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The thyroid hormone activating enzyme, type 2 deiodinase, induces myogenic differentiation by regulating mitochondrial metabolism and reducing oxidative stress.

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1. Summary

1.1 *The thyroid hormone activating enzyme, type 2 deiodinase, induces myogenic differentiation by regulating mitochondrial metabolism and reducing oxidative stress.*

Thyroid hormone (TH) is a key metabolic regulator that acts by coordinating short- and long-term energy needs. Accordingly, significant metabolic changes are observed depending on thyroid status. Although it is established that hyperthyroidism augments basal energy consumption, thus resulting in an enhanced metabolic state, the net effects on cellular respiration and generation of reactive oxygen species (ROS) remain unclear.

Reactive oxygen species (ROS) are bioproducts of various ubiquitous cellular processes, and were long considered deleterious moieties that generate oxidative stress, disease and aging [1]. However, it is now recognized that ROS is a molecular signal that regulates physiological cellular processes [2]. Skeletal muscle is one of the most active ROS generating tissues, which is in line with its intense metabolic action. In physiologic conditions, mitochondrial ROS production is enhanced during muscle contraction, and superoxide generation can increase up to ∼100-fold during aerobic contractions [3]. However, excess ROS levels promote mitochondria fragmentation and dysfunction, thereby altering the physiological turnover of muscle fibers [3,4]. During muscle repair, ROS are physiologically active signaling molecules that trigger the cascade of events that enable correct muscle repair and activation of muscle stem cells ("satellite cells") [5]. However, prolonged exposure to elevated ROS levels can exacerbate muscle injury by inducing oxidative toxic damage to regenerating myofibers [5]. Indeed, as in many other tissues, excess of ROS in skeletal muscle exacerbates muscular dystrophy [1], and other muscle diseases [4-7]. The relative prevalence of the aforementioned "positive" or "negative" effects of ROS depends on complex intracellular mechanisms that maintain the ROS threshold within physiological concentrations.

Thyroid hormone has a major impact on whole-body energy metabolism and on tissue-specific energy balance [8]. Thyroid hormone modulates all metabolic signaling pathways and consequently, altered TH concentrations in humans are associated with profound changes in energy status [8]. The extent of intramuscular TH action is determined both by the systemic levels of TH and by local regulation that modulates the nuclear availability of the hormone [9]. Indeed, although the hypothalamic-pituitary-thyroid axis efficiently regulates TH homeostasis, thus maintaining circulating TH levels in a constant steady-state, its intracellular concentration can rapidly be attenuated or increased in- dependent of serum TH blood levels by the enzymatic control of the selenodeiodinases (D1, D2 and D3) that catalyze TH activation and catabolism [10]. The actions of the three deiodinases, together with the uptake of T3 and T4 into the cell by

specific transporters, constitute a mechanism of pre-receptor control of TH action at cellular level regardless of the constant serum T3 levels [10]. The metabolic relevance of the deiodinases is exemplified by the effects of D2 on thermo-regulation and the consequent energy expenditure in brown adipose tissue (BAT) [11,12]. Cold exposure causes an increase in the sympathetic activity of BAT, which, in turn, increases lipolysis, mitochondrial uncoupling and D2 activity [13]. In the absence of D2-generated T3, there is a decrease in *Ucp-1* gene expression and impaired adaptive thermogenesis [11]. In skeletal muscle, D2 expression is barely detectable under basal conditions, however its expression is markedly increased during muscle regeneration [14]. We previously demonstrated that D2 is functionally relevant in muscle stem cells and that loss of D2 severely impairs muscle repair after injury [14,15]. These findings raise the intriguing possibility that, similar to BAT, D2 is a "master regulator" of overall energy metabolism in skeletal muscle during the regeneration process. To determine the metabolic relevance of intracellular D2-mediated TH changes in muscle cells, we generated a muscle cell line (pTRE-D2) in which D2 can be reversibly induced by doxycycline. We found that induction of D2 induces a shift toward glycolytic metabolism. Importantly, D2 induction decreased basal levels of ROS and induced a parallel up-regulation of the detoxifying gene *Sod2*. The D2-mediated TH activation led to detoxifying effects that were essential for differentiation. Taken together, these data reveal a novel metabolic role of TH in which this hormone regulates redox balance by inducing the transcription of Sod2 and by inducing a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis.

2. Introduction

2.1 *Thyroid hormone action and deiodinases.*

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3), are pleiotropic agents that regulate the metabolism and homeostasis of many tissues in vertebrates [16]. In the bloodstream, the steady-state level of THs concentration is regulated by the hypothalamus-pituitary-thyroid axis, that determines the set point of TH production. Hypothalamic thyrotropin-releasing hormone (TRH) stimulates the synthesis and secretion of pituitary thyrotropin (thyroid-stimulating hormone, TSH), which acts at the thyroid to stimulate all steps of THs biosynthesis and secretion. In turn, the THs control the secretion of TRH and TSH by negative feedback to maintain physiological levels of the main hormones of the HPT axis.

Although T4 is produced by the thyroid gland, the major quote of T3 originates from the extra-thyroidal metabolism of T4 into T3. In fact, while around 20% of T3 is directly secreted by the thyroid gland, the residual percentage of T3 is derived from outer ring deiodination of T4 in peripheral tissues, through a potent mechanism of pre-receptor control of TH action at cellular level. This reaction, called 5′-deiodination, is catalyzed by two selenoproteins, the type 1 or type 2 iodothyronine deiodinase (D1 or D2), which act as TH-activating enzymes.

T4 can also be metabolized by conversion to reverse T3 (rT3), which is an inactive metabolite due to its nearly complete inability to bind the thyroid hormone nuclear receptors. rT3 is generated by the removal of the inner ring [16] iodine from T4, through an inactivation step catalyzed mostly by the type 3 iodothyronine deiodinase (D3), which acts as TH-inactivating enzyme.

To exert its functions at cellular level, the TH access to the intracellular compartment is mediated by four different families of TH-transporting proteins, that have been shown to be involved in the traffic of iodothyronines across the cell membrane, with different patterns of tissue expression [17,18]; they are monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), transporters of organic anions (OATPs), and L-amino acid transporters (LATS) [19,20]. MCT8 is probably the most relevant transporter that transports both T4 and T3 and is expressed in liver, muscle, kidney and in many brain areas [21,22]. MCT10, instead, preferentially transports T3 and is expressed in kidney, liver and muscle [18].

Once transported inside cells, the THs levels can be regulated by the activity of deiodinases, that are responsible, in part, for the regulation of intracellular TH action because they can activate (types 1 and 2) or inactivate (types 1 and 3) THs depending on the type of deiodinase expressed and how active the enzyme is in each tissue.

The selenodeiodinases are membrane-anchored proteins of 29-33 kDa that share substantial sequence homology, catalytic properties and contain the selenocysteine (Sec) aminoacid as the key residue within their catalytic center. All the three deiodinases are homodimers whose dimerization is required for full catalytic activity [23, 24]. The unique feature of selenoproteins in general, and deiodinases in particular, lies in the recoding of the UGA codon from a stop codon to Sec-insertion codon by the presence of the SECIS element in the 3′-UTR of the respective mRNAs. Iodinated contrast agents such as iopanoic acid inhibit all three deiodinases, while propylthiouracil is a relatively specific inhibitor for D1.

Type 1 iodothyronine deiodinase (D1) is localized in the plasma membrane and is present in almost all human cells, but the highest concentrations were found in the thyroid, liver and kidneys [25]. D1 is able to exert both activating and inactivating functions.

D2 is considered the main T4-activating enzyme, given its high substrate affinity. D2 is a classical type-1 membrane protein, approximately of 31kDa, residing on the endoplasmic reticulum (ER) membrane, with a half-life of ~ 45 minutes [24]. Its relatively short half-life is due to ubiquitination and proteasome uptake, a feature that is accelerated by D2 interaction with its natural substrate, T4 [26]. Responsible for the specific deiodination of T4 into T3, D2 enzyme is considerably more efficient than D1 in the removal of an outer ring iodine atom from the pro-hormone T4 to generate the physiologically active product T3 [27,28]. D2 is expressed in many brain areas and is considered to play a major role in local T3 production in the brain. D2 is also expressed in pituitary, brown adipose tissue, placenta and in skeletal muscle [29]. In contrast to D1, D2 is involved in outer-ring deiodination exclusively. The preferred substrate for D2 is T4 and, although to a lesser extent, rT3. D2 is regulated by thyroid hormones both pre- and post-transcriptionally as T3 downregulates D2 mRNA expression [30], while T4 as well as rT3 (the substrates of D2) increase D2 ubiquitination and subsequently proteasomal degradation, resulting in decreased D2 activity. D2 is required for normal mouse skeletal muscle differentiation of muscle stem cells (satellite cells) and regeneration [31].

Type 3 iodothyronine deiodinase (D3) is localized in the plasma membrane and is highly expressed in pregnant uterus, placenta, fetal and neonatal tissues, playing an important role during embryonic development protecting the fetus from excessive exposure to active thyroid hormone. It is also expressed in the brain and skin, but in adult healthy tissues expression levels are very low [32]. Recent studies have revealed reactivation of D3 expression in specific pathophysiological contexts correlated with hyperproliferation conditions as cancer.

Inside the cells, the effects of THs are mediated mostly by genetic alterations mainly through the T3 binding to thyroid hormone receptors (THRs, TRα and TRβ, which are encoded by

Thra1, *Thrb1*, and *Thrb2* genes, respectively) [33,34], a family of ligand-dependent transcription factors that modify gene expression by enhancing or inhibiting the expression of target genes by binding to specific DNA sequences, known as TH response elements (TREs). T3 is the preferred ligand of THRs therefore, since the serum concentration of T4 is 100-fold higher than that of T3, this undergoes extra- thyroidal conversion to T3. The importance of T3 generation is that the affinity of THRs for T4 is 10-fold lower than that for T3, and thus the conversion activates T4.

2.2 *Metabolic role of thyroid hormone.*

Thyroid hormones have long been known to regulate energy metabolism [35] and different pathways that are involved in the metabolism of carbohydrates, lipids and proteins in several target tissues. Indeed, TH actions in the liver, white adipose tissue, skeletal muscle, and pancreas influence plasma glucose levels, insulin sensitivity, and carbohydrate metabolism. Patients with TH dysfunction often have symptoms of metabolic dysregulation, including fatigue and weight changes [36].

Pathological excess of THs in humans raises the basal metabolic rate (BMR) while TH deficiency is accompanied by a decreased BMR [36]. BMR is the primary source of energy expenditure in humans, and reductions in BMR can result in obesity and weight gain [37]. BMR correlates with lean body mass [38] and thyroid hormone levels [39]. Cold and heat intolerance are hallmark clinical features of patients with hypothyroidism and hyperthyroidism, respectively. In addition, resting energy expenditure (REE) is remarkably sensitive to TH, especially in athyreotic individuals [40]. TH stimulates BMR by increasing ATP production for metabolic processes and by generating and maintaining ion gradients [41].

In healthy individuals, variations in serum TSH are associated with body weight and body weight change in both men and women [42]. In fact, TSH and TRH exert thyroidal and non-thyroidal effects and thus integrate signals from nutritional status and the adrenergic nervous system with a fine regulation of THs production [43].

A peculiar feature of TH-dependent metabolic regulation is the acceleration of the rates of anabolic and catabolic reactions [44]. For instance, TH increases fat mobilization thereby leading to increased concentrations of fatty acids in plasma as well as to enhanced oxidation of fatty acids. THs stimulate insulin-dependent glucose uptake, and both gluconeogenesis and glycogenolysis. Particularly, inactivation of *Dio2* gene is associated with insulin resistance and diet-induced obesity [45]. The correlation of a Thr92Ala polymorphism in the *Dio2* gene with

altered glycemic control, obesity and type 2 diabetes mellitus (T2DM) [46], as well as the association of genetic variants of the *Dio1* gene with insulin resistance [47], reinforces the clinical relevance of the peripheral T4-to-T3 conversion in metabolic control.

TH is also a key regulator of mitochondria respiration and biogenesis, stimulate ion cycling by altering membrane permeability, the expression of ion pumps and the characteristics of these pumps [41,48]. Therefore, the action of THs culminates in promoting futile cycles that contribute significantly to the increase oxygen consumption seen in thyrotoxicosis ("hyperthyroidism").

Notably, hyperthyroidism induces a hyper-metabolic state characterized by increased resting energy expenditure, reduced cholesterol levels, increased lipolysis and gluconeogenesis followed by weight loss, whereas hypothyroidism induces a hypo-metabolic state characterized by reduced energy expenditure, increased cholesterol levels, reduced lipolysis and gluconeogenesis followed by weight gain. TH critically influences BAT activity [49]. During cold exposure, the sympathetic nervous system induces D2 expression in brown adipocytes, thereby promoting local T4-to-T3 conversion, and activation of the transcription of target genes involved in the thermogenic program [50]. Loss of function of D2 reduces the level of UCP-1, which is normally up-regulated at RNA level by TH. D2 is thus considered a marker of BAT activity [35].

Thus, the pleiotropic effects of TH can fluctuate among tissues and strictly depend on the cell-autonomous action of the deiodinases.

2.3 *Effects of intracellular regulation of thyroid hormone in skeletal muscle.*

Although each cell of the body is virtually a TH target, the TH signal is differentially integrated in each tissue depending on the cell-autonomous machinery. Therefore, the action of TH on whole body metabolism is best evaluated by examining the specific contribution of TH and its modulating enzymes to energy metabolism in the context of each target tissue. The relative roles of most components of the TH signaling pathways have been assessed in mouse models of inducible, tissue-specific activation or inactivation of deiodinases, receptors and transporters [35]. These studies revealed how different TH-induced processes contribute to regulating metabolic homeostasis in humans.

Skeletal muscle represents 40-50% of the total body mass in humans and is crucial for metabolism, heat generation and maintenance of posture. TH influences skeletal muscle contraction, regeneration and metabolism [10]. All components of the TH signaling process, from

TR to TH transporters and D2 and D3, are expressed in the skeletal muscle of rodents and humans [31].

During skeletal muscle development, D2 is up-regulated, particularly during the first postnatal days, and decreases at day 30, although its activity returns to high levels during differentiation of muscle stem cells [51-53]. During embryo development, adequate levels of thyroid hormones are essential for the formation and differentiation of muscle progenitor cells from the endoderm and are subsequently also required for the shift from producing neonatal myosin isoforms to producing adult myosin isoforms [54]. In adult life, T3 regulates muscle homeostasis by ensuring the correct expression of structural and metabolic muscle proteins [55,10]. D2 and D3 are expressed at a very low level in healthy adult muscle [10]. Consequently, the role of the deiodinases in this tissue was largely neglected until we found that D2 and D3 are abundantly expressed in muscle stem cells and have a major role in muscle regeneration and in the formation and maturation of new fibers [56,57]. Muscle stem cells (also known as 'satellite cells') play a key role in muscle regeneration [58]. They are normally dormant in adults and are located beneath the basal lamina in the quiescent phase of the cell cycle [58]. Satellite cells are activated when muscle is damaged, at which point they enter the cell cycle and activate a myogenic programme that is orchestrated by the sequential expression of a hierarchy of muscle-specific transcription factors [59].

Accordingly, coordinated D2-D3 expression is required to finetune intracellular TH availability during muscle stem cell differentiation, and in vivo, during muscle regeneration (31). While D2 is essential for a correct T3 surge and the subsequent differentiation of muscle stem cells, D3 fosters muscle stem cell proliferation by lowering TH availability during the early phases of the myogenic program [31]. D2-mediated TH in skeletal muscle influences also muscle fibers. High TH levels induce a shift from type I fibers (slow) to type II fibers (fast), which results in up-regulation of sarcoendoplasmic reticulum Ca2+ATPase, of glucose transporter 4 (GLUT4) and of uncoupling protein 3 (UCP3) thereby producing heat and increasing energy expenditure [55]. Interestingly, D2-dependent T3 activation influences insulin response in skeletal muscle [35]. Moreover, D2 is a target of FOXO3, which is a protein involved in myocyte fusion and metabolism as well as in atrophy and autophagy [31], and it is up-regulated in response to such metabolic signals as bile acids [60] and during exercise under β-adrenergic stimulus in order to amplify TH signaling and regulate PGC-1α expression [61,62]. These studies highlight the crucial role of the intracellular TH coordination by the deiodinases in muscle physiology.

2.4 *Reactive oxygen species in skeletal muscle.*

Reactive oxygen species (ROS) are by-products of numerous enzymatic reactions in various cell compartments, including the cytoplasm, cell membrane, endoplasmic reticulum, mitochondria, and peroxisome, as part of basal metabolic function. They are also generated specifically by enzymes such as NOXes (nicotinamide adenine dinucleotide phosphate [NADPH] oxidases) and serve a signaling function in the cell [63]. ROS have been long considered as merely deleterious species to skeletal muscle tissue. Indeed, since the 1980s abundant evidence clearly indicated that ROS play a pathogenic role in muscular dystrophies [64] and have then been identified as con-causal factors in various muscular diseases [65–67,7]. Thereafter, accumulating evidence indicated that ROS, at least within concentrations emerging from physiological conditions, could also play a positive role in physiologically relevant processes in muscle cells. As an example, inflammation-derived ROS play a contradictory role in muscle repair [65]: in combination with other actors such as growth factors and chemokines, ROS participate in a cascade of events leading to muscle regeneration and repair; on the contrary, the local persistence of ROS sustained by infiltrated neutrophils may cause further injury by oxidatively damaging differentiating myoblasts and myotubes thus delaying the restitutio ad integrum. Similarly, ROS generated during exercise promote mitochondria-genesis (a key factor in muscle differentiation) via PGC-1α activated signal transduction pathway [66] but, at higher and persistent levels, they might target mitochondria and mitochondrial DNA (mtDNA) turning into blockers of myogenic differentiation [67,7]. The prevalence of each of the two actions, that is, beneficial or detrimental, depends on the coincidence of various intrinsic and extrinsic factors among which the most prominent is the level and the duration of ROS targeting muscle cells; other variables are the source or the site of ROS generation, the antioxidant status of target cells, and their DNA repair capacity.

Mitochondria are commonly considered as the predominant source of ROS in skeletal muscle cells [68, 69]. Mitochondria are central regulators of aerobic energy production. Proper respiratory chain function requires a delicate balance between pro-oxidant and antioxidant systems. Importantly, mitochondrial respiration relies on electron transfer and a proton gradient to drive ATP production. ROS are a natural by-product of this process; however, inflammatory and metabolic diseases are associated with perturbed mitochondrial ROS production [70,71]. Mitochondrial ROS are generated by numerous mechanisms such as in the course of intense contractile activity [72] or in response to cytokines such as tumor necrosis factor-α (TNF-α) [73]. Recent studies indicated that complexes I and III of the electron transport chain are the main

sites of mitochondrial superoxide production [74,75]. During exercise, the increased ROS generation in the course of contractile activity is due to the high oxygen consumption that takes place during increased mitochondrial activity. Indeed, superoxide generation in skeletal muscle increases to about a 50- or 100-fold during aerobic contractions [76, 77].

2.5 *Reactive oxygen species and myogenic differentiation.*

Many recent studies indicate that ROS are important messengers in signaling pathways relevant to skeletal muscle cells homeostasis and adaptation. Increasing evidence indicates that ROS are capable of affecting mostly reducing the efficiency of myogenic differentiation. The integrity/alteration of myogenic differentiation is central to many physiological and pathological processes. Successful differentiation of satellite-derived myoblasts into functioning and integrated myotubes is a fundamental prerequisite for muscle regeneration, a repair process which is of primary importance in maintaining muscle function [78]. Notably, oxidative stress is known to play a deleterious role in a variety of multifactorial muscular pathologies characterized by proliferation/differentiation imbalance such as Duchenne dystrophy [79], myotonic dystrophy [80], sarcopenia [81], and cachexia [82]. Is well known that ROS elicit a wide spectrum of cellular responses, depending on their intracellular level [81]. A low dose of ROS controls normal cellular signaling pathways while an intermediate dose results in either temporary or permanent growth arrest [84]. Obviously, a high dose of ROS causes cell death via either apoptotic or necrotic mechanisms [85].

3. Results

3.1 *D2 up-regulation induces a metabolic shift toward glycolysis and reduces oxidative phosphorylation.*

To determine the metabolic effects of D2 in muscle cells and its contribution to the rate of cellular respiration and ROS production, we generated an inducible C2C12 cell line (pTRE-D2) in which the D2 gene can be turned on by tetracycline administration. Doxycycline treatment (2 μg/ml) (Fig. 1A) induced up-regulation of D2 mRNA (Fig. 1B), protein (Fig. 1B and C) and enzymatic activity (Fig. 1D). The D2 surge determined increased TH action as indicated by activation of the TRE-LUC-response plasmid (TRE3TK-Luc) transiently transfected in the pTRE-D2 cells (Fig. 1E). This effect was strongly reduced when cells were cultured in the absence of TH (charcoal stripped medium, CH, Fig. 1F), thus confirming that the increased promoter activity is effectively due to the D2-mediated T4-to-T3 conversion. Accordingly, the TH-target genes *MyoD*, *Myogenin* and *FoxO3a* were up regulated in C2C12 cells grown with normal TH-containing FBS after D2 induction, but not in C2C12 cells cultured in the absence of TH (Fig. 1G). Consistent with the finding that TH favors a shift from type I (oxidative) fibers to type II (glycolytic) fibers [10], induction of D2 promoted glycolytic metabolism as demonstrated by a reduced oxygen consumption rate (OCR), and an increased acidification rate (ECAR) (Fig. 1A and S1A and B). Coupling efficiency and spare capacity were reduced, which indicates that an increase in D2, and the consequent production of active TH, down-modulated the overall contribution of OXPHOS to the cellular energy demand (Fig. S1C and D). Maximal respiration was accordingly reduced, thus confirming the reduced ability to address the energy demands in conditions of increased D2- mediated TH up-regulation (Fig. 2B). Furthermore, the mitochondrial uncoupling protein UCP3 was strongly upregulated, thus supporting the reduced respiration rate in D2-overexpressing muscle cells (Fig. 2C). D2 did not affect total ATP production as demonstrated by both Seahorse assay and quantitation of ATP levels (Fig. 2D). However, the differential contribution to the ATP production rate of OXPHOS, glycolysis and other metabolic pathways, differed between control cells and D2-overexpressing cells. Treatment with 2-deoxyglucose, an inhibitor of ATP production by glycolysis had a more potent effect on ATP production in D2-expressing cells, while conversely, treatment with oligomycin which inhibits ATP production by OXPHOS did not decrease ATP production in D2-expressing cells (Fig. 2D). Overall, these data indicate that D2 induction reduced the contribution of OXPHOS to ATP production and increased the contribution of glycolysis (Fig. 2E). Analysis of myosin

heavy chain expression showed that D2 induction drastically reduces the expression of the slow, oxidative fibers (type I fibers, MHC I) and increases the expression of the fast, glycolytic fibers (type II, MHC IIa and IIb) (Fig. 2F). These results confirmed that the metabolic shift induced by D2 up-regulation is associated with changes in MHC expression towards faster, more glycolytic isoforms. Taken together, the data reported above demonstrate that D2 reduces the rate of OXPHOS in muscle C2C12 cells.

3.2 *Basal ROS production is reduced by D2 induction.*

We evaluated if the observed metabolic shift from OXPHOS to glycolysis induced by D2 affects ROS homeostasis. We found that the intracellular basal ROS level was significantly reduced by D2 induction (Fig. 3A). Moreover, the rate of ROS production induced by doxorubicin was similarly reduced by D2 (Fig. 3B). These findings suggest that D2 and TH activation protects against basal and doxorubicin-induced oxidative stress. Using the mitochondrial indicator, mitoSOX, we also evaluated the effects of D2 on mitochondrial ROS. Unsurprisingly, mitochondrial ROS levels were reduced by D2 induction (Fig. 3C). To determine whether the effects exerted by D2 on endogenous ROS production were due to enhanced antioxidant potential (scavenger pathway) or to a reduced ROS production rate (oxidative stress pathway), we measured the expression of a panel of antioxidant genes and OXPHOS genes. While OXPHOS gene expression was unchanged by D2 induction, the expression of three antioxidant genes (*Sod2*, *Gpx1* and *Gclc*) was up- regulated by D2 (Fig. 3D and E).

3.3 *D2-mediated TH activation potentiates antioxidant fluxes by up-regulating SOD2.*

The analysis of ChIP-Seq data in T3-treated C2C12 cells versus un-treated C2C12 cells demonstrated that, among the three up-regulated genes in Fig. 3D, superoxide-dismutase SOD2 is a putative direct target of T3 (Fig. 4A). Chromatin immunoprecipitation assay confirmed that the thyroid receptor TRα physically binds to the *Sod2* promoter region (Fig. 4B). Moreover, TH treatment and D2 induction caused up-regulation of the SOD2 protein in muscle cells (Fig. 4C-E). Accordingly, in vivo SOD2 levels were higher in hyperthyroid gastrocnemius (GC) muscles and lower in hypothyroid GC muscles than in control muscles, (Fig. 4F and G). SOD2 expression was also higher and mitochondrial ROS expression lower in muscle fibers isolated from hyperthyroid muscles (Fig. 4H, I and S2). These data reveal that the scavenger protein SOD2 is a novel TH-target gene, which, in turn, suggests that TH plays a specific role in the

regulation of oxidative stress. Given that FoxO proteins play a role in inducing muscle differentiation (86) thereby repressing oxidative stress and regulating SOD2 expression (87), and also given the reciprocal cross talk between TH- FoxO3 and D2 (14), we asked whether FoxO3 is involved in the mechanism by which TH regulates ROS production in muscle cells. To this aim, we transfected C2C12 cells with FoxO3 dominant negative (FoxO DN) or empty vector (CMV-Flag) and treated them with TH. We also transfected C2C12 cells with a FoxO3 shRNA or control shRNA (as described in [14]) and treated them with TH. Although, as expected, basal levels of SOD2 and GCLC were reduced by FoxO3 inhibition, TH treatment induced the expression of these antioxidant genes to a similar extent regardless of FoxO3 expression, which suggests that the expression of TH-induced detoxifying genes is independent of FoxO3 (Figure S3A and B). Accordingly, intracellular ROS levels were similarly down-regulated by TH in the presence and absence of FoxO3 (Figure S3C). These data indicate that TH and FoxO3 exert cooperative, but not synergistic effects, in protecting muscle cells from oxidative stress (Figure S3D).

3.4 *The antioxidant action of TH augments myogenic differentiation.*

The cell-signaling role of ROS includes the ability to inhibit myo- genic differentiation [88, 81,89], whereas antioxidant genes are positive regulators of myogenic differentiation [90]. Since TH and its activation by D2 are critical mediators of myogenic differentiation, we evaluated whether reduction of ROS levels is part of the TH-dependent cascade that triggers myogenic differentiation. To this aim, we first analyzed the effects of D2 induction on muscle cell proliferation and differentiation. As expected, D2 induction led to reduced proliferation and enhanced differentiation. Indeed, cyclin D1 and EdU positive cells were down regulated (Fig. 5A-C), while the differentiation markers Myogenin, Desmin and MHC were up regulated by D2 (Fig. 5D and E). Notably, the differentiation observed in doxycycline-treated cells reflected the levels of D2 induction in a dose- and time-dependent manner. Indeed, the expression of Myogenin was up regulated by doxycycline-induced D2 up to 8 μg, 24 h after doxycycline treatment (Fig. 5F-H). ROS levels paralleled D2 expression, being down regulated by D2 induction in a dose-dependent manner (Fig. S4). Moreover, time-course induction of D2 showed that the SOD2 and ROS levels were inversely regulated by D2 in a time-dependent manner in myoblasts exposed to doxycycline for 48h, whereas prolonged exposure of myoblasts to high D2 levels reversed these effects which suggests that prolonged activation of D2 might have deleterious effects on oxidative stress (Figure S5). Finally, to assess if the antioxidant

effects of D2 and TH are mediated by the superoxide dismutase SOD2, we down-regulated SOD2 in C2C12 cells using the siRNA method. Two siRNAs (shSOD.1 and shSOD.2) were selected and validated for effective silencing of SOD2 by greater than 50% (Fig. S6). Importantly, *Sod2* silencing reduced the ability of D2 to trigger cellular differentiation, as indicated by reduced levels of myogenic differentiation markers (Fig. 6A and B). Furthermore, SOD2 inhibition potently reduced the antioxidant effects of D2 on ROS and mitochondrial ROS (Fig. 6C), which confirms that SOD2 is the effector of TH-dependent ROS scavenger activity. These results indicate that the intracellular activation of TH by D2 is a metabolic modulator of muscle cells, and that activation of D2 in proliferating muscle cells induces cell differentiation by regulating the ROS balance, thereby linking the pro-differentiative action of TH to its metabolic action (Fig. 6D).

4. Discussion

Despite the large body of data on the multiple roles of TH signaling in muscle metabolism, several fundamental questions remain unanswered: (i) Since TH increases the cellular metabolic rate, how do TH-dependent metabolic changes affect oxidative stress? (ii) What is the tissue-specific role played by TH modulators and deiodinases in cellular respiration and oxidative stress? (iii) What are the cross-implications between the metabolic and pro-differentiative roles of TH in muscle? In this study we demonstrate that TH and its activating enzyme, D2, attenuate intracellular ROS production in C2C12 muscle cells. Our study shows that TH alters the intracellular metabolism of muscle cells by reducing the extent of OCR and inducing ECAR. This is in line with the ability of TH to induce a shift from slow-to-fast muscle fibers (10). Significantly, the same shift was associated with increased uncoupling power and UCP3 expression, and a reduced rate of OXPHOS, thus suggesting that the intracellular activation of TH might reduce oxidative stress. This concept is further validated by the finding that the endogenous production of ROS was markedly reduced in muscle cells overexpressing D2. This role was unexpected given the pro-apoptotic action of TH in muscle stem cells [31]. Similarly, since TH is commonly regarded as a hyper-metabolic agent, one might expect that it should be associated with enhanced cellular respiration and increased oxidative damage, which was not confirmed by our study. Our finding that D2-mediated TH activation reduces oxidative stress in muscle cells is in line with reports that TH and its activation by D2 is critical for the maintenance of cellular homeostasis during stress responses as occurs in idiopathic pulmonary fibrosis [91]. In contrast, a study performed with hepatic HepG2 cells showed that an increased T3 signal leads to increased mitochondrial activity, OXPHOS rate and fission [92]. These discrepancies highlight the complicate inter-connections between TH and cellular metabolism, and can be partially explained by speculating that TH exerts dose-dependent control over ROS dynamics, thereby reducing ROS production under physiological circumstances, while increasing ROS production and apoptosis in conditions of thyrotoxicosis. Compatible with this speculation, we found that, while D2-TetON cells displayed ROS reduction, increased levels of proteins involved in ROS-detoxification, and greater susceptibility to the myogenic process, prolonged exposure to D2 or excessive levels of D2 induction produced the opposite effects. Mitochondria are the predominant site of ROS production in skeletal muscle [69]. Contextually, TH is a central regulator of mitochondrial biogenesis (via the peroxisome proliferator-activated receptor gamma, coactivator 1α) [93] and of dynamics (via DRP-1) [92]. Moreover, the existence of a mitochondrial-specific TH receptor (p43) emphasizes the important role of TH as a mitochondrial regulator [94]. These examples illustrate that TH affects cellular respiration in mitochondria through multiple genomic and non-genomic pathways [95]. Our finding that

mitochondrial ROS production was reduced by TH confirms the intimate relationship between TH and mitochondria, and is in accordance with the concept that TH enhances muscle cell differentiation. We also found that SOD2, the primary mitochondrial oxidative scavenger, is a novel TH-target gene. Our data show that the genomic action of TH via TRα increased SOD2 transcription and protein synthesis in vitro and in vivo and importantly, *Sod2* silencing interfered with TH-dependent decrease in ROS, which indicates that SOD2 is a TH-downstream mediator of ROS dynamics. To explore the connection between TH and ROS in protein regulation, we evaluated whether the detoxifying action of TH requires FoxO3, which is an upstream regulator of D2 expression [14], and, contextually, a TH-target gene [31]. Surprisingly, we found that, although both FoxO3 and D2 reduced intracellular ROS and that both induced SOD2 expression, the effects of D2 on ROS generation remained unchanged in the absence of FoxO3. These data indicate that the TH signal and FoxO3 play convergent roles in oxidative stress regulation, but do not synergistically reduce endogenous ROS. Radical oxygen species are no longer viewed solely as damaging agents. Indeed, in many physiological processes, ROS are intracellular signaling molecules [2]. In muscle cells, increased ROS levels inhibit myotube formation while favoring cell proliferation [2]. These effects raise the possibility that TH-dependent regulation of myogenic differentiation is linked to its detoxifying action. Our data demonstrate that D2-mediated TH activation triggers muscle differentiation by reducing oxidative stress. Indeed, SOD2 inhibition partially reduces the ability of D2 to induce muscular differentiation. In conclusion, under physiological conditions, intracellular activation of TH via D2 attenuates cellular reliance on aerobic glycolysis, thus reducing mitochondrial respiration and ROS production, which in turn minimizes oxidative stress and augments myogenic differentiation. In addition, TH induces the increased expression of the ROS scavenger SOD2. Here we describe a model whereby D2-mediated TH activation regulates redox balance by inducing SOD2 transcription and by inducing a metabolic shift from OXPHOS to glycolysis. This suggests that D2 is a key player in the induction of fast-twitch fibers in muscle tissue and that it is involved in activation of alternative, uncoupling processes.

5. Materials and methods

5.1 *Cell cultures*

C2C12 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS (Microgem), 2 mM glutamine, 50 i.u. penicillin, and 50 μg/ml streptomycin (proliferating medium). C2C12 TET-ON D2 cells were cultured in DMEM supplemented with 10% tetracycline-free Fetal Bovine Serum (FBS) (Clontech, Mountain View, CA, USA), 100μg/ml geneticin (Biowest, Nuaillé, France) and 0.8μg/ml puromycin (Invitrogen, Carlsbad, CA, USA). For studies in proliferative conditions (PROL), cells were grown at 40-50% confluence and treated with the indicated amounts of doxycycline for 16, 24 or 48 has indicated. For experiments in differentiation conditions (DIFF), cells were grown at 60%-70% confluence, treated with doxycycline and then induced to differentiate in Differentiation Medium (DMEM with 2% horse serum, insulin 10 μg/ ml and transferrin 5 μg/ml). D2 expression was turned on by 2 μg/ml doxycycline (Clontech, Mountain View, CA, USA). In some experiments, TH (T3 and T4, Sigma-Aldrich S. Louis, Missouri, USA) were added in culture medium at a 30 nM final concentration or removed from the FBS by charcoal absorption (Larsen, 1972).

5.2 *Constructs and transfections*

The set of vectors for tetracycline-inducible transgene expression, constituted by the response pTRE3G, the regulator pCMV-Tet3G and the pTRE3G-Luc control plasmids, was purchased from Clontech Laboratories. The full-length coding sequence of *Dio2* gene, the 3′-UTR containing the SECIS and three repetitions of the Flag tag at the N-terminal, was inserted into the EcoRV site of the pTRE3G vector (Fig. S1). A puromycin resistance gene was also cloned in the XhoI site of the same vector for the screening of positive clones. Direct sequencing was carried out to verify the correct sequence (Nucleic acid sequencing, CEINGE-Biotecnologie Avanzate s.c.a.r.l.).

5.3. *Conditional Dio2 expression in C2C12 cells*

Transfection experiments were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, C2C12 cells were stably transfected with the pCMV-Tet3G plasmid that encodes the reverse transactivator and the neomycin phosphotransferase gene. Transfected cells were selected in media with 100 μg/ml G418 and single clones were isolated and expanded. Clones with a low basal activity and high transactivator inducibility in the presence of doxycycline were

identified by transient transfections with the pTRE3G-Luc control vector. Two clones displaying approximately 2500-fold (clone 6) and 3800-fold (clone 8) luciferase induction were chosen for the second round of transfection with the pTRE3G-D2 plasmid. Cells were selected in media with 100 μg/ml G418 and 0.8 μg/ml puromycin, yielding a total of 90 clones. Dio2 expression was induced in all selected clones by supplementing the medium with 2 μg/mL doxycycline. Cells were recovered after 48 h of induction and Western blot using a monoclonal anti-Flag M2 anti- body (Sigma-Aldrich) was performed to detect the D2-Flag in the cell lysates. The clone with the highest fold induction of the D2 protein (clone 7) was selected for propagation and further testing. Conditional Dio2 expression was also confirmed by mRNA analysis and Luciferase (Luc) expression assays.

5.4 *Luciferase (Luc) expression assays*

The luciferase reporter plasmids TRE3-TkLuc or the pTRE3G-Luc and CMV-Renilla were co-transfected into cells. Luc activities were measured 48h after transfection with the Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA), and differences in transfection efficiency were corrected relative to the level of Renilla Luciferase. Each construct was studied in triplicate in at least three separate transfection experiments.

5.5 *D2 deiodination assays*

Cells were homogenized on ice in phosphate buffer containing 0.25 M sucrose, 1 mm EDTA, and 10 mM DTT and complete protease inhibitor cocktail from Roche. Reactions were incubated at 37 °C for 4 h in 0.1mlPE buffer with 1nM or 500nM T4 (blank), 20mM DTT, 1mM propylthiouracil, with the addition of approximately 2 × 105 cpm [3′- 5′-125I] T4. Blank values were subtracted before calculation of deiodinase activities.

5.6 *In vivo experiments*

12-week-old C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, Maine, USA) (WT) were made hyperthyroid by intraperitoneally injection of TH (5 μg/mouse T3 + 20 μg/mouse T4) 3 days before harvesting the muscles. Hypothyroidism was obtained by adding 0.1% MMI and 1% $KClO_4$ to the drinking water for 6 weeks. Animal experimental protocols were approved by the Animal Research Committee of the University of Naples Federico II.

5.7 *Western blot analysis*

Total protein extracts from cells and muscles were run on a 10% SDS-PAGE gel and transferred onto an Immobilon-P transfer membrane (Millipore, Burlington, MA, USA). The membrane was then blocked with 5% non-fat dry milk in PBS, probed with anti-SOD2 (sc-133134) and anti-Cyclin D1 (sc-20044) antibodies overnight at 4 °C, washed, and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (1:3000), and detected by chemiluminescence (Millipore, cat. WBKLS0500). After extensive washing, the membrane was incubated with anti-tubulin (sc-8035) antibody as loading control. All Western blots were run in triplicate, and bands were quantified with ImageJ software.

5.8. *Chromatin immunoprecipitation (ChIP) assay*

Approximately 2 × 10 C2C12 cells were fixed for 10 min at 37 °C by adding 1% formaldehyde to the growth medium. Fixed cells were harvested and the pellet was resuspended in 1 ml of lysis buffer containing protease inhibitors (200 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin). The lysates were sonicated to obtain DNA fragments of 200–1000 bp. Sonicated samples were centrifuged and the soluble chromatin was diluted 10-fold in dilution buffer and used directly for ChIP assays. An aliquot (1/10) of sheared chromatin was further treated with proteinase K, extracted with phenol/chloroform and precipitated to determine DNA concentration and shearing efficiency ("input DNA"). Briefly, the sheared chromatin was pre-cleared for 2 h with 1 μg of non-immune IgG (Calbiochem, Burlington, MA, USA) and 30μL of Protein G Plus/Protein A Agarose suspension (Calbiochem) saturated with salmon sperm (1mg/mL). Precleared chromatin was divided in aliquots and incubated at 4 °C for 16 h with 1 μg of anti-THR antibody (C3) (ab2743, Abcam). After five rounds of washing, bound DNA-protein complexes were eluted by incubation with 1% sodium dodecyl sulfate-0.1M NaHCO3 elution buffer. Formaldehyde cross-links were reversed by incubation in 200 mM NaCl at 65°C. Samples were extracted twice with phenol-chloroform and precipitated with ethanol. DNA fragments were recovered by centrifugation, resuspended in 50 μl H_2O, and used for real-time PCRs.

5.9 *Real-time PCR*

Messenger RNAs were extracted with Trizol reagent (Life Technologies). Complementary DNAs were prepared with Vilo reverse transcriptase (Life Technologies) as indicated by the manufacturer. The cDNAs were amplified by PCR in an iQ5 Multicolor Real Time Detector System (BioRad) with the fluorescent double-stranded DNA-binding dye SYBR Green (BioRad). Specific primers for each gene were designed to work under the same cycling conditions (95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min), thereby generating products of comparable sizes (about 200 bp for each amplification). Primer combinations were positioned whenever possible to span an exon–exon junction and the RNA digested with DNAse to avoid genomic DNA interference. Primer sequences are reported in the Supplementary material (Table 1). For each reaction, standard curves for reference genes were constructed based on six four-fold serial dilutions of cDNA. All samples were run in triplicate. The template concentration was calculated from the cycle number when the amount of PCR product passed a threshold established in the exponential phase of the PCR. The relative amounts of gene expression were calculated with Cyclophilin A expression as an internal standard (calibrator). The results, expressed as N-fold differences in target gene expression, were determined as follows: N *target = 2(ΔCt sample-ΔCt calibrator).

5.10 *Cellular bioenergetics*

Cellular bioenergetics was analyzed on a Seahorse extracellular flux bioanalyzer (XF96) according to the manufacturer's instructions. Briefly, C2C12 TET-ON D2 cells were seeded at an appropriate density in a Seahorse XF96 plate. Cells were cultured for 24h and 48h in growth media supplemented with doxycycline. Immediately prior to the assay, all cells were equilibrated in minimal assay media (Seahorse Biosciences, 37 °C, pH 7.40) supplemented with 25 mM glucose and 1 mM sodium pyruvate for 45-60 min in a non-CO_2 incubator. The basal rates of oxygen consumption (OCR) and extracellular-acidification (ECAR) were measured in triplicate for 2min (for a total of 15mins) from the rate of decline in O2 partial pressure (OCR), and the rate of change in assay pH (ECAR). Mitochondrial respiration and glycolytic activity were monitored at basal level and after sequential injection of the mitochondrial modulators oligomycin (3 μM) for the oxygen consumed for ATP production, FCCP (6 μM) for maintenance of the proton gradient and antimycin (2.5 μM)

that induce mitochondrial stress (spare respiratory capacity and non-mitochondrial respiration). ECAR is expressed in milli pH (mpH) units representing the change in pH per minute and is measured simultaneously with OCR in the Seahorse assay. ATP levels were determined using the ATPlite 1 step kit (PerkinElmer) following the manufacturer's recommendations.

5.11 *Short hairpin RNA-mediated knockdown of SOD2*

C2C12 TET-ON D2 cells were grown until they reached 60% con- fluence and then they were transfected with two SOD2 shRNAs (SOD.1 ID 152022; SOD.2 ID 152023, Thermofisher Scientific) and control shRNA (AM4635, Thermofisher Scientific). Forty-eight hours after transfection, total protein lysate was collected and analyzed by Western blot, total RNA was extracted and analyzed by real-time PCR and intracellular ROS were measured by Flow Cytometry.

5.12 *Immunofluorescence*

For immunofluorescence staining, cells were fixed with 4% formaldehyde and permeabilized in 0.1% Triton X-100, then blocked with 0.2% BSA/PBS and washed in PBS. Cells were then incubated with primary antibody overnight at 4 °C. Secondary antibody incubation was carried out at room temperature for 1 h, followed by washing in 0.2% Tween/PBS. Images were acquired with an IX51 Olympus microscope and the Cell*F Olympus Imaging Software.

5.13 *Measurement of cellular ROS*

Total and mitochondrial ROS levels were measured using the 5- (and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate CM-H2DCFDA probe and mitoSOX dye, respectively (Molecular Probes, USA), according to the manufacturer's instructions. Briefly, after trypsinization with 0.25% (w/v) Trypsin–EDTA (Life Technologies), cells were collected and rinsed with PBS. Cells were then resuspended and incubated in pre-warmed PBS containing 5 μM CM-H2DCFDA or 5 μM mitoSOX in the dark for 20 min at 37 °C. As positive control, total ROS levels were evaluated after treating with 200μM H_2O_2. Intracellular fluorescence was then quantified using a FACS Canto2 (Becton Dickinson,

USA). To evaluate the contribution of D2 on induced ROS production, cells were treated for 16 h with 0.5 μM, 1 μM and 2.5 μM of doxorubicin (Sigma Aldrich).

5.14 *Confocal microscopy*

C2C12 TET-OND2 cells were grown on coverslips and treated with doxycycline for 48 h. Cells were labeled with mitoSOX Red reagent, which fluoresces when oxidized by superoxide, and nuclei were stained with the blue fluorescent dye Hoechst 33342 (Sigma Aldrich). Images were acquired on a Zeiss 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) using an x60 1.4 NA oil immersion objective using the same instrument settings for each image.

5.15 *Isolation and loading of single skeletal muscle fibers with CM-H2DCFDA and mitoSOX*

Male C57BL/6 mice, 3 months old, were euthanized and the extensor digitorum longus (EDL) muscles were removed and placed into 0.1% type 1 collagenase (C0130, Sigma–Aldrich Co. St. Louis, MO) solution in DMEM. Both EDL muscles from each mouse were incubated in collagenase solution at 37 °C for 1 h. Fiber bundles that had not been released during the incubation were separated using a wide-bore glass pipette. The fibers were washed four times in fresh culture medium. Cleaned fibers were plated onto 60-mm dishes in medium for satellite cells (50% DMEM, 50% MCDB, 20% FBS, 1% Ultroser G, 2 mM glutamine, 50 i.u. penicillin, and 50 μg/ml streptomycin). Fibers were incubated for 30 min at 37 °C. The medium was then replaced by PBS containing 5 μM mitoSOX and incubated for 20 min at 37 °C. The fibers were then washed and analyzed by fluorescence microscopy. Animal experimental protocols were approved by the Animal Research Committee of the University of Naples Federico II.

5.16 *Statistics*

The results are shown as means ± SD throughout. Differences between samples were assessed by the Student's two-tailed t-test for independent samples. Relative mRNA levels (in which the control sample was arbitrarily set as 1) are reported as results of real-time PCR, in which the expression of cyclophilin A served as housekeeping gene. In all experiments, differences were considered significant when p was less than 0.05. Asterisks indicate significance at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ throughout.

6. Figures and tables

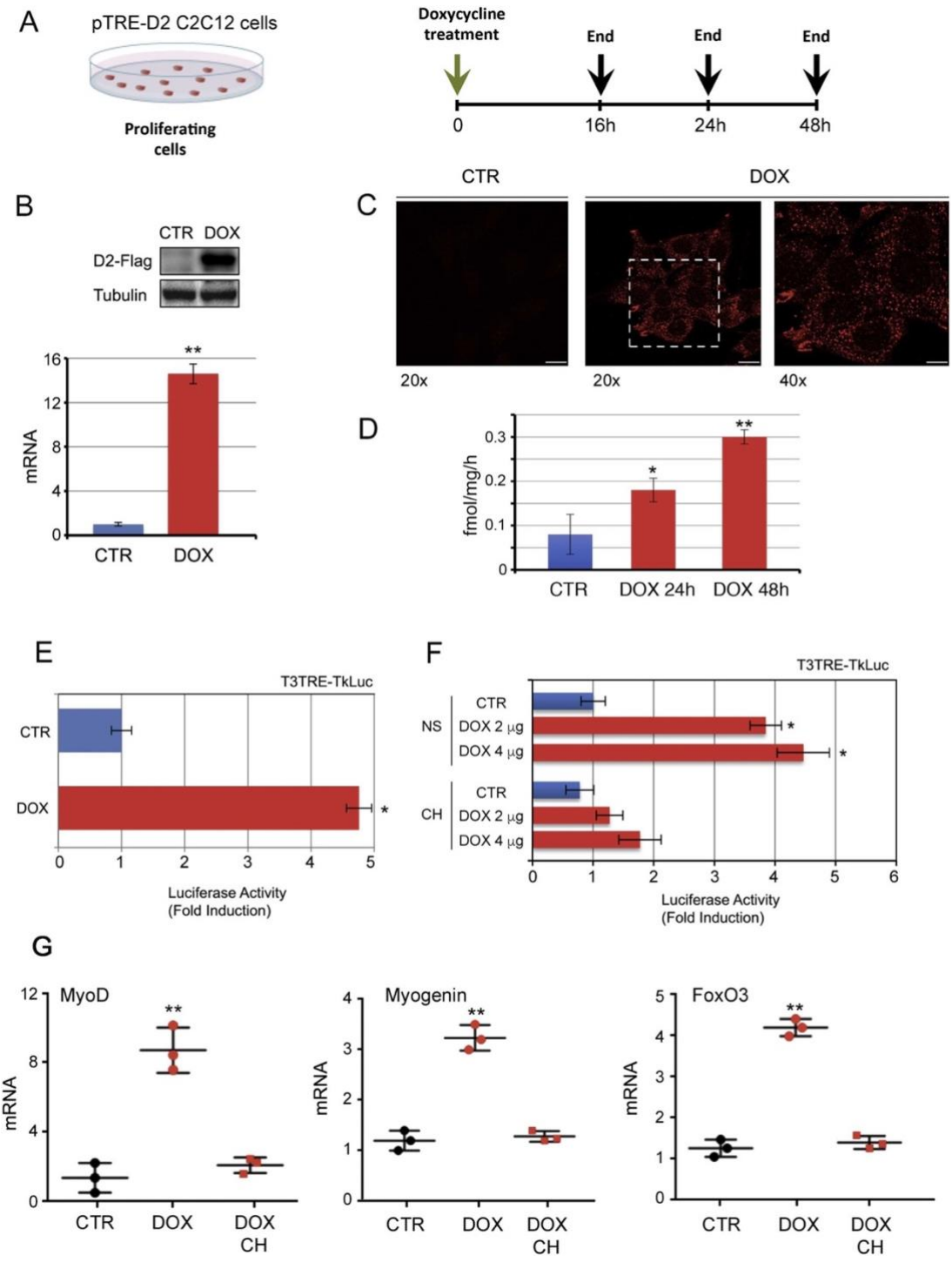

Fig. 1. *D2 is upregulated in C2C12 cells at mRNA and protein level by the tetracycline regulation system, TET-ON.* **(A)** Schematic representation of the doxycycline treatment of proliferating pTRE-D2 C2C12 cells. **(B)** D2 mRNA and D2-Flag protein expression was evaluated by real time PCR and Western blot analysis in C2C12 pTRE-D2 cells ± 2 μg/ml doxycycline for 24 h. **(C)** Immunofluorescence analysis of D2-Flag protein (red) in C2C12 pTRE-D2 cells treated as in **A** revealed the correct expression and localization of D2 by confocal microscopy. **(D)** D2 enzymatic activity was measured in pTRE-D2 cells ± 2 μg/ml doxycycline for 24 h and 48 h as described in Material and Methods. **(E)** T3 transactivation activity was measured indirectly by using the T3-reporter plasmid T3TRE-Tk-Luc. C2C12 pTRE- D2 ± 2 μg/ml doxycycline for 24 h were transfected with the T3TRE-TkLuc and CMV-Renilla as internal control. The results are shown as means ± SD of the luciferase/renilla (LUC/Renilla) ratios from at least 3 separate experiments, performed in duplicate. **(F)** pTRE-D2 cultured in normal serum (NS) or in TH-depleted medium (Charcoal stripped, CH) ± 2 and 4 μg/ml doxycycline for 24 h were transfected with the T3TRE-TkLuc and CMV-Renilla as internal control. **(G)** mRNA expression of the indicated genes was measured by real time PCR in C2C12 pTRE-D2 cells grown in normal serum or in TH-depleted serum (CH). All the experiments were performed in proliferating cells. The results are shown as means ± SD from at least 3 separate experiments. *P < 0.05, **P < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

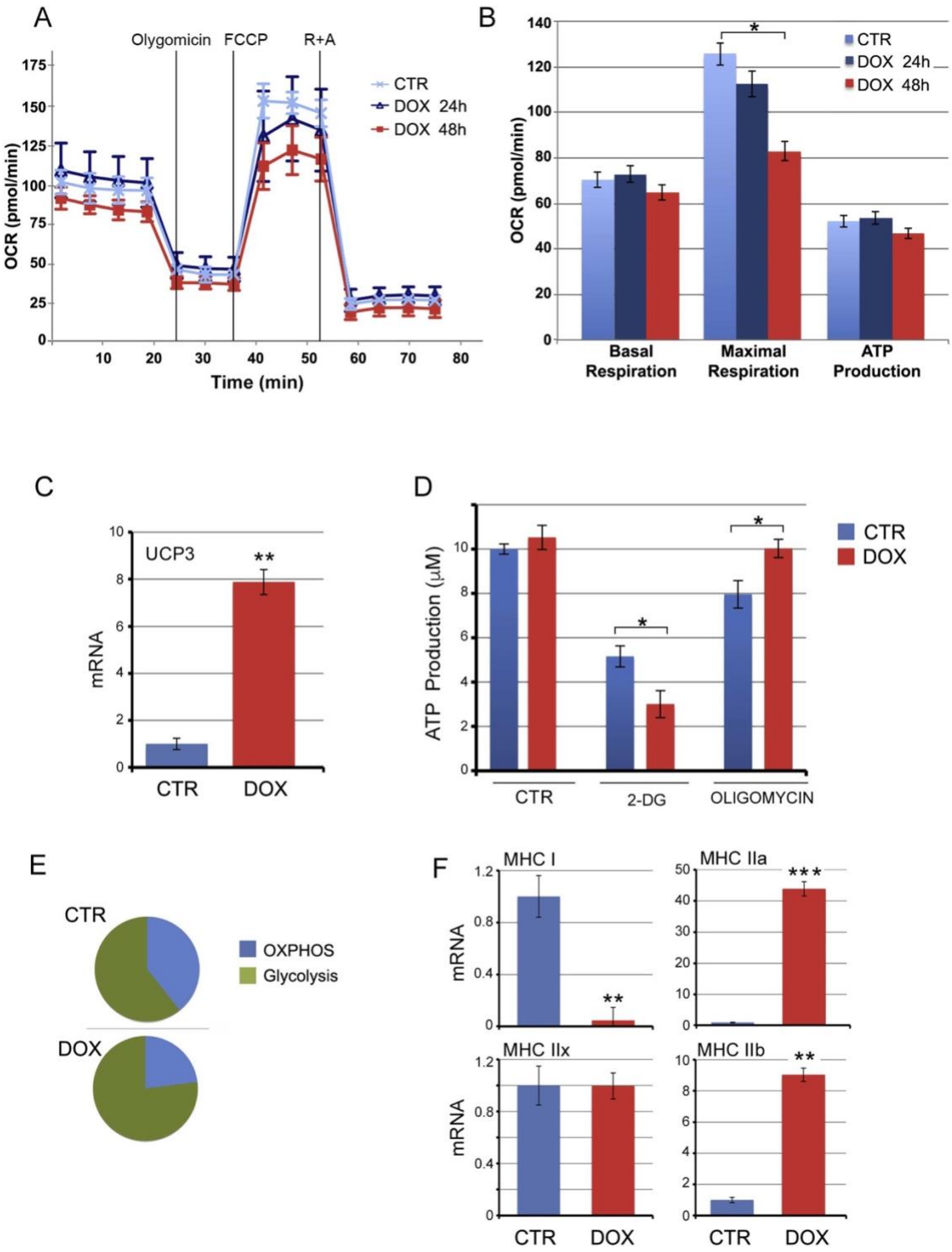

Fig. 2. *Thyroid hormone activation via D2 induces a shift from OxPhos to glycolysis.* **(A, B)** pTRE-D2 cells were treated with 2 μg/ml doxycycline for 24 h and 48 h and the rate of OXPHOS and glycolysis was measured with the Seahorse analyzer. OCR was measured continuously throughout the experiment at baseline and in the presence of the indicated drugs. **(C)** UCP3 mRNA was measured by real time PCR analysis in pTRE-D2 cells treated with doxycycline for 48 h. Cyclophilin A served as internal control. Normalized copies of UCP3 in untreated cells (CTR) were set as 1. **(D, E)** ATP production was measured in pTRE-D2 cells treated with doxycycline for 48 h by using the ATPlite kit. **(F)** Expression levels of the myosin heavy chain isoforms (MHC) were measured by real time PCR analysis in pTRE-D2 cells treated with 2 μg/ml doxycycline for 24 h. All the experiments were performed in proliferating cells; the results are shown as means ± SD from at least 3 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

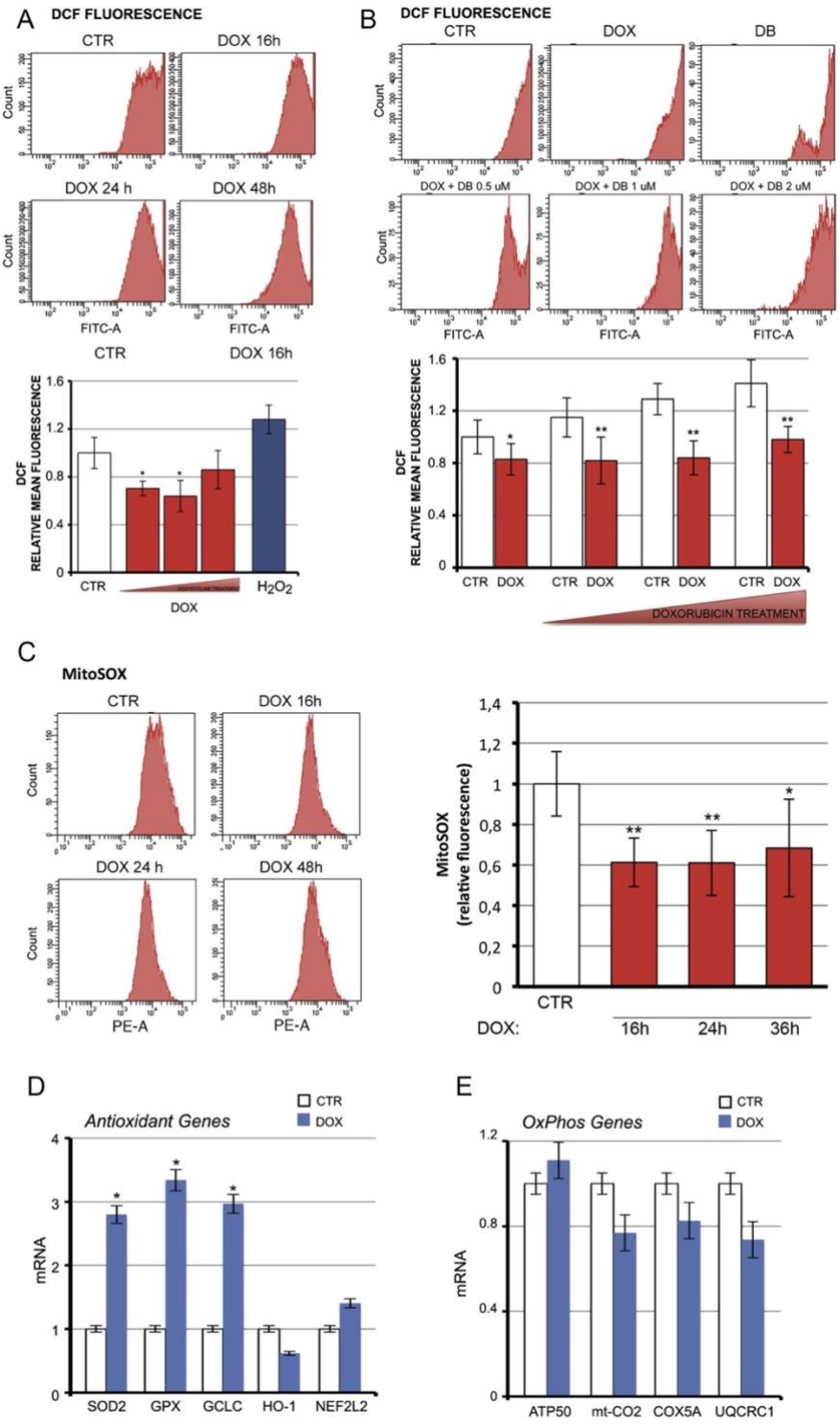

Fig. 3. *D2 reduces endogenous ROS produc- tion.* **(A)** Basal ROS were measured by FACS analysis in pTRE-D2 cells treated with doxycycline (DOX) and in C2C12 control cells. Bottom panel shows the relative mean fluorescence compared to CTR cells arbi- trarily set as 1. **(B)** ROS production was evaluated as in A in pTRE-D2 cells treated with doxycycline and/or with 0.5, 1 or 2 μM doxorubicin (DB) as indicated. Bottom panel represents relative mean fluorescence compared to CTR cells arbitrarily set as 1. **(C)** Mitochondrial ROS were measured by fluoresecence-activated cell sorting using MitoSOX dye in pTRE-D2 cells treated with doxycycline for the indicated time points, and in non-treated pTRE-D2 cells (controls). Right panel shows the relative mean fluor- escence versus CTR cells arbitrarily set at 1. **(D, E)** mRNA expression of a panel of antioxidant genes **(D)** and OXPHOS genes **(E)** was measured in pTRE-D2 cells treated with doxycycline for 24h and in control cells. Cyclophilin A was used as internal control. Normalized copies of the indicated gene in untreated cells (CTR) were set as 1. All the experiments were performed in pro- liferating cells; the results are shown as means ± SD from at least 3 separate ex- periments. *P < 0.05, **P < 0.01.

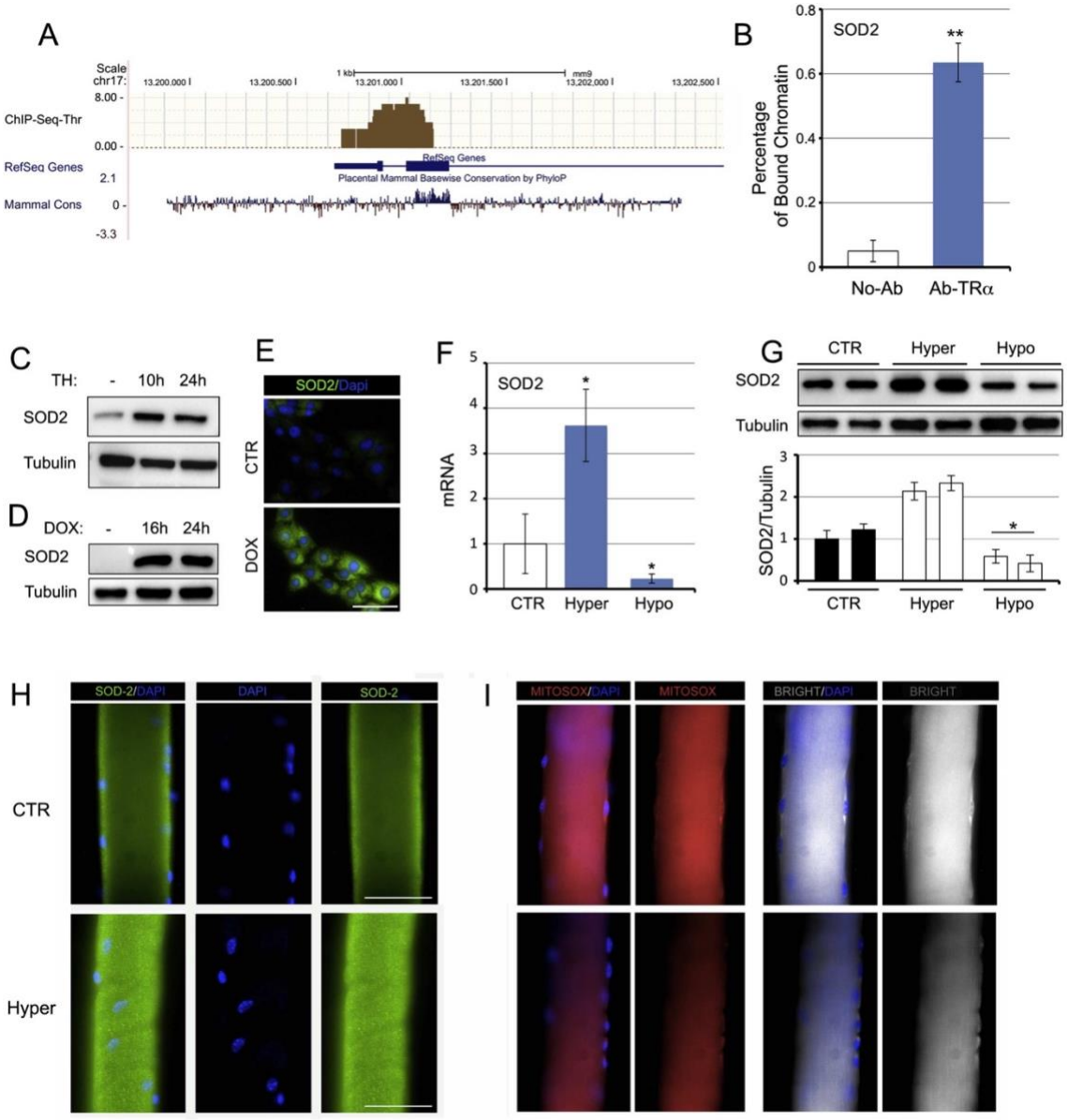

Fig. 4. *Thyroid hormone is a positive regulator of SOD2 transcription.* **(A)** ChIP seq analysis of T3-target genes performed in C2C12 cells showed that the *Sod2* gene is a direct target of T3. The mammalian conservation, the gene structure and position from Genome Browser and the ChIP Seq results (black bars) are indicated for the *Sod2* gene. **(B)** Chromatin immunoprecipitation assay was performed in C2C12 cells. Immunoprecipitation of the chromatin using the anti-TRα antibody revealed that the *Sod2* gene is a direct target of T3. **(C, D)** Protein levels of SOD2 were measured by Western blot analysis in pTRE-D2 cells treated or not with doxycycline and in cells treated with TH for 10 h and 24 h. **(E)** Immunofluorescence analysis of SOD2 expression in pTRE-D2 cells treated or not with doxycycline. **(F, G)** Real time PCR analysis and Western blot analysis of SOD2 expression in gastrocnemius (GC) muscles of hyper- and hypothyroid mice (see Methods). **(H)** SOD2 expression was analyzed in muscle fibers isolated from control (euthyroid) and hyperthyroid mice. **(I)** mitoSOX analysis in the same muscle fibers as in **H** was assessed by immunofluorescence. The results are shown as means ± SD from at least 3 separate experiments. *P < 0.05, **P < 0.01.

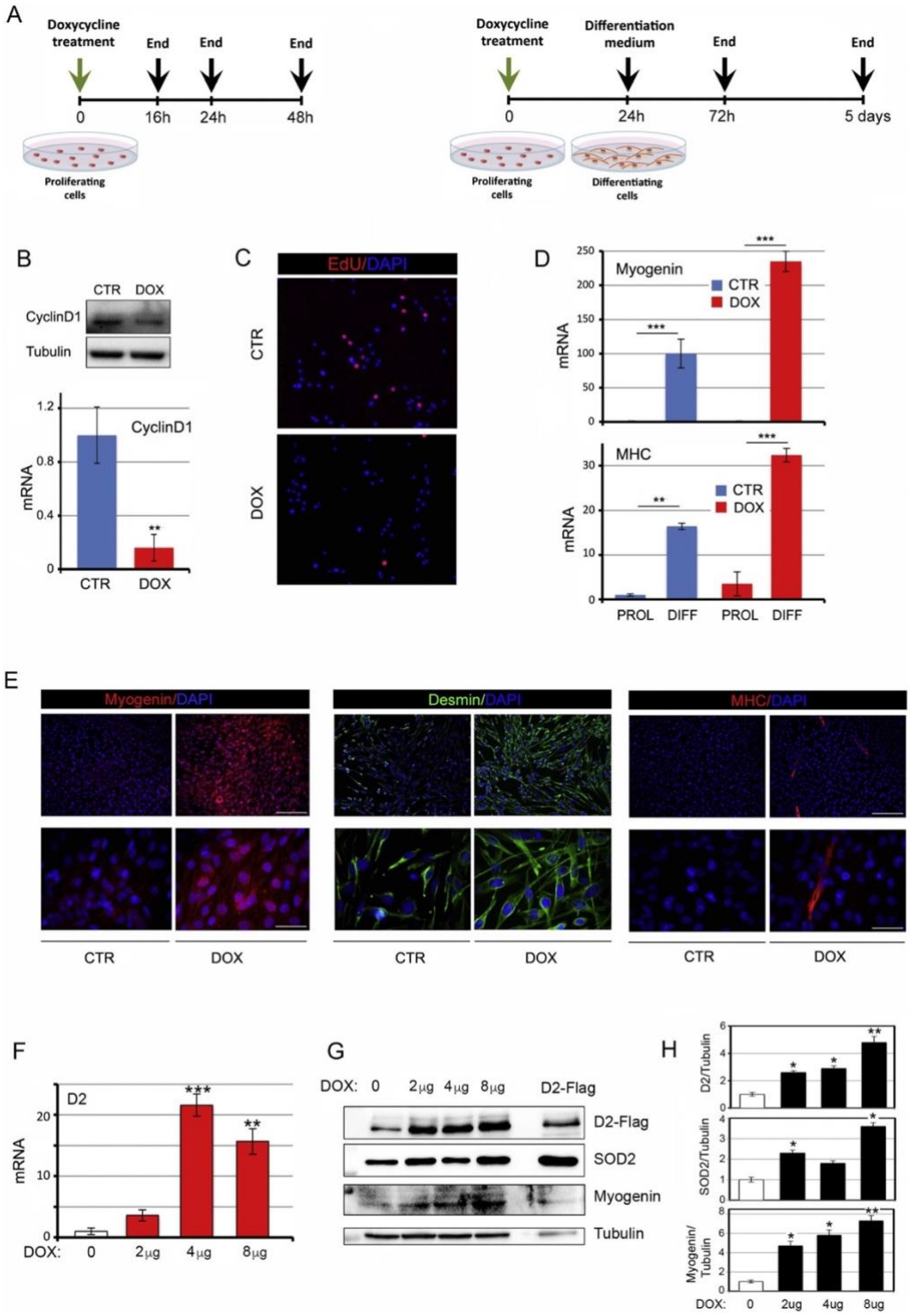

Fig. 5. *D2 upregulation induces myogenic differentiation.* **(A)** Schematic representation of the doxycycline treatment of proliferating and differentiating pTRE-D2 C2C12 cells. **(B)** Cyclin D1 mRNA and protein levels were measured in proliferating pTRE-D2 cells treated with 2 μg/ml doxycycline for 24 h. **(C)** EdU incorporation was assessed by immunofluorescence analysis of the same cells as in **A**. **(D)** Myogenin and MHC mRNA levels were measured in pTRE-D2 cells grown in proliferative or differentiative conditions as indicated in **A** and treated or not with 2 μg/ml doxycycline for 24 h. **(E)** Immunofluorescence analysis of Myogenin, Desmin and MHC expression was performed in differentiated pTRE-D2 cells grown as shown in **A**. Scale bars represent 200 μm and 50 μm, respectively. **(F)** mRNA D2 expression levels were measured in proliferating pTRE-D2 cells treated for 24 h with doxycycline at the indicated concentrations and in pTRE-D2 non treated cells (control cells). **(G)** D2, Myogenin and SOD2 protein levels were measured by Western blot in the same cells as in **F**. **(H)** Quantification of the single protein levels versus tubulin levels is represented by histograms. The results are shown as means ± SD from at least 3 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

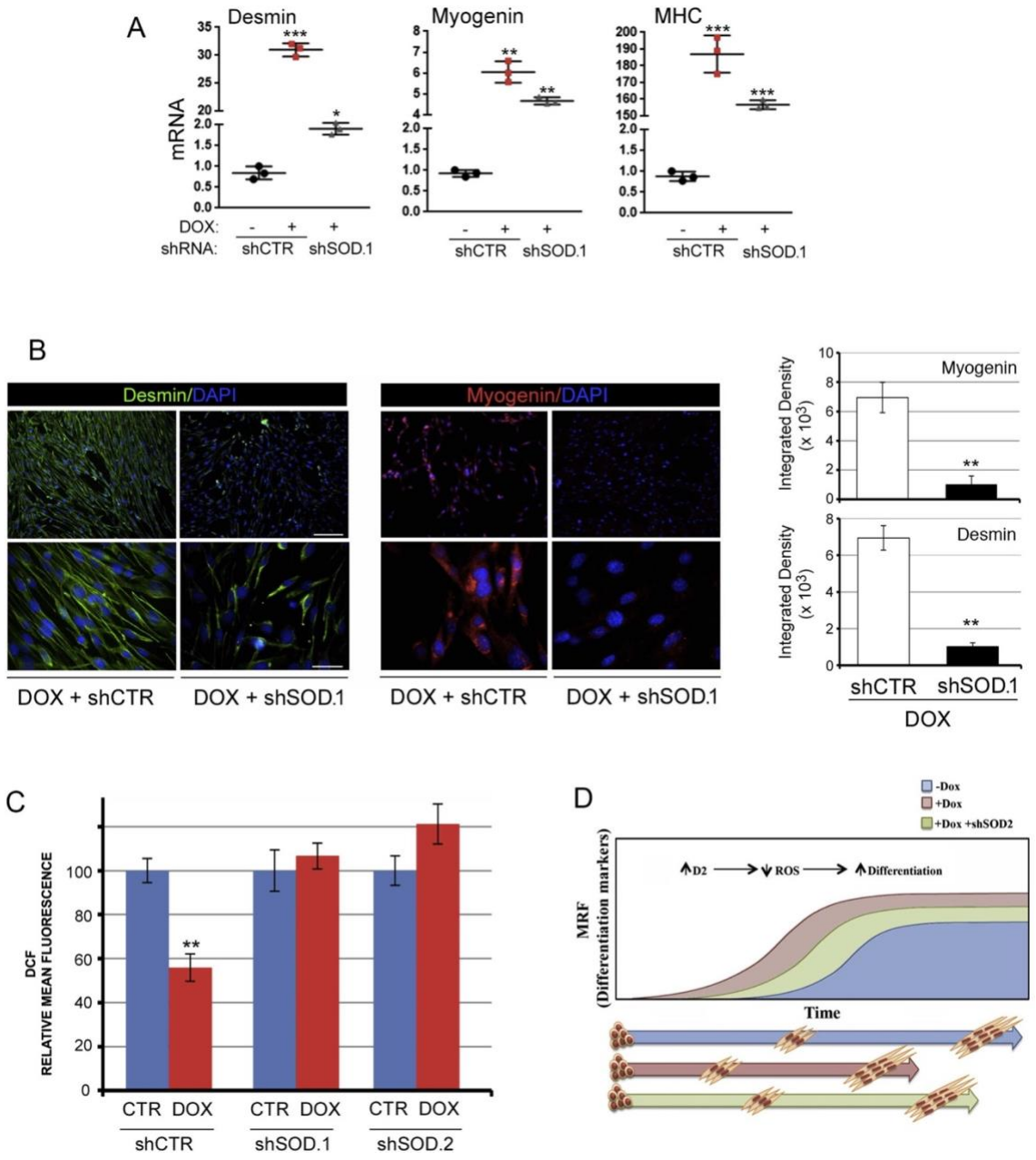

Fig. 6. *SOD2 silencing attenuates TH-mediated myogenic differentiation.* **(A)** mRNA expression of Desmin, Myogenin and MHC were measured in differentiated pTRE-D2 cells treated or not with doxycycline and transfected with SOD2.1 shRNA or CTR shRNA as indicated. **(B)** Immunofluorescence analysis of Desmin and Myogenin expression of the same cells as in **A**. Scale bars represent 200 μm and 50 μm, respectively. **(C)** Levels of total ROS and mitochondrial ROS were measured by FACS analysis of the same cells as in **A**. **(D)** D2 up-regulation increases myogenic differentiation and reduces ROS production. SOD2 silencing attenuates the pro-differ- entiative ability of D2. **P < 0.01, ***P < 0.001.

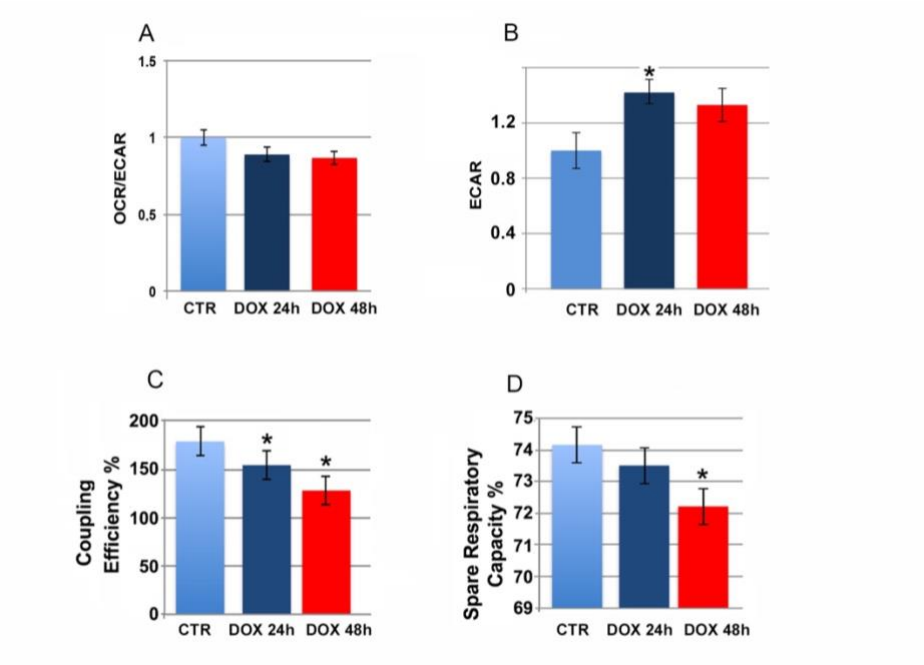

Fig. S1. *D2 up-regulation induces a metabolic shift from OXPHOS to glycolysis.* **(A)** Ratio between OXPHOS and glycolysis is reported as OCR/ECAR in the same experiment as in Figure 2A. OCR/ECAR was arbitrarily set at 1 in control cells (CTR). **(B)** The rate of glycolysis was measured with the Seahorse analyzer in the same experiment as in Figure 2A. **(C)** Coupling efficiency was calculated as the fraction of basal mitochondrial OCR used for ATP synthesis. **(D)** Spare Respiratory Capacity was measured by subtracting basal respiration from maximal respiratory capacity, respectively from the same experiment as in Figure 2A. The experiments were performed in proliferating cells. The results are shown as means ± SD from 3 separate experiments. *P < 0.05.

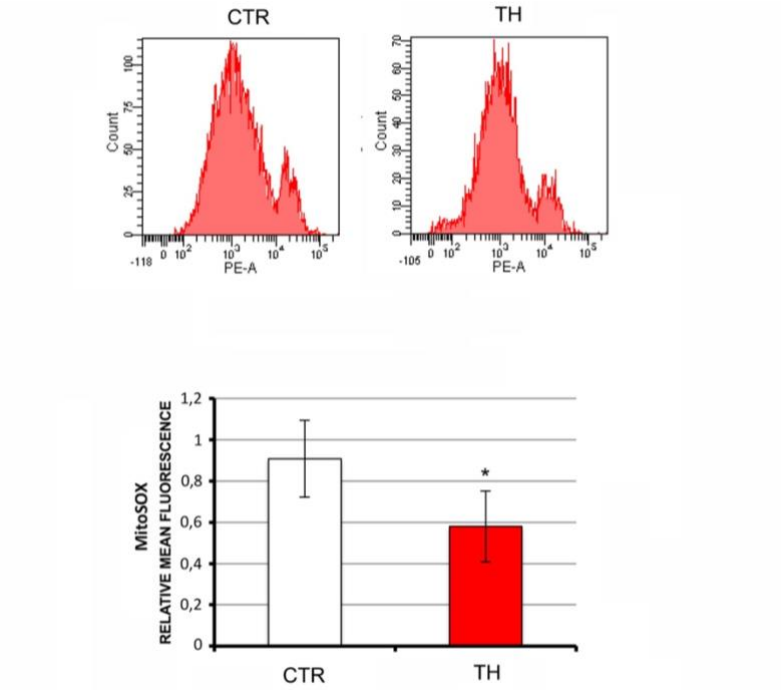

Fig. S2. *Mitochondrial ROS levels are reduced* in vivo *in hyperthyroid muscles.* Mitochondrial ROS levels were measured by fluorescence-activated cell sorting using the MitoSOX dye in gastrocnemius muscles from C57/BL7 mice treated or not with thyroid hormone (TH) (5 µg/mouse T3 and 20 µg/mouse T4) for 7 days. Six animals were used for each condition (n=6). Bottom panel shows relative mean fluorescence versus CTR muscles arbitrarily set at 1. The results are shown as means ± SD, *P < 0.05.

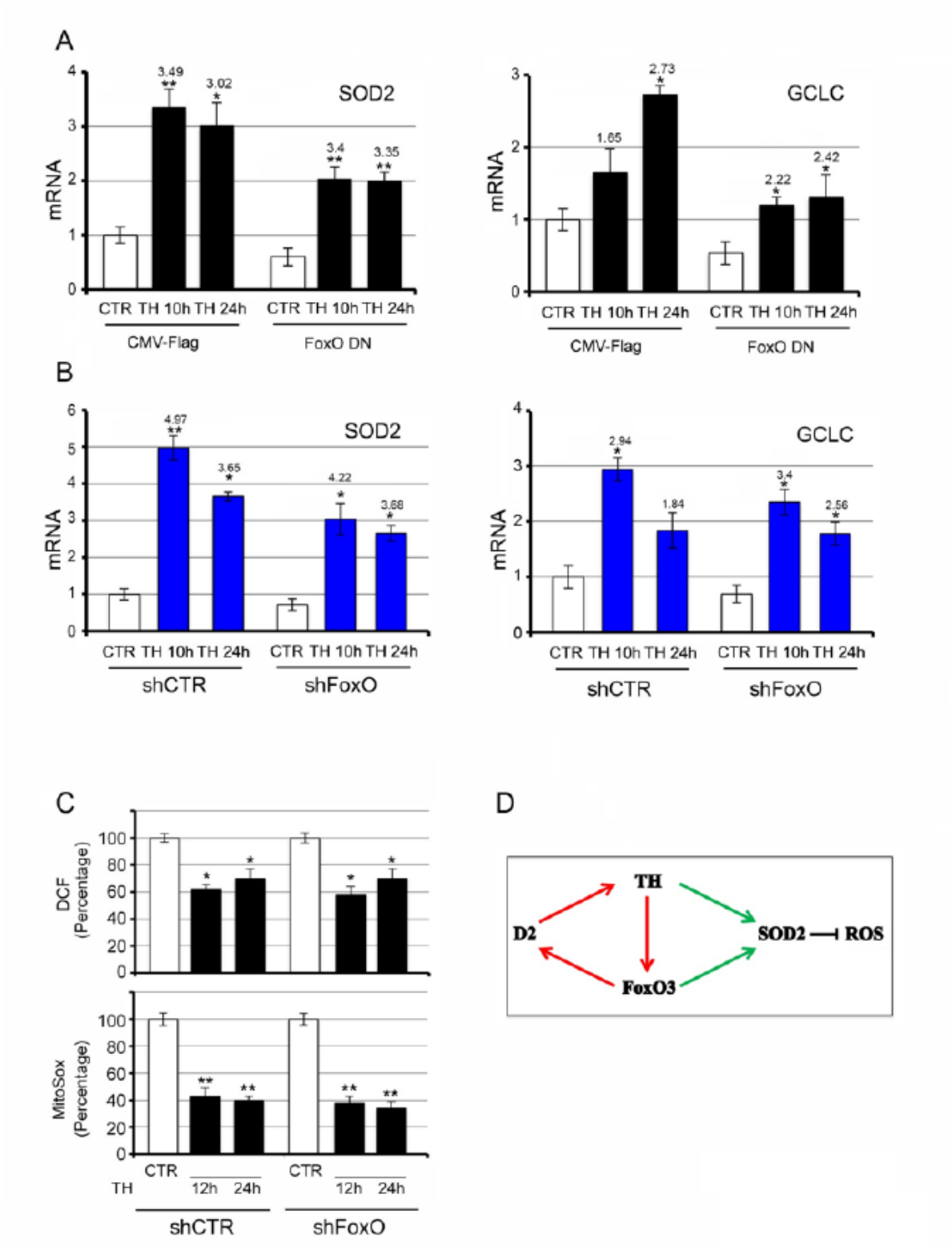

Fig. S3. *TH induces ROS attenuation independently of FoxO3.* (**A**) mRNA expression levels of SOD2 and GCLC were measured in C2C12 cells transfected with CMV-Flag (control cells) or FoxO3 dominant negative (FoxO-DN) plasmid and treated with TH for 10 and 24 hours. Relative mRNA levels are reported as results of the ÄCT in the antioxidant gene versus cyclophilin (used as housekeeping gene) in which the control sample was arbitrarily set at 1. (**B**) mRNA expression levels of SOD2 and GCLC were measured in C2C12 cells transfected with shCTR or shFoxO3 and treated with TH for 10 and 24 hours. **(C)** FACS of cellular ROS and mitochondrial ROS in C2C12 cells transfected with shCTR or shFoxO3 and treated with TH for 10 and 24 hours. (**D**) Schematic representation of the TH-FoxO3-D2 axis and of the independent effects of TH and FoxO on SOD2 expression and ROS dynamics. The experiments were performed in proliferating cells. The results are shown as means ± SD from 3 separate experiments. *P < 0.05, *P < 0.05.

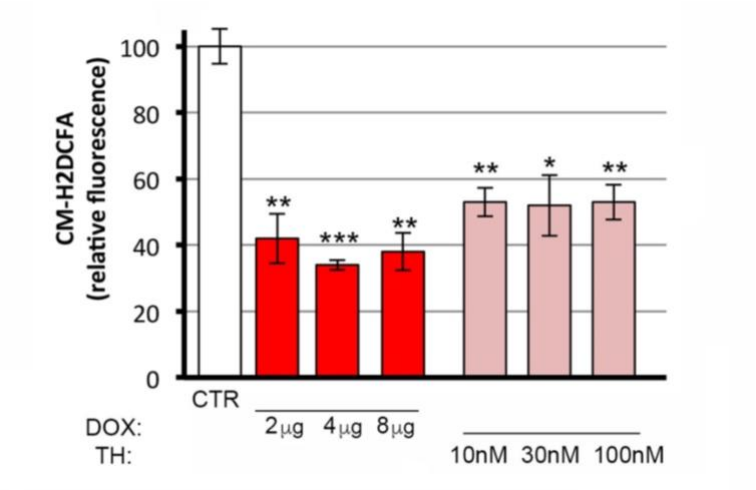

Fig. S4. *ROS attenuation correlates with D2 induction in a dose-dependent manner.* Endogenous ROS were measured by FACS analysis in pTRE-D2 cells treated with the indicated amounts of Doxycycline (DOX) for 24 hours and in C2C12 control cells. C2C12 cells were also treated with increasing amounts of T3+T4 for 24 hours. Results are reported as relative mean fluorescence compared to CTR cells arbitrarily set as 100. The experiments were performed in proliferating cells. The results are shown as means ± SD from 3 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

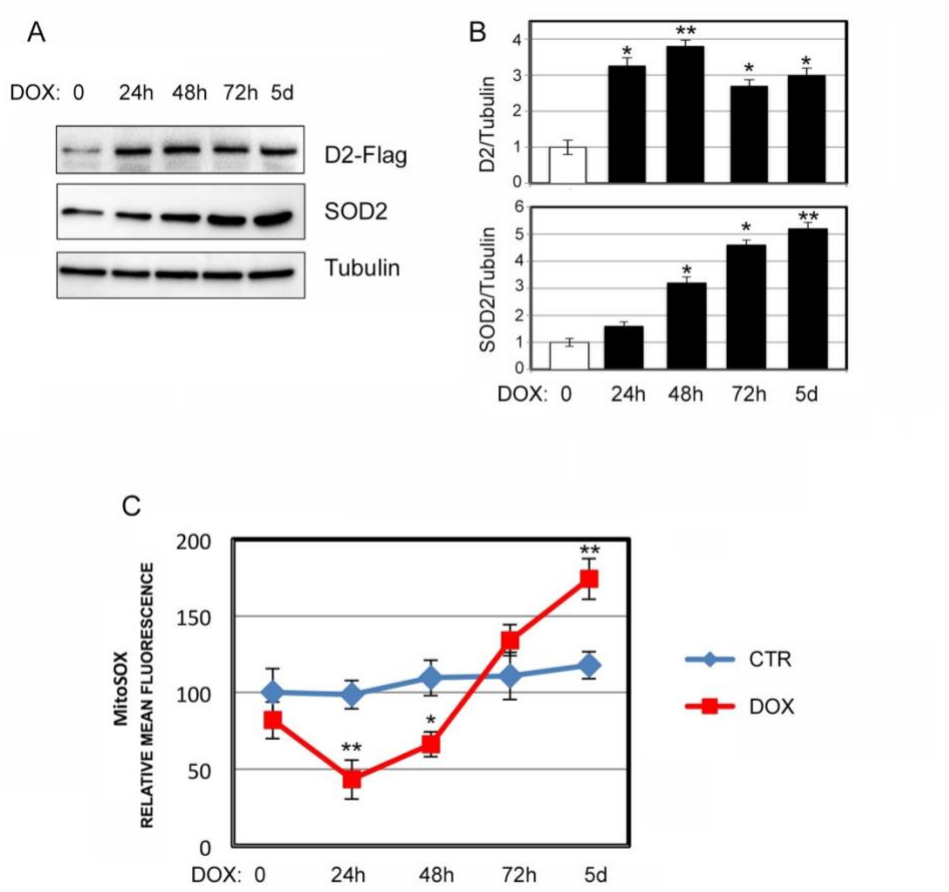

Fig. S5. *Muscle differentiation and ROS attenuation correlate with D2 levels in a time-dependent manner.* (**A**) D2 and SOD2 protein levels were measured by western blot in pTRE-D2 cells treated with 2 μg/ml Doxycycline (DOX) for the indicated times. (**B**) Quantification of the ratio between D2 and tubulin levels and SOD2 and tubulin levels in the same experiment as in **A**. (**C**) Mitochondrial ROS levels were measured by FACS analysis in pTRE-D2 cells treated with 2 μg/ml doxycycline for the indicated times, and in pTRE-D2 non treated cells (control cells). The experiments were performed in proliferating cells. The results are shown as means ± SD from 3 separate experiments. *P < 0.05, **P < 0.01.

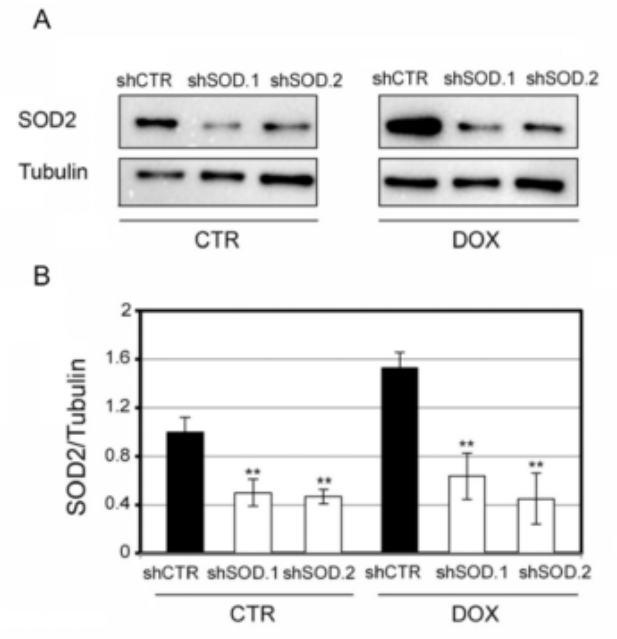

Fig. S6. *Effective silencing of SOD2 by iRNA.* (**A**) Western blot analysis of SOD2 expression in C2C12 cells transiently transfected with two different siRNA for SOD2 silencing. **(B)** Quantification of the ratio between SOD2 expression and tubulin levels in the same experiment as in **A**.

Table 1: List of Oligonucleotides

Oligonucleotides used for real-time PCR		
Gene	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**
Cyclophilin A(CypA)	CGCCACTGTCGCTTTTCG	AACTTTGTCTGCAAACAGCTC
FlagDio2	CAAGGATGACGATGACAAGGGA	GGCATAATTGTTACCTGATTCAGG
Sod2	ATCAGGACCCATTGCAAGGA	AGGTTTCACTTCTTGCAAGCT
Gpx	CTCGGTTTCCCGTGCAATCAG	GTGCAGCCAGTAATCACCAAG
Gclc	CCTGGAGCCTCTGAAGAACA	AGACTCGTTGGCATCATCCA
Nfe2l2	TGCCCACATTCCCAAACAAG	CTGCCAAACTTGCTCCATGT
Ho-1	TTCAGAAGGGTCAGGTGTCC	CAGTGAGGCCCATACCAGAA
Myod	GACCTGCGCTTTTTTGAGGACC	CAGGCCCACAGCAAGCAGCGAC
Myog	TTGCTCAGCTCCCTCAACCAGGA	TGCAGATTGTGGGCGTCTGTAGG
Foxo3a	TTGTCCCAGATCTACGAGTGGA	CGTGCCTTCATTCTGAACGCGCA
MhcI	CGCTCCACGCACCCTCACTT	GTCCATCACCCCTGGAGAC
MhcIIa	CCATTCAGAGCAAAGATGCAGGG	GCATAACGCTCTTTGAGGTTG
MhcIIx	AGGACCAAGTGAGTGAGCTG	CTTTTCGTCTAGCTGGCGTGA
MhcIIb	GCTAGGGTGAGGGAGCTTGAA	AGACCCTTGACGGCTTCGA
Desmin	TACACCTGCGAGATTGATGC	ACATCCAAGGCCATCTTCAC
CycD1	GCTCCTGTGCTGCGAAGTGGA	TCATGGCCAGCGGGAAGACCT
Ucp3	GCCTACAGAACCATCGCCAG	GCCACCATCTTCAGCATACA
Cox5a	GCCGCTGTCTGTTCCATTC	GCATCAATGTCTGGCTTGTTGAA
Mt-co2	AACCGAGTCCTTCTGCCAAT	CTAGGGAGGGGAACTGCTCAT
UQCRC1	AGACCCAGGTCAGCATCTTG	GCCGATTCTTTGTTCCCTTGA
Atp50	TCTCGACAGGTTCGGAGCTT	AGAGTACAGGGCGGTTGCATA
Oligonucleotides used for ChIP analysis		
Gene	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**
Sod2	GAGCAGCGGTCGTGTAAAC	GCGTTAATGTGTGGCTCCAG

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