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Type 2 Transglutaminase, mitochondria and Huntington's disease: Menage a trois

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ABSTRACT

Mitochondria produce the bulk of cellular energy and work as decisional "hubs" for cellular responses by integrating different input signals. The determinant in the physiopathology of mammals, they attract major attention, nowadays, for their contribution to brain degeneration. How they can withstand or succumb to insults leading to neuronal death is an object of great attention increasing the need for a better understanding of the interplay between inner and outer mitochondrial pathways residing in the cytosol. Of the latter, those dictating protein metabolism and therefore influencing the quality function and control of the organelle are of our most immediate interest and here we describe the Transglutaminase type 2 (TG2) contribution to mitochondrial function, dysfunction and neurodegeneration. Besides reviewing the latest evidences we share also the novel ones on the IF_1 pathway depicting a molecular conduit governing mitochondrial turnover and homeostasis relevant to envisaging preventive and therapeutic strategies to respectively predict and counteract deficiencies associated with deregulated mitochondrial function in neuropathology.

1. Introduction

Transglutaminase type 2 (TG2 or tissue transglutaminase) (Grosso and Mouradian, 2012; Kim et al., 1999) is a multifunctional ubiquitously expressed member of TG (TGs) family that catalyses post-translational modifications of proteins through Ca^{2+} dependent reactions (Fesus and Piacentini, 2002) (Fig. 1A). The multi-functionality of TG2 is dependent on its structural features. The structure of the enzyme, crystallised in a dimer form in a complex with GDP, is composed of 4 domains, a N-terminal β-sandwich domain with fibronectin and integrin binding sites (aa 1–140), the catalytic core containing the catalytic triad for the acyl-transfer reaction (aa 141–460) and two C-terminal β-barrel domains (aa 461–586 and 587–687) (Fig. 1A).

In eukaryotic cells, TG2 is regulated by reversible conformational changes (Fig. 1B) that include Ca^{2+}-dependent activation, which shifts TG2 to the "open" conformation thereby unmasking the enzyme's active centre, and inhibition by GTP, GDP, and ATP, which constrains it in the "closed" conformation (Nurminskaya and Belkin, 2012). The site of transamidating activity is composed of the catalytic triad of cysteine proteases: cysteine 277 (C277), histidine 335 (H335) and aspartate 358 (D358), which are the critical residues for the enzymatic activity.

Although it was initially identified and studied as a typical cytoplasmic protein, TG2 was later described to localise in other compartments, including the nucleus, mitochondria, endolysosomes and the extracellular space (Lorand and Graham, 2003; Malorni et al., 2008; Zemskov et al., 2006; Gundemir and Johnson, 2009; Park et al., 2010).

By its transamidating activity, TG2 is able to incorporate amines into glutamine residues forming a polyamine bond leading to the conversion of the substrate glutamine residue into glutamate (Beninati and Piacentini, 2004). TG2 has also a protein disulphide isomerase as well as isopeptidase activity and can hydrolyse the isopeptide bond. Beyond its main transamidating activity, TG2 presents other several enzymatic functions. Its GTPase activity has been shown to impact signal transduction as well as the ability to bind and hydrolyse GTP distinguishing TG2 from other TGs (Fesus and Piacentini, 2002).

Under normal physiology, TG2 is inactive in high concentration of GTP/GDP and low Ca^{2+} levels. GDP binding thus leads to a closed conformation of TG2 that reduces the affinity of the enzyme for Ca^{2+} becoming catalytically inactive (Fig. 1B). However, major changes in

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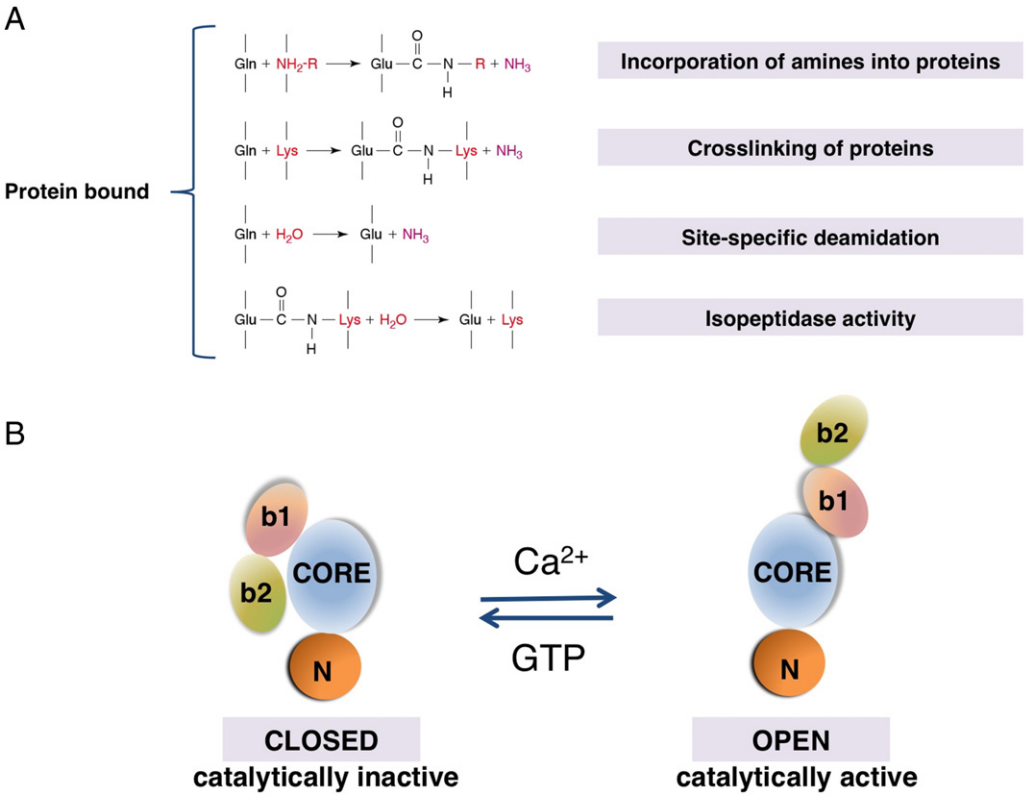

Fig. 1. A) Transamidation and deamidation mechanisms of tissue transglutaminase. B) Ca^{2+} and guanine nucleotide binding inversely regulate the transamidating activity of TG2. GTP bound TG2 has a closed conformation and it is catalytically inactive. Binding of Ca^{2+} is essential to acquire a catalytically active 'open' or 'extended' conformation.

cellular homeostasis leading to the increase of Ca^{2+} in the cytoplasm shift the enzyme towards an open active conformation (Klöck et al., 2012) (Fig. 1B). Recently we focussed our attention on the impact of TG2 expression level on mitochondrial homeostasis and highlighted its relevance to preserving quality of the organelle by manipulating mitochondrial autophagy and its ability to interplay with core regulatory pathways of mitochondrial respiration. Tightly linked to basic energy-dependent functions as well as being associated with more specialised cellular activities, mitochondria undergo active turnover dictated by the autophagy-related events.

We shall summarise the available data and present unpublished ones that will sustain the TG2 fine-tuning role in mitochondria by impacting the core of organelle bio-energy and the upstream regulation of cell mitophagy. This will be done by linking TG2 to the molecular physiology of IF_1, the endogenous regulator of the F_1Fo-ATP synthase, and showing how this is disrupted when TG2 is ablated. Specific evidences will be given on mitochondrial morphology, membrane potential ($\Delta\Psi_m$) and reactive oxygen species (ROS) framing a functional interplay that lies at the heart of cellular homeostasis influencing mitochondrial physiology and quality control.

2. TG2-dependent regulation of the mitochondrial ADP/ATP exchange activity

Almost a decade ago, Hasegawa and his co-workers found that TG2 has relatively low but detectable protein disulphide isomerase (PDI) activity. This activity does not require the presence of either Ca^{2+} or GTP (Hasegawa et al., 2003). They showed that TG2 converted completely inactive RNAase molecules to the native active enzyme that requires free sulfhydryl groups of the protein for catalysis. An interesting role for the PDI activity of TG2 came by analysing TG2 knockout ($TG2^{-/-}$) mice which exhibit glucose intolerance due to impaired glucose induced insulin secretion after intraperitoneal glucose loading (Bernassola et al., 2002). It is also known that impaired glucose-induced insulin secretion often results from impaired glucose-induced ATP elevation or from defective respiratory chain (Fujimoto et al., 2007). Based on these observations, our group provided the first in vivo evidences showing that TG2 acts as PDI and contributes to the correct assembly of the respiratory chain complexes (Mastroberardino et al., 2006). Thus, $TG2^{-/-}$ mice display impairments in mitochondrial energy production and decreased ATP levels after physical challenge. The molecular mechanism behind this phenotype may reside in the defective disulphide bond formation in ATP synthase complex and other key components of the respiratory chain after TG2 deletion (Mastroberardino et al., 2006; Battaglia et al., 2007; Sarang et al., 2009). Additionally, it was found that mitochondrial ADP/ATP transporter adenine nucleotide translocator 1 (ANT1) was incorrectly assembled and was dysfunctional in the absence of PDI activity of mitochondrial TG2 (Malorni et al., 2009). ANT1 is the most abundant protein in mitochondria involved in ADP/ATP exchange across the mitochondrial inner membrane (Liu and Chen, 2013). It serves as a core component of the permeability transition pore complex in the inner mitochondrial membrane (IMM) and also mediates basal proton leak. ANT1 mutations cause severe human diseases including early-onset diseases (Vartiainen et al., 2014). Mice lacking TG2 displayed an increased thiol-dependent ANT1 oligomer formation as well as higher ADP/ATP exchange activity of ANT1 in heart mitochondria. Thereby, PDI activity of TG2 decreased oligomer formation of ANT1 and inhibited its transporter activity by directly binding to ANT1 and sequestering its monomers. These data correlate with current experiments, carried out in our laboratory, showing a higher proton leak, paralleled by augmented oxygen consumption, in cell knockouts for TG2 suggesting that higher respiration rate is required to maintain equal membrane potential (Fig. 2). These findings showed that TG2's

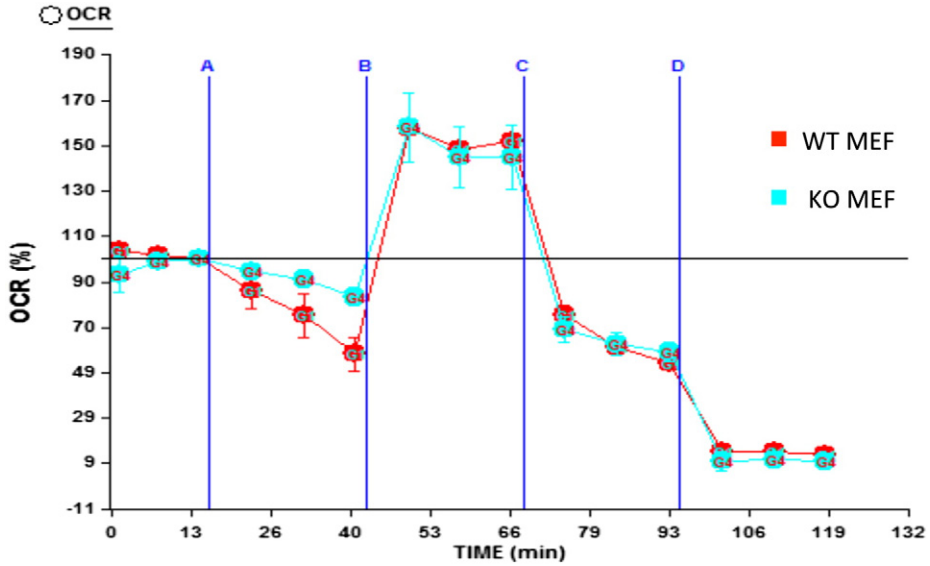

Fig. 2. Oxygen consumption rate (OCR) was measured in primary WT and TG2 KO MEFs using Seahorse XF technology. According to the standard protocol, the cells are metabolically perturbed by addition of four different compounds (in succession) that shift the bioenergetic profile of the cell: a) oligomycin, b) FCCP, c) 3-NP, d) antimycin.

protein PDI activity could be involved in the maintenance of the mitochondrial physiology, by modulating the level of ANT1 polymers. All these evidences highlighted for the first time the importance of TG2-PDI enzymatic activity in vivo and indicated a direct link with the regulation of mitochondrial pathophysiology.

3. TG2 in quality control mechanisms

There are two major pathways for clearing misfolded proteins, namely, the ubiquitin–proteasome system (UPS) and the autophagy–lysosome system. The UPS identifies misfolded or unfolded proteins and targets them for proteasomal degradation and generally it is involved in the clearance of short-lived proteins by tagging them with ubiquitin. On the other hand, autophagy is the major mechanism to eliminate long lived abnormal proteins and damaged organelles (Hideshima et al., 2005). During autophagy, cellular components are sequestered by double-membrane vesicles called autophagosomes, and degraded after fusion with lysosomes. However, the function of autophagy is not only the simple elimination of materials, but also, the dynamic recycling system that produces new building blocks and energy for cellular renovation and homeostasis (Levine et al., 2011). It has been reported that neurodegenerative diseases like Huntington's disease (HD) could be related to the impairments in the capacity of the UPS so that these abnormal proteins cannot be cleared. In the absence of a proper quality control system, cells transform abnormal proteins into aggregates as a last attempt to prevent toxicity. Since aggregates are not completely harmless, they interfere with cellular metabolism. When they get trapped in brain inclusions, these aggregates cause toxicity and lead to neuronal death. This progressive neuronal loss eventually results in neurodegenerative disorder (Martinez-Vicente and Cuervo, 2007).

Autophagy has a beneficial role for the clearance of various mutant proteins and increases in response to nutrient deprivation, hypoxia and mitochondrial dysfunction (Mizushima, 2007). The defects in this system may lead in cellular malfunctions such as neurodegeneration, tumorigenesis, bacterial infections and severe myopathies (Levine et al., 2011).

In HD, the presence of damaged mitochondria leads to increased production of reactive oxygen species (ROS) that participate in cell death pathways (Mastroberardino and Piacentini, 2010). Although autophagy is responsible for nonspecific, bulk degradation of cytoplasmic components, it is also the only mechanism to degrade unhealthy organelles such as mitochondria, ribosomes and peroxisomes through selective degradation (Chen and Klionsky, 2011). Under stressful conditions, the amount of TG2 is highly increased inside the cells and Ca^{2+}-dependent activation of its crosslinking activity leads to the formation of insoluble protein aggregates (Ricotta et al., 2010). We demonstrated the involvement of the enzyme in the autophagosome maturation showing that its absence is associated with impaired clearance of damaged mitochondria (D'Eletto et al., 2009; Rossin et al., 2011). Additionally, we have recently shown that TG2 knockout mice displayed impairments in autophagy and accumulation of ubiquitinated protein aggregates upon starvation. Furthermore, TG2-dependent post-translational modification of its substrate proteins is increased by autophagy induction and further potentiated upon proteasome inhibition. Interestingly, TG2 is present in protein complexes containing either p62 or NBR1 (D'Eletto et al., 2012). A growing amount of data has drawn attention to these autophagy cargo receptors and their possible role in connecting autophagy with the UPS. p62 and NBR1 are both selective autophagy substrates and cargo receptors known to mediate the clearance of ubiquitinated proteins. These cargo proteins contain UBA domains, allowing them to interact with the aggregated protein complexes and organelles that have to be degraded. Subsequently, by a LIR domain these cargo proteins interact with the ATG8-family members inside the nascent pre-autophagic vesicles.

4. Is TG2 involved in mitophagy?

We have recently demonstrated a TG2-protective role in the turnover and degradation of dysfunctional mitochondria (Rossin et al., 2014). In fact, in cells lacking TG2 we observed an accumulation of dysfunctional mitochondria, suggesting an important role for the enzyme in the regulation of mitochondrial homeostasis. In keeping with this assumption, we detected a mitochondria-specific activation of the enzyme transamidating activity upon mitochondrial damage by treatment with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Table 1). We showed that cells lacking TG2 display altered mitochondrial morphology and functionality, as indicated by the presence of fragmented and depolarized mitochondria. In line with this notion cell knockouts for TG2 displayed, before and after damage, a sustained higher mitochondrial level of the Drp1 protein level, a primary regulator of mitochondrial fission. Interestingly, we demonstrated the interaction of TG2 with Drp1; this physical interaction, however, significantly decreased after sustained mitochondrial damage as well as the

Table 1
A) Expression levels of mitochondrial proteins in cells expressing or lacking TG2: Drp1 involved in mitochondria fragmentation, PINK1 a key regulator of mitophagy and Tom-20, a structural mitochondrial protein. The absence of TG2 leads to a general increase in the expression levels of these proteins under basal condition and especially following mitochondrial damage (using CCCP), due to the impairment in the mitophagy process and accumulation of dysfunctional mitochondria. B) TG2 protein expression in the cytosol and on mitochondria either in basal condition or after CCCP treatment to induce mitochondrial damage. TG2 transamidating activity is largely induced on damaged mitochondria.

A

	TG2 protein expression	Drp1	PINK1	Tom-20
Physiological conditions	+	1.0	1.0	1.0
	-	1.6	1.4	1.1
Mitochondrial damage	+	0.9	0.9	0.8
	-	7.0	1.9	1.3

B

	Subcellular localization	Physiological conditions	Mitochondrial damage
TG2 protein expression	Cytosol	1.0	0.8
	Mitochondria	1.0	0.3
TG2 protein activity	Cytosol	Undetectable	3.0
	Mitochondria	Undetectable	8.0

interaction of TG2 with Fis1, the Drp1 mitochondrial receptor. Based on these evidences we hypothesised that TG2 interferes with Drp1 binding to its mitochondrial receptors, thereby delaying mitochondrial fission preceding mitophagy. Interestingly, in order to survive, cells lacking TG2 switch to a higher rate of aerobic glycolysis and are more sensitive to the glycolytic inhibitor 2-deoxy-D-glucose (Rossin et al., 2014). This important metabolic change indicates a compensatory alteration in the absence of the TG2, suggesting a role for the enzyme in the regulation of the cellular metabolism from mitochondrial respiration to aerobic glycolysis.

It is known that fission is a key prerequisite to remove damaged mitochondria, leading to their degradation by autophagy. However, when this protective mechanism is defective, mitochondrial fission can lead to cell death as well. In keeping with this assumption the accumulation of fragmented mitochondria, detected in cells lacking TG2, leads to caspase 3 activation and PARP cleavage indicating the induction of apoptosis. By contrast, wild type cells undergo apoptosis only when the degradation of mitochondria by autophagy is pharmacologically inhibited, thus further indicating the importance of active mitophagy for cell survival. In line with the protective role displayed by TG2 upon mitochondrial damage, we demonstrated that the activity of TG2 leads to inhibition/delay of apoptosis by crosslinking caspase 3 and thus consequently impairing its activation. In support of this notion is the observation that upon mitophagy induction there is a substantial increase of free calcium ions that may result in the activation of the transamidating activity of TG2 (East and Campanella, 2013; Zhang et al., 2014).

5. TG2, mitochondria and Huntington's disease

The majority of TG2 in mitochondria is found to be associated with outer mitochondrial membrane and inner membrane space while inner mitochondrial membrane has 5–10% of the enzyme. The initial observations coming from TG2 knockout mice, whose hearts appeared more sensitive to ischemia/reperfusion injury, have shown that TG2 acts as an important regulator of energy metabolism and mitochondrial functions (Szondy et al., 2006). Moreover, when TG2 was overexpressed in neural cells, this resulted in their apoptosis very rapidly (Piacentini et al., 2002). Importantly, the chemotherapeutic agent staurosporine induced apoptosis via mitochondria leading to a rapid loss of mitochondrial membrane potential in the cells overexpressing TG2 only, an evidence which could also be important in the context of neurodegenerative diseases including HD patients who display impaired mitochondrial function and increased TG2 activity in their brains (Lesort et al., 2000). In addition, it has been shown that an increase of transglutaminase activity in HD caudate may contribute to mitochondrial dysfunction by incorporating mitochondrial aconitase, a TG2 substrate, into inactive polymers (Kim et al., 2005). Interestingly, the TG2 inhibition, achieved either via pharmacological or genetic approaches, has been shown by various research groups to be beneficial for the treatment of HD in animal models (Mastroberardino and Piacentini, 2010) opening novel therapeutic avenues.

Neurodegenerative diseases are characterised by progressive neuronal loss and the aggregation of disease-specific pathogenic proteins in the central nervous system (CNS). Growing evidences show that TG2 crosslinks many of these proteins, including Huntingtin, and its expression and activity is increased in affected brain regions in these diseases (Mastroberardino and Piacentini, 2010). All these observations, along with experimental evidence in vivo and in vitro, suggest that TG2 can contribute to the abnormal aggregation of proteins causing disease and consequently neuronal damage (Badarau et al., 2013).

HD is a progressive autosomal dominant neurodegenerative disorder resulting from the expansion of a polyglutamine [poly(Q)] tract in the Huntingtin protein (Cattaneo et al., 2005). It is one of the best-characterised disorders by the accumulation and aggregation of N-terminal Huntingtin (Htt) proteins in CNS, causing the death of neurons. HD is caused by the expansion of a CAG tract beyond 35 repeats in exon 1 of the Huntingtin (Htt) gene rendering Huntingtin prone to aggregate into insoluble inclusion bodies (Chen et al., 2002).

Inclusion bodies are one of the earliest pathological changes in the brain, and some studies have found that they can cause cellular toxicity and organ damage and mitochondrial dysfunction (Rubinsztein and Carmichael, 2003), however, other studies have shown that they could be part of cell survival mechanisms (Arrasate et al., 2004). Currently, there is no underlying mechanism being fully elucidated. However, there is clear evidence of transcriptional deregulation and mitochondrial dysfunction contributing to the pathogenesis of HD (Browne, 2008). These pathological pathways may lead to fragile neurons which are vulnerable to oxidative injury, pro-apoptotic signals and energy depletion, causing the death of neurons in HD (Kim et al., 2010). As neurons are in need of excess amount of energy they are also vulnerable to mitochondrial impairment.

Starting from very early studies, evidence for mitochondrial abnormalities in HD was first observed in a defect in succinate dehydrogenase, involved in Krebs cycle, found in HD brains (Caraceni et al., 1977). Afterwards, it had been hypothesised that HD may bring energetic impairments throughout the cells. Patients with HD are more susceptible to progressive weight loss or metabolic defects in brain and muscle in spite of caloric intake. Additional studies revealed that HD patients showed abnormal mitochondria morphology during early studies of cortical biopsies (Quintanilla and Johnson, 2009). Experimental models have also shown that during HD, mitochondrial fission and fusion may be altered and lead to neuronal death (Su et al., 2010). As there is a balance between an organelle's fusion and fission, modulation of both mitochondrial shape and size change the mitochondrial trafficking throughout the cell (Scott et al., 2003). Even though polyglutamine repeats give rise to higher mitochondrial fragmentation and lower ATP levels, promotion of fusion and repression of fission

can increase ATP leading to death in HeLa cells (Wang et al., 2009). Lastly, based on the observations on *mHtt* transgenic (*mHtt-Tg*) mice, binding of mHtt to p53 increases levels of nuclear p53 as well as p53 transcriptional activity. The inhibition of p53 has been shown and proposed to prevent mitochondrial membrane depolarization and cytotoxicity by mHtt (Bae et al., 2005).

Interestingly, in a recent study it has been shown that TG2 can exacerbate α-synuclein toxicity in mouse models. In fact, compared with α-synuclein transgenic mice, animals double transgenic for human α-synuclein and TG2 manifested greater high-molecular-weight insoluble species of α-synuclein in the brain (Grosso et al., 2014). This correlated with an increased inflammation in astrocytes and microglia. The pathogenic role of TG2 in neurodegenerative disease is further suggested by the evidence that the enzyme accelerates neuroinflammation in amyotrophic lateral sclerosis (ALS) through interaction with misfolded superoxide dismutase 1. In fact, the inhibition of spinal TG2 by cystamine (a putative TG inhibitor) significantly delayed the progression and reduced SOD1 oligomers and microglial activation (Oono et al., 2014). These results suggest a role of TG2 in SOD1 oligomer-mediated neuroinflammation, as well as involvement in the intracellular aggregation of misfolded SOD1 in ALS. Taken together all these findings indicate a key role of TG2 in the pathogenesis of neurodegenerative disease, thus suggesting that its inhibition could represent a possible therapeutic approach.

6. TG2 and IF_1 interplay in cell mitophagy and molecular physiology

Homeostasis of cells and tissues depends on the fine balance between energy and quality priming the research on mitochondria and autophagy. Autophagy was recently discovered to target dysfunctional mitochondria to preserve the whole mitochondrial reticulum: a process named mitophagy. This mechanism involves selective sequestration and subsequent degradation of the dysfunctional mitochondrion within a process known as mitochondrial autophagy or mitophagy (Kubli and Gustafsson, 2012). J. Lemasters and colleagues (Kim et al., 2007) forged this term and the "cleaning up" role of dysfunctional mitochondria was further corroborated by the work of O. Shirihai's group who positioned mitophagy at the heart of mitochondrial dynamics (Twig et al., 2008). The seminal work by R. Youle and colleagues demonstrated the selectivity of the process by explaining how the PTEN-induced putative kinase protein 1 (PINK1) recruits the E3 ubiquitin-protein ligase Parkin on depolarized mitochondria and mediates their engulfment and elimination by autophagosomes (Narendra et al., 2008). The latest advancements in the field have addressed the connection between cellular stress and energy-sensing mediators (Egan et al., 2011) but leaving still unaddressed the role played by mechanisms governing mitochondrial bio-energy and those mediating efficient ubiquitination of defective mitochondria to be targeted and disposed via autophagy. The experimental work we have led in recent years links mitochondrial homeostasis and its quality control via mitophagy to IF_1 (Campanella et al., 2008; Lefebvre et al., 2013).

IF_1 is a small, basic, heat-stable protein of approximately 10 kDa predominantly compartmentalised inside the mitochondrial environment. Discovered in 1963 by Pullman and Monroy in mitochondria from bovine hearts (Pullman and Monroy, 1963), homologues of IF_1 have been isolated from other mammals (e.g., rat and human), yeasts and plants (Faccenda et al., 2013). IF_1 interacts with the catalytic subunit of the F_1Fo-ATP synthase, inhibiting the hydrolysis of ATP under conditions that favour the reversion of the enzyme activity (Faccenda et al., 2013). This regulatory protein is therefore an indispensable component to protect cells from ATP depletion-driven damage and demise (Campanella et al., 2008, 2009a, b). Thus IF_1 completely inhibits, through a non-competitive mechanism, the ATP hydrolysing activity of the F_1Fo-ATP synthase without affecting the synthesis of ATP during oxidative phosphorylation; nevertheless, IF_1 is reported to be largely active only at low pH (Panchenko and Vinogradov, 1985) hence in conditions of ATP hydrolysis. This is important for maintaining cellular ATP by preventing its hydrolysis, when the H^+ electrochemical gradient across the mitochondrial inner membrane is lost (e.g., during hypoxic/ischaemic conditions) and the enzyme reverses its activity to transiently restore $\Delta\Psi_m$ (Campanella et al., 2008). Besides avoiding the consumption of ATP it promotes the dissipation of the $\Delta\Psi_m$ within these organelles facilitating the recruitment of Parkin (Lefebvre et al., 2013).

Quite notably, mitochondria of mouse embryonic fibroblasts (MEFs) knocked out for TG2 display scarce inclination to mitophagy, lower $\Delta\Psi_m$ and are devoid of IF_1 (Rossin et al., 2014). The most recent data in our hands also demonstrate that TG2 KO cells present a mitochondrial phenotype that is aligned with the reversion of the F_1Fo-ATP synthase and disruption of the enzyme function (Fig. 3A) underlying a pathological accumulation of reactive oxygen species (ROS) (Fig. 3B): evidence that fits well with the increased glycolytic metabolism registered in TG2 KO cells (Rossin et al., 2014). In the same way, IF_1 is also absent in MEF cells in which mutated isoforms of the Huntingtin are expressed (Fig. 3C). This is an important finding as highlights a consistent outcome on mitochondrial physiopathology, read by IF_1 deletion, in conditions such as HD cellular models in which TG2 physiology/expression is altered. All this makes us speculate that TG2 and Atpif1 may be opposite but interconnected checkpoints of mitochondrial homeostasis regulation and therefore sustains a novel function for TG2 that has direct impact on the maintenance of the intact mitochondrial respiratory function and quality control dependent on the efficiency of the F_1Fo-ATP synthase.

7. Concluding remarks

The scientific evidences here present a flag that TG2 sets the modulation of mitochondrial efficiency as one of its functions beyond the catalysis of post-translational modifications of proteins. Core aspects of the mitochondrial wellbeing such as i) ADP/ATP exchange activity, ii) F_1Fo-ATP synthase molecular regulation and ultimately iii) the autophagy regulation of its quality are indeed all undermined by alterations in TG2 expression or mutations in its activity.

Interestingly, TG2 seems to impinge on mitochondria by playing a dual role that occurs both inside and outside the organelle by modulating the essential bio-energetic adaptation of the organelle as well as the post-translational modifications of proteins key to the autophagy clearance exploited by mitophagy. The hierarchy of the mitochondrial events combined with TG2's lost efficiency is far from being understood and we picture this as the far-reaching and ambitious goal we shall face in the years to come.

What is instead well known is the physiological outcome for the TG2 deregulation with regard to the onset of neurodegenerative conditions and specifically that of thein Huntington's disease (HD) that presents a core-binding link with TG2 and consequently the abnormal protein accumulation with which the disease is associated. In HD models we indeed envisage the paradigm pathology via which a three-way interplay between TG2, HD and mitochondria takes place opening up to a novel scenario to score and target the pathology.

8. Materials and methods

8.1. Quantitative imaging of $\Delta\Psi_m$ and ROS

These analyses were performed as described in (Campanella et al., 2008, 2009a, 2009b). In brief, cells were loaded with 50 nM tetramethyl rhodamine methyl ester (TMRM) (Sigma-Aldrich, T5428) and 10 μM Verapamil HCl (Sigma, V4629) (which is required to inhibit TMRM export from the cells on the multidrug transporter) in HEPES-buffered salt solution (156 mM NaCl; 3 mM KCl; 2 mM $MgSO_4$ (M2643); 2 mM $CaCl_2$; 10 mM glucose; 10 mM HEPES, all from Sigma-Aldrich, UK). TMRM accumulates in mitochondria, and its signal intensity is a

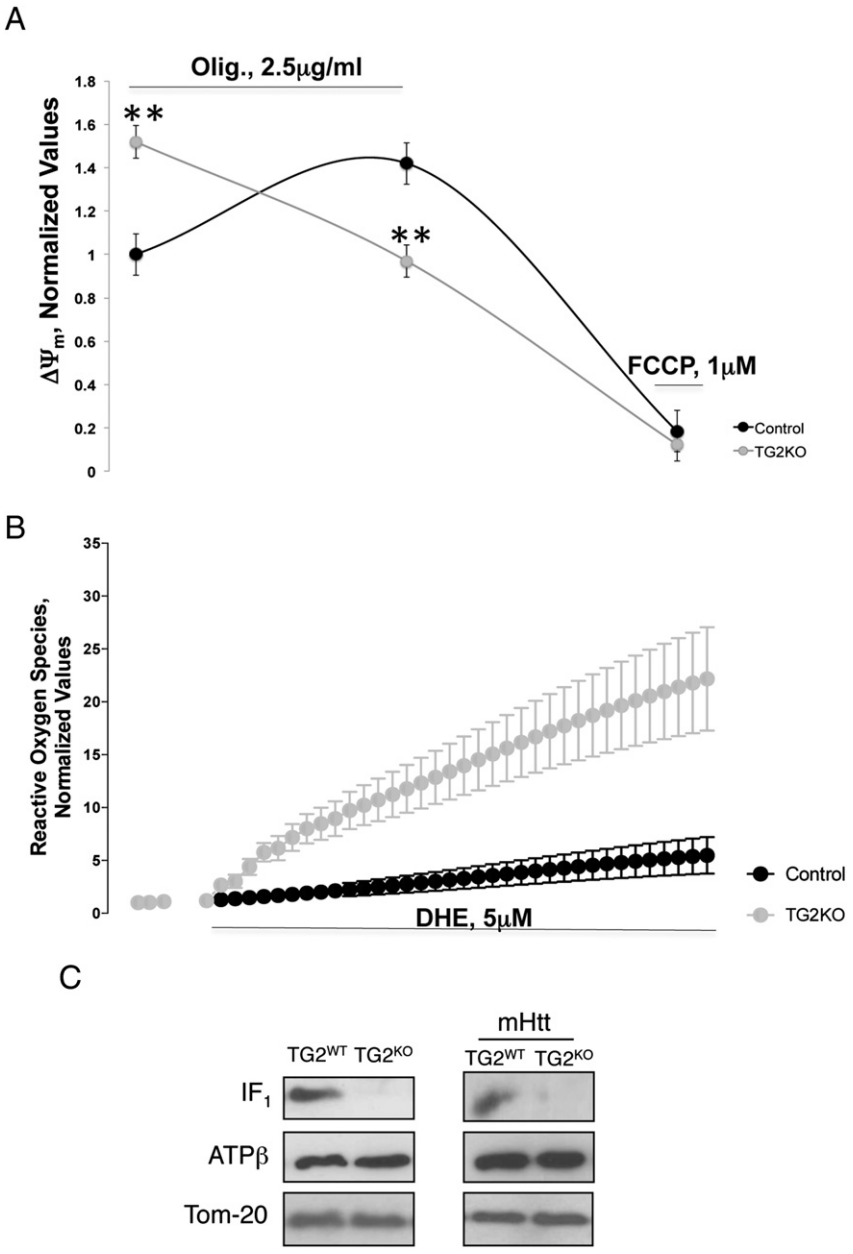

Fig. 3. A) Data points of ΔΨm in WT and TG2 KO MEFs in response to the pharmacological inhibition of the F1Fo-ATP synthase with Oligomycin (2.5 μg/ml) (n = 4). The lack of TG2 in KO MEFs correlates with the functional analysis of F1Fo-ATP synthase that is indeed acting in reverse. In fact, the reversion of F1Fo-ATP synthase is unmasked by oligomycin that, by dropping the $\Delta\psi_m$, highlights an inverse way of rotating the enzyme. B) Acute accumulation of reactive oxygen species in the cytosolic environments in the TG2 KO cells compared to the WT cohort of MEFs (n = 2) thus outlining a post-mitochondrial outcome on cell signalling, metabolism recorded in TG2 KO MEFs. C) Representative Western blot of IF_1 protein. Protein levels in the mitochondrial fraction of WT and TG2 KO MEFs (left) and in the same cells expressing the mutated htt protein (right). Tom-20 and ATPβ were used as loading control (n = 3).

function of membrane potential. Cells were allowed to equilibrate the dye for at least 30 min at room temperature before they were transferred to a Zeiss LSM 510 confocal microscope (40× objective) for imaging. Fields of transfected cells were selected before time series imaging. After several minutes of continuous recording at basal conditions, 2.5 mg/ml Oligomycin was added to block the F_1Fo-ATP synthase and 20 μM FCCP at the end of the experiment to induce depolarisation. Settings were kept constant between experiments. Mitochondrial regions of interest (ROIs) were demarcated and the corresponding TMRM fluorescence intensities calculated. Dihydroethidium (DHE) is sensitive to O_2^- (superoxide) and may be oxidised to the red fluorescent molecule, ethidium. Once oxidised, ethidium binds to DNA, which results in the amplification of the red signal, within the nucleus. The rate at which the signal increases in intensity is dependent on cytosolic levels of O_2^-, thereby enabling the quantification of ROS levels within the cell. Cells were transferred to the Zeiss LSM 510 confocal microscope and 5 μM DHE (Life Technologies, D11347), diluted in RM, was added and the increase in fluorescence intensity was measured through continuous recording for at least 10 min.

8.2. Gel electrophoresis and immunoblotting analyses

Sample proteins were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific 23227). Equal amounts of protein (10 μg for mitochondrial fractions) were resolved on Any kD TGX™ (Bio-Rad, Hemel Hempstead, UK), 10 or 12% polyacrilamide gels and transferred to nitrocellulose membranes. The membranes were blocked in 2% non-fat dry milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h then incubated with the appropriate diluted primary antibody at 4 °C overnight. Membranes were washed in TBST

(3 × 15 min at RT) and then incubated with corresponding peroxidase-conjugated secondary antibodies for 1 h at RT. After further washing in TBST, blots were developed using an ECL Plus Western blotting detection kit (Amersham, RPN2133). Immunoreactive bands were analysed by performing densitometry with ImageJ software (NIH).

8.3. Measure of mitochondrial respiration

Rate of oxygen consumption (OCR) was measured using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). Primary mouse embryonic fibroblasts were seeded at a density of 60,000 cells/well and analysed after 24 h. Optimal cell densities were determined experimentally to ensure proportional oxygen consumption with cell number in response to FCCP (data not shown). The system also allows the injection of up to four drugs during the experimental run and monitoring of their effects over time. According to the standard protocol,we injected in the following order (final concentrations are indicated): 1 μM oligomycin (ATP-synthase inhibitor); 0.4 μM carbonyl, cyanide-4-(trifluoromethoxy) phenylhydrazone (or FCCP, oxidative phosphorylation uncoupler); 0.5 μM rotenone (complex I inhibitor) and 1 μM antimycin A (complex III inhibitor). Five measurements were taken for both OCR at baseline and four measurements after each toxin injection. The cumulative OCR was calculated at baseline and over time after each drug injection. The values of OCR at any time point was calculated as the average of multiple measurements for the same cell line (n = 5). No cell death was observed throughout the experiment.

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References

Arrasate, M., Mitra, S., Schweitzer, E.S., Segal, M.R., Finkbeiner, S., 2004. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431, 805–810.

Badarau, E., Collighan, R.J., Griffin, M., 2013. Recent advances in the development of tissue transglutaminase (TG2) inhibitors. Amino Acids 44, 119–127.

Bae, B.I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S.D., Moran, T.H., Montell, C., Ross, C.A., Snyder, S.H., Sawa, A., 2005. p53 Mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. Neuron 47, 29–41.

Battaglia, G., Farrace, M.G., Mastroberardino, P.G., Viti, I., Fimia, G.M., Van Beeumen, J., Devreese, B., Melino, G., Molinaro, G., Busceti, C.L., Biagioni, F., Nicoletti, F., Piacentini, M., 2007. Transglutaminase 2 ablation leads to defective function of mitochondrial respiratory complex I affecting neuronal vulnerability in experimental models of extrapyramidal disorders. J. Neurochem. 100, 36–49.

Beninati, S., Piacentini, M., 2004. The transglutaminase family: an overview: minireview article. Amino Acids 26, 367–372.

Bernassola, F., Federici, M., Corazzari, M., Terrinoni, A., Hribal, M.L., De Laurenzi, V., Ranalli, M., Massa, O., Sesti, G., McLean, W.H., Citro, G., Barbetti, F., Melino, G., 2002. Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient. FASEB J. 16, 1371–1378.

Browne, S.E., 2008. Mitochondria and Huntington's disease pathogenesis: insight from genetic and chemical models. Ann. N. Y. Acad. Sci. 1147, 358–382.

Campanella, M., Casswell, E., Chong, S., Farah, Z., Wieckowski, M.R., Abramov, A.Y., Tinker, A., Duchen, M.R., 2008. Regulation of mitochondrial structure and function by the F1 Fo-ATPase inhibitor protein, IF1. Cell Metab. 1, 13–25.

Campanella, M., Parker, N., Tan, C.H., Hall, A.M., Duchen, M.R., 2009a. IF1: setting the pace of the F1 Fo-ATP synthase. Trends Biochem. Sci. 7, 343–350.

Campanella, M., Seraphim, A., Abeti, R., Casswell, E., Echave, P., Duchen, M.R., 2009b. IF1, the endogenous regulator of the F1 Fo-ATPsynthase, defines mitochondrial volume fraction in HeLa cells by regulating autophagy. Biochim. Biophys. Acta 5, 393–401.

Caraceni, T., Calderini, G., Consolazione, A., Riva, E., Algeri, S., Girotti, F., Spreafico, R., Branciforti, A., Dall'olio, A., Morselli, P.L., 1977. Biochemical aspects of Huntington's chorea. J. Neurol. Neurosurg. Psychiatry 40, 581–587.

Cattaneo, E., Zuccato, C., Tartari, M., 2005. Normal huntingtin function: an alternative approach to Huntington's disease. Nat. Rev. Neurosci. 6, 919–930.

Chen, Y., Klionsky, D.J., 2011. The regulation of autophagy — unanswered questions. J. Cell Sci. 124, 161–170.

Chen, S., Ferrone, F.A., Wetzel, R., 2002. Huntington's disease age-of-onset linked to polyglutamine aggregation nucleation. Proc. Natl. Acad. Sci. U. S. A. 99, 11884–11889.

D'Eletto, M., Farrace, M.G., Falasca, L., Reali, V., Oliverio, S., Melino, G., Griffin, M., Fimia, G. M., Piacentini, M., 2009. Transglutaminase 2 is involved in autophagosome maturation. Autophagy 5, 1145–1154.

D'Eletto, M., Farrace, M.G., Rossin, F., Strappazzon, F., Giacomo, G.D., Cecconi, F., Melino, G., Sepe, S., Moreno, S., Fimia, G.M., Falasca, L., Nardacci, R., Piacentini, M., 2012. Type 2 transglutaminase is involved in the autophagy-dependent clearance of ubiquitinated proteins. Cell Death Differ. 19, 1228–1238.

East, D., Campanella, M., 2013. Ca2+ in quality control: an unresolved riddle critical to autophagy and mitophagy. Autophagy 9, 1710–1719.

Egan, D.F., Shackelford, D.B., Mihaylova, M.M., Gelino, S., Kohnz, R.A., Mair, W., Vasquez, D. S., Joshi, A., Gwinn, D.M., Taylor, R., Asara, J.M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., Hansen, M., Shaw, R.J., 2011. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456–461.

Faccenda, D., Tan, C.H., Seraphim, A., Duchen, M.R., Campanella, M., 2013. IF1 limits the apoptotic-signalling cascade by preventing mitochondrial remodelling. Cell Death Differ. 20, 686–697.

Fesus, L., Piacentini, M., 2002. Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem. Sci. 27, 534–539.

Fujimoto, S., Nabe, K., Takehiro, M., Shimodahira, M., Kajikawa, M., Takeda, T., Mukai, E., Inagaki, N., Seino, Y., 2007. Impaired metabolism-secretion coupling in pancreatic beta-cells: role of determinants of mitochondrial ATP production. Diabetes Res. Clin. Pract. 77.

Grosso, H., Mouradian, M.M., 2012. Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications. Pharmacol. Ther. 133, 392–410.

Grosso, H., Woo, J.M., Lee, K.W., Im, J.Y., Masliah, E., Junn, E., Mouradian, M.M., 2014. Transglutaminase 2 exacerbates α-synuclein toxicity in mice and yeast. FASEB J. pii: fj.14-251413.

Gundemir, S., Johnson, G.V., 2009. Intracellular localization and conformational state of transglutaminase 2: implications for cell death. PLoS One 4.

Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y., Saito, Y., 2003. A novel function of tissue-type transglutaminase: protein disulphide isomerase. Biochem. J. 373, 793–803.

Hideshima, T., Bradner, J.E., Chauhan, D., Anderson, K.C., 2005. Intracellular protein degradation and its therapeutic implications. Clin. Cancer Res. 11, 8530–8533.

Kim, S.Y., Grant, P., Lee, J.H., Pant, H.C., Steinert, P.M., 1999. Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in Alzheimer's disease. J. Biol. Chem. 274, 30715–30721.

Kim, S.Y., Marekov, L., Bubber, P., Browne, S.E., Stavrovskaya, I., Lee, J., Steinert, P.M., Blass, J.P., Beal, M.F., Gibson, G.E., Cooper, A.J., 2005. Mitochondrial aconitase is a transglutaminase 2 substrate: transglutamination is a probable mechanism contributing to high-molecular-weight aggregates of aconitase and loss of aconitase activity in Huntington disease brain. Neurochem. Res. 30, 1245–1255.

Kim, I., Rodriguez-Enriquez, S., Lemasters, J.J., 2007. Selective degradation of mitochondria by mitophagy. Arch. Biochem. Biophys. 462, 245–253.

Kim, J., Moody, J.P., Edgerly, C.K., Bordiuk, O.L., Cormier, K., Smith, K., Beal, M.F., Ferrante, R.J., 2010. Mitochondrial loss, dysfunction and altered dynamics in Huntington's disease. Hum. Mol. Genet. 19, 3919–3935.

Klöck, C., Diraimondo, T.R., Khosla, C., 2012. Role of transglutaminase 2 in celiac disease pathogenesis. Semin. Immunopathol. 34, 513–522.

Kubli, D.A., Gustafsson, Å.B., 2012. Mitochondria and mitophagy: the yin and yang of cell death control. Circ. Res. 111, 1208–1221.

Lefebvre, V., Du, Q., Baird, S., Ng, A.C., Nascimento, M., Campanella, M., McBride, H.M., Screaton, R.A., 2013. Genome-wide RNAi screen identifies ATPase inhibitory factor 1 (ATPIF1) as essential for PARK2 recruitment and mitophagy. Autophagy 11, 1770–1779.

Lesort, M., Tucholski, J., Zhang, J., Johnson, G.V., 2000. Impaired mitochondrial function results in increased tissue transglutaminase activity in situ. J. Neurochem. 75, 1951–1961.

Levine, B., Mizushima, N., Virgin, H.W., 2011. Autophagy in immunity and inflammation. Nature 469, 323–335.

Liu, Y., Chen, X.J., 2013. Adenine nucleotide translocase, mitochondrial stress, and degenerative cell death. Oxid. Med. Cell Longev. 146860.

Lorand, L., Graham, R.M., 2003. Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat. Rev. Mol. Cell Biol. 4, 140–157.

Malorni, W., Farrace, M.G., Rodolfo, C., Piacentini, M., 2008. Type 2 transglutaminase in neurodegenerative diseases: the mitochondrial connection. Curr. Pharm. Des. 14, 278–288.

Malorni, W., Farrace, M.G., Matarrese, P., Tinari, A., Ciarlo, L., Mousavi-Shafaei, P., D'Eletto, M., Di Giacomo, G., Melino, G., Palmieri, L., Rodolfo, C., Piacentini, M., 2009. The

adenine nucleotide translocator 1 acts as a type 2 transglutaminase substrate: implications for mitochondrial-dependent apoptosis. Cell Death Differ. 16, 1480–1492.
Martinez-Vicente, M., Cuervo, A.M., 2007. Autophagy and neurodegeneration: when the cleaning crew goes on strike. Lancet Neurol. 6, 352–361.
Mastroberardino, P.G., Piacentini, M., 2010. Type 2 transglutaminase in Huntington's disease: a double-edged sword with clinical potential. J. Intern. Med. 268, 419–431.
Mastroberardino, P.G., Farrace, M.G., Viti, I., Pavone, F., Fimia, G.M., Melino, G., Rodolfo, C., Piacentini, M., 2006. "Tissue" transglutaminase contributes to the formation of disulphide bridges in proteins of mitochondrial respiratory complexes. Biochim. Biophys. Acta 1757, 1357–1365.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.
Narendra, D., Tanaka, A., Suen, D.F., Youle, R.J., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell Biol. 183, 795–803.
Nurminskaya, M.V., Belkin, A.M., 2012. Cellular functions of tissue transglutaminase. Int. Rev. Cell Mol. Biol. 294, 1–97.
Oono, M., Okado-Matsumoto, A., Shodai, A., Ido, A., Ohta, Y., Abe, K., Ayaki, T., Ito, H., Takahashi, R., Taniguchi, N., Urushitani, M., 2014. Transglutaminase 2 accelerates neuroinflammation in amyotrophic lateral sclerosis through interaction with misfolded superoxide dismutase 1. J. Neurochem. 128, 403–418.
Panchenko, M.V., Vinogradov, A.D., 1985. Interaction between the mitochondrial ATP synthetase and ATPase inhibitor protein: active/inactive slow pH-dependent transitions of the inhibitor protein. FEBS Lett. 2, 226–230.
Park, D., Choi, S.S., Ha, K.S., 2010. Transglutaminase 2: a multi-functional protein in multiple subcellular compartments. Amino Acids 39, 619–631.
Piacentini, M., Farrace, M.G., Piredda, L., Matarrese, P., Ciccosanti, F., Falasca, L., Rodolfo, C., Giammarioli, A.M., Verderio, E., Griffin, M., Malorni, W., 2002. Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress. J. Neurochem. 81, 1061–1072.
Pullman, M.E., Monroy, G.C., 1963. A naturally occurring inhibitor of mitochondrial adenosine triphosphatase. J. Biol. Chem. 238, 3762–3769.
Quintanilla, R.A., Johnson, G.V., 2009. Role of mitochondrial dysfunction in the pathogenesis of Huntington's disease. Brain Res. Bull. 80, 242–247.
Ricotta, M., Iannuzzi, M., Vivo, G.D., Gentile, V., 2010. Physio-pathological roles of transglutaminase-catalyzed reactions. World J. Biol. Chem. 1, 181–187.
Rossin, F., D'Eletto, M., Macdonald, D., Farrace, M.G., Piacentini, M., 2011. TG2 transamidating activity acts as a rheostat controlling the interplay between apoptosis and autophagy. Amino Acids 42, 1793–1802.
Rossin, F., D'Eletto, M., Falasca, L., Sepe, S., Cocco, S., Fimia, G.M., Campanella, M., Mastroberardino, P.G., Farrace, M.G., Piacentini, M., 2014. Transglutaminase 2 ablation leads to mitophagy impairment associated with a metabolic shift towards aerobic glycolysis. Cell Death Differ. http://dx.doi.org/10.1038/cdd.2014.106.
Rubinsztein, D.C., Carmichael, J., 2003. Huntington's disease: molecular basis of neurodegeneration. Expert Rev. Mol. Med. 5, 1–21.
Sarang, Z., Tóth, B., Balajthy, Z., Köröskényi, K., Garabuczi, E., Fésüs, L., Szondy, Z., 2009. Some lessons from the tissue transglutaminase knockout mouse. Amino Acids 36, 625–631.
Scott, S.V., Cassidy-Stone, A., Meeusen, S.L., Nunnari, J., 2003. Staying in aerobic shape: how the structural integrity of mitochondria and mitochondrial DNA is maintained. Curr. Opin. Cell Biol. 15, 482–488.
Su, B., Wang, X., Zheng, L., Perry, G., Smith, M.A., Zhu, X., 2010. Abnormal mitochondrial dynamics and neurodegenerative diseases. Biochim. Biophys. Acta 1802, 135–142.
Szondy, Z., Mastroberardino, P.G., Váradi, J., Farrace, M.G., Nagy, N., Bak, I., Viti, I., Wieckowski, M.R., Melino, G., Rizzuto, R., Tósaki, A., Fesus, L., Piacentini, M., 2006. Tissue transglutaminase (TG2) protects cardiomyocytes against ischemia/reperfusion injury by regulating ATP synthesis. Cell Death Differ. 13, 1827–1829.
Twig, G., Elorza, A., Molina, A.J., Mohamed, H., Wikstrom, J.D., Walzer, G., Stiles, L., Haigh, S. E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B.F., Yuan, J., Deeney, J.T., Corkey, B.E., Shirihai, O.S., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27, 433–446.
Vartiainen, S., Chen, S., George, J., Tuomela, T., Luoto, K.R., O'Dell, K.M., Jacobs, H.T., 2014. Phenotypic rescue of a *Drosophila* model of mitochondrial ANT1 disease. Dis. Model Mech. 7, 635–648.
Wang, H., Lim, P.J., Karbowski, M., Monteiro, M.J., 2009. Effects of overexpression of huntingtin proteins on mitochondrial integrity. Hum. Mol. Genet. 18, 737–752.
Zemskov, E.A., Janiak, A., Hang, J., Waghray, A., Belkin, A.M., 2006. The role of tissue transglutaminase in cell–matrix interactions. Front. Biosci. 11, 1057–1076.
Zhang, C., Yuan, X.R., Li, H.Y., Zhao, Z.J., Liao, Y.W., Wang, X.Y., Su, J., Sang, S.S., Liu, Q., 2014. Downregulation of dynamin-related protein 1 attenuates glutamate-induced excitotoxicity via regulating mitochondrial function in a calcium dependent manner in HT22 cells. Biochem. Biophys. 443, 138–143.