College of Medicine). *Ucp1*^{-/-}/*Tigar*^{-/-} double knockout (UTKO) mice were generated by breeding *Ucp1* knockout mice with TKO, which resulted in the production of WT, TKO, UKO, and UTKO mice.

Mouse husbandry and rectal temperature measurement

Mice were housed in a facility equipped with a 12-h light/dark cycle. Animals were fed a normal chow diet that contains 62.3% (kcal) carbohydrates, 24.5% protein, and 13.1% fat (5053, LabDiet). The mice at 12-16 weeks of age were used for rectal temperature measurement using TH-8 Thermometer (Physitemp Instruments, Clifton, NJ) per manufacturer's instructions. For cold exposure, the mouse was individually put into a pre-chilled cage with bedding in a cold chamber (4°C). For tissue collection, the mice

1031 were killed, and the tissues were collected and snap-frozen in liquid nitrogen and stored
1032 in a -80°C freezer. All studies were approved by and performed in compliance with the
1033 guidelines of the Albert Einstein College of Medicine Institutional Animal Care and Use
1034 Committee.

1035

1036 ***Blood plasma collection***

1037 Mice were briefly anesthetized by isoflurane and the blood sample was collected from
1038 mouse orbital sinus into microtube with EDTA (Microvette 500 K3E, SARSTEDT,
1039 Germany) on ice. The plasma sample was collected by centrifugation (1,000 g, 30 min)
1040 and was stored at -80°C .

1041

1042 ***Gastrocnemius and quadricep skeletal muscle samples isolation***

1043 Gastrocnemius and quadricep white muscle were collected and snap-frozen in liquid
1044 nitrogen and stored at -80°C . The whole piece of the muscle was ground using a
1045 mortar-pestle in liquid nitrogen and the muscle powder (30 mg) was used for
1046 metabolomics analysis.

1047

1048 ***Tubocurare and cyclopiazonic acid treatments***

1049 Tubocurare (T2379, Sigma-Aldrich) was given as an intraperitoneal injection in a total
1050 volume of 100 μl in saline solution reaching at various doses and times indicated in the
1051 figure legends and the median lethal dose (LD50) was calculated with LD50 Calculator,
1052 a online tool from AAT Bioquest (Sunnyvale, CA). Cyclopiazonic acid (C1530, Sigma-
1053 Aldrich) was given as an intraperitoneal injection in a total volume of 100 μl in saline

solution reaching a dose of 10 mg/kg body weight for the time indicated. In both cases, the same volume of 100 µl of saline were injected as vehicle controls.

TIGAR knockout in human SH-SY5Y neuroblastoma cells

Human SH-SY5Y neuroblastoma cell line was purchased from ATCC (ATCC® CRL-2266). The SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin. Cell lines was maintained in a 5% CO₂ incubator at 37 °C and were routinely tested to exclude *Mycoplasma* contamination.

The CRISPR 3xsgRNA/Cas9 all-in-one expression clone targeting *Tigar* (NM_020375.2) (catalogue number: HCP215394-CG04-3) and scrambled sgRNA control for pCRISPR-CG04 (catalogue number: CCPCTR01-CG04-B) were purchased from GeneCopoeia (Rockville, MD). The plasmid DNAs were transformed and amplified in Mix & Go Competent Cells-Strain HB 101 (Zymo Research, catalog no. T3013, Irvine, CA) and purified using PowerPrep HP Plasmid Maxiprep kits with prefilters (Origene, Rockville, MD). The SH-SY5Y neuroblastoma cells were transfected with 10 ug of plasmid DNAs when 60% confluence in 100-mm dishes using transfection reagents GenJet II (SignaGen Laboratories, Rockville, MD per the manufacturer's instructions). The transfected cells were in culture for 48 hours and were collected in 500 µl of MACS buffer (PBS containing EDTA) containing 1% fetal bovine serum in Falcon 5 ml polystyrene round-bottom tube filtered with cell-strainer cap. The GFP positive cells were selected using FACS GFP sorting and were cultured and proliferated in 10% FBS

1076 DMEM. The cell descent was collected and cell lysate was immunoblotted with a TIGAR
1077 antibody.

1078 **Method details**

1079 ***In vivo ¹⁸O isotopic labeling of cellular phosphoryls of quardricep white muscle of*** 1080 ***the mice***

1081 Wildtype (WT) and *Tigar* knockout (TKO) male mice (n= 3-4 per condition) were at room
1082 temperature or shifted to 4°C for 1 h and then given an oral gavage of 0.3 ml pure H₂¹⁸O
1083 for 10 min at 4°C and an IP injection of 1 ml saline H₂¹⁸O (0.9% NaCl in ¹⁸O water) for
1084 another 10 min at 4°C. The dosing and timing of this protocol was selected from pilot
1085 studies to assure steady-state labeling of the ATP pool. The quadricep muscles were
1086 freeze-clamped with liquid N₂ and extracts prepared for mass spectroscopic analyses for
1087 identification of the position of ¹⁸O into the phosphates of ATP, that can be extrapolated
1088 to ADP and AMP (Nemutlu, Gupta et al. 2015).

1089

1090 ***Purification and Isotopic Analysis of ¹⁸O-Labeled Cellular Phosphoryls***

1091 Quadricep muscle were freeze-clamped and pulverized in mortar with liquid nitrogen,
1092 and extracted in 80% methanol solution. The samples were centrifuged at 10,500 rpm
1093 for 10 minutes at 4°C to precipitate proteins. The supernatants were transferred to a
1094 LCMS sampling vial pending for injection. Metabolites separation was performed on a
1095 ZIC-pHILIC column (Merk). Data was collected with ABSciex 6500+ QTrap MS/MS with
1096 a Multiple Reaction Monitoring (MRM) mode. The ions monitored were listed in as
1097 below:

Metabolites	Isotope Labels	Q1	Q3
AMP	M0	346	79
AMP	M2	348	81
AMP	M4	250	83
ADP	M0	426	79
ADP	M2	428	81
ADP	M4	430	83
ADP	M0	426	159
ADP	M2	428	161
ADP	M4	430	163
ADP	M6	432	165
ATP	M0	506	79
ATP	M2	508	81
ATP	M4	510	83
ATP	M0	506	159
ATP	M2	508	161
ATP	M4	510	163
ATP	M6	512	165
ATP	M0	506	239
ATP	M2	508	241
ATP	M4	510	243
ATP	M6	512	245

CP	M0	210	79
CP	M2	212	81
CP	M4	214	83

1098 The ^{18}O concentration in mouse body was determined with GC-MS according to the

1099 MMPC protocol:

1100 (<https://www.mmpc.org/shared/document.aspx?id=275&docType=Protocol>).

1101 Briefly, 10 μl of plasma from the matched quadricep muscle isolated mice were added

1102 to 2 μl of 5N NaOH and 4 μl of % acetone in acetonitrile to allow for ^{16}O to ^{18}O

1103 exchange (Aksnes, Aksnes et al. 1966). The samples were at room temperature for

1104 overnight (at least 10 hours). After chloroform extraction, the samples were analyzed on

1105 an Agilent GC-MS system with EI mode. Selected ion monitoring (SIM scan with ions of

1106 58, 59, and 60) was used for the detection. A linear curve of different percentage (from

1107 0 to 10%) of H_2^{18}O in water prepared along with the samples. ^{18}O enrichment was

1108 calculated with the linear curve. The enrichment of ^{18}O in phosphates in skeletal

1109 muscle (see below) was normalized to the ^{18}O water enrichment in the plasma.

1110

1111 ***Stable isotope enrichment and acetylcholine analyses in culture media of SH-***

1112 ***SY5Y control and TKO neuroblastoma cells***

1113 The SH-SY5Y neuroblastoma cells were cultured in 10 cm dishes until confluent and

1114 the culture media was removed, and the cells were washed with DMEM medium

1115 (Biological Industries, Boston, catalogue number 01-057-1A, without glucose,

1116 glutamine, and pyruvate). Ten ml of DMEM containing either 10 mM D-glucose-1,2- $^{13}\text{C}_2$

1117 (Sigma-Aldrich 453188-1G) or 10 mM D-glucose- $^{13}\text{C}_6$ (U- ^{13}C]-glucose) and 10 mM D-

1118 glucose (unlabeled : labeled = 1:1), 1 mM glutamine, without pyruvate was added to the
1119 cultured cells and medium was collected at 4, 8, and 24-hour time points. The medium
1120 was centrifuged at 1,000 g for 20 min at 4°C and stored at -80°C. The plates at 24-hour
1121 time point were put on ice and gently rinsed with 1.5 ml of ice-cold 150 mM ammonium
1122 acetate. The cells were scraped and transferred into a 2-ml screw cap tube (Cat#
1123 02682558, ThermoFisher). The cell pellets were collected by 1000 g centrifugation at 4
1124 °C for two minutes and stored at -80°C for cellular acetyl-CoA and acetyl carnitine
1125 measurement.

1126 Following thawing on ice, the medium was subjected to an ethyl chloroformate
1127 (ECF) derivatization according to our developed procedure with minor modification (Xie,
1128 Qiu et al. 2007). Briefly, a volume of 700 µl of cell culture media was added to 500 µl of
1129 ethanol: pyridine = 4:1, and 100 µl of ECF. After a brief vortex, the samples were
1130 sonicated for 1 min. The derivatized metabolites were extracted with 300 µl of
1131 chloroform and injected 1 µl into the GC-MS system in splitless mode (GC-MS, Agilent,
1132 USA). Helium was used as carrier gas at a consistent flow of 1 ml/min. The injection
1133 temperature was set at 260 °C and metabolites separation was performed on an Agilent
1134 DB-17MS column. The oven program started at 60°C for 0.5 min, and rose to 100°C at
1135 a rate of 20°C/min, and then to 300°C at a rate of 10 °C/min, and then maintained at
1136 300°C for 4.5 min. The data was analyzed with MassHunter Quantitative Analysis
1137 software (Agilent, USA).

1138 The m0 glutamate fragment m/z of 128 contains 3 carbons (carbon 2-4) out the 5
1139 carbons, while fragment m/z of 202 contains 4 carbons (carbon 2-5) from the whole
1140 glutamate molecule. M1 of glutamate at m/z 129 reflects the presence of ¹³C at the 4th

1141 carbon position. This reflects acetyl units originating from glucose->pyruvate entering
1142 through pyruvate dehydrogenase for eventual oxidation (glycolytic flux for energy
1143 generation). M2 of glutamate m/z 130 reflects the presence of ^{13}C at the 2nd and 3rd
1144 carbon position, reflects pyruvate entering the oxaloacetate pool or through pyruvate
1145 carboxylase (Madhu, Boneski et al. 2020, Silagi, Novais et al. 2020). M2 of glutamate at
1146 204 is the overall TCA cycle flux. Those two ^{13}C come from either pyruvate entering
1147 from pyruvate carboxylase or pyruvate dehydrogenase. For lactate, the m/z of M0 to M2
1148 for carbon 2 and 3 position will be 145 to 147 (Madhu, Boneski et al. 2020). A pooled
1149 quality control (QC) sample was injected six times for coefficient of variation (CV)
1150 determination. Metabolites with CVs lower than 30% were included for quantification
1151 while loss with CVs greater than 30% were considered as indeterminant. These data
1152 were obtained using a Sciex 6500+ QTRAP with ACE PFP and MERCK Zic-pHILIC
1153 columns as previously described (Hoshino, Matoba et al. 2012).

1154

1155 ***Acetylcholine enrichment in culture media of SH-SY5Y control and TKO***

1156 ***neuroblastoma cells:***

1157 The cell culture medium samples were thawed on ice and 200 ul was added to 800 ul of
1158 acetonitrile plus 10ul of 10ng/ml acetylcholine_d9 (deuterium 9 as an internal control).
1159 The samples were vortex, centrifuged at 14000rpm for 10 min and supernatants were
1160 transferred into glass vials. The samples were analyzed with ABSciex 6500+ with ACE
1161 PFP column. The MRMs for m0, m1, m2 for acetylcholine and the internal standard are
1162 as following table. Data was processed with multiquanta software (ABSciex). The

enrichment was calculated after subtracted from the background of the non-labeled treatment samples.

MRM table

		Q1 mass (Da)	Q3 mass (Da)
1	Acetylcholine	146.3	87.0
2	Acetylcholine_m1	147.3	88.0
3	Acetylcholine_m2	148.3	89.0
4	(d9) Acetylcholine	155.3	87.0

Cellular acetyl-CoA and acetyl-carnitine measurement in SH-SY5Y control and TKO neuroblastoma cells

The cell pellet samples were added 80% methanol with internal standards (malonyl CoA-¹³C₃, and acetyl-carnitine-d₃) extraction with 3 cycles of frozen (liquid nitrogen)-thaw (sonication). The samples were vortex, centrifuged at 21,000 g for 10 min and the supernatants were transferred into glass vials. The samples were analyzed with ABSciex 6500+ with iHILIC-p column (HILICON). Data was processed with multiquanta software (ABSciex).

Total RNA extraction and quantitative RT-PCR

Cellular and tissue total RNA was extracted using TRI reagent and Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA). First-strand cDNA was synthesized using the SuperScriptTM IV VILOTM cDNA synthesis kit (Thermo Fisher Scientific). TaqMan RT-PCR was performed for measurement of mRNA using the $\Delta\Delta C_t$ method. Gene expression was adjusted by comparison with *Rpl7* expression. The quantitative RT-PCR results were analyzed by RG Manager version 1.2.1 (Applied Biosystems). Primer-probe mixtures for genes of *Ucp1*, *Atp2a1* (*SERCA1*), *Atp2a2* (*SERCA2*), *Sln*

1184 (*sarcolipin*), *Mrln* (*myoregulin*), *Chrna1*, *Chrn1b*, *Chrnd*, *Chrne*, and *Chrng* were listed
1185 as below:

Gene name	Gene name	Taqman Assay ID
Uncoupling protein 1 (mitochondrial, proton carrier)	<i>Ucp1</i>	Mm01244861_m1
ATPase, Ca ⁺⁺ transporting, cardiac muscle, fast twitch 1 (SERCA1)	<i>Atp2a1</i>	Mm01275320_m1
ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2 (SERCA2)	<i>Atp2a2</i>	Mm01201431_m1
Sarcolipin	<i>Sln</i>	Mm00481536_m1
Myoregulin	<i>Mrln</i>	Mm01175781_m1
Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	<i>Chrna1</i>	Mm00431629_m1
Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	<i>Chrn1b</i>	Mm00680412_m1
Cholinergic receptor, nicotinic, delta polypeptide	<i>Chrnd</i>	Mm00445545_m1
Cholinergic receptor, nicotinic, epsilon polypeptide	<i>Chrne</i>	Mm00437411_m1
Cholinergic receptor, nicotinic, gamma polypeptide	<i>Chrng</i>	Mm00437419_m1

1186
1187 Primer-probe mixture for *Rpl7* was customized, and other primer-probe mixtures were
1188 obtained from Thermo Fisher Applied Biosystems.

1189 1190 ***Immunoblotting***

1191 Commercial primary antibodies were purchased for the detection of TIGAR (Cat#
1192 AB10545, Millipore/Sigma; Cat# sc-677290, Santa Cruz), ChAT (Cat# AB144P,
1193 Millipore/Sigma), VACHT (Cat# 24286, ImmunoStar), choline transporter (ChT) (Cat#
1194 PA5-77385, ThermoFisher), c-fos (2250S, Cell Signaling; MA5-15055, Invitrogen),
1195 phosphor-c-Fos^{Ser32} (5348S, Cell Signaling), AMPK α (2532S, Cell Signaling), phospho-
1196 threonine 172 AMPK α (2535S, Cell Signaling), UCP1 (ab209483, abcam), vinculin (sc-
1197 73614), and β -actin (sc-47778, Santa Cruz). Samples were prepared from culture cells
1198 (washed by cold PBS) or tissues by homogenization with a radioimmune precipitation
1199 assay lysis buffer (sc-24948, Santa Cruz Biotechnology) containing HaltTM protease and

1200 phosphatase inhibitor mixture (catalog no. 78442, Thermo Fisher Scientific), 100
1201 μM MG132, and 100 μM ALLN (EMD Millipore, Darmstadt, Germany) using Ceria
1202 stabilized zirconium oxide beads (MidSci, Valley Park, MO). Homogenates were
1203 centrifuged for 15 min at $21,000 \times g$ at 4°C , and supernatants were collected for the
1204 protein assay using BCA method. Protein samples were separated by SurePAGE (Bis-
1205 Tris) precasted gel (GenScript, Piscataway, NJ) and transferred to nitrocellulose
1206 membrane using iBlot Blotting System (Thermo Fisher Scientific). The immunoblot
1207 membrane was blocked with Pierce Protein-Free T20 (TBS) blocking buffer (product no.
1208 37571, Thermo Fisher Scientific) and incubated with the first antibody indicated in the
1209 blocking buffer. Blots were washed in TBS with Tween 20 (TBST) and incubated with
1210 either IRDye 800CW secondary antibody (LI-COR, Lincoln, NE) or horseradish
1211 peroxidase-conjugated secondary antibody in blocking buffer. The Membrane was
1212 washed with TBST and visualized either by the Odyssey Imaging System (LI-COR) or
1213 enhanced chemiluminescence (ECL) (Thermo Fisher Scientific Pierce) method. ImageJ
1214 was used to quantify protein bands on the membrane.

1215

1216 ***Fluorescence imaging of nicotinic acetylcholine receptors clustering***

1217 ***at neuromuscular junctions***

1218 Whole Extensor Digitorum Longus (EDL) muscle were fixed 30 min with 4% w/v
1219 paraformaldehyde, after dissection. After PBS washing 5 min X 3 times , EDL muscle
1220 were incubated with 1:1000 dilution of Alexa Fluor™ 488 conjugate α -Bungarotoxin
1221 (Invitrogen B13422) for 2 hours at room temperature, and mount onto 35 mm glass
1222 bottom dish (MatTek Corporation, Cat# P35G020C.S) with Vectashield Antifade

1223 Mounting Medium with DAPI (Cat# H1200, Vector Laboratories, Inc., Burlingame, CA).

1224 The neuromuscular junction was visualized using a z-series projection on a confocal
1225 microscope (Leica SP8) with 40X objective. Velocity software was used for image
1226 analysis.

1227

1228 ***Immunofluorescence imaging of superior cervical ganglion (SCG)***

1229 The *Chat*^{Cre} and chTKO male mice (forteen-week old) were euthanized and the fresh
1230 SCG tissues were removed (Figure 4-figure supplement1) and embedded in optimal
1231 cutting temperature compound. The frozen tissue cross-sections (10 µm) were blocked
1232 with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 60 min at room
1233 temperature. For vesicular acetylcholine transporter (VChAT) and TIGAR
1234 immunofluorescence staining, The SCG section was incubated with the mouse
1235 monoclonal anti-TIGAR (E-2) Alexa Fluor 488 (sc-166290 AF488, Santa Cruz
1236 Biotechnology) and Goat polyclonal anti-VChAT (Cat# 24286, ImmunoStar, Hudson,
1237 WI) for 2 hours at room temperature. After washing PBS 5 min X 3 times, Alexa Fluor
1238 594 donkey anti-goat IgG (H+L) (1:1000, Cat# A-11058, ThermoFisher Scientific) was
1239 added to the sections for 30 min at room temperature. The slides were mounted cover
1240 slips with Vectashield Antifade Mounting Medium with DAPI (Cat# H1200, Vector
1241 Laboratories, Inc., Burlingame, CA), and visualized using a z-series projection on a
1242 confocal microscope (Leica SP8) with 40X objective.

1243

1244 ***Synchronized measurement of core body temperature, blood pressure, heart***
1245 ***beating rate, and electromyography.***

1246 *Chat^{Cre}* and chTKO mice were anesthetized with isoflurane and implanted with DSI HD-
1247 X11 telemetric transponder probes according to manufacturer's guidelines, with the
1248 blood pressure probe placed in the descending aorta, the EMG leads run
1249 subcutaneously to the neck muscles bilaterally, and the core temperature probe placed
1250 intraperitoneally. Animals were returned to a standard temperature housing room
1251 maintained at 21-22°C on a 12h/12h light dark cycle, lights on at 0700. Mice were
1252 individually housed on cob bedding in standard polycarbonate home cages that were
1253 placed on telemetric DSI platforms to continuously monitor arterial blood pressure, heart
1254 rate, core body temperature, and neck EMG muscle activity from the implanted DSI XD-
1255 11 probes using LabChart 8.1 software for Macintosh. After 1 week recovery from
1256 surgery, animals' home cage bedding was removed and replaced by stainless steel
1257 mesh flooring 1 inch above the base of the cage. Food but not water was then removed
1258 from the home cage, and animals in their home cages on DSI platforms were then
1259 placed in a 10'x10' environmental cold room maintained at 4°C, 35% humidity for 1 hour
1260 from 10:00-11:00 am, and continuous measurements of heart rate, blood pressure, core
1261 temperature and EMG activity continued. At the end of the 1 hour cold challenge, mice
1262 in their individual cages were then returned to the standard temperature housing room,
1263 and food and bedding were replaced.

1264

1265 ***Mitochondrial oxidative phosphorylation (OXPHOS) efficiency and capacity***

1266 Respiration of permeabilized muscle fibers was performed as previously described
1267 (Heden, Ryan et al. 2017, Johnson, Ferrara et al. 2018, Heden, Johnson et al. 2019).
1268 Briefly, a small portion of freshly dissected red gastrocnemius muscle tissue was placed

1269 in 7.23 mM K₂EGTA, 2.77 mM Ca K₂EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM
1270 ATP, 14.3 mM phosphocreatine, 6.56 mM MgCl₂·6H₂O, and 50 mM K-MES (pH 7.1).
1271 Fiber bundles were separated and permeabilized for 30 min at 4°C with saponin (30
1272 µg/ml) and immediately washed in 105 mM K-MES, 30 mM KCl, 10 mM K₂HPO₄, 5 mM
1273 MgCl₂·6H₂O, BSA (0.5 mg/ml), and 1 mM EGTA (pH 7.4) for 15 min. After washing,
1274 high-resolution respiration rates were measured using an OROBOROS Oxygraph-2k.
1275

1276 ***Muscle contraction/relaxation***

1277 The extensor digitorum longus muscle was dissected and tied into the Horizontal Tissue
1278 Bath System from Aurora Scientific Inc. as previously described (Ferrara, Verkerke et
1279 al. 2018). The muscles were stimulated with a 20-V twitch train and stretched until
1280 optimal length for force production was reached. Muscles were then allowed to
1281 equilibrate for 5 min. For force-frequency curves, muscles were stimulated with
1282 frequencies ranging from 10 to 200 Hz (0.1-ms pulse, 330-ms train, and 2 min between
1283 trains). Forces produced by electrically stimulated muscle contractions were recorded in
1284 real time via a force transducer (model 400A, Aurora Scientific Inc.). Specific force was
1285 calculated using cross-sectional area of the muscle tissue (millinewton per square
1286 millimeter), as estimated from the weight and length of the muscle.
1287

1288 ***Gastrocnemius muscle acetylcholine assay***

1289 Fresh gastrocnemius muscle was collected and snap-frozen in liquid nitrogen and the
1290 whole piece of the muscle was ground to powder using a mortar-pestle in liquid
1291 nitrogen. The muscle powder was then homogenized in PBS (pH 7.4) using Ceria

1292 stabilized zirconium oxide beads (MidSci, Valley Park, MO) and the homogenate was
1293 centrifuged for 20 minutes at 3,000 rpm at 4°C degree. The supernatant was collected
1294 and the protein assay was performed using the Pierce BCA Protein Assay kit
1295 (Cat#23225, ThermoFisher Scientific). The supernatants from different samples were
1296 adjusted to the same protein concentration of protein and the same volume of the
1297 supernatants were subjected to acetylcholine assay using QuickDetect™ Acetylcholine
1298 (ACh) (Mouse) ELISA Kit (Cat#E4453-100, Biovision, Milpitas, CA).

1300 ***Body composition and calorimetry***

1301 Male wild type C57BL/6J (WT) and TKO mice (n=4) were internal transferred to Animal
1302 Energy Balance Phenotyping Core of the New York Obesity Research Center at the
1303 Albert Einstein College of Medicine. *In vivo* body composition was determined by
1304 magnetic resonance spectroscopy using an ECHOMRI instrument (Echo Medical
1305 Systems). The mice were then individually housed in metabolic chambers maintained at
1306 21–22°C on a 12-hour light/12 dark cycle with lights on at 7 am and were provided with
1307 nutritionally complete standard powdered diet (NIH 07) and tap water ad libitum for five
1308 consecutive days. Metabolic and behavioral measurements (oxygen consumption, food
1309 intake, locomotor activity) were obtained continuously using a CLAMS (Columbus
1310 Instruments) open-circuit indirect calorimetry system (Li, Wu et al. 2011).

1312 ***Measurement of OCR in SH-SY5Y control and TKO neuroblastoma cells***

1313 Real-time oxygen consumption rate (OCR) was simultaneously assessed in SH-SY5Y
1314 control and TKO neuroblastoma cells using a Seahorse Bioscience XF96 extracellular

flux analyzer (Zhang and Zhang 2019). Three batches of control CTL (10 wells/batch) and TKO (10 wells/batch) cells, respectively, were performed for OCR assay. The cell suspension of 5×10^4 in 50 μ l of DMEM with 10% fetal bovine serum was seeded into a well of the XF 96-well microplates to reach confluence growing an even monolayer. Sixteen-hour later, 200 μ l/well of medium were added. The cells were at 95%-100% confluence and subjected to Seahorse analysis at the Stable Isotope & Metabolomics Core Facility at the Albert Einstein College of Medicine. Briefly, cells were rinsed and incubated with XF base medium (Agilent, 102353–100) supplemented with 25 mM glucose, 2 mM L-glutamine (Thermo Fisher Scientific, 25030081) and 1 mM sodium pyruvate (Thermo Fisher Scientific, 11360070), pH 7.4. After 45 min incubation in a CO₂-free incubator at 37°C, OCR was recorded at baseline, followed by sequential additions of 1 μ M oligomycin (Sigma Aldrich, 75351), 1 μ M FCCP (Sigma Aldrich, C2920), and 1 μ M rotenone (Sigma Aldrich, R8875). The OCR was automatically recorded and calculated by the Seahorse XF24 software, and the data were exported into Prism 8.1 software for graph production and statistical analysis.

Soleus and extensor digitorum longus (EDL) muscles crossed section HE staining

Fresh Soleus and EDL skeletal muscles from four-month old male WT and TKO mice were isolated and were fixed in 10% formalin for 48 hours at 4°C. The muscle samples were then switched to 70% alcohol and subjected to hematoxylin and Eosin (H&E) staining at the Histology and Comparative Pathology Core at the Albert Einstein College of Medicine. Briefly, fixed muscle samples were embedded in cassettes perpendicularly

with paraffin. The block was sectioned 10 μ m-thick and the sections were mounted on slides, H&E stained, and covered with clips. The H&E stained images were captured with a digital microscope camera.

Quantification and statistical analyses

Prism (8.1)-GraphPad software was used for data processing, analyses, and graph productions in the experiments. To analyze the differences of body temperature at different time points among two to four groups of the mice, the tabs of Grouped Analyses/Two-way ANOVA (or mixed model)/Multiple comparison were selected. The subtab "Compare each cell mean with the other cell mean in that row" was chosen to show the "Sidak's multiple comparison test", which summarized the statistical test results. The number of independent experimental replications and the average with standard deviation were provided in the figure legends. Unpaired two-tailed P value *t* tests was used for the statistical tests between two groups. The statistical analyses were made at significance levels as follows: ns, not statistically significant; **p*<0.5; ***p*<0.1; ****P*<0.001, and *****p*<0.0001.

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Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (<i>E. Coli</i>)	Mix & Go Competent Cells-Strain HB10	Zymo Research	T3013	
genetic reagent (Mus. musculus)	<i>Tigar</i> ^{-/-} (TKO) (C57BL/6J)	PMCID: PMC5961042	MMRRC, Cat# 063577-UCD	
genetic reagent (Mus. musculus)	C57BL/6N- <i>Tigar</i> ^{tm1b(EUCOMM)} _{Wtsi} /Wtsi	Welcome Trust Sanger Institute (Hinxton Cambridge, U.K.)	IMSR Cat# EM:09836, RRID:IMSR _EM:09836	
genetic reagent (Mus. musculus)	<i>Myf1</i> ^{Cre} . <i>Myf1</i> ^{tm1(cre)Sjb} /J	The Jackson Laboratory	RRID:IMSR _JAX:024713	

genetic reagent (Mus. musculus)	<i>Chat</i> ^{Cre} ; B6J.129S6- <i>Chat</i> ^{tm2(cre)Lowl} /MwarJ	The Jackson Laboratory	RRID:IMSR JAX:028861	
genetic reagent (Mus. musculus)	<i>Tigar</i> ^{fl/fl} (C57BL/6J)	This study	N/A	Described in "Mice models"
genetic reagent (Mus. musculus)	<i>Tigar</i> ^{fl/fl} <i>Adipoq</i> ^{Cre} (C57BL/6J)	This study	N/A	Described in "Mice models"
genetic reagent (Mus. Musculus)	<i>Tigar</i> ^{fl/fl} <i>Myf1</i> ^{Cre} (C57BL/6J)	This study	N/A	Described in "Mice models"
genetic reagent (Mus. musculus)	<i>Tigar</i> ^{fl/fl} / <i>Chat</i> ^{Cre} (C57BL/6J)	This study	N/A	Described in "Mice models"
genetic reagent (Mus. musculus)	<i>Ucp1</i> ^{-/-} (UKO) (C57BL/6J)	Laboratory of Victor L. Schuster	Albert Einstein College of Medicine	Described in "Mice models"
genetic reagent (Mus. musculus)	<i>Tigar</i> ^{-/-} / <i>Ucp1</i> ^{-/-} (UTKO) (C57BL/6J)	This study	N/A	Described in "Mice models"
cell line (Homo-Sapiens)	SH-SY5Y neuroblastoma	ATCC	ATCC CRL-2266	
cell line (Homo-Sapiens)	SH-SY5Y Control neuroblastoma	This study	N/A	Materials and Methods
cell line (Homo-Sapiens)	SH-SY5Y TIGAR knockout neuroblastoma	This study	N/A	Materials and Methods
antibody	Rabbit monoclonal anti-c-Fos (9F6)	Cell Signaling Technology	Cat# 2250S; RRID: AB_2247211	(1:1000)
antibody	Rabbit anti-AMPK α	Cell Signaling Technology	Cat# 2532S; RRID: AB_330331	(1:1000)

antibody	Rabbit anti-AMPK α phopho (Thr172)	Cell Signaling Technology	Cat# 2535S; RRID: AB_331250	(1:1000)
antibody	Rabbit monoclonal anti-UCP1 (EPR20381)	abcam	Cat# ab209483; RRID: AB_2722676	(1:2000)
antibody	Rabbit polyclonal anti-TIGAR	Millipore/Sigma	Cat# AB10545; RRID: AB_1080718 1	(1:1000)
antibody	Goat polyclonal anti-VChAT (IF)	ImmunoStar	Cat# 24286; RRID: AB_572269	(1:100)
antibody	Rabbit polyclonal anti-VChAT	Thermo Fisher Scientific	Cat# PA5-95404; RRID: AB_2807207	(1:1000)
antibody	Rabbit monoclonal anti-ChAT	Millipore Sigma	Cat# AB144P, RRID:AB_2079751)	(1:1000)
antibody	Rabbit anti-choline transporter	Thermo Fisher Scientific	Cat# PA5-77385; RRID: AB_2736619	(1:1000)
antibody	Mouse monoclonal anti-TIGAR (E-2)	Santa Cruz Biotechnology	Cat# sc-166290; RRID: AB_2066582	(1:250)
antibody	Mouse monoclonal anti-TIGAR (E-2) Alexa Fluor 488 (IF)	Santa Cruz Biotechnology	Cat# sc-166290 AF488	(1:100)
antibody	Alexa Fluor 594 donkey anti-goat IgG (H+L)	Thermo Fisher Scientific	Cat# A-11058; RRID: AB_2534105	(1:1000)

antibody	Mouse monoclonal anti- β -actin (C4)	Santa Cruz Biotechnology	Cat# sc-47778; RRID: AB_626632	(1:300)
antibody	Mouse monoclonal anti-vinculin (7F9)	Santa Cruz Biotechnology	Cat# sc-73614; RRID: AB_1131294	(1:300)
antibody	Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	Cat# sc-2005	(1:5000)
antibody	Goat anti-Rabbit IgG (H+L), HRP	Fisher Scientific	Cat# PI32460	(1:5000)
antibody	Goat anti-rabbit 800CW	Li-Cor	Cat# 926-32211	(1:6000)
antibody	Goat anti-mouse 800CW	Li-Cor	Cat# 926-32210	(1:6000)
antibody	Donkey anti-goat 800CW	Li-Cor	Cat# 926-32214	(1:6000)
recombinant DNA reagent	3 $\times$ sgRNA/Cas9 all-in-one expression clone targeting TIGAR (NM_020375.2)	GeneCopoeia	Cat# HCP-215394-CG04-3	
recombinant DNA reagent	3 $\times$ sgRNA/Cas9 all-in-one expression clone scrambled sgRNA (control)	GeneCopoeia	Cat# CCPCTR01-CG04-B	
sequence-based reagent	<i>Tigar</i> <fl/fl> genotyping forward	This study	IMSR Cat# HAR:7395, RRID:IMSR_ HAR:7395 (9514-9534)	5'-AGGGGG TTGCACC TCTATCT C

sequence-based reagent	<i>Tigar</i> genotyping reverse	This study	IMSR Cat# HAR:7395, RRID:IMSR_ HAR:7395 (16933-16954)	5'-CACACAA GAAGGAA GCTGTTG G
commercial assay or kit	Direct-zol RNA miniprep with 200 ml TRI Reagent	Zymo Research	Cat# R2053	
commercial assay or kit	QuickDetect TM Acetylcholine (ACh) (Mouse) ELISA Kit	BioVision Inc.	Cat# E4453-100	
commercial assay or kit	PowerPrep HP Plasmid Maxiprep Kits with Prefilters	Origene	NP100025	
commercial assay or kit	Ceria Stabilized Zirconium Oxide Beads	MidSci	Cat# GB01	
commercial assay or kit	ProLong [®] Gold Antifade Reagent with DAPI	Cell Signaling Technology	Cat# 8961	
commercial assay or kit	SuperScript IV VILO Master Mix	Thermo Fisher Scientific Invitrogen	Cat# 11756050	
commercial assay or kit	Taqman Universal Master Mix II, no UNG	Thermo Fisher Scientific Invitrogen	Cat# 4440049	
chemical compound, drug	Tubocurarine hydrochloride pentahydrate	Millipore/Sigma	Cat# T2379; CAS: 6989-98-6	
chemical compound, drug	Cyclopiazonic acid from <i>Penicillium cyclopium</i>	Millipore/Sigma	Cat# C1530; CAS: 18172-33-3	

chemical compound, drug	D-Glucose-1,2- ¹³ C ₂	Millipore/Sigma	Cat# 453188; CAS: <u>138079-87-5</u>	
chemical compound, drug	D-Glucose- ¹³ C ₆	Millipore/Sigma	Cat# 389374; CAS: 110187-42-3	
chemical compound, drug	MG-132	Millipore/Sigma	Cat# 474787; CAS# 133407-82-6	
chemical compound, drug	ALLN	Millipore/Sigma	CAT# 208719-25MG; CAS:110044-82-1	
chemical compound, drug	RIPA lysis buffer	Santa Cruz Biotechnology	Cat# sc-24948	
chemical compound, drug	Halt Protease and Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific	Cat# 78442	
chemical compound, drug	Pierce Protein-Free T20 (TBS) Blocking Buffer	Thermo Fisher Scientific	Cat# 37571	
chemical compound, drug	SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific	Cat# 34080	
chemical compound, drug	SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific	Cat# 34096	

chemical compound, drug	4% paraformaldehyde solution	Electron Microscopy Sciences	Cat# RT157-4	
chemical compound, drug	Vectashield Antifade Mounting Medium with DAPI	Vector Laboratories, Inc.	Cat# H1200	
chemical compound, drug	DMEM medium without glucose, glutamine, and sodium pyruvate	Biological Industries USA	Cat# 01-0571-1A	
chemical compound, drug	DMEM medium no glucose, glutamine, and sodium pyruvate	Biological Industries USA	Cat# 01-0571-1A	
software, algorithm	Odyssey Application Software 2.0	Li-Cor	N/A	
software, algorithm	GraphPad Prism 8	GraphPad Software	http://www.graphpad.com/scientific-software/prism/	
software, algorithm	RQ Manager 1.2.1.	Thermo Fisher Scientific Applied Biosystems	N/A	
software, algorithm	LD50 Calculator	AAT Bioquest	https://www.aatbio.com/tools/ld50-calculator	
software, algorithm	Velocity software	Velocity Software, Inc.	https://www.velocitysoftware.com/	

software, algorithm	MassHunter	Agilent Technologies	Version: B08.00	
software, algorithm	MultiQuant	ABSciex	Version: 3.0.2	
software, algorithm	LabChart 8.1 for Macintosh	ADInstruments	https://www.adinstruments.com/products/labchart	
other	Thermalert Model TH-8 Temperature Monitor	Physitemp Instruments LLC.	Thermalert Model TH-8	
other	HD-X11 telemetric transponder probe	DSI™, Harvard Bioscience, Inc.	https://www.datasci.com/products/implantable-telemetry/small-animal-telemetry	
other	DSI Implantable Telemetry	DSI™, Harvard Bioscience, Inc.	https://www.datasci.com/products/implantable-telemetry/small-animal-telemetry	
other	GC-MS instrument	Agilent Technologies	Model: 5975C	
other	DB-5MS column	Agilent Technologies	Cat# 122-5532G	
other	LC-MS/MS instrument	ABSciex	Model: 6500+	
other	ZIC-pHILIC column	Merck	Cat# 1.50460.0001

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 1-figure supplement 1

Figure 1-figure supplement 2

Figure 2-figure supplement

Figure 3-figure supplement 1

Figure 3-figure supplement 2

Mouse Superior Cervical Ganglion (SCG) Dissection

Figure 4-figure supplement

A [1,2-¹³C] glucose

B Glutamate m/z 128 C2-C4

Figure 6-figure supplement