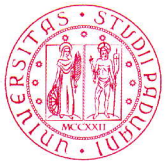

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NUTRIENT DEPENDENT CONTROL OF MITOCHONDRIAL Ca^{2+} SIGNALING

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1. Abstract

Eukaryotic cells are able to continuously adapt to fluctuations in external conditions. Furthermore, when metabolites availability varies, cells undergo rapid changes in order to adapt their metabolism and protect themselves against potential damages. These rapid changes are regulated through different nutrient dependent pathways. The most important proteins, known so far, involved in these pathways are AMPK and Sirtuins. These proteins, that have a key role in the cells response to caloric stress, are activated when the cells are under nutrient deprivation (Dilova et al. 2007).

Ca^{2+} is a fundamental second messenger that enters the cytosol upon the opening of a variety of plasma membrane and endoplasmic/sarcoplasmic reticulum (ER/SR) channels and controls numerous cell functions also at the mitochondrial site (Rizzuto and Pozzan 2006). Foskett'group recently identified a new role of constitutive Ca^{2+} transfer from ER to mitochondria. They demonstrated that this represents a crucial intracellular signal for AMPK activation and autophagy induction. On the other hand still unknown are the precise physiological signals inside the cell that can translate fluctuation of metabolites concentration into a specific regulation of mitochondrial Ca^{2+} content (Cardenas et al. 2010).

During my PhD, I measured mitochondrial Ca^{2+} uptake using targeted recombinant aequorin (Pinton et al. 2007). I found that in HeLa cells, after 2 hours of glucose deprivation, mitochondrial Ca^{2+} uptake is drastically reduced. This physiological response appears to be transient and reversible. Indeed, after glucose deprivation, cells show a reduced mitochondrial Ca^{2+} uptake up to 4 hours, but after this period it returns to the levels measured in normal feeding conditions. I also investigated the possible involvement of a newly identified regulator of mitochondria Ca^{2+} uptake, MICU1, and we found that after 2 hours of glucose deprivation this regulator is quickly degraded. Based on its short half-life, we wondered whether during glucose deprivation MICU1 could be ubiquitylated and rapidly degraded. I also found that the

proteasome inhibitor MG132 inhibits MICU1 degradation during glucose deprivation and it also increases MICU1 half-life. High-resolution mass spectrometry data reveal five lysines in MICU1 protein sequence that are reported to be ubiquitylated. Thus, I decided to substitute each one of these lysines with one arginine (K>R) in order to generate a MICU1 ubiquitylation incompetent mutant ($MICU1^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$). Importantly, I found that the overexpression of $MICU1^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$ partially abolishes the effect of glucose deprivation on mitochondrial Ca^{2+} uptake. Further experiments will allow us to understand how MICU1 influences the modulation of the activity of mitochondrial Ca^{2+} transport system. The analysis of this mechanism will allow us to understand if mitochondria can be the link that directly connects glucose availability with the modulation of physio-pathological processes such as autophagy and apoptosis.

2. Riassunto

Le cellule eucariotiche hanno la necessità di adattarsi a cambiamenti nella disponibilità di metaboliti. Quando i livelli di nutrienti cambiano, il metabolismo cellulare si adatta rapidamente per proteggere la cellula stessa da eventuali danni. Questi rapidi cambiamenti sono regolati attraverso proteine che sono sensibili alla disponibilità di metaboliti. Le più importanti proteine coinvolte in questa risposta sono AMPK e le sirtuine (Dilova et al. 2007).

Il Ca^{2+} è un secondo messaggero fondamentale che controlla numerose funzioni cellulari e il mitocondrio è uno degli organelli più importanti nel mantenimento dell'omeostasi del Ca^{2+} intracellulare (Rizzuto and Pozzan 2006). Recentemente, il gruppo di ricerca di Foskett ha identificato un nuovo ruolo per il trasferimento di Ca^{2+} che normalmente avviene dal reticolo endoplasmatico/sarcoplasmatico (ER/SR) ai mitocondri. Hanno quindi dimostrato che il Ca^{2+} trasferito è un segnale fondamentale per l'attivazione intracellulare di AMPK e per l'induzione di una risposta adattativa alla mancanza di nutrimenti qual è l'autofagia. Rimane ancora sconosciuto il segnale fisiologico all'interno della cellula che converte cambiamenti nella disponibilità di nutrimenti con variazioni nell'ampiezza dei transienti Ca^{2+} mitocondriali.

Durante il mio dottorato di ricerca ho utilizzato l'equorina come sonda per misurare il Ca^{2+} all'interno dei vari comparti intracellulari (Pinton et al. 2007). Le nostre ricerche hanno dimostrato che cellule HeLa, private per due ore di un metabolita fondamentale qual è il glucosio, presentano transienti Ca^{2+} mitocondriali drasticamente ridotti. Misurare anche altri parametri mitocondriali ci ha fatto capire che questa risposta è fisiologica e reversibile e che avviene in molti tipi cellulari diversi.

Inoltre ho indagato il ruolo di MICU1, un regolatore dei livelli di Ca^{2+} mitocondriale recentemente identificato, quale modulatore dei transienti Ca^{2+} mitocondriali durante l'assenza di glucosio. I nostri esperimenti dimostrano chiaramente come, dopo 2 ore di deprivazione del glucosio dal mezzo di coltura, questo fondamentale regolatore risulta essere rapidamente

degradato. Mi sono quindi chiesta, vista la sorprendentemente breve semi-vita di MICU1, se durante la deprivazione di glucosio MICU1 potesse essere ubiquitinato e rapidamente degradato. A supporto di questa ipotesi, ho dimostrato che il trattamento delle cellule con l'inibitore del proteasoma MG132 inibisce la degradazione di MICU1 e ne aumenta la semi-vita. Inoltre, dati pubblicati di spettrometria di massa hanno rivelato cinque lisine nella sequenza proteica di MICU1 che sono predette essere ubiquitinate. Abbiamo quindi deciso di sostituire ognuna di queste lisine con arginine (K>R) in modo da generare un mutante incompetente per l'ubiquitinizzazione ($MICU1^{K102R, K103R, K104R, K296R, K359R}$). La sovraespressione di questo mutante in cellule HeLa abolisce parzialmente l'effetto della deprivazione del glucosio sull'entrata di Ca^{2+} mitocondriale.

Esperimenti futuri ci permetteranno di capire come MICU1 influenzi la modulazione dell'attività del trasporto di Ca^{2+} a livello mitocondriale. L'analisi di questo meccanismo ci permetterà di comprendere se il mitocondrio rappresenti un anello di congiunzione tra la disponibilità di glucosio e la modulazione di processi fisiopatologici quali l'autofagia e l'apoptosi.

3. Introduction

3.1 Metabolism and metabolites

Metabolism is the set of life-sustaining chemical reactions, precisely catalyzed by enzymes, present in all living organisms in order to provide energy for growth, reproduction and adaptation to the environment. Metabolism is usually a balance between anabolic and catabolic processes. Catabolism breaks down organic molecules in order to provide energy whereas anabolism uses this energy to build cellular components such as proteins and nucleic acids.

The equilibrium between anabolism and catabolism is maintained by precise pathways in order to maintain intracellular homeostasis. These reactions are organized and linked to each others through specific metabolic pathways (Spirin et al. 2006), in which one metabolite is transformed through a series of steps into another metabolite by a sequence of enzymes.

Metabolites are the intermediates and products of metabolism (Schmidt et al. 2003). They are low molecular weight organic molecules. They have an important function: indeed, they are fuel to provide energy to the organism. Discoveries in biochemistry during the late nineteenth and twentieth centuries have provided a complex series of interactions between these metabolites (Rennie 1999). It is thus very difficult to assess the contribution of single metabolites to intracellular homeostasis. Object of intense investigations were the most known metabolites like glucose, amino acids and growth factors.

Glucose is a simple monosaccharide that it is used as primary source of energy (Lienhard et al. 1992), representing the main fuel for cellular energy production. The fate of this simple monosaccharide is through three different ways. It can be stored; it can be converted through glycolysis into pyruvate in order to provide ATP and metabolic intermediates (Boiteux and Hess 1981); it can be also oxidized through the pentose phosphate pathway to ribose 5-phosphate, a key step for acid nucleic synthesis.

Amino acids are made from amine (-NH_2) and carboxylic acid (-COOH) functional groups, along with side-chains specific to each amino acid. About 500 amino acids are known. Approximately half are essential in humans, as we cannot manufacture them ourselves. 22 are the most important which are combined into peptide chains to build blocks of a vast array of proteins (McNurlan 2012). When taken up into the human body from the diet, the 22 standard amino acids are either used to synthesize proteins and other biomolecules or they are oxidized to urea and carbon dioxide as a source of energy. Since amino acids are required for protein biosynthesis, it is logical that amino acids should control components involved in the regulation of protein synthesis (mRNA translation). Glucogenic amino acids can also be converted into glucose, through gluconeogenesis. For their biological significance, amino acids are important in nutrition and are commonly used as nutritional supplements.

Growth factors are proteins capable of stimulating cellular growth, proliferation and cellular differentiation (Sporn and Roberts 1988, Frazier et al. 1996). They are very important in the regulation of a variety of cellular processes. They carry out their function during development including regulating tissue morphogenesis, angiogenesis, cell differentiation and neurite outgrowth. They also play important roles in the maintenance of tissue homeostasis and wound healing in the adult (Klenkler and Sheardown 2004). Their activities are mediated via binding transmembrane receptors that often contain cytoplasmic tyrosine kinase domains. When unregulated, many growth factors and their receptors have been implicated in tumor formation.

Metabolites as signaling molecules

The different types of metabolites have a central role in maintaining intracellular balance; indeed, the amount of metabolites reflects the feeding status of the cell and determines which metabolic reactions can occur (Ebenhoh and Heinrich 2001, Kapahi et al. 2010)).

During the last years, many researchers have found a new role for metabolites. They are not only substrates for metabolic pathways, they also act as signaling molecules that regulate and

integrate the metabolic status of the cells with other fundamental cellular events such as cell growth, replication and adaptive response to nutrient withdrawal. Cells thus use metabolites not only as energy source but also like molecules that are able to connect the feeding status of the cells with the ongoing cellular processes. This information is very important, and metabolites can create a link between the changes in nutrient availability and the regulation of different cellular processes.

Nutrient dependent pathways

Metabolites control a large variety of cellular pathways that are called “nutrient dependent pathways”. These pathways trigger specific actions like cell growth, proliferation, differentiation, apoptosis and autophagy that represent all metabolic adaptations. In these pathways, sensor proteins play key roles.

Fundamental sensors of nutrient availability include mTOR, AMPK and Sirtuins. They can detect changes in specific metabolites concentration.

The mammalian Target of Rapamycin (mTOR) is a large polypeptide with several distinct functional domains: a kinase domain, several HEAT repeats, which are likely to be involved in protein-protein interactions, and a domain for Rapamycin binding. mTOR binds several other proteins and forms two major complexes: mTORC1 and mTORC2. The activity of mTORC1 is tuned by amino acids and regulates the translational machinery of mammalian cells (Kapahi et al. 2010). mTORC1 controls the phosphorylation of several components of the translational machinery and also regulates ribosome biogenesis. mTORC2 is an important regulator of the cytoskeleton (Oh and Jacinto 2011). These different complexes act as a critical nodal point in a signaling network that regulates multiple cellular processes and several other inputs in response to nutrient conditions (Proud 2007). The decreased mTOR activity under nutrient-poor conditions results in the removal of nutrient transporter from the cell surface. This loss of external nutrient supply is compensated by increased production of nutrients from intracellular

stores via autophagy. This type of response plays a key role in cell survival under stress conditions (Chang et al. 2009).

Another fundamental sensor of nutrient availability is AMPK that controls a sophisticated pathway acting to maintain and monitor the ratio of ATP/AMP/ADP (Hardie 2003). AMPK is a heterotrimeric protein kinase. The reaction between ADP and ATP is catalyzed by a very active adenylate kinase that converts 2ADP into ATP and AMP. As a consequence, the ratio of AMP/ATP varies as the approximate square of the ADP/ATP ratios. This means that it is sufficient a small decrease in cellular ATP level to result in a large increase in AMP, making it an efficient signaling molecule of the energy status of the cell (Kahn et al. 2005). AMP is an allosteric activator of AMPK by enhancing the phosphorylation of its T-loop by its upstream protein kinase (Hardie 2003). Under glucose starvation, AMPK promotes autophagy by directly activating Ulk1 through phosphorylation of its Ser317 and Ser777. This phosphorylation can be prevented by nutrient availability through high mTOR activity.

Sirtuins are deacetylases involved in metabolic regulation and longevity (Blander and Guarente 2004, Michishita et al. 2005, Haigis et al. 2006). The activity of Sirtuins is controlled by a tight redox regulation by the [NADH/NAD^+] ratio, which is a major sensor for metabolite availability conserved from invertebrates to vertebrates. Sirtuins have different levels of NAD^+-dependent deacetylase activity (North and Verdin 2004). SIRT1, SIRT2 and SIRT3 have a strong activity, whereas the others are weak in enzymatic activity but highly selective in their targets. Reversible protein acetylation occurs at the ε-amino group of lysine residues and it is one of the most common post-translational modifications that regulate DNA-protein interaction, subcellular localization, protein stability and enzymatic activity during metabolic adaptations. Mitochondrial proteins involved in the regulation of energy metabolism are subject to extensive lysine acetylation. Indeed three sirtuins, SIRT3, SIRT4 and SIRT5 localize to mitochondria. SIRT3 expression is activated during nutrient deprivation (Scher et al. 2007). The increased

expression of SIRT3 help the switch toward fasting metabolism, as tissues move away from glucose instead of lipids and amino acids as source of energy (Nogueiras et al. 2012).

From metabolites to intracellular signaling: the glucose pathway

As mentioned above, glucose is one of the most important metabolites. It is also a crucial primary messenger molecule (Sheen et al. 1999), signalling optimal growth conditions to the cellular machinery. Its intracellular signalling involves specific glucose transporters, the activation of protein kinase C (PKC) and the mitogen-activated kinases (MAPK), with the consequent stimulation of gene expression of specific genes (i.e. TGFβ) (Haneda et al. 2003). Blood glucose levels are maintained constant and independent from food intake, due to the critical importance of this metabolite (Triplitt 2012).

Glucose deregulation is a pathological relevant event. Indeed, diabetes is one of the well-known pathology caused by imbalance of glucose metabolism (Bouche et al. 2004). It affects glucose availability by interfering with signals triggered by two hormones that maintain constant blood glucose level, insulin and glucagon (Triplitt 2012). Understanding the cellular responses to glucose withdrawal could lead to the discovery of new strategies to overcome the diabetes damage. On the other hand, cancer cells base their metabolism on glycolysis in a phenomenon termed “Warburg effect” (Vander Heiden et al. 2009). This metabolic adaptation allows cancer cells to overcome the growth factor dependence that normally controls nutrients intake. Independently from the growth factor stimulation, cancer cells uptake an excess of nutrients, particularly glucose, that exceed the bioenergetics demands (DeBerardinis et al. 2008). This ability enables cancer cells to proliferate and survive in hypoxic areas. The clarification of the link between glucose availability and metabolic adaptation in cancer cells may ultimately lead to more efficient treatments for human cancers.

For many years researchers have focused their studies on trying to uncover the effects of glucose withdrawal on intracellular signalling. Many proteins that are involved in energy and

nutrient sensing, including AMPK and mTORC1, trigger metabolic adaptations like autophagy. The determination of the proteins that control these metabolic adaptations will allow interfering with this process and block the protection against glucose withdrawal-mediated cell death.

Thus, by choosing our model of nutrient deprivation, we decided to focus our studies on the dissection of glucose signalling pathways. Indeed, we decided to completely remove all metabolites and to add only glucose to control cells. In our experimental system, HeLa cells were starved for 2 hours in KRB and control HeLa cells were incubated in KRB with 25mM glucose.

Adaptive response to nutrient withdrawal

For any organism, the most important skill that allows surviving is the ability of sensing the extracellular and the intracellular environment. The signals generated by these stimuli are coded in order to respond appropriately in terms of intracellular modifications. There is a plethora of different types of signaling, but we focused our attention on the metabolic ones that give information about the feeding status of the cells. In this regard, metabolites play a central role because they act as signaling molecules and they create a connection between nutrient availability and key sensors of nutrient dependent pathways that are able to trigger cellular adaptations to the availability of nutrients. Metabolic adaptations are intracellular events that allow cells to maintain minimal cellular metabolism even in the absence of normal nutrients by tuning ongoing chemical reactions.

One of the most important responses to the loss of external nutrient supply is the increased production of nutrients from intracellular stores via autophagy.

3.2 Autophagy

Autophagy, or cellular self-degradation, is a pathway involved in protein and organelle degradation. Christian de Duve firstly described this phenomenon in the late 60's (Deter and De Duve 1967), providing the clear biochemical proof of the involvement of lysosomes in this process. However, a clear molecular description of this cellular event remained unresolved until the isolation of the first autophagy-deficient yeast mutants (Tsukada and Ohsumi 1993) and the consequent genetic dissection of the pool of regulatory genes (the so-called ATG genes), thus ascribing autophagy among the tightly regulated and genetically programmed cellular processes. There are three primary forms of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. CMA is a secondary response to starvation and, unlike the other two processes, involves direct translocation of the targeted proteins across the lysosomal membrane (Massey et al. 2006). Microautophagy is the least-characterized process but is used to sequester cytoplasm by invagination and/or septation of the lysosomal/vacuolar membrane (Wang and Klionsky 2003). By contrast, the most prevalent form, macroautophagy, involves the formation of cytosolic double-membrane vesicles that sequester portions of the cytoplasm (Klionsky and Ohsumi 1999). During macroautophagy, the sequestering vesicles, termed autophagosomes, are not derived from the lysosome/vacuole membrane. Fusion of the completed autophagosome with the lysosome or vacuole results in the delivery of an inner vesicle (autophagic body) into the lumen of the degradative compartment. Subsequent breakdown of the vesicle membrane allows the degradation of its cargo and eventually the recycling of the amino acids and other nutrients. Although autophagy and autophagy-related processes are highly dynamic, they can be divided into several discrete steps for the purpose of discussion: (1) induction; (2) cargo selection and packaging; (3) nucleation of vesicle formation; (4) vesicle expansion and completion; (5) retrieval; (6) targeting, docking and fusion of the completed vesicle to the lysosome and (7) breakdown of the intraluminal vesicle and its cargo and recycling of the macromolecular constituents.

One of the major regulatory component for sensing the extracellular milieu and transducing it in an appropriate signal to sensors that allow the induction of autophagy is mTOR. mTORC1 complex is a critical nutrient sensor and it causes the hyper-phosphorylation of the Atg13 protein (Funakoshi et al. 1997). This modified form of Atg13 shows a lower affinity for its interacting kinase Ulk/Atg1 and this impaired interaction might inhibit autophagy (Kamada et al. 2000). mTORC1 sequesters Ulk/Atg1 in a complex with Atg13 and Fip200 in an inactive state. Inhibition of mTOR through starvation or treatment with rapamycin results in a partial dephosphorylation of Atg13 and allows the induction of autophagy (Noda and Ohsumi 1998). DAP1 usually inhibits macroautophagy preventing abnormal activation of this pathway. DAP1 is usually phosphorylated and inactivated by mTORC1 (Koren et al. 2010). Reduced mTOR activity results also in reduced DAP1 phosphorylation (Singh and Cuervo 2011).

AMPK is another crucial regulatory component for autophagy induction (Kahn et al. 2005). As sensor of ATP/AMP/ADP ratio, this protein can translate the energetic state of the cell into a metabolic adaptation like autophagy. AMPK interacts with Ulk/Atg1 in a nutrient-dependent manner. AMPK phosphorylates Ulk/Atg1 and favors its release from mTORC1 (Singh and Cuervo 2011, Wong et al. 2013).

Once autophagy is initiated, a portion of cytoplasm is randomly sequestered into the autophagosomes, even if some reports show specificity in the cargo selection (Onodera and Ohsumi 2004). The subsequent vesicles nucleation process represents probably the least understood step in autophagy, but likely it originates from a pre-autophagosomal structure (PAS) already present in the cytoplasm. Vesicle expansion and completion require an ubiquitin-like system mediating protein lipidation through the Atg8 protein (also known as LC3) (Ichimura et al. 2000). Only two proteins are known to remain associated with the completed autophagosomes, the specific receptor Atg19 and Atg8; other proteins that are involved in vesicle formation presumably recycle from the PAS or the vesicles during formation, thus enabling the retrieval of autophagy components. Of course, the timing of vesicle fusion with the

lysosome must be tightly regulated. Indeed, if the fusion process begins prior to completion of the double-membrane vesicle, the cargo will remain in the cytosol. However the molecular machinery mediating this complex process still remains in part obscure, even if several members of the SNARE protein family have been demonstrated to be necessary (Darsow et al. 1997). Lastly, the whole process must break down the single-membrane subvacuolar vesicles that result from the fusion of the autophagosome with the lysosome, a step that mainly depends on the acidic pH of the organelle (Nakamura et al. 1997).

Autophagy covers several physiological functions, ranging from a basal housekeeping role to response to metabolic stress and regulation of cell death. Moreover, the relevance of this cellular process at whole organism level is underlined by the observation that the genetic ablation of many Atg genes leads to organism death due to impaired cell differentiation (Sandoval et al. 2008), embryonic lethality or reduction of survival during peri-neonatal starvation (Kuma et al. 2004). The repertoire of routine housekeeping functions performed by autophagy includes the elimination of defective or damaged proteins and organelles, the prevention of abnormal protein aggregate accumulation and the removal of intracellular pathogens (Mizushima and Klionsky 2007). Such functions are critical for autophagy-mediated protection against aging, cancer, neurodegenerative diseases, and infection. Although some of these functions overlap with those of the ubiquitin-proteasome system (the other major cellular proteolytic system) the autophagy pathway is uniquely capable of degrading entire organelles such as mitochondria (in a process called mitophagy), peroxisomes and ER, as well as intact intracellular microorganisms (Kim et al. 2007, Zhang et al. 2007). Furthermore, the relative role of the autophagy-lysosome system in protein quality control may be greater than it was previously thought. Moreover, autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stresses, including nutrient deprivation, growth factor depletion and hypoxia. This bulk form of degradation generates free amino and fatty acids that can be recycled in a cell-autonomous fashion or delivered systemically to distant sites within the

organism. Presumably, the amino acids generated are used for the *de novo* synthesis of proteins that are essential for stress adaptation. It is presumed that the recycling function of autophagy is conserved in mammals and other higher organisms, although direct data proving this concept are lacking. The amino acids liberated from autophagic degradation can be further processed and, together with the fatty acids, used by the tricarboxylic acid cycle (TCA) to maintain cellular ATP production. The importance of autophagy in fueling the TCA cycle is supported by studies showing that the phenotype of autophagy-deficient cells can be reversed by supplying them with a TCA substrate such as pyruvate (or its membrane-permeable derivative methylpyruvate). For example, methylpyruvate can maintain ATP production and survival in growth factor-deprived autophagy-deficient cells that would otherwise quickly die (Lum et al. 2005). Moreover, it can restore ATP production and the generation of engulfment signals in autophagy-deficient cells during embryonic development (Qu et al. 2007).

Ca^{2+} dependent control of autophagy

The first report on Ca^{2+}-dependent regulation of autophagy dates back to 1993 (Gordon et al. 1993), and it suggested a complex role for Ca^{2+}, since chelation of either intra- and extracellular Ca^{2+} as well as elevating cytosolic [Ca^{2+}] ($[Ca^{2+}]_{cyt}$) suppressed autophagy. Only recently this topic returned to be of great interest but the published results are in conflict regarding the role of intracellular Ca^{2+} in autophagy inductions.

Many reports describe Ca^{2+} as an inhibitor of autophagy. They are focused on the inositol 1,4,5-triphosphate (IP_3) receptor (IP_3R), a ubiquitously expressed intracellular Ca^{2+}-releasing channel, located mainly in the endoplasmic reticulum (ER). IP_3Rs mediate Ca^{2+} release from the ER into the cytoplasm in response to elevations in cytoplasmic [IP_3] produced in cells after stimulation for example by hormones, growth factors or antibodies (Berridge 2009). In 2005, Sakar et al. reported the use of Li^+ for autophagy stimulation (Sarkar et al. 2005). Li^+ acts through inhibition of inositol monophosphatase (IMPases), thereby reducing the IP_3 levels. Also

chemical inhibition of IP_3Rs with xestospongin (XeB) or suppression of its expression using siRNA, also induced autophagy in HeLa cells (Criollo et al. 2007). The IP_3R-mediated inhibition of autophagy was also verified in IP_3R triple knock out (TKO) chicken DT40 B lymphocytes, which showed higher autophagy levels (Cardenas et al. 2010, Khan and Joseph 2010). Different downstream mechanism and effectors have been proposed for the inhibitory role of IP_3Rs and Ca^{2+} in autophagy. In a recent study Cardenas and coworkers showed increased glucose and O_2 consumption, pyruvate dehydrogenase and AMPK activation in TKO cells, suggesting a mechanism whereby constitutive Ca^{2+} release through IP_3Rs fuels into the mitochondria, thereby increasing mitochondrial bio-energetics and ATP production (Cardenas et al. 2010). When these essential Ca^{2+} signals are abolished there is an increased AMP/ATP ratio with a consequent AMPK activation and a subsequent stimulation of autophagy. In this study, the authors can not exclude a scaffold function for the IP_3R.

On the other hand, treatments of cells with ER/SR Ca^{2+} ATPase (SERCA) inhibitors resulted in increased autophagy. Of course, prolonged treatment with these agents leads to ER Ca^{2+} depletion and subsequent ER stress, which itself might also be a trigger for autophagy (Hoyer-Hansen and Jaattela 2007).

These models do not necessarily represent conflicting ideas, but may represent different Ca^{2+}-signaling modes that depend on the cellular state of the cells. While spontaneous Ca^{2+} signals may suppress basal autophagy in healthy cells through mitochondrial pathways, stress conditions may promote Ca^{2+} signaling and thus elevate Ca^{2+} in the cytoplasm resulting in autophagy stimulation through cytoplasmic effector molecules (Decuypere et al. 2011). This is especially true for stressors that already induce a Ca^{2+} release into the cytoplasm, like the SERCA inhibitor thapsigargin.

3.3 Ca^{2+} signaling: the general framework

Intracellular signaling requires messengers whose concentration rapidly and efficiently varies with time. One of the most important messengers is Ca^{2+} and cells invest much of their ATP energy to affect changes in [Ca^{2+}]. This high consumption of ATP is balanced by the fact that Ca^{2+} is one of the most important second messenger in the cell and ATP is the energy source to allow modifications in Ca^{2+} concentration ([Ca^{2+}]) in space and time (Clapham 2007). These rapid modifications in intracellular [Ca^{2+}] are required because Ca^{2+} can not be chemically altered and for this reason changes in [Ca^{2+}] require the binding to buffering proteins, the compartmentalization into intracellular stores or the extrusion outside the cell (Berridge 2009).

Ca^{2+} binding triggers changes in protein shape and charge and consequently activates or inhibits protein functions. The best known protein that buffers Ca^{2+} is calmodulin. This buffering protein and others can control the amplitude and the timing of Ca^{2+} signaling (Hoeflich and Ikura 2002).

Ca^{2+} signaling in cells consists in dynamic variations of the cytosolic [Ca^{2+}]. These variations are provoked by fluxes of ions coming from two main sources: the extracellular medium and the internal stores. The most important Ca^{2+} store in the cell is ER/SR, but recent works demonstrated that also other organelles (such as Golgi apparatus, endosome and lysosome (Pinton et al. 1998), (Calcraft et al. 2009)) are able to participate in Ca^{2+} signaling.

The signals that triggers Ca^{2+} changes generate Ca^{2+} waves within the cytoplasm where it can stimulate numerous Ca^{2+} sensitive processes (Hajnoczky et al. 1995).

Most of the cells have a cytosolic [Ca^{2+}] that fluctuates from 0.1 μM of resting condition to 2-3 μM after release from the stores. Cells use different types of mechanisms to access to the different intracellular sources of Ca^{2+}. These pathways are not exclusive and most cells express combination of them. The best known pathway involves the release of IP_3 after stimulation with a hormone, and the consequent release of Ca^{2+} from the ER through the binding to the IP_3R.

Once Ca^{2+} has carried out its signaling functions, it is rapidly extruded from the cytoplasm by various pumps and exchangers and intracellular [Ca^{2+}] returns to resting conditions.

The extrusion from the cells or the compartmentalization of Ca^{2+} is due to the action of ATPase pumps that use ATP like energy sources to maintain intracellular low intracellular [Ca^{2+}] by extruding Ca^{2+} from the cells or into intracellular Ca^{2+} stores. In the plasma membrane there are exchangers that exchange two Na^{+} with one Ca^{2+} and pumps (Ca^{2+}-ATPase PMCA) that use ATP like an energy source to extrude Ca^{2+} out of the cells. Other pumps return back Ca^{2+} into intracellular stores, like the endoplasmic reticulum Ca^{2+}-ATPase SERCA.

Given that the message decoded by Ca^{2+} is given to the cells like an oscillatory difference of [Ca^{2+}], it is simple to understand the high complexity of pumps and channels that, with their activity, modulate the Ca^{2+} message. During the last years, many scientists focused their attention on the identification of all the import/out-port mechanisms for Ca^{2+} signaling, but in spite of this large effort, the whole scenario is not yet complete.

Mitochondria: the basics

ER and mitochondria are the main actors of Ca^{2+} signaling. Importantly, they are not homogenously distributed within the cell. Indeed, they are spatially organized in order to optimize intracellular Ca^{2+} signaling: some mitochondria are located in close proximity of ER-Ca^{2+} source, and for this reason a major portion of the Ca^{2+} released into the cytoplasm is absorbed by mitochondria. On the other hand, ER and mitochondria are strategically located near the Ca^{2+} entry sites in the plasma membrane, where micro-domains of high [Ca^{2+}] are generated (Rizzuto et al. 1993).

The capability of mitochondria to buffer Ca^{2+} was first described by two key experiments that took advantage of the possibility to target genetically encoded proteins into specific intracellular organelles. By targeting Ca^{2+} sensitive photoprotein aequorin into mitochondria, Rizzuto and coworkers demonstrated that a small increase of Ca^{2+} into the cytoplasm was

paralleled by a high increase of Ca^{2+} into mitochondrial matrix (Rizzuto et al. 1992). Furthermore, labeling mitochondria with two different colored green fluorescent proteins (GFPs), allowed demonstrating the existence of small regions (about 20% of mitochondria surface) that are in close proximity to both ER and mitochondria (Rizzuto et al. 1998). The very fine subcellular organization of these organelles into the cytoplasm is due to the fact that Ca^{2+} waves from ER regulates mitochondrial function, movement and viability and mitochondria themselves are able to modulate the cellular response to different stimuli.

Further studies performed by my laboratory clarified the complexity of mitochondria morphology that reflects the multiplicity of roles in the control of the intracellular response to different stimuli. Mitochondria are composed by two membranes, the external one, that is named outer mitochondrial membrane (OMM) that is a highly permeable membrane to ions and small molecules, and the internal one, the inner mitochondrial membrane (IMM) that is ion impermeable. More representative channels in the OMM are VDACs. They cluster at ER/mitochondrial contact sites and they appear limiting for the Ca^{2+} uptake capacity of the organelle (Rapizzi et al. 2002, Tan and Colombini 2007). On the other hand, IMM is an ion impermeable membrane and it was shown that Ca^{2+} uptake machinery presents low affinity for the ion. This low affinity would allow only small increase in mitochondrial [Ca^{2+}], which could not explain the observed rapid and huge increase of Ca^{2+} into mitochondrial matrix after cellular stimulation. This apparent discrepancy was solved by the demonstration that, as mentioned above, mitochondria are strategically located in close proximity of Ca^{2+} release source; this generates micro-domains of high [Ca^{2+}] near the mitochondrial Ca^{2+} uptake channels (Rizzuto et al. 1998, Csordas et al. 1999). On the other hand, mitochondria extrude protons to create the electrochemical gradient that allows ATP synthesis, thus generating a steep driving force for the accumulation of the ion into the organelle.

The chemiosmotic theory of energy transfer was first demonstrated by Mitchell (Mitchell 1967) that showed that the electrochemical gradient across the IMM is utilized by the F1/F0

ATPase to convert the energy of NADH and $FADH_2$, generated by the breaking down of energy rich molecules, such as glucose, into ATP. This gradient is characterized, for the most part, by electrical charge across the membrane ($\Delta\psi$) and, in minor part it is a H^+ concentration difference between the two compartments (ΔpH). This difference of membrane potential generates a huge driving force that allows the passage of cations through the low sensitive Ca^{2+} channels into the matrix. This gradient is normally maintained in the range of -120/-200 mV.

Role of mitochondria

Mitochondria are very important components of intracellular Ca^{2+} signalling. Importantly Ca^{2+} regulates key enzymes of the TCA cycle (McCormack et al. 1990), thus determining the rate of ATP production and in addition it can regulate cellular metabolic adaptation to nutrient levels and it could initiate the apoptosis process (Rasola and Bernardi 2011).

Different [Ca^{2+}] in the mitochondrial matrix regulate aerobic metabolism, tuning mitochondrial ATP production in the needs of a stimulated cell by the control of metabolic enzymes. There are two Kreb cycle's dehydrogenases (isocitrate deidrogenase and αketoglutarato deidrogenase) that are Ca^{2+} sensitive since they directly bind Ca^{2+} and pyruvate dehydrogenase that undergoes a dephosphorylation step that in a Ca^{2+} dependent manner (Melendez-Hevia et al. 1996). Thus, the increase in Ca^{2+} level into the matrix modulates the activity of Kreb cycle's enzymes and therefore the passage of electrons through the respiratory chain with the subsequent generation of the gradient across the IMM that is needed for ATP production.

When Ca^{2+} has carried out its functions in the mitochondria, it is necessary to rapidly extrude it in order to renew the resting balance into mitochondria. Ca^{2+} extrusion is finely regulated by different exchangers. There are two major mechanisms for Ca^{2+} extrusion from mitochondria; they match the exit of Ca^{2+} with the entrance of Na^+ or H^+. Recently, Sekler and

coworkers identified an essential component of Na^+/Ca^{2+} exchanger, inhibited by CGP37157 (Palty et al. 2010).

If this mechanism for the regulation of mitochondria [Ca^{2+}] fails and high levels of Ca^{2+} are reached in the mitochondria, apoptosis is initiated. These observations started from the observation that Bcl-2 has a role in the modulation of Ca^{2+} ions fluxes (Pinton and Rizzuto 2006).

This protein, like other anti-apoptotic proteins, reduces mitochondrial Ca^{2+} response to extracellular stimuli by reducing the ER Ca^{2+} levels. On the other hand, pro-apoptotic proteins exert their effect by increasing mitochondrial sensitivity. Massive Ca^{2+} entry into mitochondria causes PTP opening that leads to modifications in mitochondrial morphology and the release of pro-apoptotic factors, such as cytochrome c, that initiate the complex cascade of apoptosis.

As written above, Ca^{2+} was shown to play a more extended role in cellular bioenergetics, by controlling autophagy, the adaptive response to nutrient deprivation. Constitutive Ca^{2+} transfer from ER to mitochondria is required for autophagy suppression (Cardenas et al. 2010) and its absence induces prosurvival mTOR independent macroautophagy, mediated by the activation of AMPK. It is not yet clear if this effect on autophagy is dependent from the release from IP_3R or on the amount of Ca^{2+} into mitochondria. Indeed, the overexpression and the silencing of the recently identified mitochondrial calcium uniporter (MCU) exert an effect in the AMPK activation and autophagy induction. Furthermore, it is not clear if the activation of AMPK is simply through a metabolic effect on ATP/ADP/AMP ratio or directly on Ca^{2+} levels. This could be a nice example of the activation of AMPK, sensor of nutrient availability, and the consequent induction of a metabolic adaptation like autophagy. But still unknown are the physiological signals that can translate fluctuations in metabolite concentration with a specific regulation of mitochondrial Ca^{2+} content. I focused my PhD research on this aspect of control of metabolism.

The study of the cellular processes mediated by mitochondrial calcium was severely limited by the lack of the molecular identity of the channel responsible of Ca^{2+} entry into the organelle. The electrophysiological properties of this channel, named mitochondrial calcium uniporter (MCU) have been described in details about thirty years ago (Bernardi et al. 1984). Indeed, it was shown its dependence on mitochondrial membrane potential, its sensitivity to ruthenium red and its activity when extra-mitochondrial [Ca^{2+}] are in the μM range. In this seminal study, Clapham and coworkers in 2004 were the first to define that Ca^{2+} uptake was carried out through a channel and not through a carrier. He showed that the current through this channel is inwardly rectifying and gradually increases with the increase of free [Ca^{2+}]. It defined also that there is not a Ca^{2+} dependent inactivation because the amplitude of the current is not altered with different [Ca^{2+}]. Ca^{2+} is the primary charge carrier; indeed the channel binds Ca^{2+} with high affinity (Kd<2 nM) despite the low amount of cytoplasmic [Ca^{2+}] and the abundant cytoplasmic Mg^{2+} and K^{+} ions (Kirichok et al. 2004).

After this seminal work, many groups tried to describe this channel from the molecular point of view. Graier and coworkers proposed in 2007 that uncoupling proteins 2 and 3 (UCP2 and UCP3) mediate mitochondrial calcium uptake (Trenker et al. 2007). This study was controversial because the overexpression of these proteins increased the capacity of mitochondrial Ca^{2+} uptake but they are non ubiquitously expressed in all tissues, as expected from this channel, and UCP2 and UCP3 knockout mice still retain mitochondrial Ca^{2+} uptake.

Two years later, Clapham group's identified by genome-wide RNAi screen in Drosophila, Letm1 as a Ca^{2+}/H^{+} antiporter (Jiang et al. 2009). This identification triggered a long discussion because Letm1 knockdown cells demonstrate a Letm1 independent mitochondrial Ca^{2+} uptake and also Letm1 containing liposomes rapidly accumulate Ca^{2+} in a ruthenium red dependent mode. This antiporter is also sensitive to CGP37157, a non-selective inhibitor of Na^{+}/Ca^{2+} (NCX) and H^{+}/Ca^{2+} (HCX) exchangers. Moreover, these data are in contrast with previous results on K^{+}/H^{+} exchanger (Nowikovsky et al. 2004) (Dimmer et al. 2008).

Identification of the mitochondrial Ca^{2+} uniporter (MCU) and of its crucial regulator Micu1

In the past years, the investigation of the molecular identity of the MCU benefited from the genome-wide approach. In this respect, in a seminal piece of work, Mootha and co-workers reported in 2008 the generation of a mitochondrial "genoteque" (MitoCarta) by performing mass spectrometry analyses on both highly purified and crude mitochondrial preparations from 14 different mouse tissues to discover genuine mitochondrial proteins, validated by GFP tagging (Pagliarini et al. 2008). By considering only the proteins localized into inner mitochondrial membrane, expressed in the majority of mammalian tissue and with homologues in vertebrates and kinetoplastids but not in the yeast, he identified a protein with unknown function, named "mitochondrial calcium uptake 1" (MICU1). This is a mitochondrial EF hand protein required for mitochondrial Ca^{2+} uptake (Perocchi et al. 2010). Its silencing abolishes mitochondrial Ca^{2+} uptake but it was clear that this protein was not the long searched MCU as it presents only one transmembrane domain in the protein sequence. The two canonical EF hands are essential for its activity in Ca^{2+} sensing and gating the activity of MCU. In our laboratory we demonstrated that the overexpression of MICU1 increases mitochondrial Ca^{2+} uptake. Furthermore, we also found that in the presence of μM $[Ca^{2+}]$, MICU1 is able to increase the open probability of the uniporter and thus an increase in overall Ca^{2+} uptake into mitochondria.

Recently, Mallilankaraman K. and coworkers demonstrated that MICU1 is required to preserve normal mitochondrial $[Ca^{2+}]$ under basal conditions. In its absence mitochondria become constitutively loaded with Ca^{2+}, triggering excessive reactive oxygen species generation (Mallilankaraman et al. 2012).

The MitoCarta database and the identification of MICU1 laid the foundation stone for the identification of the MCU. Indeed, our group and Mootha's identified in 2011 a protein CCDC109A, that fulfills all the requirements to be the mitochondrial calcium uniporter (Baughman et al. 2011, De Stefani et al. 2011). This identification has been supported by two

different approaches based both on MitoCarta database. Mootha group compared MICU1 phylogenetic profile with unknown proteins profiles in order to find the channel. We used a strategy based on the well-known properties of MCU. Among 529 candidates with ubiquitous expression in mammalian tissues, we have selected proteins with a structure compatible with a gated ion channel activity (i.e. two transmembrane domains), absent in *Saccaromyces Cervisiae* and significantly conserved also in lower eukaryotes, such as kineetoplastids.

Mootha's laboratory and ours demonstrated that MCU overexpression in HeLa cells strongly increases mitochondrial Ca^{2+} uptake while its silencing by siRNA drastically reduces it. The effects of overexpression are also correlated with MCU physiological role; indeed MCU-expressing cells are more efficiently killed after apoptotic stimuli. Importantly, Mootha's group performs MCU silencing of MCU *in vivo*. They knockdown MCU in mice livers and they found a complete loss of Ca^{2+} uptake in response to extramitochondrial pulses of Ca^{2+}. Our laboratory was able to show that MCU is necessary and sufficient to mediate Ca^{2+} uptake. Indeed, purified MCU was able to form a RuR dependent channel in planar lipid bilayers.

Very recently, Mallilankaraman and coworkers identified also a new component of this complex. Indeed, it showed that the mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1) is an IMM integral membrane protein that is required for MCU-dependent mitochondrial Ca^{2+} uptake. Furthermore, they demonstrated that this protein is involved in the regulation of oxidative phosphorylation and in the activation of AMPK dependent pro-survival autophagy (Mallilankaraman et al. 2012).

3.4 Fine regulation of mitochondrial proteins

Mitochondrial proteins, once they have carried out their purpose or in response to damage, have to be efficiently removed. Mitochondria have in place multiple quality control mechanisms.

Usually damaged mitochondria are removed by mitophagy, a process where damaged and non functional mitochondria are removed via a selective process involving the autophagosome (Pattingre et al. 2005, Narendra et al. 2008, Geisler et al. 2010, Suen et al. 2010). In this case, the entire content of mitochondria including its proteins is subjected to lysosomal degradation.

Usually the quality control of mitochondrial proteins is ensured by ATP dependent oligomeric proteases that survey protein quality control within the organelle and aid in the removal of non assembled and misfolded proteins (Koppen and Langer 2007). These proteases are found in the mitochondrial matrix (PIM1/Lon and ClpXP proteases), the inner membrane facing the matrix (m-AAA protease) and the inner membrane facing the intermembrane space (i-AAA protease) (Varabyova et al. 2013).

Protein degradation through the ubiquitin-proteasome system is the major pathway of non-lysosomal proteolysis of intracellular proteins. It plays important roles in a variety of fundamental cellular processes and the central element of this system is the covalent linkage of ubiquitin to targeted proteins, which are then recognized by the proteasome (Livnat-Levanon and Glickman 2011). Ubiquitynilation involves the covalent attachment of an ubiquitin molecule or a chain of ubiquitin molecules to a lysine residues of a target protein. Like phosphorylation, ubiquitinylation is a reversible process due to the activity of deubiquitinylation enzymes (Millard and Wood 2006). The attached molecule or chain can affect the localization, activity, structure or interaction partners of the target protein. One of the main functions of ubiquitinylation is to target a protein for degradation by the 26S proteasome (Hershko and Ciechanover 1998). Until recently it was not known whether mitochondrial inner membrane proteins were subjected to ubiquinylation. However, recent reports suggest that ubiquitinylation may play an important role in mitochondria protein quality control (Margineantu et al. 2007)((Radke et al. 2008). Indeed, many proteins critical to mitochondrial function such as cytochrome oxidase subunits I, III and IV accumulate upon proteasome inhibitor treatment (Margineantu et al. 2007) and moreover, the cytosolic 26S proteasome has been implicated in the turnover of the uncoupling proteins 2 and 3

(UCP2 and UCP3) (Azzu et al. 2010, Azzu et al. 2010) and recently also the uncoupling protein 1 (UCP1) has been shown to be targeted to the proteasome (Clarke et al. 2012).

4. Aim

Mitochondria are unique organelles within the complex system of subcellular compartments. They are involved in the regulation of a plethora of physiological and pathological conditions. The numbers of cellular processes that are controlled by mitochondria are object of deep investigation by several groups.

The variation of the metabolic availability induces intracellular adaptations in order to maintain the energy status for the different cellular functions. The contribution of mitochondria in the regulation of intracellular metabolism is well known. Due to the ATP production, mitochondria provide energy for the cell and connect two important intracellular signals: ATP and Ca^{2+}. It is still unknown, which are the specific mitochondrial proteins that function as sensors of nutrient availability and how they can translate the variation of nutrient concentrations into modulation of Ca^{2+} uptake, which in turn induces intracellular adaptive processes like autophagy.

The focus of my PhD research was to investigate the nutrient dependent control of mitochondrial Ca^{2+} uptake. In particular, we took advantage from the long-standing experience of our group in the analysis of cellular Ca^{2+} signals in order to precisely investigate mitochondrial Ca^{2+} homeostasis during glucose deprivation. By using the most modern technologies based on both fluorescent and bioluminescent Ca^{2+}-probes, we also correlated the effect of nutrient deprivation on Ca^{2+} signals to their consequences on induction of autophagy. Moreover we also analyzed the possible involvement of MICU1 in this process.

5. Materials and Methods

5.1 Cell culture, transfection and proteomic analysis

All the experiments were performed in HeLa cells cultured in Dulbecco's modified Eagle's medium (DMEM) (Lifetechnologies), supplemented with 10% Fetal bovine serum (FBS) (Lifetechnologies). For minor experiment we used also MEF (mouse embryonic fibroblast), Hek293A cultured in DMEM 10% FBS or C_2C_{12} coltured in DMEM 10% FBS and differenciate in DMEM 2% Horse serum (HS) (Lifetechnologies) for 5 days, MDA cultured in DMEM/F12 (1:1) (Lifetechologies, supplemented with 10% FBS.

Cells were transfected with a standard Ca^{2+}-phosphate procedure. For Ca^{2+}-phosphate transfection procedure the following stock solution need to be prepeared and conserved at -20°C until used.

- $CaCl_2$ 2.5 M.
- HEPES Buffered Solution (HBS): 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.12.

All solutions were sterilized by filtration using 0.22 μm filters. Just before the transfection procedure, cells are washed with fresh medium. For one 13 mm coverslip, 5 μl of 2.5 M $CaCl_2$ were added to the DNA disolved in 45 μl of H_2O. Routinely, 4 μg of DNA were used to transfect 1 coverslip. The solution was then mixed under vortex with 50 μl of HBS and incubated for 20 to 30 minutes at room temperature. For one 24 mm coverslip the amount of solution and DNA were duplicated while for 10 cm dishes was used 50 μl of 2.5 M $CaCl_2$, 20 μg of DNA in 450 μl of H_20 and 500 μl of HBS. The solution was then added directly to the cell monolayer. Sixteen hours after addition or the DNA, cells were washed with PBS (two or three times until the excess precipitate is completely removed). Experiments were carried out 24-36 hours after transfection.

MICU1 was silenced using specific siRNAs:

siMICU1 #1 TCTGAAGGGAAAGCTGACAAT

siMICU1 #2 GACTTCGCTTTACCCAAACAG

For glucose deprivation experiments culture medium were washed four times and then cells were incubated for 2 hours in Krebs–Ringer modified buffer (KRB: 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 20 mM HEPES, pH=7.4) with or without 25 mM glucose. Most experiments were performed adding 1 mM $CaCl_2$.

Cells were lisated in lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1% Triton X-100) and after a brief sonication 20-40 μg of total proteins were separated by SDS-PAGE gel electrophoresis in 4-12% SDS-bis,tris,acrylamide gels (Lifetechnologies), transferred to Nitrocellulose membrane (Lifetechnologies), stained with Ponceau S solution and immunoblotted against MICU1 (1:1000), Actin (1:7500), HA (1:1000; IF; 1:100), FLAG (1:1000). These antibodies were purchased from Sigma-Aldrich. TOM20 (1:5000), (WB, 1:10000) were purchased from Santa Cruz Biotechnologies. P-AMPK (1:500), AMPK (1:1000), Acetyl-lysine (1:1000) were purchased from Cell Signaling. VDAC2 (1:1000) was purchased from Abcam. Secondary, HRP-conjugated antibodies (1:10000) were purchased from BioRad.

All chemicals were purchased from Sigma-Aldrich, unless specified.

5.2 MICU1 expression constructs

The pcDNA3.1MICU1-HA, pcDNA3.1MCU-HA and pcDNA3.1MCU$^{D260N,\ E263Q}$-HA expression constructs were provided by De Stefani et al. 2011 (De Stefani et al. 2011).

- The generation of the pcDNA3.1MICU^{K102R}-HA was performed by mutagenesis PCR using the wild type pcDNA3.1-MICU-HA as template and the mutagenesis primer:

GCCTTATCCTGAGGACAAG**AGG**AAGAAGCGCTCTGGATTCA

-The generation of the pcDNA3.1MICU^{K103R}-HA was performed by mutagenesis PCR using the wild type pcDNA3.1-MICU-HA as template and the mutagenesis primer:

CTTATCCTGAGGACAAGAAG**AGG**AAGCGCTCTGGATTCAGAG

- The generation of the pcDNA3.1MICU^{K104R}-HA was performed by mutagenesis PCR using the wild type pcDNA3.1-MICU-HA as template and the mutagenesis primer:

ATCCTGAGGACAAGAAG**AAG**AGGCGCTCTGGATTCAGAGAC

- The generation of the pcDNA3.1MICU^{K296R}-HA was performed by mutagenesis PCR using the wild type pcDNA3.1MICU-HA as template and the mutagenesis primer: CTCAAAGGGAAACTGACCATC**AGA**AACTTCCTGGAATTTCAGCGC

- The generation of the pcDNA3.1MICU^{K359R}-HA was performed by mutagenesis PCR using the wild type pcDNA3.1-MICU-HA as template and the mutagenesis primer:

AGAAGCACTTCAAAGATGGG**AGG**GGCCTGACTTTCCAGGAG

- The generation of the pcDNA3.1MICU$^{D233A,\ E244K}$-HA was performed by mutagenesis PCR using the wild type pcDNA3.1MICU-HA as template and the mutagenesis primer:

TGAAATTGCTTTCAAGATGTTT**GCC**TTGAATGGAGACGGAGAGGTAGACATGGAG**A**

AGTTTGAGCAGGTTCAGAGC

- The generation of the pcDNA3.1MICU$^{D233A,\ E244K,\ D423A,\ E434K}$-HA was performed by mutagenesis PCR using the wild type pcDNA3.1MICU$^{D233A,\ E244K}$-HA as template and the mutagenesis primer:

GTGGTGTTCGCGCTCTTT**GCC**TGTGATGGCAATGGGGAGCTGAGCAACAAG**AAG**TT

CGTTTCCATCATGAAGC

The protocol for one step direct mutagenesis were described in Marianne Ratcliffe & Sergei Sokol, (Ratcliffe et al. 2000), and adapted from Makarova et al.,(Makarova et al. 2000); and Chen et al, (Chen et al. 2000).

For the cloning of MICU1^{K296R}-HA in pEGFP-N1 the cDNA was subloned from pcDNA3.1MICU1^{K296R}-HA to pEGFP-N1.

5.3 Aequorin as a Ca^{2+} indicator

Aequorin is a 22 KDa photoprotein isolated from jellyfish *Aequorea Victoria* which emits blue light in the presence of Ca^{2+}. The aequorin originally purified from the jellyfish is a mixture of different isoforms called "heterogeneous aequorin" (Shimomura 1995). In its active form the photoprotein includes an apoprotein and a covalently bound prosthetic group, coelenterazine. The apoprotein contains four helix-loop-helix "EF hand" domains, three of which are Ca^{2+}-binding domains (Inouye and Tsuji 1993). These domains confer to the protein a particular globular structure forming the hydrophobic core cavity that accommodates the ligand coelenterazine. The binding cavity for coelenterazine is situated in the centre of the protein; this cavity is closed to a spherical probe, indicating that the outside molecules have no access to the coelenterazine moiety. When Ca^{2+} ions bind to the three high affinity EF hand sites, coelenterazine is oxidized to coelenteramide, with a concomitant release of CO_2 and emission of light (Head et al. 2000). Although this reaction is irreversible, an active aequorin can be obtained *in vitro* by incubating the apoprotein with coelenterazine in the presence of oxygen and 2-mercaptoethanol. Reconstitution of an active aequorin (expressed recombinantly) can be obtained also in living cells by simple addition of coelenterazine into the medium. Coelenterazine is highly hydrophobic and has been shown to permeate cell membranes of various cell types. Different coelenterazine analogues have been synthetized and are now commercially available from Molecular Probes.

The possibility of using aequorin as Ca^{2+} indicator is based on the existence of a well-characterized relationship between the rate of photon emission and the $[Ca^{2+}]$.

The first method used to correlate the amount of photons emitted to the $[Ca^{2+}]$, was that described by Allen and Blinks (Allen and Blinks 1978). In the following years, this system was improved to achieve a simple algorithm for converting aequorin luminescence into $[Ca^{2+}]$ values. This mathematical approach reposes on an accurately relationship between $[Ca^{2+}]$ and the logarithm of L/Lmax, where L is the instant rate of light emission and Lmax is the maximal

values of light emission measured in saturated conditions. For the native jellyfish photoprotein, in this logarithmic scale the response are linear in the physiological range of cytosolic [Ca^{2+}], i.e. between 10^7 and 10^5, thus allowing careful calibration of the luminescence signal into absolute [Ca^{2+}] values.

The rate of aequorin luminescence is independent of [Ca^{2+}] at very high (>10^{-4} M) and very low (<10^{-7} M) [Ca^{2+}]. However, as described below in more details, it is possible to expand the range of [Ca^{2+}] that can be monitored with aequorin.

Ion concentration affects the luminescence of aequorin because ions are competitive inhibitors of Ca^{2+}. pH was also shown to affect aequorin and for this reason experiments with aequorin need to be done in well-controlled conditions of pH and ionic concentration.

Recombinant aequorins

Aequorin began to be widely used when the cDNA encoding the photoprotein was cloned, thus circumventing the need for purification of the native polypeptide and microinjection and also open the possibility of molecular engineering the protein sequence; introducing specific targeting sequences and thus directing the Ca^{2+} probe to a defined subcellular compartment. Indeed, the analysis of a large number of amino acids sequences has highlighted short presequences shared by proteins with the same subcellular location. Deletion analysis experiments have shown that these amino acids presequences are sufficient and necessary for a correct intracellular sorting of the protein (Hartl et al. 1989, Nothwehr and Gordon 1990, Garcia-Bustos et al. 1991).

By introducing minimal sequences (as for mitochondrial targeting) or larger targeting information (such as the entire hormone-binding domain of the glucocorticoid receptor, thus retaining the property of shuttling from the cytosol to the nucleus in a hormone-dependent manner), a large panel of aequorin chimeras with defined subcellular distribution was

constructed. The selective localization allowed monitoring for the first time Ca^{2+} dynamic in specific subcellular compartment.

Below we briefly describe the constructs used in our experiments (Brini 2008):

- Cytoplasmic (cytAEQ): an unmodified aequorin cDNA encodes a protein that, in mammalian cells is located in the cytoplasm and, given its small size, also diffuses into the nucleus.
- Mitochondria (mtAEQ): mtAEQ was generated to measure the [Ca^{2+}] of the mitochondrial matrix of various cell types. This construct includes the targeting presequence of subunit VIII of human cytochrome c oxidase fused to the aequorin cDNA. To expand the range of Ca^{2+} sensitivity that can be monitored the photoprotein was also mutated (Asp119>Ala). This point mutation affects specifically the second EF hand motive of wild type aequorin. The affinity for Ca^{2+} of this mutated aequorin (mtAEQmut) is about 20 fold lower than that of the wild type photoprotein.

Luminescence detection

The aequorin detection system is derived from that described by Cobbold and Lee (Cobbold and Bourne 1984) and is based on the use of a low noise photomultiplier placed in close proximity (2-3 mm) of aequorin expressing cells. The cell chamber, which is on the top of a hollow cylinder, is adapted to fit 13-mm diameter coverslip. The volume of the perfusing chamber is kept to a minimum (about 200 μl). The chamber is sealed on the top with a coverslip, held in place with a thin layer of silicon. Cells are continuously perfused via peristaltic pump with medium thermostated via a water bath at 37°C. The photomultiplier (Hamamatsu H7301) is kept in a dark box. The output of the amplifier-discriminator is captured by C8855-01 photon-counting board in an IBM compatible microcomputer and stored for further analysis.

Experimental procedures

Cytoplasmic [Ca^{2+}] and mitochondrial [Ca^{2+}] were measured in cells using appropriate cytosolic (cytAEQ) and mitochondrial (mtAEQmut) chimeras (Pinton et al. 2007). Cells were seeded 24 hours before transfection onto 13 mm glass coverslips and allowed to grow to 50% confluence before transfection. Cells were transfected with CytAEQ or mtAEQmut probe (as previously described (Pinton et al. 2007)) together with the indicated siRNA or plasmid. pcDNA3.1 was used as control for transfection. The coverslip with the cells was incubated with 5 μM coelenterazine for 2 hours in KRB with or without 25 mM glucose, supplemented with 1mM $CaCl_2$, and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB. Agonists and other drugs were added to the same medium. The most common stimuli used for maximal stimulation are: 100 μM histamine, 100 μM ATP, 0.5 mM carbachol, 100 μM norepinephrine, 100 nM bradikynin. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into [Ca^{2+}] values by an algorithm based on the Ca^{2+} response curve of aequorin at physiological conditions of pH, [Mg^{2+}] and ionic strength, as previously described (Pinton et al. 2007). Representative traces are shown in the figures whereas column graphs are the results of the full dataset.

Statistical data are presented as mean ± S.E.M. unless specified, significance was calculated by Student's t test, *$p<0.05$, **$p<0.001$.

In the experiments with permeabilized cells, a buffer mimicking the cytosolic ionic composition, (intracellular buffer [IB]) was employed: 130 mM KCl, 10 mM NaCl, 2 mM K_2HPO_4, 5 mM succinid acid, 5 mM malic acid, 1 mM $MgCl_2$, 20mM HEPES, 1 mM pyruvate, 0.5 mM ATP and 0.1 mM ADP (pH 7 at 37°C). IB was supplemented with either 100 μM EGTA

(IB/EGTA) or a 2 mM EGTA and 2 mM HEEDTA-buffered [Ca] of 1 or 2 μM (IB/Ca^{2+}), calculated with Chelator software (Schoenmakers et al. 1992).

HeLa cells were permeabilized by a 1 min perfusion with 50 μM digitonin (added to IB/EGTA) during luminescence measurements. Mitochondrial Ca^{2+} uptake speed was calculated as the 1st derivative by using the SLOPE excel function and smoothed for 3 time points. The higher value reached during Ca^{2+} addition represents the maximal Ca^{2+} uptake speed.

Also for capacitative Ca^{2+} influx experiment cells were reconstituted and starved for 2 hours before the experiment. This procedure allows evaluating Ca^{2+} influx through store operated channels if we were measuring cytoplasmic Ca^{2+} level with CytAEQ or mitochondrial Ca^{2+} uptake independently to ER Ca^{2+} release if we were measuring mitochondrial [Ca^{2+}] with mtAEQmut. ER was emptied by treatment with cyclopiazonic acid (CPA), a highly selective reversible inhibitor of Ca^{2+}-ATPase in the intracellular Ca^{2+} storage sites. Cells were perfused with KRB without Ca^{2+} and with 100 μM EGTA and then for 3 minutes with KRB without Ca^{2+} and with 100 μM EGTA and 20 μM CPA. During this period ER was emptied and a little and slow increase in cytoplasmic Ca^{2+} was observed. This cytoplasmic [Ca^{2+}] is directly correlated with the amount of ER Ca^{2+} released.

After that, cells were perfused with KRB with 2 mM Ca^{2+} and 20 μM CPA. During this perfusion the Ca^{2+} influx through the plasma membrane (CytAEQ) and into mitochondria (mtAEQmut) could be evaluated.

5.4 Imaging techniques

All imaging experiments were carried out on three digital imaging systems. FRET measurements were carried out on an inverted Zeiss Axiovert 100 TV equipped with a 63x/1.4N.A. objective. The probe was excited by a LED-based illumination device (OptoLED, Cairn Research) with a 436/20 nm bandpass filter. Donor and acceptor wavelength were

separated by a beamsplitter device (Optosplit, Cairn Research) using a 480/40 nm filter for the CFP, a D505 dichroic mirror and a 535/30 nm filter for the cpVenus. Images were collected with a front-illuminated CCD camera (Photometrics CoolSnap ES2). TMRM and SypHer imaging were performed on an inverted Zeiss Axiovert 200 equipped with a 40x 1.3N.A. objective. SypHer was excited by a 75W Xenon lamp equipped with a prism-based monochromator (PTI) at 430nm and 485 nm. Probe emission was collected through a 525/30 nm bandpass filter. Images were collected with a back-illuminated EMCCD camera (Photometrics Cascade 512B) at 200-300 ms exposure time. TMRM was imaged on the same microscope but using a spinning disk confocal head (BD CarvII). Probe was excited by a 300W Xenon arc lamp (Sutter Lambda LS) with a 543/22 nm filter and collected through a 593/40 nm emission filter. ER/mitochondria contact sites were imaged on a Leica TCS-SP5-II confocal system equipped with a PlanApo 100x/1.4 N.A. objective. For all images, pinhole was set to 1 airy unit, pixel size was about 100 nm and a Z-stack was acquired for the whole depth of the cell by sampling at 130 nm in the Z plane. 488 nm Ar-laser line was used to excite GFP and its signal collected in the 492-537 nm range, while RFP fluorescence was excited by the 543 nm HeNe laser and its emission was collected in the 555-700 nm range. For each image, PMT gain was slightly adjusted in order to maximize signal and avoid saturation.

Cameleon as Ca^{2+} probe

Cameleons are FRET-based ratiometric Ca^{2+} probe. The molecular structure is based on two variant of GFP (having differing excitation and emission characteristics), calmodulin (CaM), and the calmodulin-binding domain of myosin light chain kinase (M13). It was created by Roger T. Tsien and coworkers (Palmer and Tsien 2006). The excitation energy of one fluorophore (the donor) is transferred to another (the acceptor) by dipolar interactions, without fluorescence emission (FRET). The donor emission and acceptor absorption spectra must overlap for FRET to occur. Calmodulin is able of bind Ca^{2+} ions and the M13 chain can bind with calmodulin after it

has bound the Ca^{2+} ions. The bindind of Ca^{2+} by the calmodulin moiety of cameleon produces a conformational change of the entire molecule and the consequent positions of the two fluorescent proteins into close spatial proximity. In this conformation, dipolar energy transfer by the excited donor protein stimulates the acceptor to produce secondary fluorescence. Intracellular [Ca^{2+}] can be determined by fluorescence ratio imaging. Also cameleon based probe can be targeted into different intracellular compartment. The first cameleon developed had high pH-sensitivity but during the years cameleon were engineered in order to overcome this problem. There are several cameleon probes available that differ for the Ca^{2+}-binding affinity and for the GFP variants used.

In our experiments we used two mitochondrially-targeted cameleons with two different affinities for Ca^{2+}-binding. The GFP variants used are CFP (the donor) and cpVenus (the acceptor).

HeLa cells were grown on 24 mm coverslips and transfected with 4mtD1cpv and 4mtD3cpv (Palmer et al. 2004, Palmer et al. 2006). 24-48 hours after transfection HeLa cells were mounted into an open-topped chamber and maintained in KRB. Cells were stimulated by applying 10 μM histamine as stimulus; thereafter, Ca^{2+} ionophore ionomycin (5 μM) with a $CaCl_2$ saturating concentration (5 mM) were applied to completely discharge the stores and to verify the dynamic range of each probe. Image analysis was performed by the public domain ImageJ program (developed at the U.S. National Institutes of Health by Wayne Rasband and available on the Internet at http://rsb.info.nih.gov/ij/). Regions of interest (ROIs) were selected covering essentially mitochondria (identified based on their morphology). Subsequently, a ratio between cpVenus and CFP emission was calculated. Data are presented as normalized ratio for 4mtD1cpv or for the ratio difference between the starting point (R_{min}) and the point reach after agonist stimulation (R) for 4mtD3cpv.

5.5 Measurement of Mitochondrial Membrane Potential

The measurement of mitochondrial membrane potential is based on the distribution of lipofilic cations. They are fluorescent and membrane permeable and their distribution into intracellular compartment is due only to electrochemical gradients (Scaduto and Grotyohann 2000). The mitochondrion-selective tetramethylrhodamine dye (TMRM) was used to measure mitochondrial membrane potential in HeLa cells. The accumulation of TMRM in mitochondria at low concentrations was shown to be driven by their membrane potential (-150/-180mV). In order to promote the correct distribution of the probe, cells are loaded with very low concentration (10-40 nM). Changes in mitochondrial membrane potential will cause a redistribution of the dye between mitochondria and cytoplasm. Data are expressed as percentage difference between the TMRM fluorescence before and after FCCP (10μM) depolarization. Confocal laser microscope (Zeiss Axiovert 200, objective PlanFluar 40X/1.3) was used in TMRM experiment.

Cells were starved in KRB for 1 hour and 30’ and then were loaded with TMRM stock solution for 30’ at 37°C. The probe was excited at 560 nm and the emission light was recorded in the 590-650 nm ranges.

5.6 SypHer

The measurement of mitochondrial pH gradient is the sole driving force for the electroneutral transport of many ions and metabolites in and out of the mitochondrial matrix. In order to determine the mitochondrial matrix pH, a ratiometric circularly permuted YFP was used. This probe allow us to real time measure the mitochondrial matrix pH in intact cells (Poburko et al. 2011). HeLa cells were grown onto 24 wells until 50% and then transfected with 4 μg of mtSypher with a standard Ca^{2+}-phosphate procedure. 24 hours after transfection SyPher expressing cells were starved for 2 hours and then mounted into an open-topped chamber and

maintained in KRB with or without 25 mM glucose. After KRB whashout, pH was stepped between 5.5 and 10 by turnover the bath solution. The solutions added were a Ca^{2+}-free intracellular buffer: 130 mM KCl, 10 mM NaCl, 2 mM K_2HPO_4, 1mM $MgCl_2$ supplemented with 20 mM MES (adjusted to pH 5.5 and 6.5 with KOH) or 20 mM HEPES (adjusted to pH 7.0 and 7.5 with KOH) or TRIS (adjusted to pH 8.0 and 9.0 with HCl) or Acid Boric (adjusted to pH 9.5 and 10 with KOH). For each experiment monensin (ionophore 5 μM) and nigericin (N^+/H^+ antiporter 1 μM) were also added to these Ca^{2+}-free intracellular buffers in order to increase the velocity to reach equilibrium between the external medium and the pH into the intermembrane space.

Fluorescence ratio (F480/430) was calculated in MetaFluor 6.3 (Universal Imaging) and analysed in Excel (Microsoft). For each cell, a 8-point calibration curve was fitted to a variable slope sigmoid equation

5.7 Morphological analysis

ER-mitochondria colocalization

ER-mitochondria interactions are studied by confocal images of HeLa cells coexpressing a mitochondria-targeted RFP (mit-RFP) and an ER-targeted GFP (ERD1cpv). Cells were excited separately at 488 nm or at 543 nm, and the single images were recorded. Single channel images were then merged. Statistical quantification of the overlapping signal area (OSA) was performed from single confocal images by the public domain ImageJ program with Jacop plugin (Bolte and Cordelieres 2006). Pearson's correlation index, an overlap coefficient that is commonly used to measure the amount of overlap between two image pairs, was obtained to determine the amount of localization between ER and mitochondria (Manders et al. 1992).

Immunofluorescence

HeLa cells were grown on 24 mm coverslips and transfected with MICU-HA and/or mit-RFP encoding plasmid. After 24 hours, cells were washed with PBS, fixed in 4% formaldehyde for 10 minutes and quenched with 50 mM NH_4Cl in PBS. Cells were permeabilized for 10 minutes with 0.1% Triton X-100 in PBS and blocked in PBS containing 2% BSA for 1 hour. Cells were then incubated with primary antibodies (anti-HA) for 3 hours at room temperature and washed 3 times with 0.1% Triton X-100 in PBS. The appropriate isotype matched AlexaFluor conjugated secondary antibodies (Lifetechnologies) were used and coverslips were mounted with ProLong Gold Antifade reagent (Lifetechnologies).

Confocal images were recorder and quantification of the number and the volume of the mitochondria were performed from ImageJ program.

5.8 Luciferin-Luciferase system

There are different methods to measure intracellular ATP production and the major difference is if they can discriminate ATP production from different subcellular site or not. We used an approach based on specific targeting luciferase into different subcellular compartments.

Luciferase as an ATP probe: The direct measurement of mitochondrial and cytosolic ATP levels is based on specifically targeted chimeras of the ATP-dependent photoprotein luciferase. The cDNA of luciferase was cloned into different plasmid in order to have different intracellular localization (Jouaville et al. 1999). Cells were seeded for transfection onto 13-mm coverslip and grown to 50% confluence. Transfection with cytLUC cDNA was carried out according to a standard Ca^{2+}-phosphate procedure. The day after, cells were tripsinized and transferred onto 96wells (one 24well divided onto eight 96wells). 48 hours after transfection cell luminescence was measured in a multimode plate reader (Perkin Elmer Envision). During the experiment cells are maintained in KRB with or without 25 mM glucose, 1 mM $CaCl_2$ and 20 μM lucifern. As control were used the inhibitor of ATP synthase oligomycin (10 μM), and the glycoslisis blocker

2-deoxiglucose (10 mM).

5.9 ROS production measurement

ROS production was measured using a 2',7'-dichlorfluorescein diacetate (DCFDA). This dye is fluorogenic and after diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescin (DCF). DCF is highly fluorescent compound, which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively. HeLa cells were growth in 96 wells until 80% confluence. Just before the experiment the dye was reconstituted in DMSO (stock solution 5 mM) and then HeLa cells were loaded with 5 μM DCFDA for 30 minutes at 37°C. HeLa cells were washed four times and then treated with KRB with or without 25 mM glucose. H_2O_2 dilution scale was used as positive control. Fluorescence emission was measured at Envision plate reader.

6. Results

6.1 Role of mitochondria as sensors of nutrient availability

Intracellular signals can be translated through mitochondria into specific actions in order to maintain intracellular homeostasis. A good example of this important function mediated by mitochondria is the identification of the role of constitutive Ca^{2+} transfer from ER to this organelle. Indeed, constitutive Ca^{2+} release from ER was shown to suppress a prosurvival mTOR independent macroautophagy; in its absence cells activates this adaptive response mediated by the activation of AMPK (Cardenas et al. 2010). Until now it is still unknown if this effect on macroautophagy is dependent on Ca^{2+} release from IP_3R per se or if the amount of Ca^{2+} into mitochondria is the real determinant of this function. In support of the latter mechanism, the overexpression and the silencing of the recently discovered mitochondrial calcium uniporter (MCU) (De Stefani et al. 2011) exerts an effect in the AMPK activation and autophagy induction. Furthermore, it is not clear if the activation of AMPK is a metabolic consequence of the effect on ATP/ADP/AMP ratio or if it is directly mediated by Ca^{2+} levels. What is clear is that AMPK is a key protein sensor of nutrient availability and its activation involves also mitochondrial Ca^{2+} signalling. It is unknown if metabolic availability can also be translated into specific metabolic adaptations through mitochondria. During my PhD project we analyzed the changes of mitochondrial [Ca^{2+}] after nutrient deprivation.

Mammalian cells need different metabolites to support their growth. The most important are amino acids, growth factors and glucose. Glucose and its metabolites are also important as primary messenger molecules, signalling optimal growth conditions to the cellular machinery. Its intracellular signalling involves specific glucose transporter, the activation of protein kinase C (PKC) and the mitogen-activated kinases (MAPK) with the consequent stimulation of gene expression (i.e. TGFβ) (Haneda et al. 2003).

We decided to focus our attention on the study of the contribution of glucose to cellular signalling pathways. Indeed, we decided to completely remove all metabolites and to add to control cells only glucose. The model of glucose deprivation is thus HeLa cells starved for 2 hours in KRB compared to HeLa cells in KRB with 25 mM glucose. Two hours of glucose deprivation induce a constant decrease in total protein deacetylation as consequence of Sirtuins activation and a strong phosphorylation of AMPK (Figure 1A). This result further underlines that these proteins are key sensors of nutrient availability. We wondered whether there is a common converging signalling pathway that links changes of nutrient availability with the activation of these proteins. We therefore asked whether mitochondrial Ca^{2+} content plays a key role in this process.

Our main working hypothesis is that there is a feedback loop: on one hand some protein sensors of nutrient availability can sense mitochondrial Ca^{2+} content; on the other hand, they can sense nutrient levels and in turn control the amplitude of mitochondrial Ca^{2+} response.

My research was focused on the regulation of Ca^{2+} content by post-translational modifications occurring in mitochondria after nutrient deprivation and the intracellular processes that are activated by this modification.

The amount of mitochondrial Ca^{2+} determines the activation of AMPK

We first verified if changes in the amplitude of the mitochondrial Ca^{2+} transient could tune the activation of AMPK. 80% confluent HeLa cells were treated with and AMP mimetic (AICAR 0.5 mM) that is an AMPK activator. In order to change the amplitude of the mitochondrial Ca^{2+} transient, HeLa cells were treated with CGP37157 (20 μM). CGP37157 is a selective inhibitor of the mitochondrial Na^{+}/Ca^{2+} exchanger, the major mechanism of Ca^{2+} extrusion from mitochondria to cytoplasm. After sixteen hours of treatment, HeLa cells were lysed and 20 μg were separated by SDS-PAGE. As expected, an AMP mimetic (AICAR) treatment induces a strong phosphorylation and thus activation of AMPK. Interestingly, AMPK

activation by AICAR can be blocked by an increase of mitochondrial Ca^{2+} transient, by blocking the mitochondrial Ca^{2+} extrusion (Figure 1B). Thus, it is clear that the amount of Ca^{2+} into mitochondria determines the AMPK activation.

This effect on AMPK could be direct or a consequence of the known modulation of mitochondrial ATP production.

SIRT1 and SIRT3 overexpression controls mitochondrial Ca^{2+} uptake

Sirtuin proteins are a family of NAD^{+}-dependent protein deacetylases that are important regulators of cellular metabolism (Blander and Guarente 2004). Indeed protein acetylation is a post-translational modification that has a key role in the control of important metabolic pathways. As discussed in the introduction, three sirtuins, SIRT3, SIRT4 and SIRT5 localize to mitochondria and SIRT3 expression is activated during nutrient deprivation (Nogueiras et al. 2012). This increase in SIRT3 expression helps the switch to fasting metabolism, as tissues move away from glucose instead of lipids and amino acids as a source of energy.

We started to study the role of Sirtuins as sensors of nutrient availability and we first asked if they play a role in the control of mitochondrial Ca^{2+} uptake. To assess a direct role of Sirtuins on mitochondrial Ca^{2+} uptake we co-expressed in HeLa cells different Sirtuin proteins and mitochondrial targeted aequorin-based Ca^{2+} probe (mtAEQmut), and we evaluated organelle Ca^{2+} responses to agonist stimulation. After reconstitution with the aequorin co-factor coelenterazine for 2 hours, cells were challenged with histamine and luminescence was measured and converted to $[Ca^{2+}]$. We found that SIRT1 and SIRT3 have a role in the control of the magnitude of Ca^{2+} transients inside mitochondria. Indeed the mitochondrial Ca^{2+} rise in SIRT3 and SIRT1 overexpressing HeLa cells was reduced (Figure 1C).

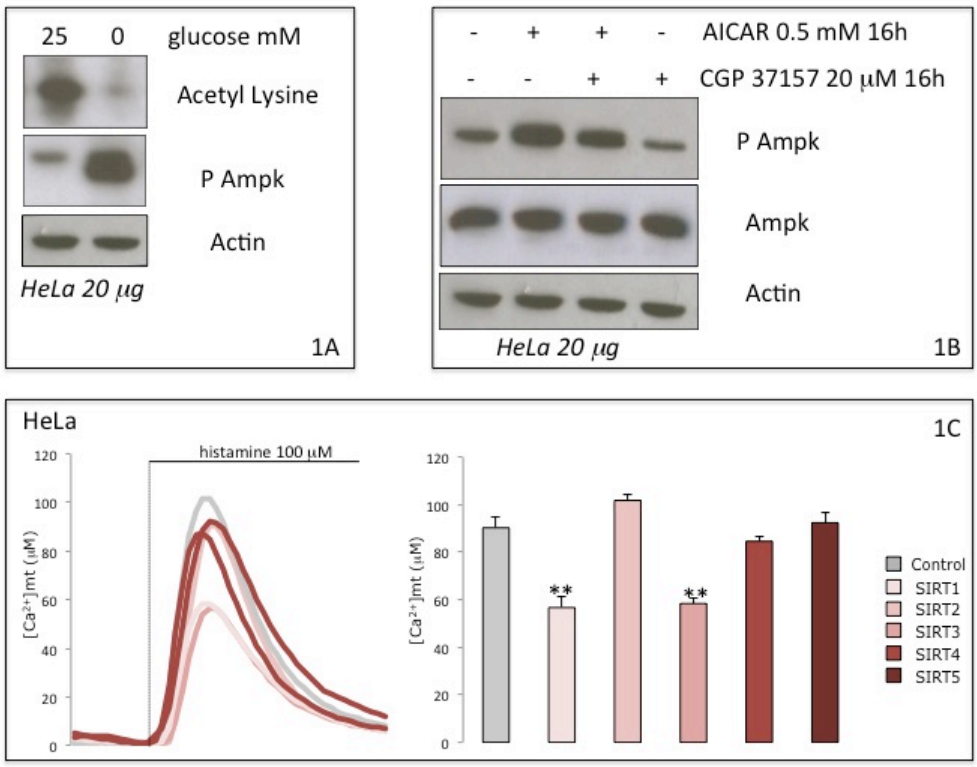

Figure 1A, B, C. Effect of AMPK and Sirtuins activations. (A) Immunoblot analysis of phosphorylation of AMPK prepared from HeLa cells. HeLa cells were starved for 2 hours in KRB with or without 25mM glucose. (B) Immunoblot analysis of phosphorylation of AMPK prepared from HeLa cells treated for 16 hours with AICAR 0.5 mM and/or 20 µM CGP1375. (C) HeLa cells were transfected with mitochondrial targeted aequorin and $[Ca^{2+}]$ was measured as described in the Materials and Methods section. During Ca^{2+} experiment cells were maintained in KRB + 1 mM Ca^{2+} + 5 mM glucose. Control trace is shown in grey; traces from cells co-transfected with Sirtuins are shown in pink scale. Mean ± S.E.M. of values are shown.

6.2 Glucose deprivation decreases mitochondrial Ca^{2+} uptake

Glucose ($C_6H_{12}O_6$) is a simple monosaccharide that it is used as the primary source of energy, representing the main fuel for cellular energy production, as discussed in the introduction section. The fate of this simple monosaccharide is through three different ways. It can be stored; it can be converted through glycolysis into pyruvate in order to provide few ATP molecules; finally, the product of glycolysis, pyruvate, can then enter mitochondria where it is further processed by TCA cycle. Reducing equivalents derived by TCA cycle fuel the oxidative phosphorylation and lead to efficient ATP production.

Glucose deprivation is a pathologically relevant event. Diabetes is one of the well-known pathology of deregulation of glucose metabolism. It affects body's ability to use glucose because

it interferes with two hormones that maintains constant blood glucose level. Understanding cellular response to glucose withdrawal could bring out new strategies to overcome the diabetes damage. On the other hand, cancer cells base their metabolism on glycolysis in a phenomenon termed “Warburg effect”. This metabolic adaptation allows cancer cells to overcome the growth factor dependence that normally controls the nutrients intake. Independently of the growth factor stimulation, cancer cells uptake an excess of nutrients, particularly glucose, that exceeds the bioenergetics demands. This ability enables cancer cells to proliferate and survive in hypoxic areas. Better understanding the link between glucose availability and metabolic adaptation in cancer cells, may ultimately lead to better treatments for human cancer.

For many years researchers have focused their studies on the understanding of the effects of glucose withdrawal on intracellular signalling. There are proteins that are involved in energy and nutrient sensing, including AMPK and mTORC1, that trigger metabolic adaptation like autophagy.

In cancer cells modification in cellular microenvironment (i.e., nutrient availability) can alter the cellular redox balance, provoking a positive feedback loop that amplifies ROS levels above a toxicity threshold resulting in cell death (Graham et al. 2012). Furthermore, understanding which proteins are involved in these systems offers a scaffold for synergistic combination of therapeutics targeting signalling, metabolism and redox homeostasis.

Given the central role of mitochondria in the maintenance of intracellular homeostasis, we focused our attention on the identification of the proteins that in different feeding conditions can modulate mitochondrial Ca^{2+} levels and which intracellular response to different feeding conditions are triggered by this modification. It is known that ROS production by mitochondria triggers the response of glucose availability (Li et al. 2011), but we hypothesize the existence of a more precise response that involves sensor proteins and triggers autophagy as survival mechanism instead of cell death caused by apoptosis for excessive ROS production.

To assess a direct role of glucose deprivation on mitochondrial Ca^{2+} uptake, we expressed in HeLa cells mitochondrial targeted aequorin-based Ca^{2+} probe (mtAEQmut), and evaluated organelle Ca^{2+} responses to agonist stimulation. After reconstitution with the aequorin co-factor coelenterazine and the simultaneous glucose deprivation for 2 hours, cells were challenged with histamine and luminescence was measured and converted to $[Ca^{2+}]$. We found that, after 2 hours of glucose deprivation, mitochondrial Ca^{2+} uptake drastically decreases (60% reduction in mitochondrial $[Ca^{2+}]$, Figure 2A). We performed the same experiment also in other cell type such C_2C_{12} myoblasts (data not shown), myotubes (Figure 2B) or MEFs (mouse embryonic fibroblasts) (data not shown) with the same results. These data suggest that the inhibition of mitochondrial Ca^{2+} transient is a common signalling event that correlates with glucose deprivation.

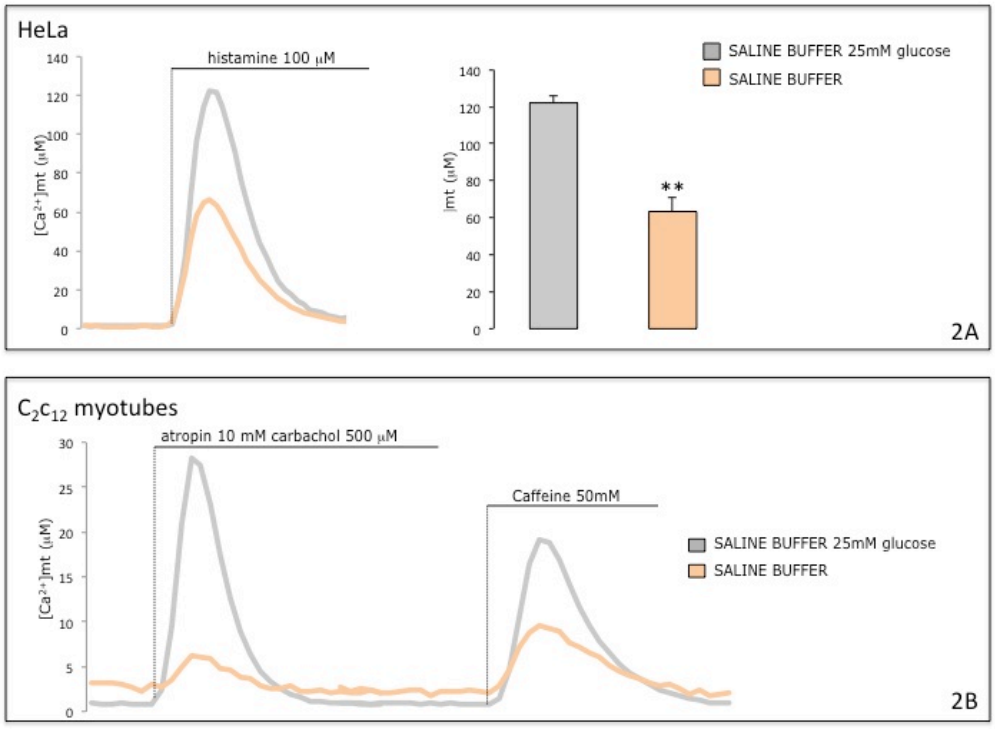

Figure 2A, B. Effect of glucose withdrawal on mitochondrial Ca^{2+} uptake. (A) HeLa cells were transfected with mitochondrially targeted aequorin and $[Ca^{2+}]$ was measured as described in the Materials and Methods section. HeLa cells were starved for 2 hours. (B) C_2C_{12} were grown until 80% and then infected with Ad-mtAEQmut and differentiated in DMEM 2% Horse serum for five days. Myotubes were starved for 2 hours in KRB with or without 25 mM glucose and then were challenged with 500 μM carbachol or 50 mM caffeine. Control trace is shown in grey; the trace from starved cells is shown in orange. Mean ± S.E.M. of values are shown.

We confirmed this data also with a FRET-based Ca^{2+} probe Cameleon (54% reduction in mitochondrial [Ca^{2+}] Figure 2C) (Palmer and Tsien 2006). We used two mitochondrially targeted Cameleons (4mtD1cpv or 4mtD3cpv), that differ for their Ca^{2+} binding affinity (Palmer et al. 2004, Palmer et al. 2006). These probes belong to a new class of indicators for Ca^{2+} ion concentration in living cells which operate through a conformational change that results in fluorescence resonance energy transfer (FRET) in presence of Ca^{2+} ions.

We used 4mtD1cpv to measure the mitochondrial Ca^{2+} increase after cell stimulation with an agonist. The starting level for sensor in each experiment was considered like R_{min}: the subsequent addition of 10 μM histamine caused an increase in the FRET ratio as Ca^{2+} released form ER is uptaken by the mitochondria. Once the FRET ratio returned to baseline, cells were washed and calibration of the sensor was performed. Cells were treated with 5 μM ionomycin in 5 mM $CaCl_2$ to obtain the R_{max}, the ratio at saturating Ca^{2+} levels. We tried two different experimental approaches, first, we starved HeLa cells for 2 hours and then we performed the Ca^{2+} measurement (Figure 2C); second, we starved HeLa cells and we acquired the Cameleon probe emission under the microscope at the same time (data not shown). We used as positive control a dominant negative form of mitochondrial Ca^{2+} uniporter ($MCU^{D260N, E263Q}$) that causes a reduction in mitochondrial Ca^{2+} uptake (De Stefani et al. 2011).

We used 4mtD3cpv to measure mitochondrial Ca^{2+} resting level. We starved HeLa cells for 2 hours and then we acquired the baseline ratio of different cells for 30 sec (Figure 3A). We used MICU1 silencing as positive control of basal mitochondrial [Ca^{2+}] accumulation because it has been demonstrated that HeLa cells mitochondria become constitutively loaded with Ca^{2+} after MICU1 silencing (Mallilankaraman et al. 2012).

These data confirm the aequorin-probe based experiment (Figure 2A). After 2 hours of glucose deprivation, mitochondrial Ca^{2+} uptake drastically decreases with no difference in the resting conditions.

These data also strongly indicate that this is a physiological response because it happens in a short period of time and it is reversible. To further investigate this aspect, HeLa cells transfected with mtAEQmut were starved for 2, 4, 6, 8 and 10 hours. During the last 2 hours of starvation cells are also reconstitute with the aequorin co-factor coelenterazine.

Importantly, this response to glucose withdrawal is reversible: indeed, cells have a reduced mitochondrial Ca^{2+} uptake up to 4 hours and then mitochondrial Ca^{2+} uptake level returns to normal feeding condition (2 hours 32%, 4 hours 32%, 6 hours 14%, 8 hours 18% and 10 hours 2% reduction in mitochondrial [Ca^{2+}], Figure 3C).

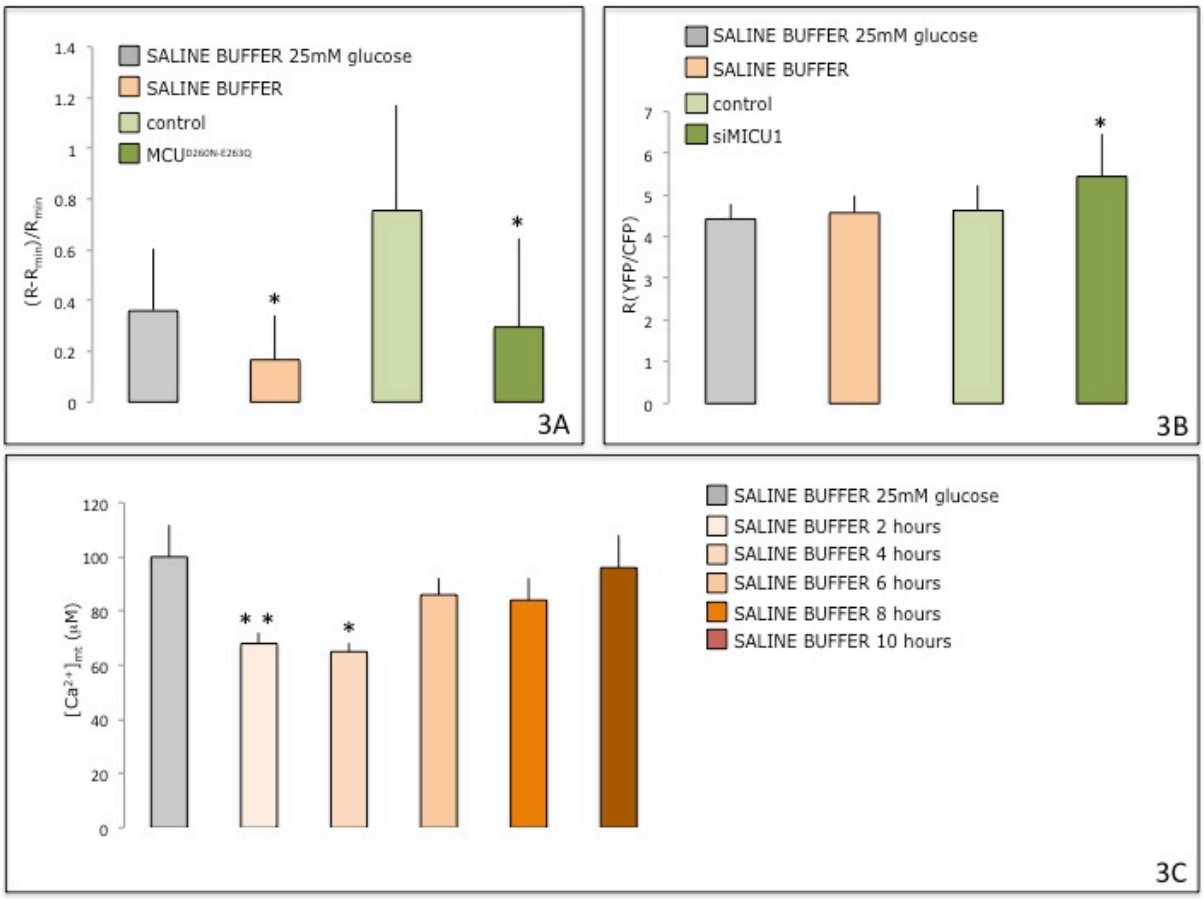

Figure 3A, B, C. Effect of glucose withdrawal on mitochondrial Ca^{2+} uptake. HeLa cells were transfected with 4mtD1cpv (A) and 4mtD3cpv (B). After 48 hours cells were starved for 2 hours in KRB with or without 25 mM glucose. Mitochondrial Ca^{2+} uptake was measured as described in the Materials and Methods section. (C) HeLa cells were transfected with mtAEQmut and 48 hours after transfection starved for 2, 4, 6, 8 and 10 hours in KRB with or without 25 mM glucose. During the last two hours cells were also reconstituted with coelenterazine.
Mean ± S.E.M. of values are shown.

We next investigated the specificity of this effect. Indeed, the observed decrease in Ca^{2+} uptake levels could also be due to modification of mitochondrial physiological parameters. This organelle is able to uptake Ca^{2+} because it is in close proximity to the ER Ca^{2+} source. IP_3R opening generates micro domains of high [Ca^{2+}] in the contact sites near to the Ca^{2+} uptake machinery. Reduced ER Ca^{2+} content or ER-mitochondria contact sites induce a decrease in mitochondrial Ca^{2+} transient. This is not the only mechanism that allows mitochondria to uptake Ca^{2+}. Indeed, the driving force due to the difference in pH and ion concentration inside and outside the mitochondrial inner membrane drives the entry of ions from the cytoplasm.

We thus investigated if one of these mechanisms is involved in the observed reduction of mitochondrial Ca^{2+} uptake during glucose deprivations.

This reduction in mitochondrial Ca^{2+} uptake is not a secondary effect to modification in ER Ca^{2+} content or positioning. HeLa cells transfected with aequorin (cytAEQ) (after 2 hours of starvation and at the same time reconstituted with the prostetic group coelenterazine) were challenged with an agonist (100 μM histamine) in order to evaluate cytoplasmic Ca^{2+} level. In starved cells cytoplasmic Ca^{2+} level does not change (Figure 4A), ruling out the involvement of the ER in this response to glucose deprivation. To further prove ER exclusion, cells were transfected with cytAEQ in order to perform "capacitative Ca^{2+} influx experiment". This procedure allows evaluating in the same experiment, ER Ca^{2+} content and Ca^{2+} influx through store operated channels. In this experiment, ER is emptied with a highly selective reversible inhibitor of Ca^{2+}-ATPase in the intracellular Ca^{2+} storage sites, Cyclopiazonic acid (CPA). 20 μM CPA (in KRB supplemented with 100 μM EGTA) is given by perfusion and aequorin light emission is measured. During this period ER compartment is emptied and a consequent little and slow increase in cytoplasmic Ca^{2+} is observed. This cytoplasmic [Ca^{2+}] is directly correlated with the amount of ER Ca^{2+} released (Figure 4B). Cells are then perfused with KRB with 2 mM Ca^{2+} and 20 μM CPA. During this perfusion Ca^{2+} influx through the plasma membrane can be evaluated (Figure 4Bbis). These data suggest that the reduction in mitochondrial Ca^{2+} uptake is

not due to a decrease in ER Ca^{2+} content or to a reduction in Ca^{2+} import from the external space inside the cell.

It is well known that the ER membrane interacts closely with mitochondria. This highly regulated interaction is endowed with key players of the Ca^{2+}-handing machinery. Many evidences indicate that the rapid Ca^{2+} uptake into mitochondria upon Ca^{2+} release relays on these close interactions. Thus, we investigated the ER-mitochondria interactions in starved HeLa cells coexpressing mitochondria-targeted RFP (mit-RFP) and an ER-targeted GFP (ERD1cpv). Cells were excited separately at 488 nm or at 543 nm and single images were recorded. Statistical quantification of the overlapping signal area (OSA) was performed from a Z-confocal stack covering the whole cell volume by the ImageJ program. Pearson's correlation index, a coefficient that is commonly used to measure the amount of overlap between two image pairs, was obtained to determine the ratio of localization between ER and mitochondria. Despite confocal microscopy can not resolve such contact sites, it has been reported that this method for quantifying areas of tethering is able to discriminate between known ER-mitochondria contact sites alterations (Manders et al. 1992). Glucose deprivation had no effect on the distribution and morphology of the mitochondrial/ER network. No significant differences were found when the regions of tethering were assessed quantitatively. As shown in Figure 4C, the area of co-localization is the same in control or starved cells. As negative control we used Mitofusin2 (MNF2) whose silencing was shown to increase the distance between two organelles. Pearson' co-localization coefficient (Manders et al. 1992) was calculated from z-axis confocal stacks and revealed no significant decrease in the ER-mitochondria juxtaposition (de Brito and Scorrano 2008). With this set of experiment we confirmed that the decreased in mitochondrial Ca^{2+} content is not due to decrease of contact sites between ER and mitochondria.

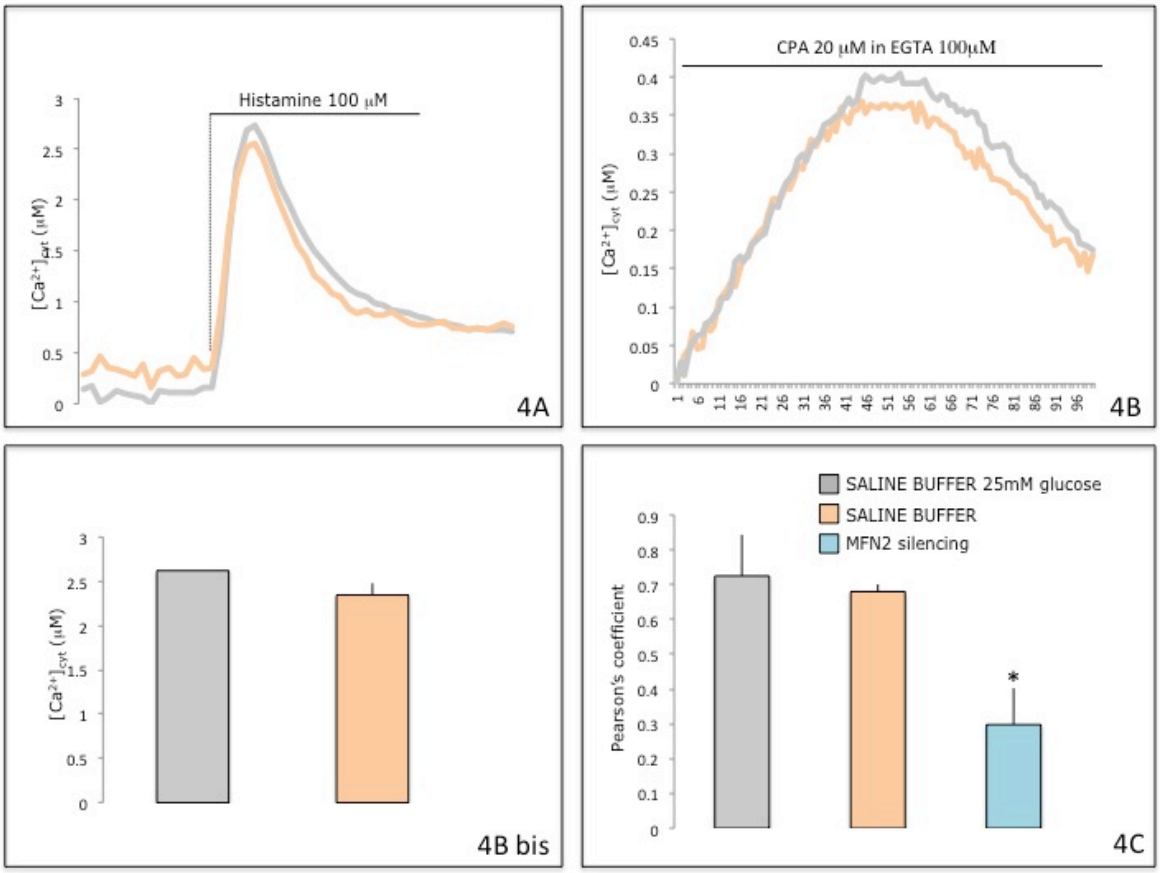

Figure 4A, B, C. Effect of glucose withdrawal on ER Ca^{2+} level and localization. (A, B, C) HeLa cells were transfected with cytAEQ and 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments for valuate cytoplasmic Ca^{2+} level and ER Ca^{2+} content were performed as described in Materials and Methods section. (A) Cytoplasmic Ca^{2+} levels. (B) ER Ca^{2+} content. (B bis) Ca^{2+} influx through the plasma membrane. (C) HeLa cells were transfected with mit-RFP and ER-D1cpv. Confocal images were acquired and Pearson's coefficient calculated with ImageJ. MFN2 silencing cells were used as positive control. Mean ± S.E.M. of values are shown.

We then focused our attention to the proper functioning of the mitochondrial Ca^{2+} uptake machinery. In order to measure the mitochondria Ca^{2+} influx independently to the ER Ca^{2+} source, we used two different strategies. First, after 2 hour of glucose deprivation in KRB supplemented with 1 mM Ca^{2+} and reconstitution with coelenterazine, cells were perfused with different solutions. ER was emptied with CPA (20 μM CPA in 100 μM EGTA) in perfusion for 240 seconds and then Ca^{2+} was given to cells (KRB with 1 mM Ca^{2+} and 20 μM CPA). During the perfusion with Ca^{2+}, we can evaluate the efficiency of mitochondria to uptake Ca^{2+} independently of the ER Ca^{2+} source. The second strategy involved cells starvation and reconstitution in KRB with 500 μM EGTA in order to induce the emptying of ER Ca^{2+} by

passive leak. After that, cells were maintained in KRB with 10 μM EGTA and subsequent addition of Ca^{2+}. After the first addition, Ca^{2+} was around 8.33 μM. In both experiments mitochondrial Ca^{2+} influx was slightly increased in starved cells compared to control but in a non-significative way (Figure 5A and 5B).

In order to investigate only the mitochondrial Ca^{2+} machinery contribution, we measured mitochondrial Ca^{2+} uptake in digitonin permeabilized cells. This experiment allows evaluating the properties of the mitochondrial Ca^{2+} uptake machinery independently of the ER Ca^{2+} release and the formation of microdomains of high [Ca^{2+}] in close proximity to mitochondrial Ca^{2+} channel. Measurements in digitonin-permeabilized cells are performed perfusing cells in IB for 60 seconds. Cells are then perfused with the same buffer with 20 μM digitonin for 60 second and washed with IB/EGTA buffer for other 60 second. The mitochondrial [Ca^{2+}] rise is then triggered by perfusing digitonin-permeabilized cells with a solution containing between 0.4 μM to 2 μM. A slow increase of Ca^{2+} into mitochondria is observed. As shown in Figure 5C, the reduction in mitochondrial Ca^{2+} uptake during starvation is not due to modification of the Ca^{2+} uptake machinery.

It is well known that the electrochemical potential ($\Delta\psi$) is formed in energized mitochondria and it is composed by an ionic potential ($\Delta\mu$) and a proton concentration gradient (ΔpH). We investigated the possible role of a modification in mitochondrial membrane potential or in changes in mitochondrial matrix pH in this reduction of mitochondrial Ca^{2+} uptake. The mitochondrion-selective tetramethylrhodamine dye (TMRM) was used to measure the mitochondrial membrane potential in HeLa cells after 2 hours of glucose deprivation. The accumulation of TMRM in mitochondria at low concentration has shown to be driven by their membrane potential (-150/-180mV). In order avoid the self-quenching of the probe, cells are loaded with very low TMRM concentration (20 nM). Changes in mitochondrial membrane potential will result in differences in absolute fluorescence intensity. The specificity of the signal

is verified by collapsing the $\Delta\psi_m$ through the treatment with the protonophore FCCP (10μM). Also after 2 hours of glucose deprivation, mitochondrial membrane potential is at the same level of control cells (Figure 5D).

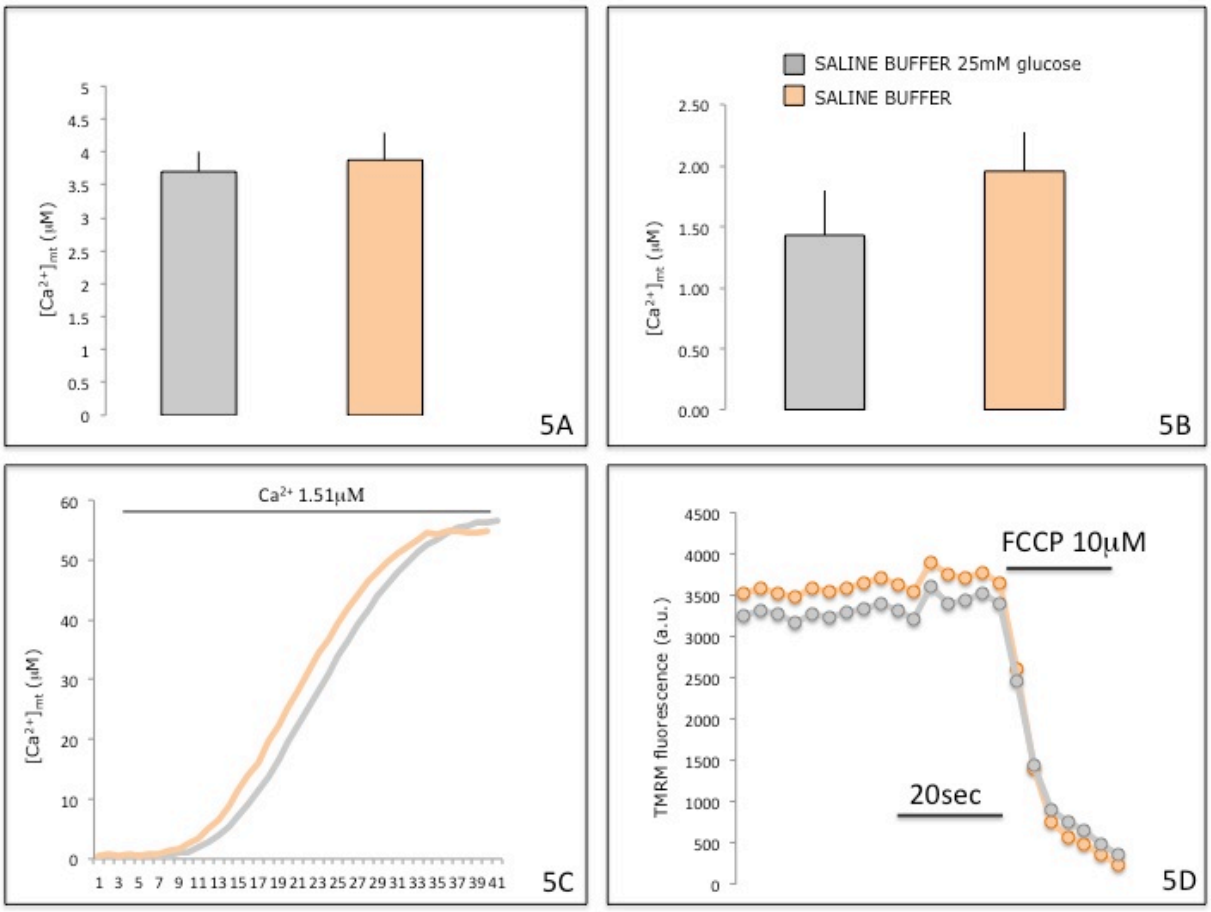

Figure 5A, B, C, D. Effect of glucose withdrawal on mitochondrial Ca^{2+} uptake machinery and mitochondrial membrane potential (A, B, C) HeLa cells were transfected with mitAEQmut and 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments for valuate mitochondrial influx and mitochondrial Ca^{2+} uptake in permeabilized cells were performed as described in Materials and Methods section. (A) Mitochondrial Ca^{2+} influx in HeLa cells by perfusing 1 mM Ca^{2+}. (B) Mitochondrial Ca^{2+} influx in HeLa cells by adding 8.33 μM Ca^{2+}. Mean ± S.E.M. of values are shown. (C) Mitochondrial Ca^{2+} uptake in permeabilized cells. (D) Measures of mitochondrial membrane potential were performed as described in Materials and Methods sections. Representative traces are shown.

Mitochondrial pH gradient (ΔpH_m) is the sole driving force for the electro neutral transport of many ions and metabolites in and out of the mitochondrial matrix. We started to study the role of ΔpH_m using a mitochondrially targeted, pH-sensitive YFP (SypHer). This is a ratiometric circularly permuted YFP. This probe allowed us to determine the real time measurement of pH

into the mitochondrial matrix in intact cells (Poburko et al. 2011). HeLa cells were grown onto 24 wells until 50% and then transfected with 4 μg of mtSypHer with a standard Ca^{2+}-phosphate procedure. 24 hours after transfection SypHer expressing cells were starved for 2 hours and then mounted into an open-topped chamber and maintained in KRB with or without 25 mM glucose. After KRB washout, pH was stopped between 5.5 and 10 by turnover of the bath solution. The added solutions were a Ca^{2+}-free intracellular buffer: 130 mM KCl, 10 mM NaCl, 2 mM K_2HPO_4, 1mM $MgCl_2$ supplemented with 20 mM MES or 20 mM HEPES or TRIS or Boric Acid (adjusted to pH 9.5 and 10 with KOH). For each experiment monensin (ionophore 5 μM) and nigericin (K^+/H^+ exchanger 1 μM) were also added to these Ca^{2+}-free intracellular buffers in order to increase the velocity to reach equilibrium between the external medium and the pH into the intermembrane space. For pH imaging, mtSypHer was alternately excited for 200-300 ms at 430 and 480 nm through a dicroic filter and fluorescence ratio (F480/430). For each cell, an 8-point calibration curve was fitted to a variable slope sigmoid equation. The resting matrix pH of HeLa cells remains stable also in starved HeLa cells (Figure 6A).

We investigated also the regulation of mitochondria morphology during glucose deprivation. Mitochondria typically form a reticular network radiating from the nucleus, creating an interconnected system that supplies the cell with essential energy and metabolites. These mitochondrial networks are regulated through the complex coordination of fission, fusion and distribution events (Ferree and Shirihai 2012). We assessed whether mitochondrial morphology is modified during glucose deprivation. It is known that autophagy induction by nutrient deprivation causes mitochondria elongation because the high level of cAMP induces the activation of protein kinase A (PKA) and the consequent reduction of the pro-fission dynamin related protein 1 (DRP1) (Gomes et al. 2011). In order to evaluate whether the strong reduction in mitochondrial Ca^{2+} uptake was due to the modification in mitochondrial morphology, HeLa cells were transfected with mit-RFP, a probe that allows the visualisation of mitochondria. Confocal images were recorder and quantification of the number and the volume of the

mitochondria were performed with ImageJ.

The observed reduction in mitochondrial Ca^{2+} uptake is not due to mitochondrial morphological changes; indeed, mitochondria number and size were unchanged after glucose withdrawal (Figure 6B). No evidence in the activation of proteins involved in the fission fusion process like Mitofusin2 and Drp1 were noticed (data not shown). Indeed HeLa cells overexpressed fission-fusion machinery proteins maintained this decrease in mitochondrial Ca^{2+} uptake during starvation.

Elongation is not the only morphological modification in mitochondria that is triggered during nutrient deprivation. Indeed, during starvation mitochondria turnover can be accelerated by an autophagic process, called mitophagy (Lee et al. 2012). Usually mitochondria are depolarized and co-localize with autophagosomes and the total amount of mitochondria drastically decreases. In the absence of extracellular nutrients cells degrade their intracellular damaged organelles in order to provide energy to cells and to overcome the low level of nutrient availability. We previously analyzed with TMRM the mitochondrial membrane potential (Figure 5D). We also investigated the amount of mitochondrial proteins. Starved HeLa cells maintained unchanged the levels of mitochondrial proteins (Figura 6C). Low mitochondrial Ca^{2+} uptake during starvation is maintained also in autophagy incompetent cells, demonstrating that the reduction in mitochondrial Ca^{2+} uptake is upstream to the induction of autophagy. The conversion from LC3I to LC3II and the phosphorylation of AMPK demonstrated that in glucose deprived HeLa cells autophagy is induced (data not shown).

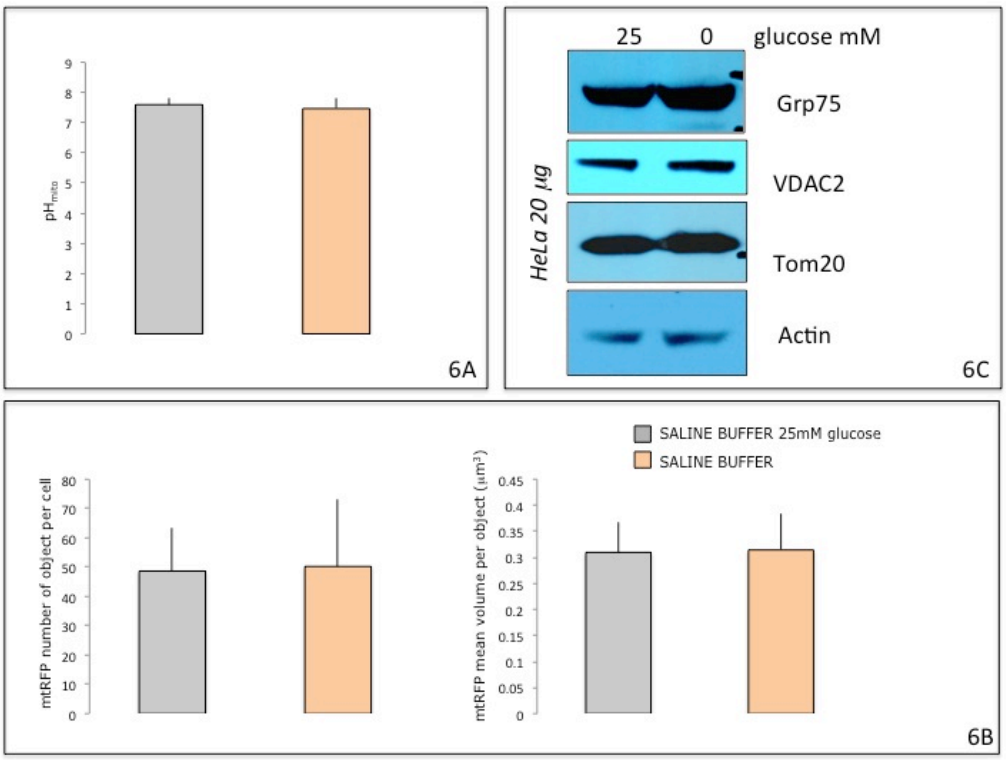

Figure 6A, B, C. Effect of glucose withdrawal on intermembrane pH (A), amount of mitochondrial proteins (C) and mitochondrial morphology (B).
(H) HeLa cells were transfected with mit-SyPher and 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments were performed as described in Materials and Methods section. (L) Immunoblot analysis of mitochondrial proteins prepared from HeLa cells. HeLa cells were starved for 2 hours in KRB with or without glucose. (I) HeLa cells were transfected with mit-RFP and after 48 hours confocal images of control (KRB + 25 mM glucose) and starved (KRB) cells were acquired. Evaluation of mitochondrial morphology was performed as described in Materials and Methods section.
Mean ± S.E.M. of values are shown.

In resting conditions, cellular ATP production is sustained mainly by glycolysis and mitochondrial respiratory chain through glucose catabolism. In mitochondria, Ca^{2+} accumulation has a role in ATP production, indeed it triggers the activation of the metabolism machinery, which increases ATP synthesis in the mitochondria and, hence, in the cytoplasm (McCormack et al. 1990, Hansford 1994). It is still controversial whether glucose withdrawal blocks ATP production due to the dependence of Krebs cycle enzymes to the amplitude of mitochondrial Ca^{2+} transient. In order to investigate the functional significance of the regulation of mitochondrial Ca^{2+} uptake by glucose deprivation, we measured intracellular changes in ATP levels after 2h of starvation in living cells through the firefly luciferase assay.

We found that cytoplasmic ATP levels decrease in starved cells compared to control cells, but in a non-significative way (Figure 7A).

Mitochondria are also the main source of reactive oxygen species (ROS) production. Excessive ROS production contributes to organelle damage and triggers redox signalling from mitochondria to the cell (Mailloux and Harper 2012). It is well known that mitochondrial ROS production is a key signal for autophagy induction (Li et al. 2011). We investigated whether the observed reduction in mitochondrial Ca^{2+} uptake during glucose deprivation can be translated into an increase of ROS production and the consequent induction of autophagy. We used DCFDA as cell-permeant indicator for reactive oxygen species. Oxidation of this probe can be detected by monitoring the increase in fluorescence. The amount of fluorescence is correlated with the amount of ROS production. Figure 7B shows that no difference in the ROS production was detected in starved HeLa cells.

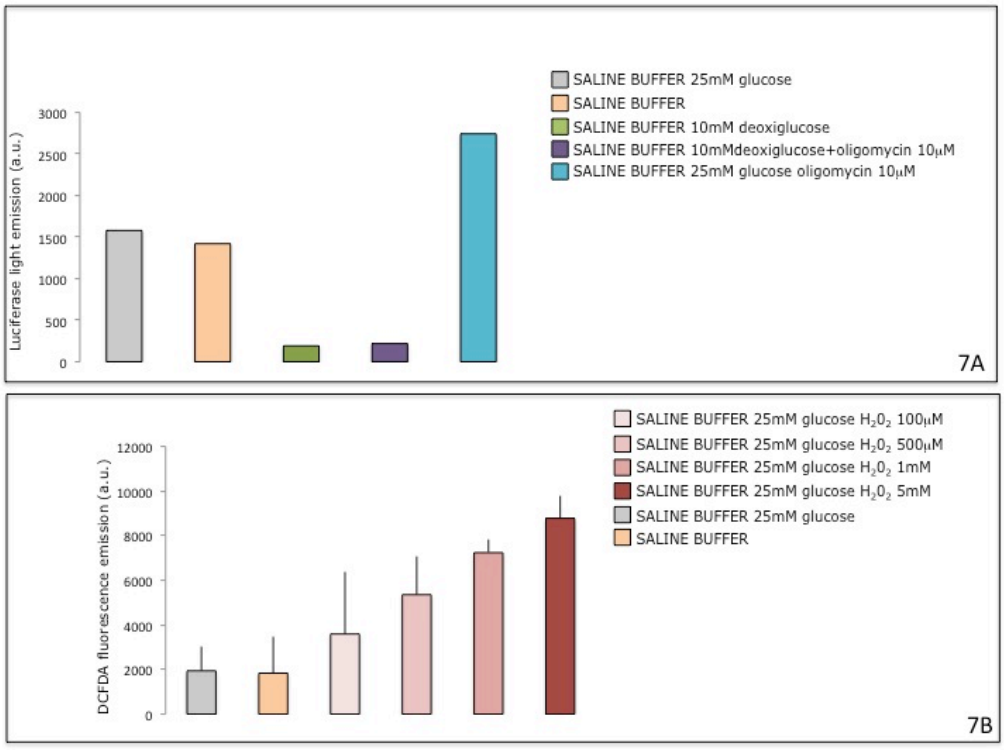

Figure 7A, B. (A) Effect of glucose withdrawal on cytoplasmic ATP levels and (B) ROS production after 2 hours of glucose deprivation.
(A) HeLa cells were transfected with cyt-LUC and 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments were performed as described in Materials and Methods section. (B) DCFDA is cell-permeant indicators for ROS production. Oxidation of this probe can be detected by monitoring the increase in fluorescence. Experiments were performed as described in Materials and Methods section. H_2O_2 concentration scale was used as positive control.

Mean ± S.E.M. of values are shown.

6.3 MICU1 controls mitochondrial Ca^{2+} uptake in response to glucose availability

Given that none of the physiological parameters of mitochondria was affected by starvation, we considered that during glucose deprivation a specific protein could control the amplitude of mitochondrial [Ca^{2+}]. We investigated the possible involvement of numerous proteins in the contact sites between mitochondria and ER (MAM) and the possible role of proteins involved in the Ca^{2+} uptake machinery. No one of the analyzed mitochondrial proteins showed modifications during glucose deprivation with one exception (Figure 6C). Indeed, we found that in our experimental condition MICU1 was rapidly degraded (Figure 8A). MICU1 is a regulator of the mitochondrial Ca^{2+} uniporter (MCU). It has been shown that it localizes to mitochondria and its loss was initially reported to abolish mitochondrial Ca^{2+} entry (Perocchi et al. 2010). This protein has two canonical EF hands that are essential for its activity in Ca^{2+} sensing and gating the activity of MCU channel. However, we found that MICU1 overexpression increases mitochondrial Ca^{2+} uptake and it has a synergistic effect with the MCU overexpression (unpublished data). Electrophysiological data revealed that in presence of μM [Ca^{2+}], MICU1 is able to increase the open probability of MCU with an increased Ca^{2+} uptake into mitochondria (unpublished data). MICU1 is also required to preserve normal mitochondrial [Ca^{2+}] under basal conditions. In its absence mitochondria become constitutively loaded with Ca^{2+}, triggering excessive ROS generation (Mallilankaraman et al. 2012).

Proteins are continually synthetized and degraded in all cells in a process called protein turnover. Every protein shows a characteristic turnover rate, due to its localization, post-translational modifications and functions. The half-life can vary from few minutes to several weeks but the half-life of a given protein in different organs and species is generally similar. The half-life of a protein is used as a quantitative measurement of this “dynamic equilibrium”.

Usually it is measured by blocking the protein synthesis by a protein synthesis inhibitor (cycloheximide) and by collecting cells at different time points in order to evaluate the relative amount of the investigated protein into the collected samples.

Based on MICU1 short half-life of around 3 hours (Figure 8B), we wondered whether during glucose deprivation it could be post-translationally modified and consequently rapidly degraded.

There are many mechanisms to degrade mitochondrial proteins. Usually damaged mitochondria are removed by mitophagy but this process, besides being slow, is not selective for the removal of a single specific protein.

Protein degradation through the ubiquitin-proteasome system is the major pathway of non-lysosomal proteolysis of intracellular proteins. It plays important roles in a variety of fundamental cellular processes and the central element of this system is the covalent linkage of ubiquitin to targeted proteins, which are then recognized by the proteasome. However, usually the quality control of mitochondrial proteins is ensured by two ATP dependent proteases, AAA proteases, which are an integral part of the inner mitochondrial membrane (Arnold and Langer 2002), although recent reports suggest that ubiquitinylation may play an important role in mitochondria protein quality control (Margineantu et al. 2007, Radke et al. 2008). Still unknown is the mechanism of the rapid degradation of MICU1.

During glucose deprivation we tried to block the MICU1 degradation by inhibiting the ubiquitin proteasome system, the autophagy induction and the mAAA activity. As shown in the Figure 8C only the treatment with MG132, a specific cell permeant proteasome inhibitor, partially blocks the reduction of agonist-evoked mitochondrial [Ca^{2+}] rises after glucose deprivation.

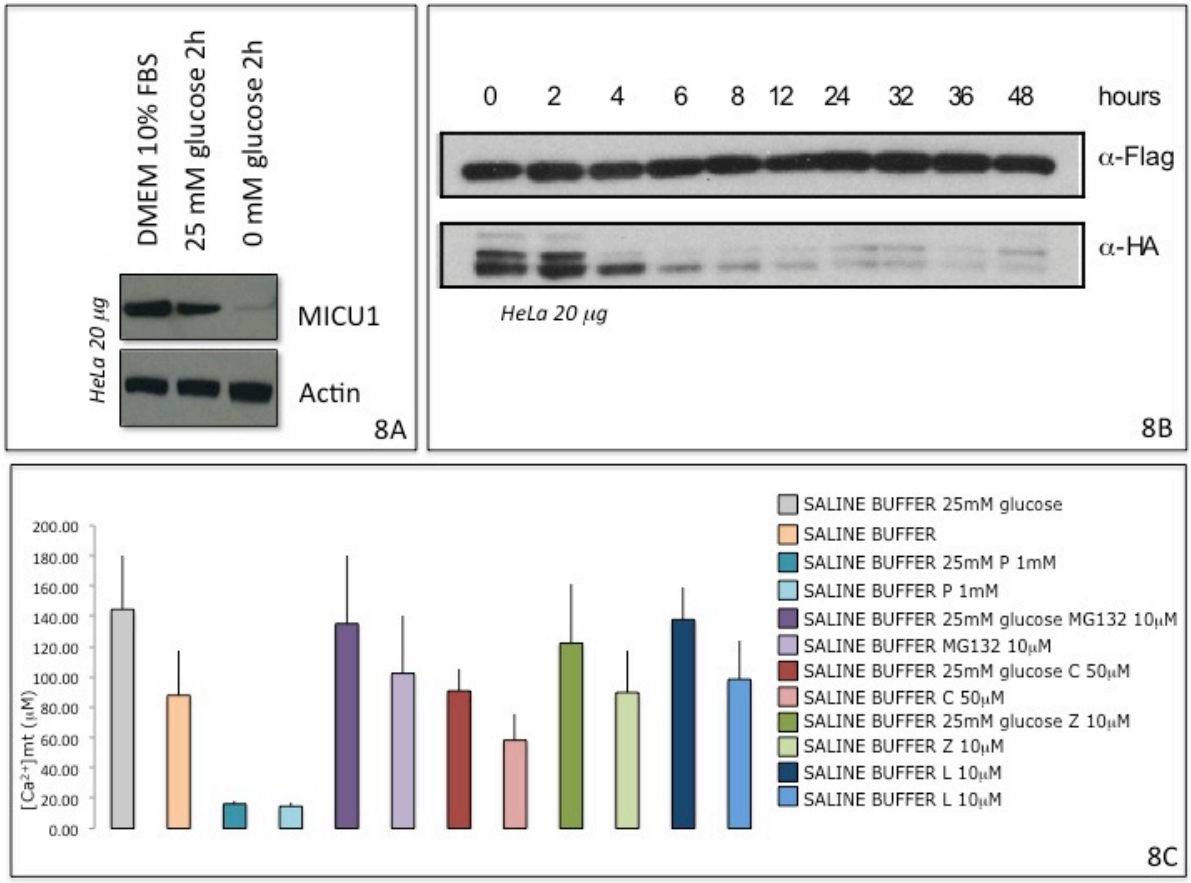

Figure 8A, B, C. Effect of glucose withdrawal on MICU1. (A) Immunoblot analysis of MICU1. Proteins were prepared from HeLa cells treated with DMEM (DMEM supplemented with 10% FBS for 2 hours), 25 mM glucose (KRB + 25 mM glucose for 2 hours) or no glucose (KRB for 2 hours). (B) Immunoblot analysis using HA and FLAG antibodies. HeLa were transfected with MCU-FLAG or with MICU1-HA. Proteins were prepared from MCU and MICU1 overexpressing HeLa cells treated with 100 µg/ml cycloheximide in DMEM supplemented with 10% FBS and collected at different time points (0, 2, 4, 6, 12, 24, 32, 36 and 48 hours). (I) HeLa cells were transfected with mtAEQmut and after 48 hours treated for 2 hours with different inhibitors of protein degradation of ubiquitin-proteasome system (MG132, Z=Z Leu Leu Phe CHO and L=Lactacistil), inhibitors of autophagy (C=cloroquine) or an inhibitor of mAAA degradation system (P=1-10-phenanthroline monohydrate). HeLa cells were also treated as control (KRB + 25 mM glucose) and starved (KRB). Mean ± S.E.M. of values are shown.

MICU1 degradation during nutrient deprivation is abolished by MG132, suggesting that MICU1 could be eliminated via ubiquitin proteasome system (Figure 9A). MICU1 half-life is increased when cells are treated with the proteasome inhibitor, thus confirming that the stability of this protein is dependent on the ubiquitin proteasome system (Figure 9B).

High-resolution mass spectrometry data reveal that five lysines in MICU1 are ubiquitylated (Hornbeck et al. 2012) and thus we decided to substitute this lysine with arginine in order to generate a MICU1 protein mutant that is not able to bind ubiquitin in this site. We generated 5 different mutant forms of MICU1 for each lysine (MICU1^{K102R}-HA, MICU1^{K103R}-

HA, MICU1^{K104R}-HA, MICU1^{K296R}-HA, MICU1^{K359R}-HA) and one mutant form of MICU1, which all the lysine mutated (MICU1$^{K102R, K103R, K104R, K296R, K359R}$-HA).

We compared the expression levels of MICU1-HA and MICU1^{K296R}-HA expression plasmids and we found that all of these ubiquitylation incompetent mutants reach a higher level of protein expression (Figure 9C), thus suggesting that these mutants are more stable per se.

At first, we investigated the intracellular distribution of these MICU1 mutated forms by performing immunofluorescence of HeLa cells overexpressing these constructs that show perfect mitochondrial localization (data not shown). We also performed a sub fractionation experiment in order to purify mitochondria from HEK293A transiently transfected with MICU1$^{K102R, K103R, K104R, K296R, K359R}$-HA (data not shown). In all these experiments, we found that the different MICU1 mutants correctly localized into mitochondria.

We focused our attention in particular to MICU1^{K296R}-HA for the higher score of mass spectrometry results and MICU1$^{K102R, K103R, K104R, K296R, K359R}$-HA. Probably also due to its low transfection efficiency, MICU1 overexpression on HeLa cells challenged with maximal agonist stimulation increase mitochondrial Ca^{2+} uptake by nearly 10%. On the contrast MICU1^{K296R}-HA overexpression increase agonist-evoked mitochondrial [Ca^{2+}] rises by nearly 100% (Figure 9D).

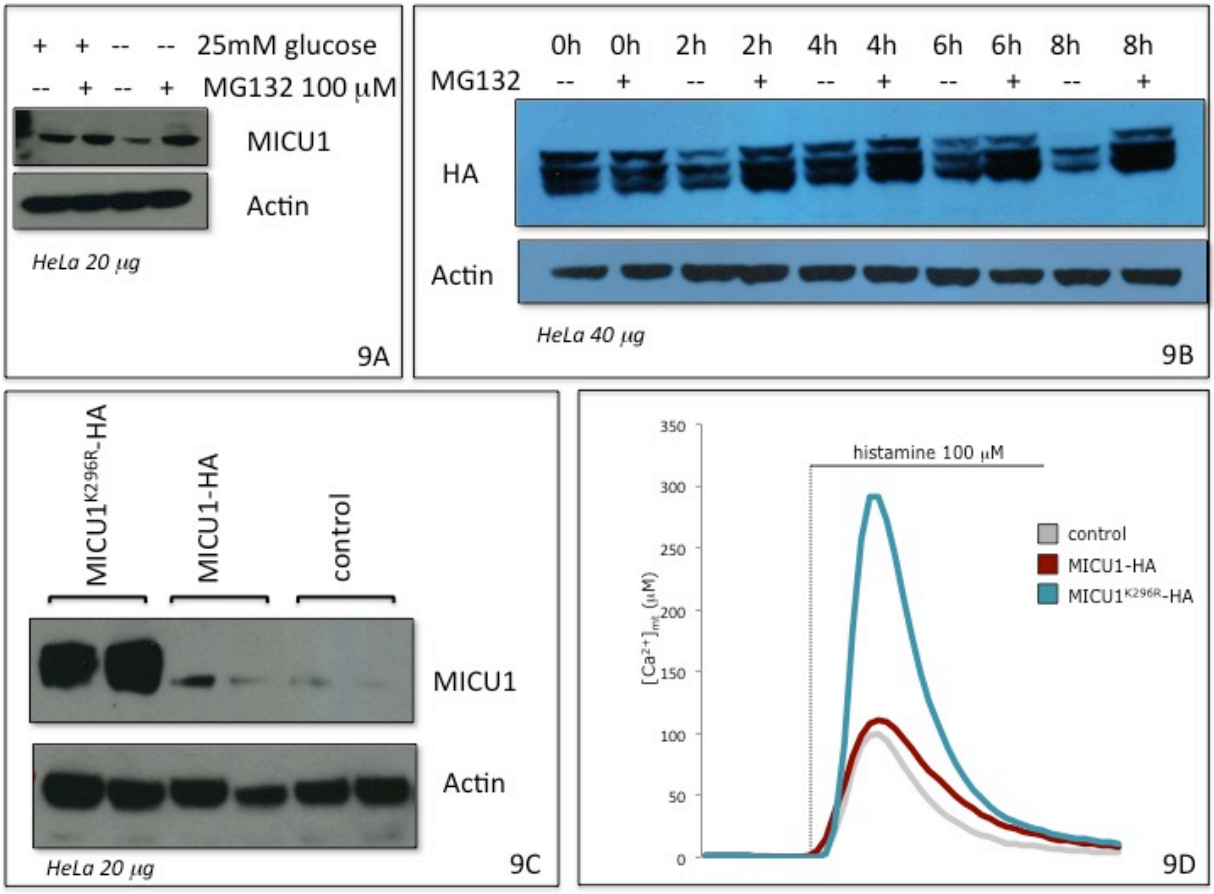

Figure 9A, B, C, D. Effect on MICU1 during the inhibition of the proteasome system and analysis of MICU1 mutants. (A) Immunoblot analysis of MICU1. Proteins were prepared from HeLa cells incubated in KRB for 2 hours with or without 25 mM glucose and 100 μM MG132 (proteasome inhibitor). (B) Immunoblot analysis of HA tag (MICU1-HA). HeLa cells were transfected with MICU1-HA. After 24 hours proteins were prepared from MICU1-HA overexpressing cells were treated with 100 μg/ml cycloheximide and 100 μM MG132 in DMEM supplemented with 10% FBS and then they were collected at different time points (0, 2, 4, 6 and 8 hours). (C) Immunoblot analysis of MICU1. HeLa cells were transfected with MICU1-HA and MICU1^{K296R}-HA and lysed after 48 hours. (D) mtAEQmut was coexpressed with MICU1-HA and MICU1^{K296R}-HA in HeLa cells. Experiments were performed as described in Materials and Methods sections.

We assumed that in glucose deprivation MICU1 degradation causes a decrease in mitochondrial Ca^{2+} uptake. To assess this hypothesis we overexpressed MICU1^{K296R}-HA and MICU1$^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$-HA in our starvation experiments in order to block the degradation of MICU1 and to prevent the uptake reduction. In order to overcome the aequorin probe saturation problems, we challenged HeLa cells with two different concentrations of agonist. We observed that the strong effect of starvation between control and starved cells is partially abolished with the overexpression of this ubiquitylation incompetent mutant (Figure 10A).

In order to understand if MICU1 has a specific role in the control of mitochondrial Ca^{2+} uptake during glucose deprivation, we generated a new mutant form of this protein. MICU1 has two canonical EF hands that are essential for its Ca^{2+} sensing activity (Perocchi et al. 2010). The EF hand is a helix loop helix structural domain found in a large family of Ca^{2+} binding proteins. It consists of two alpha helices positioned perpendicularly to one another and linked by a short loop region (usually about 12 AA) that binds Ca^{2+} ions suggesting that this motif may undergo conformational changes that enable Ca^{2+} regulated functions.

A dominant negative form of a protein is a mutated protein that can interact with the same elements as the wild-type product, but blocks its function. We generated a dominant negative form of this protein by the mutation of four residues in these EF hands ($MICU1^{D231A, E242K, D421A, E432K}$-HA) and we overexpressed this protein during glucose deprivation. We confirmed that this mutated protein plays a dominant negative function: indeed, MICU1 capacity of increasing mitochondrial Ca^{2+} uptake is blocked by the overexpression of these mutant proteins. The overexpression of $MICU1^{D231A, E242K, D421A, E432K}$-HA abolished the effect of glucose deprivation on mitochondrial Ca^{2+} uptake (Figure 10B).

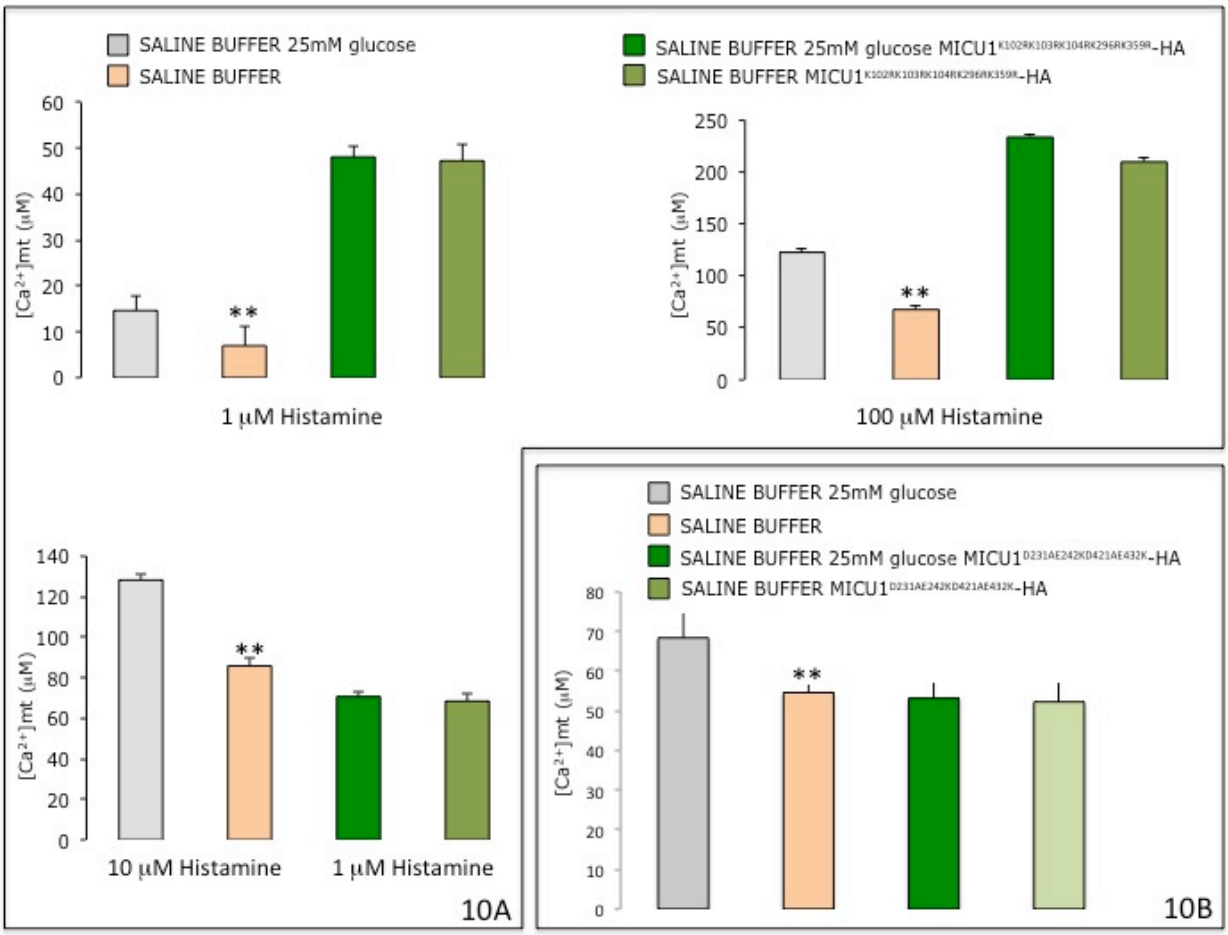

Figure 10A, B. Effect of MICU1$^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$-HA and MICU1$^{D231A,\ E242K,\ D421A,\ E432K}$-HA overexpression on glucose withdrawal (A, B) HeLa cells were transfected with mitAEQmut and 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments for valuate mitochondrial Ca^{2+} uptake were performed as described in Materials and Methods section. Mean ± S.E.M. of values are shown.

In order to mimic the effects of glucose deprivation on mitochondrial Ca^{2+} uptake machinery we silenced MICU1. We silenced MICU1 with high efficiency but the effect on mitochondrial [Ca^{2+}] is controversial, since agonist evoked mitochondrial [Ca^{2+}] rises is increased of around 40%. Furthermore, after MICU1 silencing, glucose deprivation evokes a decrease of mitochondrial Ca^{2+} uptake (Figure 11A). It is published that MICU1 silencing increases the mitochondria Ca^{2+} basal levels (as confirmed by our data: figure 5A) but the correlation between this and the increase also in agonist evoke mitochondrial [Ca^{2+}] rise is still unknown (Mallilankaraman et al. 2012).

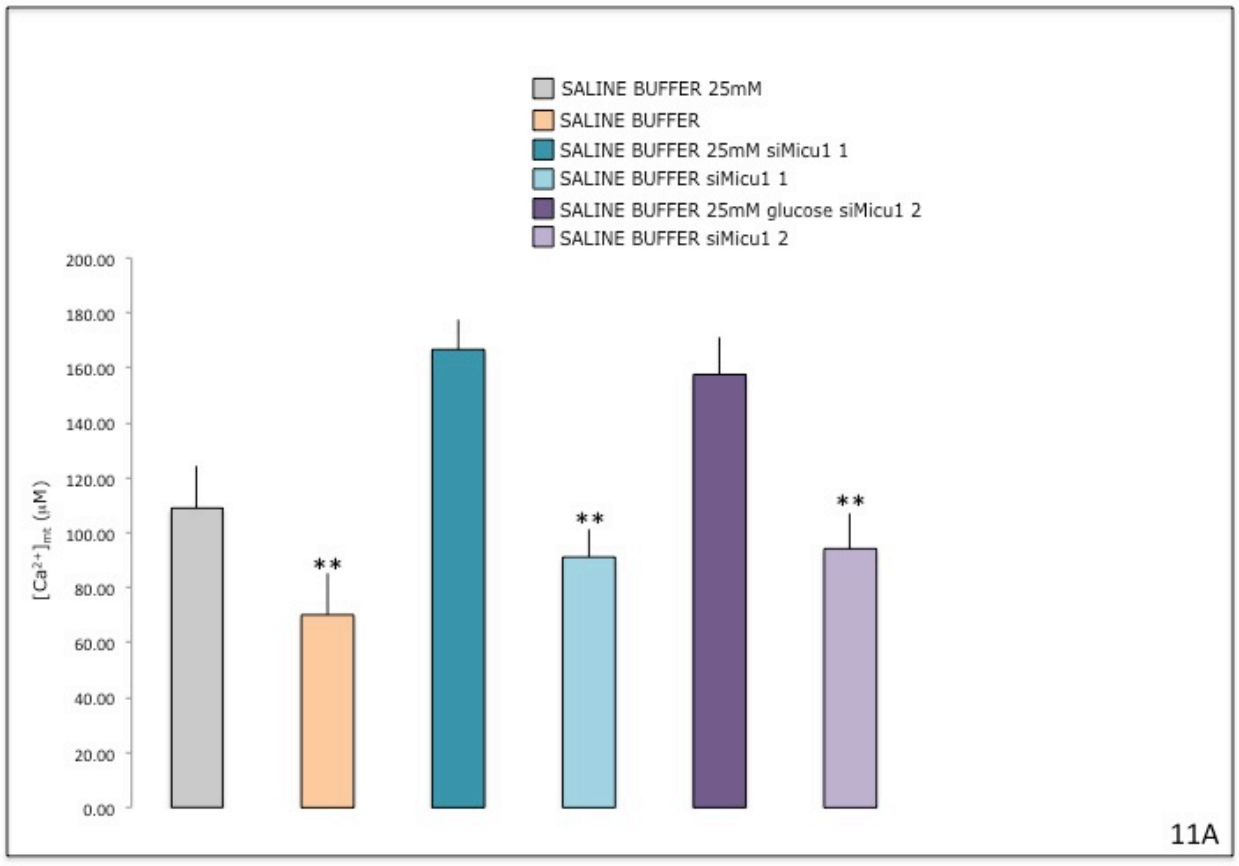

Figure 11A. Effect of glucose withdrawal on MICU1 silencing cells (A) HeLa cells were co-transfected with mitAEQmut and two different siRNA against MICU1. 48 hours after transfection starved for 2 hours in KRB with or without 25 mM glucose. Experiments to evaluate mitochondrial Ca^{2+} uptake were performed as described in Materials and Methods section. Mean ± S.E.M. of values are shown.

During my PhD, I focused my attention on the role of MICU1 protein during nutrient deprivation. We assumed that during glucose withdrawal there is a specific mechanism that connects sensing of glucose levels with the amount of the MICU1 protein. This protein appears to be unstable and its function is adjusted by its amount. In our model, we assume that blocking the degradation system of MICU1 prevents the effect of glucose deprivation in HeLa cells.

7. Discussion

The variation of the metabolic availability induces intracellular adaptations to provide energy for cellular functions. These adaptations are traduced in intracellular changes in metabolic reactions in order to overcome the decrease in feeding intake. Many data are available on the contribution of mitochondria in the regulation of intracellular metabolism (McCormack et al. 1990, Rasola and Bernardi 2011). Thanks to ATP production, mitochondria provide energy for cells and connect two important intracellular signals: ATP and Ca^{2+}. Still unknown are the specific mitochondrial proteins that sense nutrient availability and how they can translate the variation of nutrients concentration into modulation of Ca^{2+} uptake which in turn induces intracellular adaptive processes like autophagy (Deter and De Duve 1967). When triggered, autophagy provides nutrients from intracellular stores (Lum et al. 2005).

The contribution of Ca^{2+} in the activation or inhibition of autophagy is still matter of debate (Decuypere et al. 2011), but, more importantly, what is still completely unknown is the possibility of a direct link between nutrient availability and the modulation of Ca^{2+} signaling in the regulation of the autophagy process.

The goal of my PhD research was to investigate whether the adaptive response to glucose withdrawal was through the modulation of mitochondrial Ca^{2+} uptake. In particular, we focused our attention in the possible involvement of mitochondrial proteins as connection point between glucose levels and changes in mitochondrial Ca^{2+} content.

The first report on Ca^{2+}-dependent regulation of autophagy dates back to 1993 (Gordon et al. 1993), and it suggests a complex role for Ca^{2+}, since chelation of either intra- and extracellular Ca^{2+} as well as elevating cytosolic [Ca^{2+}] suppresses autophagy. Only recently this topic returned to be of great interest but the published results are again in conflict regarding the role of intracellular Ca^{2+} in autophagy induction. Another critical point is that the lack of information on the sensors of nutrient availability and their direct effects on Ca^{2+} signaling.

Many reports described Ca^{2+} as an inhibitor of autophagy (Sarkar et al. 2005, Criollo et al. 2007, Berridge 2009). These studies are focused on the inositol 1,4,5-triphosphate (IP_3) receptor (IP_3R), an ubiquitously expressed intracellular Ca^{2+}-release channel, located mainly in the endoplasmic reticulum (ER). IP_3Rs mediate Ca^{2+} release from the ER into the cytoplasm in response to elevations in cytoplasmic [IP_3] produced in cells after stimulation by hormones, growth factors or antibodies (Berridge 2009). In 2005, Sarkar et al. reported the use of Li^+ for autophagy stimulation (Sarkar et al. 2005). Li^+ acts through inhibition of inositol monophosphatase (IMPases), thereby reducing the IP_3 levels. Also chemical inhibition of IP_3Rs with xestospongin (XeB) or suppression of its expression using siRNA induce autophagy in HeLa cells (Criollo et al. 2007). The IP_3R-mediated inhibition of autophagy was also verified in IP_3R triple knock out (TKO) chicken DT40 B lymphocytes, which showed higher autophagy levels (Cardenas et al. 2010, Khan and Joseph 2010). Different downstream mechanisms and effectors have been proposed for the inhibitory role of IP_3Rs and Ca^{2+} in autophagy. In a recent study, Cardenas and coworkers showed decreased glucose and O_2 consumption, and AMPK activation in TKO cells, suggesting a mechanism whereby constitutive Ca^{2+} release through IP_3Rs fuels into the mitochondria, therefore augmenting mitochondrial bio-energetics and ATP production (Cardenas et al. 2010). When these essential Ca^{2+} signals are abolished, an increased of AMP/ATP ratio is observed with a consequent AMPK activation and subsequent stimulation of autophagy. In this study, the authors can not exclude a scaffold function for the IP_3R.

During my PhD I could benefit from the long-standing experience of our group in the analysis of cellular Ca^{2+} signals in order to precisely investigate mitochondrial Ca^{2+} homeostasis during glucose deprivation. By using the most modern technologies based on both fluorescent and bioluminescent Ca^{2+}-probes (Rizzuto et al. 1998, Pinton et al. 2007), we could also correlate the effects of nutrient deprivation on Ca^{2+} signals to their consequences on the induction of autophagy. Moreover, we also looked at the possible involvement in this process of an important

regulator of the mitochondrial Ca^{2+} uptake machinery, MICU1 (Perocchi et al. 2010, Mallilankaraman et al. 2012).

We decided to completely remove all nutrients from the cell culture medium and to add to control cells only 25 mM glucose. We noticed that 2 hours of glucose deprivation induce in HeLa cells a constant decrease in total protein deacetylation as consequence of Sirtuins activation and a phosphorylation of AMPK (Figure 1A). In order to analyze if this effect on AMPK was through modulation of mitochondrial Ca^{2+} transients, we activated AMPK by an AMP mimic and in parallel we changed the amplitude of mitochondrial Ca^{2+} transient. AMPK activation can be blocked by an increase of mitochondrial Ca^{2+} transient, by blocking the mitochondrial Ca^{2+} release. It is clear that the amount of Ca^{2+} into mitochondria determines the activation of AMPK (Figure 1B). This effect on AMPK can be direct or a consequence of the known modulation of mitochondrial ATP production (Hardie 2003). In the future, we will investigate if the regulation of this protein is simply through the regulation of the mitochondrial Ca^{2+} content or through the effect of Ca^{2+} on mitochondrial ATP production. We also found that SIRT1 and SIRT3 have a role in the control of the magnitude of Ca^{2+} transient inside mitochondria. Indeed, mitochondrial Ca^{2+} uptake in SIRT3 and SIRT1 overexpressing HeLa cells was drastically reduced (Figure 1C). The role of SIRT1 in the control of mitochondrial Ca^{2+} uptake is still controversial because it is a nuclear and cytoplasmic protein (Nogueiras et al. 2012) (Tanno et al. 2007). We hypothesize that the control is through the induction of mitochondrial biogenesis, as previously reported (Zhong and Mostoslavsky 2011). Future experiments will be focused on the analysis of the acetylation state of some members of the Ca^{2+} uptake machinery (Newman et al. 2012).

We then assessed the direct role of glucose deprivation on mitochondrial Ca^{2+} uptake and we found that after 2 hours of glucose deprivation mitochondrial Ca^{2+} uptake drastically decreases (Figure 2). We confirmed this data also with a FRET-based Ca^{2+} probe Cameleon (Palmer and Tsien 2006). Furthermore, we found no difference in resting conditions (Figure 3A

and 3B). This adaptive response to glucose deprivation is physiological because it happens in short period of time and it is reversible; indeed if starved for a long period of time (10 hours) cells have a reduced mitochondrial Ca^{2+} uptake for 4 hours and than mitochondrial Ca^{2+} uptake levels return to the one of normal feeding conditions (Figure 3C).

We next investigated the specificity of this effect. Indeed, decrease in Ca^{2+} uptake levels could be due to modifications in mitochondrial physiological parameters. This organelle is able to uptake Ca^{2+} because it is in close proximity to ER Ca^{2+} source. IP_3R opening generates micro domains of high [Ca^{2+}] in the contact sites near to the Ca^{2+} uptake machinery (Rizzuto et al. 1993). Reduced ER Ca^{2+} content or ER-mitochondria contact sites induce a decreased mitochondrial Ca^{2+} transient. We investigated the role of ER Ca^{2+} content in our model of glucose deprivation and we found that the reduction in mitochondrial Ca^{2+} uptake was not a secondary effect due to modification in ER Ca^{2+} content (Figure 4). Glucose deprivation had also no effect on the distribution and morphology of the mitochondrial/ER network. Indeed, no significant differences were found when the regions of tethering were assessed quantitatively (Figure 4C).

This is not the only mechanism that allows mitochondria to uptake Ca^{2+}. It is well known that the electrochemical potential ($\Delta\psi$) is formed in energized mitochondria and it is composed of an ionic potential ($\Delta\mu$) and a proton concentration gradient (ΔpH) (Mitchell 1967). We investigated the possible role of a modification in mitochondrial membrane potential or in changes in mitochondrial matrix pH in this reduction of mitochondrial Ca^{2+} uptake. With the TMRM dye we demonstrated also that there were no changes in mitochondrial membrane potential. Also the mitochondrial pH gradient (ΔpH_m) was maintained (Figure 5D).

Moreover, we investigated the regulation of mitochondria morphology during glucose deprivation. Mitochondria typically form a reticular network radiating from the nucleus, creating an interconnected system that supplies the cell with essential energy and metabolites. This mitochondrial network is regulated through the complex coordination of fission, fusion and

distribution events (Ferree and Shirihai 2012). We observed that the reduction in mitochondrial Ca^{2+} uptake is not paralleled by mitochondrial morphological changes (Figure 6B).

Glucose deprivation triggers autophagy without the involvement of ROS production and changes in ATP levels (Figure 7). Since none of the physiological parameters of mitochondria was modified, we considered that during glucose deprivation a specific protein could control the amplitude of mitochondrial [Ca^{2+}]. We investigated the possible involvement of a large number of proteins in the contact site between mitochondria and ER (MAM) and the possible role of proteins involved in the Ca^{2+} uptake machinery. Our evidences suggest that, in our experimental condition, only MICU1 is rapidly degraded (Figure 8A). MICU1 has a half-lives around three hours (Figure 8B). This experiment suggests that this protein has a highly dynamic equilibrium compared to the uniporter channel that appears to be highly stable. With this information, we can assume that changes in mitochondrial calcium uptake are regulated by the degradation of the regulator (MICU1) rather than of the channel (MCU).

We wondered whether during glucose deprivation MICU1 could be modified with a post-translational modification and rapidly degraded. During glucose deprivation we tried to block the MICU1 degradation by inhibiting the ubiquitin proteasome system, the autophagy induction and the mAAA activity, and we found that only the treatment with a specific cell permeant proteasome inhibitor, partially blocks the reduction of agonist-evoked mitochondrial [Ca^{2+}] rises after glucose deprivation (Figure 8C). The degradation of MICU1 during nutrient deprivation is abolished by MG132, suggesting that MICU1 could be eliminated via ubiquitin proteasome system (Figure 9A). Indeed, MICU1 half-life increased when cells were treated with the proteasome inhibitor (Figure 9B).

Based on high-resolution mass spectrometry data we generated MICU1 mutants defective for ubiquitination (Hornbeck et al. 2012). These mutant forms of MICU1 appear to be more stable and the effect on Ca^{2+} is parallel with this stability (Figure 9C).

We assumed that in glucose deprivation MICU1 degradation causes a decrease in mitochondrial Ca^{2+} uptake. To assess this hypothesis we overexpress MICU1^{K296R}-HA and MICU1$^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$-HA in our starvation experiments in order to block the degradation of MICU1 protein and to prevent the reduction of mitochondrial Ca^{2+} uptake. Our hypothesis was confirmed by the fact that the effect of starvation is partially abolished by overexpressing MICU1^{K296R}-HA and MICU1$^{K102R,\ K103R,\ K104R,\ K296R,\ K359R}$-HA (Figure 10A).

In order to understand the role of MICU1 in the control of mitochondrial Ca^{2+} uptake during glucose deprivation, we also overexpressed a dominant negative form of MICU1 (MICU1$^{D231A,\ E242K,\ D421A,\ E432K}$-HA) during glucose deprivation. The overexpression of this mutant abolishes the effect of glucose deprivation on mitochondrial Ca^{2+} uptake, further supporting the idea that MICU1 finely and dynamically regulates mitochondria Ca^{2+} uptake in different conditions (Figure 10B). This result is difficult to interpret since, as already known (Perocchi et al. 2010, Mallilankaraman et al. 2012), MICU1 plays a fundamental role in the Ca^{2+} uptake machinery. Therefore, our results using the dominant negative do not allow us to distinguish between the generic role of MICU1 on the uptake machinery per se and the specific role of this protein on the regulation of mitochondrial Ca^{2+} during glucose deprivation.

In order to mimic the effect of glucose deprivation on mitochondrial Ca^{2+} uptake machinery, we silenced MICU1 and we analysed mitochondrial Ca^{2+} homeostasis. MICU1 overexpression increased mitochondrial Ca^{2+} uptake (unpublished data) and Perocchi and coworkers (Perocchi et al. 2010) have also shown that absence of MICU1 abolishes mitochondrial Ca^{2+} entry. In contrast with these data, we found that MICU1 silencing increases mitochondrial Ca^{2+} transients of around 40% compared to control cells (Figure 11A). This result is in agreement with a recent study that demonstrated that, in absence of MICU1, mitochondria become constitutively loaded with Ca^{2+} (Mallilankaraman et al. 2012). These models suggest that the right role of MICU1 on mitochondrial Ca2+ uptake machinery is still far to be uncovered. Moreover, after MICU1 silencing, glucose deprivation still evokes a decrease of mitochondrial

Ca^{2+} uptake, strongly indicating that the picture of the adaptation to nutrient availability is more complex and in the future we will elucidate the precise pathway that controls this phenomena.

My PhD research was focused on the physiological relevance of MICU1 during nutrient deprivation. We demonstrated that during glucose withdrawal there is a specific mechanism that connects sensing glucose levels with the amount of MICU1 protein. The turnover of this protein appears to be highly dynamic and its function is adjusted by its amount.

Our data suggest that blocking the degradation of MICU1 prevents the effects of glucose deprivation in HeLa cells. In conclusion, this work presents some novel and unexpected findings on the cellular adaptations to glucose deprivation mediated by the control of mitochondrial Ca^{2+} homeostasis. To overcome the glucose dependence is a metabolic adaptation that enables cancer cells to proliferate and survive in hypoxic areas (Vander Heiden et al. 2009). The clarification of the link between the decrease in glucose availability and autophagy induction via modulation of mitochondrial Ca^{2+} content may lead to understand if MICU1 plays a central role in this process. This information may provide more efficient pharmacological intervention to treat human disorders in which mitochondria have been proposed to play a central role in the pathogenesis, for example, of metabolic disorders and cancer.

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