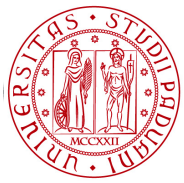

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITA' DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO NEUROBIOLOGIA

CICLO XXVIII

REGULATION OF MITOCHONDRIAL Ca^{2+} UPTAKE: ROLE OF CCDC90A AND CCDC90B

Direttore della Scuola : Ch.mo Prof. Paolo BERNARDI

Coordinatore d'indirizzo: Ch.mo Prof. Daniela PIETROBON

Supervisore : Ch.mo Prof. Rosario RIZZUTO

co-Supervisore: Dott. Diego DE STEFANI

Dottorando : Elisa PENNA

"We are not now that strength which in old days
Moved earth and heaven, that which we are, we are;
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield."

-"*Ulysses*" by Alfred, Lord Tennyson (1809-1892)

SOMMARIO

ABSTRACT

Intracellular signaling requires rapid and efficient changes in the intracellular concentration of messengers through time. One of most important second messenger is Calcium. Mitochondria are important component in intracellular signaling and Ca^{2+} is one of the key regulators of organelle physiology. Its uptake inside the organelle is driven by the potential across the inner mitochondrial membrane, through a selective ion channel, called Mitochondrial Calcium Uniporter (MCU) (De Stefani, D., et al., Nature (2011); Baughman, J.M., et al., Nature (2011)). Ca^{2+} plays a pleiotropic role into mitochondria, ranging from the regulation of ATP production, to the shaping of cytoplasmic Ca^{2+} waves and activation of apoptosis (Rizzuto, R., et al., Nat Rev Mol Cell Biol (2012)). MCU is the core element of the so-called "MCU complex". Several pathological conditions are directly or indirectly linked to mitochondrial dysfunction, and the molecular characterization of the MCU complex, that is responsible for the highly selective transport of Ca^{2+} across the inner mitochondrial membrane, gives the opportunity to modulate mitochondrial function by modifying MCU channel activity.

The mechanism by which Ca^{2+} is transported into mitochondria has been unclear for a long time and several proteins have been identified in the past years. We now know that the MCU complex is formed by MCU, the selective channel that allows Ca^{2+} entry into the matrix, its dominant negative isoform MCUb, the Essential MCU REgulator (EMRE) and two channel modulators, Mitochondrial Calcium Uptake 1 and Mitochondrial Calcium Uptake 2 (MICU1 and MICU2) (De Stefani, D. and Rizzuto,R., Biochem Biophys Res Commun (2014)). Recently, another regulator was discovered and named MCU Regulator 1 (MCUR1). MCUR1 down regulation causes a decrease in agonists-induced mitochondrial calcium transients; moreover, MCUR1 is able to bind MCU but not MICU1, thus suggesting that MCU may exists in two different complexes, one with MCUR1 and another one with MICU1 (Mallilankaraman, K., et al., Nat Cell Biol (2012)) . We found that MCUR1 (formerly known as CCDC90A) has also an isoform, named CCDC90B, whose

function is still totally unknown. This work is thus focused on the role of these two proteins in the regulation of the MCU complex activity. We found that both MCUR1 and CCDC90B are integral proteins of the inner mitochondrial membrane, they are broadly expressed among tissues, and CCDC90B mRNA is generally present at higher levels than MCUR1. From the functional point of view, silencing of MCUR1 or CCDC90B leads to a decrease of mitochondrial calcium influx. However, we could detect only a marginal functional interaction between these two proteins and the other components of the MCU complex. Accordingly, the down regulation of both these proteins decreases mitochondrial membrane potential. Overall our data suggest that MCUR1 and its isoform CCDC90B have an indirect effect on mitochondrial calcium uptake, due to a mechanism that we are currently addressing and could depend on the assembly of ETC complexes (Paupe, V., et al., Cell Metabolism (2015)).

RIASSUNTO

I meccanismi di trasduzione di segnali intracellulari richiedono cambiamenti rapidi ed efficienti della concentrazione di molecole segnale nel tempo. Uno dei più importanti secondi messaggeri è il Calcio (Ca^{2+}). I mitocondri sono fondamentali nella segnalazione intracellulare e il Ca^{2+} è a sua volta un elemento chiave della fisiologia di questi organelli. L'accumulo di Ca^{2+} all'interno dei mitocondri è guidato dal potenziale di membrana (Ψ_m), attraverso un canale ionico selettivo chiamato Mitochodrial Calcium Uniporter (MCU) (De Stefani, D., et al., Nature (2011); Baughman, J.M., et al., Nature (2011)). Il Ca^{2+} svolge un ruolo pleiotropico nei mitocondri, che va dalla regolamentazione della produzione di ATP, al controllo delle onde di Ca^{2+} citoplasmatico fino all'attivazione dell'apoptosi cellulare (Rizzuto, R., et al., Nat Rev Mol Cell Biol (2012)). MCU è l'elemento principale dell'omonimo complesso denominato "complesso MCU". Diverse condizioni patologiche sono direttamente o indirettamente legate a disfunzioni delle funzioni mitocondriali.

La caratterizzazione molecolare del complesso di MCU, responsabile del trasporto selettivo degli ioni Ca^{2+} attraverso la membrana mitocondriale interna (IMM), offre la possibilità di modulare l'attività del canale MCU, fondamentale per la funzione signaling mitocondriale.

Il meccanismo con il quale lo ione Ca^{2+} è trasportato all'interno dei mitocondri è tuttavia rimasto un mistero per molto tempo. Negli ultimi anni di ricerca diverse proteine sono state identificate come parte del complesso di MCU. Attualmente i protagonisti nell'attività di uptake di Ca^{2+} nel mitocondrio sono: MCU, il canale selettivo che permette l'accesso esclusivo agli ioni Ca^{2+} nella matrice mitocondriale; la sua isoforma MCUb, che svolge un effetto dominante negativo sull'attività del canale; EMRE, un regolatore essenziale di MCU e infine i due modulatori del canale MICU1 e MICU2, rispettivamente acronimi di Mitochondrial Calcium Uptake 1 e 2 (De Stefani, D. e Rizzuto, R., Biochem Biophys Res Commun (2014)) .

Recentemente è stato scoperto un nuovo regolatore, chiamato MCU Regulator 1 (MCUR1). È stato provato che la riduzione dell'espressione di MCUR1 causa una rilevante riduzione della concentrazione di Calcio nella matrice mitocondriale. È inoltre riportato che MCUR1 è fisicamente legato a MCU, ma non presenta alcuna interazione fisica con MICU1, suggerendo la possibile esistenza di due tipologie di complessi MCU: uno con MICU1 e un altro con MCUR1, ma non con entrambi simultaneamente (Mallilankaraman, K., et al., Nat Cell Biol (2012)).

In questo lavoro di ricerca è stato scoperto che MCUR1, conosciuto anche come CCDC90A, possiede un'isoforma, nota col nome di CCDC90B, e sua funzione è attualmente ancora sconosciuta. Pertanto abbiamo focalizzato la nostra attenzione su queste due proteine e la loro possibile funzione nel complesso di MCU. Entrambe le proteine, CCDC90A e CCDC90B, risiedono nella membrana mitocondriale interna, e sono espresse in tutti i tessuti umani. In particolare, in molti di essi l'mRNA di CCDC90B è generalmente presente in quantità superiore rispetto a CCDC90A. Dal punto di vista funzionale, il silenziamento di entrambe le proteine causa una diminuzione del flusso di Ca^{2+} mitocondriale. Tuttavia, entrambe le proteine hanno dimostrato solo una minima interazione funzionale con gli altri componenti del complesso di MCU. Inoltre, il silenziamento di CCDC90A e CCDC90B causa una significativa depolarizzazione del potenziale di membrana mitocondriale. Complessivamente, i dati raccolti in questo lavoro suggeriscono che sia CCDC90A che la sua isoforma CCDC90B hanno un effetto indiretto sull'accumulo mitocondriale di Ca^{2+}. Probabilmente, questo effetto è correlato all'azione che queste due proteine potrebbero svolgere nell'assemblaggio dei complessi della catena di trasporto degli elettroni (mETC) (Paupe, V., et al., Cell Metabolism (2015)).

INTRODUCTION

Where our body comes from? Carl Sagan, an American cosmologist, astrophysicist, author and science communicator in astronomy and other natural sciences said: "*The Nitrogen in our DNA, the Calcium in our teeth, the Iron in our blood, the Carbon in our apple pies were made in the interiors of collapsing stars. We are made of star stuff*." The story of the atoms in our bodies is the story of the universe in real sense. Neutrons from the core bombard atoms in the star's outer layers, creating heavier elements that are carried away by stellar wind. Stars spread the chemical wealth around, and the cooler environmental away from them allow the atoms to come together to form molecules like Methane and Carbon dioxide, that billion years later had become same of the precursors of life.

Ca^{2+}: One player, hundreds games

An atom of Calcium inside the cytoplasm in a skin cell of your epidermis was swept into space by stellar winds. Calcium ions (Ca^{2+}) control wide variety aspects of cellular life. Ca^{2+} is the most versatile intracellular messenger controlling a wide variety of cellular reaction and adaptive response. The discovery of the critical role of calcium in physiological events date back to a century ago, when Sydney Ringer, a British physician and physiologist, affirmed: " *I find that calcium, in the form of lime water, or bicarbonate of lime or chloride of calcium, even in minute doses produces the changes in the ventricular beat*". (Ringer,S., J Physiol (1883)). He was studying the contraction of isolated rat heart and he admitted to having used as saline medium London pipe water, which is hard: the hearts contracted beautifully. When he replaced the pipe water with distilled one, the beating of the hearts became weaker, and stopped after about 20 minutes.

Ringer did a seminal discover: Ca^{2+} was recognized as carrier of signals that initiate heart contraction. This was the first demonstration that life requires Calcium not only outside the cell to maintain skeletal and tissue architecture. Signals from the brain allow release of neurotransmitters from nerve terminals leading to muscle movement. The behavioral adjustments of eukaryotic cells, in response to internal and external stimuli, have guaranteed the evolutionary growth. What does this mean? Calcium can have a "passive" role outside the cell, but what happen inside the cells? Outside the cell, free Calcium concentration is ten thousand times greater compared to the concentration within the cell (mM Vs. μM) and here its changes activate phenomena like cell division and movement, secretion and alteration in metabolism. Changes in free and bounded Calcium inside the cells take part in the cell injury pathway by our immune system but also virus and toxins. At almost the same time, F.S. Locke, from King's College Physiological Department in London, and his colleagues discovered that Ca^{2+} is critical for the transmission between muscle and nerve (Locke, F.S., Physiol (1894)), but strangely the following 50 years did not observe any activity in Ca^{2+} signaling field. Only at the end of 1950s the situation suddenly changed. Annemarie Weber in 1959 played a pivotal role establishing the overall principles of calcium action; by showing the first direct evidence that calcium ions act as intracellular messengers by the myofibrils Ca^{2+} binding activates Actomyosin (Weber, A., J Biol Chem (1959)). In 1960s, Setsuro Ebashi made another fundamental discovery. He was able to demonstrate that muscle-relaxing effect is due to a Ca^{2+} uptalke into Sarcoplasmic Reticulum vesicles mediated by $Ca^{2+,}$ Mg^{2+}-Activated ATPase. (Ebashi,S., Lipmann,F., J Cell Biol (1962)). Thanks to Ebashi's finding, for the first time the concept of an intracellular membrane-bounded Ca^{2+} store was introduced. At the very same time Paul Fatt and Bernard Katz added another crucial element to the development of Ca^{2+} signaling ideas: the discovery of transmembrane Ca^{2+} currents. From these finding it became clear that cells have a very specific and controlled pathway for transmembrane Ca^{2+} entry and it was taking into consideration for the first time the possible existence of Ca^{2+} channels controlled by membrane voltage (Fatt, P.,

Katz, B., J Physiol (1950)). The Nernst equation allows calculating the equilibrium voltage for particular ion under specific concentration conditions. David E. Goldman from Columbia University, and the English Nobel laureates Alan Lloyd Hodgkin and Bernard Katz resolved the Nernst-Planck equation building The Goldman-Hodgkin-Katz equation that allows calculating the voltage at which the net current through passive pathways (that is, ion channel) is zero (Goldman, D.E., J General Physiol (1943); Hodgkin, A.L., Katz B., J Physiology (1949)).

Calcium signaling: General overview

"*If you don't like something, change it. If you can't change it, change your attitude*" this is what Maya Angelou (April 4,1928- May 28, 2014), American author, poet and civil rights activist, said about life. To adapt to changing environments, cells must signal, and signal requires messengers, whose concentration rapidly and efficiently changes over time. Fitting like a glove, Calcium ions (Ca^{2+}) have come to set cell signaling regulation, becoming one of the most important cellular messenger. Protein function is governed by shape and charge. Ca^{2+} binding triggers changes in protein shape and charge. The ability of Ca^{2+} ion to alter local electrostatic fields and protein conformations is a universal tool of signal transduction. Cells invest much of their energy to effects changes in Ca^{2+} concentration [Ca^{2+}] in space and time, with high consumption of ATP (Clapham, D.E.,Cell (2007)). Unlike complex molecules, Ca^{2+} cannot be chemically altered. Thus, to keep Ca^{2+} under control, cells must chelate, compartmentalize, or extrude it. Hundreds of cellular proteins have been adapted to bind Ca^{2+} over a million-fold range of affinities (nM to mM), and the local nature of Ca^{2+} signaling is strongly linked to this large range of affinities. The best-known protein that buffers Ca^{2+} is Calmodulin (CaM1-4), and a large number of proteins contain Calmodulin recruitment sites (Hoeflich, K.P. and Ikura, M., Cell (2002)). In most cells, Ca^{2+} has it major signaling function when it is elevated in the cytosolic compartment. The signal that triggers [Ca^{2+}] changes generates Ca^{2+} waves within the cytoplasm and from there, Ca^{2+} can diffuse into organelle such as

mitochondria and the nucleus, stimulating several physiological Ca^{2+} sensitive processes, like muscle contraction, hormones secretion, synaptic transmission, cell proliferation, apoptosis and many others. (Berridge, M.J. et al., Nature Molecular Cell Biology (2000); Hajnoczky, G.L. et al., Cell (1995); (Rizzuto, R., J Cell Biology (2003)). Cellular Ca^{2+} fluxes relay on two main sources: the inexhaustible supply of Ca^{2+} from the extracellular space, and channels on the Endoplasmic Reticulum and Sarcoplasmic Reticulum (respectively ER and SR) which release the finite intracellular Ca^{2+} stores. Electrical, hormonal, and mechanical stimulation of cells can produce Ca^{2+} signals by causing entry of the ion across the plasma membrane or it release from intracellular stores. There are two families of Ca^{2+} release channel: the Inositol 1,4,5-triphosphate Receptors (IP_3R_s) and Ryanodine Receptors (RyRs). Binding of hormones to G-protein-coupled receptors (GPCRs), for example, leads to generation of the second messenger Inositol 1,4,5-triphosphate (IP_3) (Bootman, M.D., CSH Perspect Biol (2012)). Increases in the intracellular [Ca^{2+}] are often result of IP_3 activation. IP_3 is soluble and diffuse through the cytoplasm to the ER or SR in muscle cells. It is made by hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) located in the plasma membrane, by Phospholipase-C (PLC). Once at ER, IP_3 binds the IP_3-Receptor (IP_3R), which is located on a ligand-gated Ca^{2+} channel on the ER surface, triggering the Ca^{2+} channel opening, and thus Ca^{2+} release into the cytoplasm. In excitable tissues (nerves and muscles), the sarcoplasmic reticulum (SR) is the main storage site for the bulk of Ca^{2+} (Strokes, D.L. and Wagenknecht, T., Eur J Biochem (2000)). The Ryanodine Receptor Ca^{2+}-release channels (RyRs) are situated in the membrane of the Endo/Sarcoplasmic reticulum (ER/SR) and control the release of Ca^{2+} from intracellular stores. The flux of Ca^{2+} from ER/SR into the cytosol trough RyRs produces a global increase in the intracellular Ca^{2+} concentration that is necessary for contraction in muscle, exocytosis in neurons or apoptosis if the Ca^{2+} levels reach the corresponding threshold. In muscles this phenomenon is referred to as excitation-contraction coupling (ECC) (Cully, T.R., et al., J Physiol (2014)). Terminated its signaling function, Ca^{2+} is rapidly extruded from the cytoplasm by pumps and exchangers to restore intracellular [Ca^{2+}]

resting conditions. The plasma membrane Ca^{2+}ATPase (PMCA) and the plasma membrane Na^{+}/ Ca^{2+} exenger (NCX) are the main mechanisms responsible for Ca^{2+} extrusion outside the cells. Alternatively, the intracellular stores are refilled to resting level trough the activity of Sarco/Endoplasmic Reticulum Ca^{2+} transport ATParse (SERCA). SERCA is the only active Ca^{2+} transporter in the SR. In muscular cells, SERCA plays a dual role: it controls the SR Ca^{2+} that can be mobilized during a muscle contraction, and it decreases the cytosolic Ca^{2+} concentration to allow muscle relaxation (Lipskala, L., et al. Expert Opin Biol Ther (2010)). During the last decades, many scientists focused their attention on the universality and versatility of Ca^{2+} as intracellular messenger. However, the modulation of the amplitude or the temporal/spatial Ca^{2+} signals by the in/out mechanisms still remains a not completely clear scenario.

Mitochondria and Ca^{2+} homeostasis

Mitochondria are unique among the cytoplasmic organelles. They contain their own DNA, which is separated and distinct from the nuclear genome of the cell. Mitochondria are surrounded by a double-membrane system, consisting of inner and outer mitochondrial membrane separated by an intermembrane space (IMS). The inner membrane (IMM) forms several folds, called cristae, which extend into the matrix of the organelle. Every single compartment plays a distinct functional role. In particular, the matrix contains the mitochondrial DNA as well as the enzymes responsible for the central reaction of oxidative metabolism. Most mitochondrial proteins are translated by cytosolic ribosomes and imported to the organelle by specific targeting signals. A new mitochondrial biology has emerged in the last few decades: these organelles have been shown to participate in many aspects of cells physiology such as amino-acid synthesis, iron- sulfur cluster assembly, lipid metabolism, Ca^{2+} signaling, reactive oxygen species (ROS) production and cell death regulation. So it is easy to understand why many pathological conditions are associated with mitochondrial dysfunction, including neurodegenerative disease as Alzheimer's, Parkinson's and Huntington's; motoneuron

disorders as Amyotrophic Lateral Sclerosis and Charcot-Marie-Tooth neuropathy; and also autosomal optic atrophy, ischemia-reperfusion injury, type-2-diabetes, ageing and cancer (Duarte, J.M. et al., Aging Dis (2014)) (Gaude, E., and Frezza, C., Cancer Metab (2014)). In the last few years, many efforts have been dedicated to tease out this complex situation, in order to lead to a better understanding of mitochondrial biology. However, despite all these efforts, many exiting challenges on mitochondrial functions still remain. As to Ca^{2+} homeostasis, it is well known that Calcium plays key roles in a variety of cellular signal transduction processes. A strict control of intracellular Ca^{2+} levels is crucial to the proper functioning of the cell. Therefore, the concerted regulation of transporters and exchangers located on the plasma and intracellular membranes is crucial (Wang, CH. et al., Ann NY Acad Sci. (2015)). Mitochondria and endoplasmic reticulum (ER) play an important role in the maintenance of intracellular Ca^{2+} homeostasis. Mitochondria/ER contact sites are essential for efficient communication between the two organelles. Mitochondria can rapidly accumulate Ca^{2+} thanks to the electrochemical gradient established by the translocation of protons thought the inner mitochondrial membrane (IMM). Mitochondrial respiration is the set of metabolic reaction and processes that takes place in mitochondria, to convert the energy stored in macronutrients to adenosine triphosphate (ATP), the universal energy donor in the cell. The mitochondrial Electron Transport Chain (mETC) is the mean way by which electrons are removed from the reduced carrier NADH and transferred to Oxygen to yield H_2O. It consists of five different protein complexes: complex I (NADH dehydrogenase), complex II (Succinate dehydrogenase), complex III (Ubiquinol Cytochrome c reductase), complex IV (Cytochrome c oxidase) and complex V constituted of the F_1F_0-ATP synthase, source for ATP production. The electrochemical gradient generated by respiration maintains high the mitochondrial membrane potential (Ψ_m) between -150 and -180 mV across the mitochondrial inner membrane, which represents the driving force for organelle cations uptake. This, in addition to the ER/mitochondrial close contacts, enables the fast and large mitochondrial Ca^{2+} uptake in living cells, and in turn, can alters both the amplitude

and spatiotemporal pattern of intracellular Ca^{2+} levels (Rimessi, A., et al., Biochim Biophys Acta (2008)). Although it has long been known that mitochondria take up Ca^{2+}, the molecular identities of the channel and transporters involved in this process were revealed only recently. In 2011, half century of intensive research peaked with the molecular identification of the mitochondrial Ca^{2+} uniporter (MCU) (Baughman, J.M. et al., Nature (2011); De Stefani, D. et al., Nature (2011)). MCU is the ion-conducting pore of the uniporter, which forms the Ca^{2+} channel together with its paralog MCUb and its regulators MICU1, MICU2, EMRE, MCUR1 and their teamwork facilitates the transport of Ca^{2+} from cytosol to the matrix.

Mitochondrial Ca^{2+} Uniporter (MCU) complex: molecular identity and regulation

As previously reported, the mitochondrial Ca^{2+} uptake machinery it is not just about MCU. MCU is part of a higher complex and its function is made from the contribution of several MCU interactors. Until now several proteins that have taken a significant place into the MCU complex have been recognized. MCUb, the MICU family members MICU1, MICU2 and MICU3, EMRE and MCUR1, but the list of the MCU complex components may grow in the future, revealing the real nature of the MCU complex and the pleiotropic role of mitochondrial Ca^{2+} signaling. MitoCarta, an inventory of mammalian mitochondrial genes, which contains 1098 mouse genes and 1013 human genes, was the landmark for the identification of the coiled-coil domain-containing protein 109A (CCDC109A), renamed MCU (Pagliarini, D.J., et al. Cell (2008)). The nuclear MCU gene is located on chromosome 10 and encodes a 40 kDa protein, with the mitochondrial target sequence that is lost during the mitochondrial import, resulting in a 35 kDa mature form (Baughman, J. M. et al., Nature (2011)). MCU is ubiquitously conserved among organism and among human tissues and it constitutes the pore-forming domain of the uniporter. Initially MCU topology was discussed, until it was achieved the conclusion that it is a multi-pass membrane protein, with two predicted transmembrane helices, which are separated by

a highly conserved linker facing the intermembrane space (Baughman, J. M. et al., Nature (2011)). The fact that MCU has only two putative transmembrane domains, suggested the possibility of the existence of MCU oligomers forming the active functional channel. MCUb, a paralog of MCU, oligomerizes both with itself as well as MCU, exerting a dominant negative effect on MCU, reducing the mitochondria Ca^{2+} uptake when over-expressed. MCU and MCUb have 50% sequence similarity and similar predicted topological features, with a distinct expression profile and a not yet identified stoichiometry (Raffaello, A. et al. EMBO J (2013)). The MCU: MCUb expression ratio is variable from tissue to tissue, from 3:1 in heart to more than 40:1 in skeletal muscle, determining a sort of tissue specific signature of mitochondrial Ca^{2+} uptake. To confirm the hypothesis that MCU oligomerizes in the IMM, blue native polyacrylamide gel electrophoresis experiment, showed that MCU takes part of a larger complex with overall molecular weight of ~480 kDa (Baughman, J. M. et al., Nature (2011)), suggesting and confirming the presence of several regulatory subunits in the MCU complex. Chronologically, few months before the final identification of MCU, MICU1 was discovered. Previously MICU1 was known as CBARA1 or EFHA3. It is a 54 kDa single-pass membrane protein, containing two EF-hand Ca^{2+} binding domains. This protein has the same evolutionary pattern of expression, and similar RNA expression in mouse tissues of MCU (Bick, A.G. at al., Science (2012)), and it was called Mitochondrial Calcium Uptake 1 (MICU1) (Perocchi, F. et al. Nature (2010)). The knockdown of MICU1 inhibits the mitochondrial Ca^{2+} uptake (Alam, M.R. at al., J Biol Chem (2012); Kamer, K.J. at al., EMBO Rep (2014)) without any inference on mitochondrial respiratory chain or membrane potential (Perocchi, F. et al., Nature (2010)). Studies performed in cells from individuals with mutations that cause the loss of MICU1 function, show high $[Ca^{2+}]_m$ and the agonist-induced mitochondrial Ca^{2+} uptake is not inhibited (Logan, C.V. et al., Nat Gen (2014)). The Mootha group used MICU1 as computational and molecular bait, to identify the core component of the uniporter MCU, because of the two proteins not only share the pattern of expression, but also physically interact (Baughman, J. M. et al., Nature (2011)). MICU1

was reported to provide a "gatekeeping" function, regulating and minimize uniporter activity in resting condition, protecting mitochondria from chronic Ca^{2+} overload. However, the interpretations of experiments based on changing MICU1 expression are complicated, and the mechanism by which MICU1 regulates MCU still remains unclear and controversial. On one hand, MICU1 interacts together with MCU to allow Ca^{2+} accumulation inside the matrix, on the other hand MICU1 stabilizes the close state of the MCU complex, reducing mitochondrial Ca^{2+} uptake under resting condition or small increase of [Ca^{2+}], by Ca^{2+} binding through its EF-hand domains (Mallilankaraman, K. at al., Cell (2012)) or independently from those ones (Csordas, G. at al, Cell Metab (2013)). The structural study revealed that MICU1 bounds 2 Ca^{2+} in its EF hands, while two pseudo-EF hands are also present, but not bind Ca^{2+}. Whereas, the overall topology of the monomers is similar in the presence or absence of Ca^{2+}, Ca^{2+} binding caused large conformational changes in the EF hands (Foskett, K.J. and Philipson B., J. Mol and Cell Cardiol (2015)). MICU1 (EHHA3) has two paralogues EFHA1 and EFHA2. Both protein have N-terminal mitochondrial target sequence and are present in several mouse tissues, sharing 25% sequence identity with MICU1, thus they were renamed MICU2 and MICU3 (Plovanich, M. at al., PLos One (2013)). In particular, MICU1 silencing leads the disappearance of MICU2 isoform, through a post-translational mechanism. This was later justified with the demonstration of the existence of a MICU1-MICU2 dimer. According to the most recent model, MICU1 and MICU2 are a unique entity that finely controls MCU gating. MICU1 stimulates the gate opening while MICU2 acts as an inhibitor. The MICU2 inhibitory effect ensures the minimal mitochondrial Ca^{2+} uptake in presence of a low extramitochondrial [Ca^{2+}] and a corresponding high driving force, that could allow a cation accumulation, preventing the dangerous effect of matrix Ca^{2+} overload. Thus, when cytosolic [Ca^{2+}] increases, Ca^{2+}-dependent MICU2 inhibition and MICU1 activation ensures the immediate mitochondrial Ca^{2+} accumulation, stimulating aerobic metabolism and increasing ATP production. (Patron, M. at al., Mol Cell (2014)). Finally, it has been demonstrated a model where MCU binds the most recently identified

components of the MCU complex, EMRE (Sancak, A.L. at al., Science (2013)), which binds the dimer MICU1-MICU2 trough MICU1 (Alam, M.R. at al., J Biol Chem (2012)) (**Fig.1**).

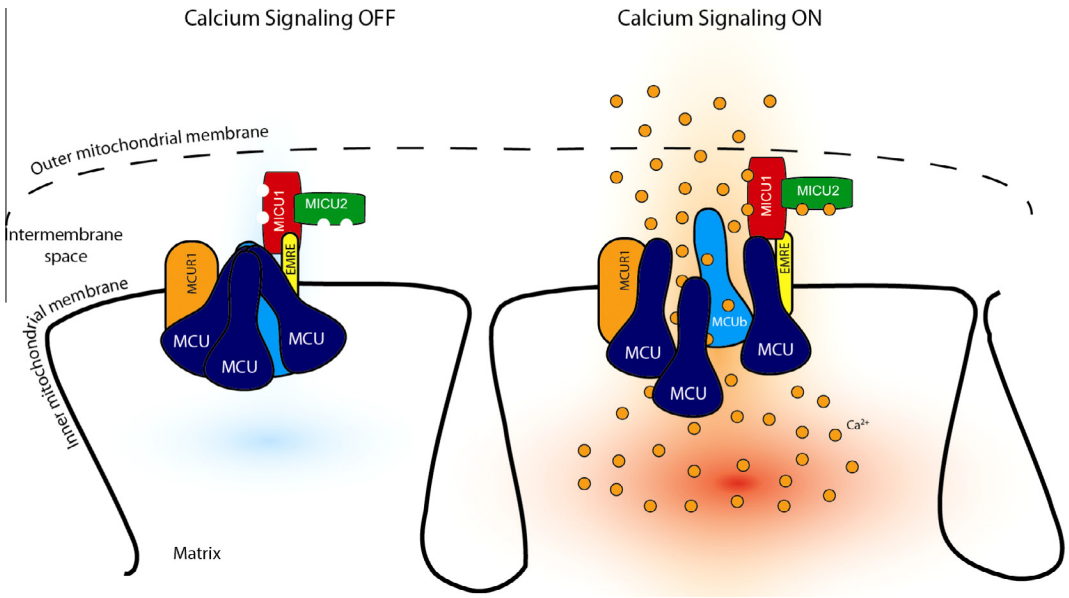

Fig.1: Schematic representation of the MCU complex: in resting condition (on left) and during activation of cellular Ca2+ signaling (on right). (De Stefani D., Rizzuto R., BBRC (2014))

Mootha and collaborators identified the Essential MCU Regulator (EMRE), previously known as C22ORF32, as a component of the uniporter complex (Sancak, A.L. at al., Science (2013)). EMRE is 10 kDa single pass membrane protein, situated at IMM. EMRE interacts with MICU1 at intermembrane space and with MCU oligomers in the IMM by a "bridging activity" between MICU1/MICU2 and the channel MCU. EMRE protein expression level strongly depends on MCU level, suggesting a partnership comparable to that of MICU1/MICU2. Loss of EMRE induces reduction of Ca^{2+} uptake as much as loss MCU, indicating that MCU requires EMRE for *in vivo* Ca^{2+} conductance (Sancak, A.L. at al., Science (2013)). Accordingly, the best-recognized protagonists of the MCU complex actually are MCU, MCUb, MICU1, MICU2 and EMRE (Sancak, A.L. at al., Science (2013)). However, MCUR1 has not been found using this experimental approach, maybe

suggesting a role of this protein in Ca^{2+} handling outside the MCU complex (Marchi, S. and Pinton P., J Physiol rev (2014)).

MCUR1…Who aRe yoU?

The Mitochondrial Ca^{2+} uptake regulator 1 (MCUR1), previously known as CCDC90A, is a ~40 kDa protein discovered by RNAi screen of 45 mitochondrial membrane protein predicted to be integral mitochondrial inner membrane proteins. CCDC90A is predicted to possess two transmenbrane helices, with N and C- termini facing into the IMS with a loop connecting the helices located in the matrix (Mallilankaraman, K., et al., Nat Cell Biol, (2012)). MCUR1 silencing resulted in dramatic reduction in the mitochondrial Ca^{2+} concentration with no changes on the cytosolic [Ca^{2+}] and its knockdown abolished uniporter activity without affecting MCU or MICU1 proteins expression. MCUR1 biochemically interact with MCU. In particular, MCUR1 physically interacts with MCU, but the immunoprecipitation did not show the presence of the endogenous MICU1, which is well-known to be also physically connected to MCU, suggesting that MCU may exists in a complex with either MICU1 or MCUR1, but not both simultaneously (Mallilankaraman, K., et al., Nat Cell Biol (2012)). Mitochondrial Ca^{2+} uptake primary driving force is the IMM potential (Ψ_m), maintained in constant equilibrium by the ETC_m and oxidative phosphorylation. It was originally reported that MCUR1 knockdown in HeLa cells did not alter $\Delta\Psi_m$, mitochondrial DNA copy number and did not affect the normal mitochondrial localization (Mallilankaraman, K., et al., Nat Cell Biol (2012)). The strong correlation between MCU activity and the ER Ca^{2+} release is essential for regulation of cellular bioenergetics, by providing sufficient reducing equivalent to support oxidative phosphorylation. Knockdown of MCUR1, and the resulting absence of Ca^{2+} transfer, reduced basal O_2 consumption rates and ATP level and activation of AMP kinase (AMPK), reflecting diminished oxidative phosphorylation and induced macroautophagy. MCUR1 knockdown shows bioenergetics abnormalities in cells, and this aspect opened a

challenge. On one hand this effect on the AMPK activation were individuated also in stable MCU knockdown condition, thus suggesting that the energetic defect could be due to the direct impairment of Ca^{2+} signaling (Cárdenas, C. et al., Cell (2010)). However, very recently CCDC90A (alias MCUR1) was also proposed to act as a Cytochrome c Oxidase assembly factor rather than a regulator of the mitochondrial Calcium uniporter (Paupe, V. et al., Cell Metab (2015)). Mallilankaraman et al. reported that silencing of CCDC90A causes a deficiency of mitochondrial respiration (Mallilankaraman, K., et al., Nat Cell Biol (2012)), but this molecular defect was not characterized. It is well known that Ca^{2+} is required to maintain the efficiency of Krebs cycle in the mitochondrial matrix (Wan, B., J Biol Chem (1989)). The first step of the Krebs cycle is catalyzed by Pyruvate dehydrogenase (PHD) that is strongly sensitive to matrix Ca^{2+} concentration. This enzyme activity is under the control of a Ca^{2+}-dependent phosphatase (PDPC1), which maintains PHD not phosphorylated to ensure the maximal activity (Denton, R.M., Biochem J (1972)). Inhibition of $InsP_3$ receptor at ER prevents constitutive Ca^{2+} entry into the mitochondria, and results in a marked increase in the P-PDH/PDH ratio (Cárdenas, C. et al., Cell (2010)). However, in CCDC90A silenced cells no increase of the P-PDH/PDH ratio was found (Paupe, V. et al., Cell Metab (2015)), thus disproving the previously suggested hypothesis that the bioenergetics alterations is caused from the decreased mitochondrial Ca^{2+} uptake (Mallilankaraman, K., et al., Nat Cell Biol (2012)). Alkaline carbonate extraction on isolated mitochondria showed that a significant CCDC90A fraction is present not only in the membrane pellet, but also in the supernatant, indicating that this protein might exist in two different functional pool or transiently interacts with the mitochondrial membrane. In addition, OXPHOS complexes analysis by BN-PAGE, showed that suppression of CCDC90A resulted in a specific defect in Complex IV or cytochrome c oxidase (COX), while the assembly of the other complexes was unaffected (Paupe, V. et al., Cell Metab (2015)). To rule out the possibility that the COX alteration may result from a mitochondrial translation defect, the rates of synthesis of mtDNA encoded COX subunits were measured. Marked differences in COX subunits turnover were found. COXI is the first

subunit to enter the assembly pathway, then COXII and COXIII being part of the COX complex, after the formation of COXI, COX4 and COX5a sub-complex (Stiburek,L. Biochem J (2005)). Instability in synthesized COXII subunit in CCDC90A knockdown cells compared to the other COX subunits may suggest that CCDC90A might function in chaperoning COXII into COXI sub-complex or could be the prosthetic factor that stabilize the COX complex assembly (Paupe, V. et al., Cell Metab (2015)). Another important aspect is the significant reduction on mitochondria membrane potential ($\Delta\Psi_m$) detected by Paupe V. et al. (Paupe, V. et al., Cell Metab (2015)). CCDC90A-defective cell lines showed a consistent 60%-65% reduction in the TMRE/MtDRFM ratio compare to control, while no significant changes were detected in cell in which MCU was silenced. These results suggest that CCDC90A is not directly implicated in MCU function, and that the Ca^{2+} uptake defect associated with loss of CCDC90A function is likely secondary to the respiratory chain deficiency (Paupe, V. et al., Cell Metab (2015)), contradicting the previously described Mitochondrial Uptake Regulator 1 (MCUR1) functions (Mallilankaraman, K., et al., Nat Cell Biol (2012)). In spite of that, direct Ca^{2+} currents recording in cells with MCUR1 (CCDC90A) knockdown showed a correlation between the level of MCUR1 expression and the magnitude of MCU-mediated Ca^{2+} currents. Indeed, the patch-clamp technique enables the direct measurement of Ca2+ current irrespective of membrane potential (Kirichok, Y. et al., Nature (2004)). These last results suggest again, that MCUR1 could function as a direct regulator of the mitochondrial Ca^{2+} uniporter (Vais, H., et al., Cell Metab, (2015)), and the challenge still remains open.

AIM

Mitochondrial dysfunction is at the core of a surprising range of very common diseases and conditions. Mitochondria are indeed responsible for energy production, and any disease accompanied by energy defect can be related to the mitochondria. Only in USA is estimated that over 50 million people suffer from chronic degenerative disorders. Comparing it to the entire worldwide population, is easy to imagine how degenerative disturbs are common and widespread. Many degenerative disorders resulted associated with mitochondrial dysfunction Defects in mitochondrial functions are reported in several degenerative disorders, such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Dementia, Parkinson's disease, Huntington Disease, deafness and blindness, mental retardation, diabetes, obesity, cardiovascular disease, stroke, and wide range of solid tumors. Although mitochondrial defects may not be the direct cause of these disorders, it is clear that mitochondria are involved and represent a potential target for therapy. There is indeed an increasing interest in the possibility that biochemical alterations associated with mitochondrial functions linked to specific alteration in mitochondrial protein expression profiles that might play an important role in the etiology of brain and muscles disorders.

Mitochondrial Ca^{2+} homeostasis has a key role in many aspect of cell physiology (Duchen, M.R. and Szabadkai, Essays Biochem (2010)). Mitochondrial membrane potential ($\Delta\Psi_m$) drives Ca^{2+} transport trough the mitochondrial Ca^{2+} uniporter (MCU) (De Stefani, D. et al. Nature (2011)). In addition, MICU1 was identified as a regulator of MCU-mediated Ca2+ uptake in HeLa cells (Perocchi, F. et al., Nature (2010)). Patients with a genetic mutation that causes MICU1 protein loss-of-function, show brain and muscles disorder. This defines a primary defect in mitochondrial Ca2+ uptake as a new mechanism underlying neuromuscular disease (Logan, V.C. et al. Nature Genetic (2013)). Changes on MCU Ca^{2+} uptake activity could be crucial. Its regulation contributes to modulate mitochondrial bioenergetics function, buffers cytosolic Ca^{2+} signals and

regulates Ca^{2+} mediated pathway leading to program a necrotic cell death (Giacomello, M., et al., Cell Death differ. (2007)). Since the molecular characterization of MCU, several proteins have been described to be necessary for MCU function *in situ*. In particular, the recently discovered protein CCDC90A, also known as Mitochondrial Calcium Uptake Regulator 1 (MCUR1), have been shown to inhibit mitochondrial Ca^{2+} uptake (Mallilankaraman, K. et al., Nat. Cell Biol. (2012)). However, this notion was challenged by the demonstration that suppression of CCDC90A produces a specific cytochrome c oxidase (COX) assembly defect, resulting in decreased mitochondrial membrane potential an reduced mitochondrial calcium uptake capacity (Paupe V., et al., Cell Metabolism (2015)). We thus decided to solve this discrepancy by looking at CCDC90A and all related proteins. Indeed, we discovered a closely related gene belonging to the same family. Coiled-coil domain-containing protein 90B (CCDC90B), belong to CCDC90 protein family and likely represents an isoform of CCDC90A. We here investigate the role of CCDC90A and CCDC90B to test whether they direct participate in the regulation of the MCU complex.

RESULTS

CCDC90A and CCDC90B similarity

Colided-Coil Domain-Containing Protein 90A (CCDC90A) was recently identified as a key regulator of the mitochondrial calcium uniporter, required for Ca^{2+} entry into mitochondria (Mallilankaraman,K. et al. Nat. Cell Biol. (2012)). CCDC90A seems to have a direct role in uniporter-mediated for Ca^{2+} uptake, probably *via* a direct interaction with MCU, and for this reason it was renamed Mitochondrial Calciun Uptake Regulator 1 (MCUR1) (Mallilankaraman,K. et al. Nat. Cell Biol. (2012)). However, the conclusion that MCUR1 directly regulates MCU was quickly disproved (Paupe,V., et al., Cell Metabolism (2015)). Both team agreed regarding the effect of CCDC90A knockout on mitochondrial Ca^{2+} uptake, even if Paupe et al. (2015) proposed that CCDC90A is primarily a cytochrome c oxidase (COX) assembly factor, demonstrating that the Ca^{2+} transport observations in Mallilankaraman et al. (2012) were secondary effect due to a reduced mitochondrial membrane potential ($\Delta\Psi_m$) due to a defect in COX subunits assembly. Starting from this debate, we decided to focus our work on Colided-Coil Domain Containing Protein 90B (CCDC90B), an apparent paralog of CCDC90A. Much less is known about CCDC90B isoform, compared to CCDC90A. Using Basic Local Alignment Search Tool (BLAST) to find region of similarity between CCDC90A and CCDC90B, we found that the two proteins have 49% identity and 67% of similarity (**Fig1**) (blast.ncbi.nlm.nih.gov). CCDC90B is predicted to have a single-pass transmembrane domain located at its C-terminal and its function is totally unknown (**Fig2**) (uniprot.org).

Score	Expect	Method	Identities	Positives	Gaps
238 bits(607)	1e-80	Compositional matrix adjust.	124/252(49%)	169/252(67%)	6/252(2%)

```
Query  109  AWRC--SPGVAAAAGALPQYHGPAPALVSCRRELSLSAGSLQLERKRRDFTSSGSRKLYF  166
            AWR   S G      + P+ H  +PAL   RRE   +      +R+  D T    RKL F
Sbjct  6    AWRLFLSQGRGDRWVSRPRGHF-SPAL---RREFFTTTTKEGYDRRPVDITPLEQRKLTF  61

Query  167  DTHALVCLLEDNGFATQQAEIIVSALVKILEANMDIVYKDMVTKMQQEITFQQVMSQIAN  226
            DTHALV  LE +GF   QAE IVSAL  +   ++D +YK+MVT+ QQEIT QQ+M+ +
Sbjct  62   DTHALVQDLETHGFDKTQAETIVSALTALSNVSLDTIYKEMVTQAQQEITVQQLMAHLDA  121

Query  227  VKKDMIILEKSEFSALRAENEKIKLELHQLKQQVMDEVIKVRTDTKLDFNLEKSRVKELY  286
            ++KDM+ILEKSEF+ LRAENEK+K+EL Q+KQQ+M E  ++R D KLD NLE+SRV +++
Sbjct  122  IRKDMVILEKSEFANLRAENEKMKIELDQVKQQLMHETSRIRADNKLDINLERSRVTDMF  181

Query  287  SLNEKKLLELRTEIVALHAQQDRALTQTDRKIETEVAGLKTMLESHKLDNIKYLAGSIFT  346
            +  EK+L+E  TE      Q    +++T  KI+ E+A LKT++ES+KL+ I+YLA S+FT
Sbjct  182  TDQEKQLMETTTEFTKKDTQTKSIISETSNKIDAEIASLKTLMESNKLETIRYLAASVFT  241

Query  347  CLTVALGFYRLW  358
            CL +ALGFYR W
Sbjct  242  CLAIALGFYRFW  253
```

Fig.1:CCDC90B and CCDC90A proteins alignment. BLAST finding about CCDC90A and CCDC90B proteins sequences. They share 49% of identity, with 67% of query coverage (blast.ncbi.nlm.nih.gov).

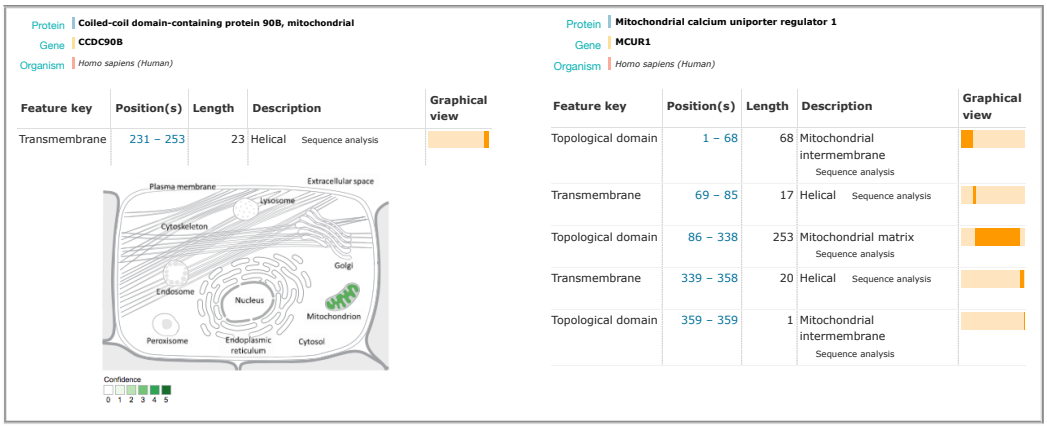

Fig.2: CCDC90B and CCDC90A topology and predicted localization. From Protein knowledgebase, both proteins localize to the inner membrane of mitochondria. The CCDC90B topology is predicted to be a single pass transmebrane protein, while CCDC90A has two transmembrane domains (UniProtKB uniprot.org).

CCDC90B is ubiquitously expressed in mammalian tissues

Based on sequence similarity, we can consider CCDC90A and CCDC90B as two isoforms belonging to the same protein family. We then looked to their respective gene expression levels. mRNA sequencing from 16 human tissues type including white blood cells, shows that CCDC90B is ubiquitously expressed in all tissue, similar to MCUR1, MCU and MICU1 (**Fig.3**). The transcription profile was generated by Illumina's Human BodyMAp 2.0 sequencing project (HiSeq™ 2000 Sequencing System, illumina.com). MCUR1 and CCDC90B show similar expression levels among tissues with few exceptions (e.g. testis and ovary)

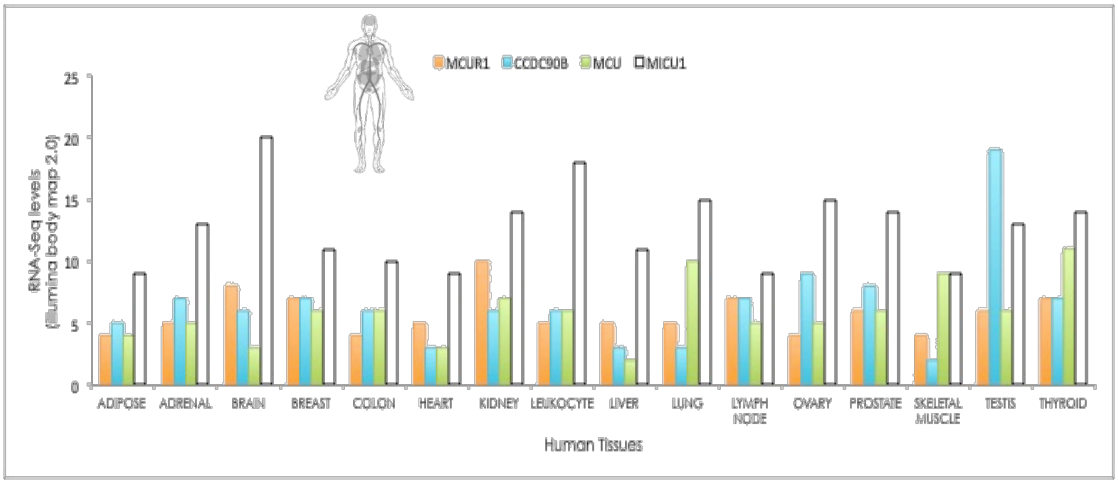

Fig.3: CCDC90B tissue distribution. RNA-Sequencing data set largely agree in terms of ubiquitously CCDC90B gene expression in human tissues (HiSeq™ 2000 Sequencing System, illumina.com).

CCDC90B and CCDC90A silencing and overexpression

In order to verify if the sequence similarity found between MCUR1 and CCDC90B could be paralleled by similar function, we decided to modulate their expression through RNA interference. We thus designed siRNAs sequences (Sigma-Aldrich) and transfected in HeLa cells by Ca^{2+}-Phosphate technique. We then verified by Western blot and showed a significant reduction of CCDC90B and CCDC90A expression level after 72 hours, without compromising the MCU protein expression level (**Fig.4A**). In parallel, we used an expression plasmid encoding the CCDC90B-Myc-Flag chimera in order to perform a successful protein overexpression (**Fig.4B**).

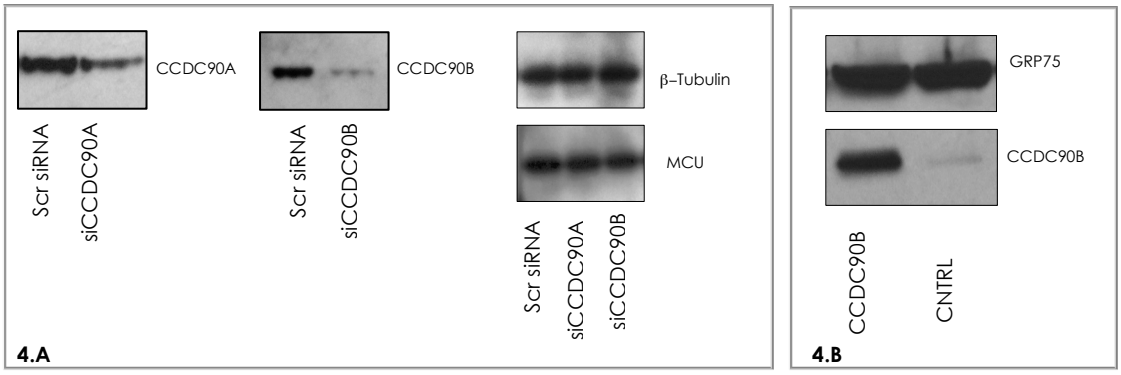

Fig.4A-B: CCDC90A and CCDC90B silencing. A) CCDC90B and CCDC90B were silenced using siRNAs, and their down-regulation does not affect MCU expression protein expression level. **B)** Western Blot analysis show also a positive result for CCDC90Bmyc-Flag over-expression, with a significant increase of the protein expression level.

CCDC90B is a mitochondrial protein

Proteins functional activities strictly depend on their subcellular localization and the existence of molecular interactions. Therefore, when the goal is a protein characterization, a good starting point is identifying where the protein resides by subcellular fractionation. The protein subcellular fractionation allows us to demonstrate in which cellular compartment a protein is located. Generally, different cellular compartments and organelles show different physical properties like shape, size and density and just these differences make a subcellular fractionation possible. Sample preparation is the key point for a successfully subcellular fractionation, because this technique requires fresh tissues and unfortunately, often the freezing process affects organelles integrity (Stahl,W L. and Swanson, P.D., Neurobiol (1975)). For this experiment was chosen a fresh liver from CD-1 mouse. Liver is enriched in mitochondrial compare to the others tissues and it is also a soft tissue, and then easier to chop up.

Mitochondrial fractions were separated by Percoll density-gradient centrifugation. CCDC90B protein results present only in crude and pure mitochondrial fractions, while it is totally absent in the others (**Fig.5**). Considering that protein functions are often correlated to where proteins reside, this result draws another analogy between CCDC90B and CCDC90A. To further confirm our subcellular fractionation studies, immunofluorescence on fixed cells provides evidence on protein localization. Immunofluorescence was performed in HeLa cells grown on rounded 13mm diameter coverslip to minimize the amount of antibody needed. CCDC90B-Myc-Flag and mitochondrial matrix-targeted Red Fluorescent Protein (mtRFP) were co-transfected in HeLa cells by Ca^{2+}-Phosphate technique. Cells were first fixed in 4% formaldehyde and then incubated in 50mM NH_4Cl to block the unreacted groups. Permealbilization was performed using 0.1% Triton X-100 in PBS. Considering that CCDC90B plasmid contains Flag sequence as tag, primary antibody Anti-Flag M2 (Sigma-Aldrich) and secondary antibody conjugated to green fluorescence AlexaFluor488 (Thermofisher) were used to detect the CCDC90B-Myc-Flag

protein localization. Images collected by Confocal Laser Scanning Microscopy (Leica, TCS-SP5), clearly prove the efficient expression and the mitochondrial localization of CCDC90B (**Fig.6**). These data confirmed the hypothesis that CCDC90B belongs to mitochondria. Moreover, by siRNA and DNA plasmid, the modulation of CCDC90A and CCDC90B protein expression levels is possible. These are the molecular tools needed to investigate the impact of CCDC90 proteins on mitochondrial Ca^{2+} activity.

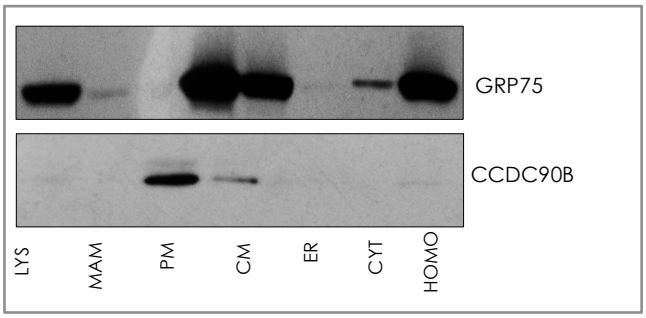

Fig.5: Mouse liver density gradient subcellular fractionation. Western Blot analysis detected CCDC90B protein using its specific antibody (Sigma-Aldrich). GRP75 protein is used as control. Fractions are respectively: LYS (Lysosomes), MAM (Mitochondria Associated Membranes), PM (Pure Mitochondria), CM (Crude Mitochondria), ER (Endoplasmic Reticulum), CYT (Cytosol), HOMO (Homogenate).

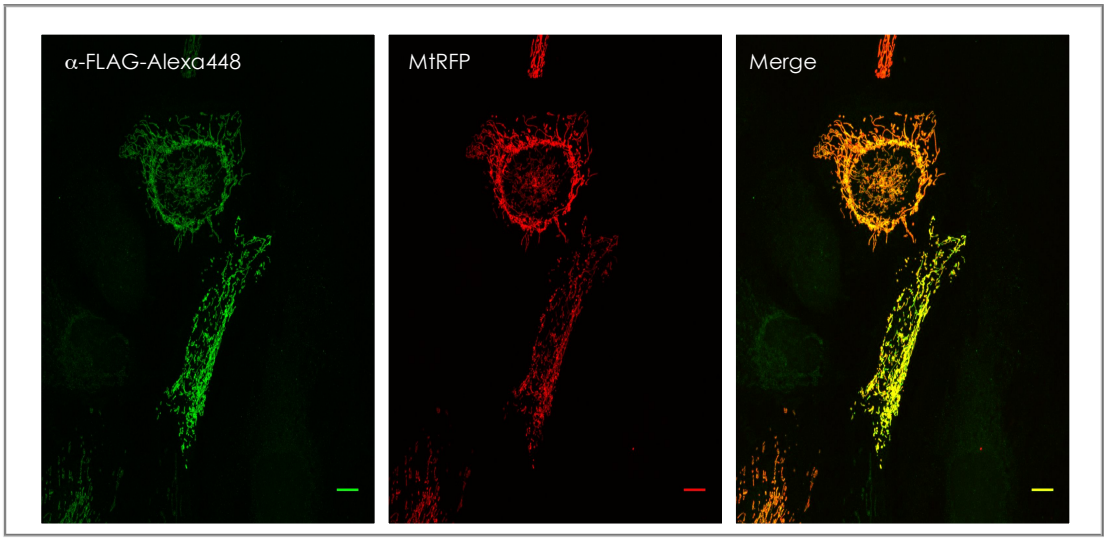

Fig.6: **Immunofluorescence confirms CCDC90B localization**. HeLa cells co-transfected with CCDC90B-Myc-DDK and mtRFP to perform Immunofluorescence. The images obtained confirm CCDC90B mitochondrial localization.

CCDC90B silencing shows a stronger reduction of mitochondrial Ca^{2+} uptake than CCDC90A silencing

Ca^{2+} influx into the mitochondria matrix plays important roles in the regulation of cell death pathways, bioenergetics and cytoplasmic Ca^{2+} signal. During the last few years, a lot of efforts have been spent to find out the molecular identification of the Mitochondrial Calcium Uniporter (MCU) complex. MCU is the ion-conducting pore of the uniporter (De Stefani, D. et al., Nature (2011)). MCUb, a homolog of MCU, is a dominant-negative regulator of the channel (Raffaello, A. et al. EMBO J (2013)). MICU1 is a protein that localizes to the mitochondrial inter membrane space (IMS). It biochemically interacts with MCU and it is required for the uniporter-mediated Ca^{2+} uptake (Perocchi, F. et al. Nature (2010)). MICU2 is a paralog of MICU1, and it is also part of the MCU complex (Plovanich, M. et al. PLos One (2013)). EMRE seems to mediate the interaction between MICU1 and MICU2 proteins with MCU, and it is required for the MCU-mediated Ca^{2+} uptake (Sancak, Y. et al. Science (2013)). CCDC90A is predicted to be integral to the mitochondrial inner membrane (IMM). It has a well-documented physical interaction with MCU, and it is demonstrated to be necessary for the MCU-mediated mitochondrial Ca^{2+} uptake (Mallilankaraman, K. et al. Nat. Cel Biol (2012)). However, nothing is known about CCDC90B functions, and this is the missing point where this work is focused.

Mitochondrial Ca^{2+} uptake was measured in HeLa cells co-transfected using Ca^{2+}Phosphate technique with siRNAs against CCDC90A or CCDC90B together with mitochondrial Aequorin mutated Mt(AEQ)mut. One hour after reconstitution with the prostetic group Coelenterazine, HeLa cells were stimulated with Histamine 100 μM as agonist, in order to evaluate the mitochondrial Ca^{2+} concentration. During Ca^{2+} experiment cells were maintained in Krebs-Ringer Buffer solution (KRB) with 1 mM Ca^{2+} and 5 mM glucose.

In line whit the data already reported (Mallilankaraman, K. et al. Nat. Cel Biol (2012)), when CCDC90A (also named MCUR1) is downregulated, we observe a decrease by 20% in mitochondrial Ca^{2+} responses, in line with previous reports. Most importantly, also

CCDC90B silencing led to a decrease of mitochondrial Ca^{2+} transient, and this was even higher comported to CCDC90A silencing (34% vs 20%) (**Fig.7A-B**).

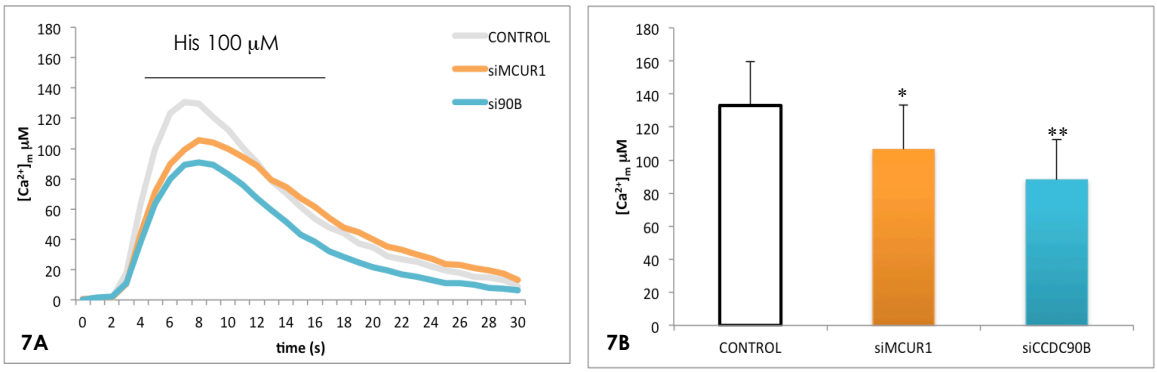

Fig.7A-B: silencing of CCDC90A (MCUR1) and CCDC90B affect mitochondrial Ca^{2+} uptake. A) Representative traces from HeLa cells co-transfected with mt[AEQ]mut, siRNAs against CCDC90B and CCDC90A (MCUR1). $[Ca^{2+}]$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05; **p<0.01).

In order to evaluate the functional interaction between CCDC90A/CCDC90B and the other MCU complex components, mitochondrial Ca^{2+} uptake was also measured in HeLa cells co-transfected with MCU, MCUb, MICU1, MICU2 DNA plasmids together with mitochondrial Aequorin mutated Mt(AEQ)mut, by Ca^{2+}Phosphate technique.

This test was useful to evaluate the effective effects of every single component of the MCU complex on mitochondrial Ca^{2+} uptake activity. The MCU overexpression shows a strong increase in Ca^{2+}uptake, while MCUb overexpression causes the opposite effect, with less Ca^{2+} into mitochondrial matrix. MICU1 and MICU2, which both regulate the MCU-mediated Ca^{2+} uptake, show two opposite effect: the first increase Ca^{2+} access into mitochondria, while its paralog MICU2 decreases it. All our data are in line with the results in literature. (**Fig.8A-B**).

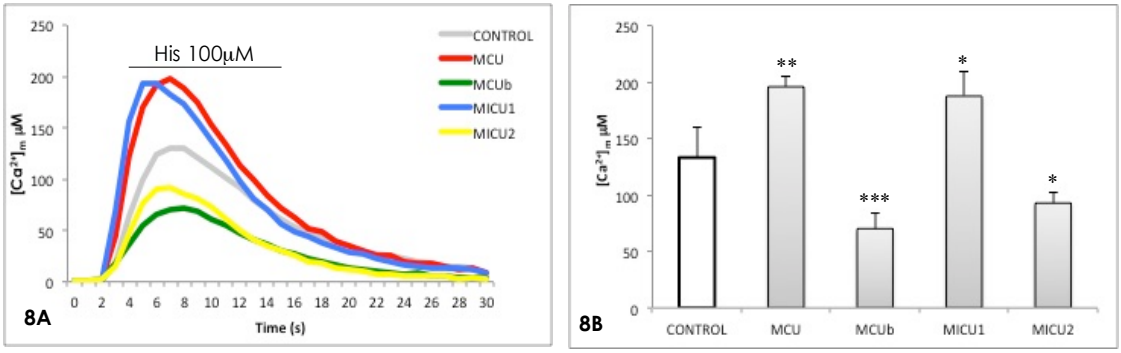

Fig.8A-B: **Effects of the overexpression of the MCU complex components on mitochondrial Ca^{2+} uptake. A)** Representative traces of HeLa cells co-transfected with MCU, MCUb ,MICU1, MICU2 and mt[AEQ]mut. Control trace is shown in grey; traces from cells co-transfected with MCU, MCUb, MICU1 and MICU2 are shown in colors. 48 hours later $[Ca^{2+}]_m$ was measured using Histamine 100 μM stimulation**. B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05, **p<0.01,***p<0.001).

CCDC90B and CCDC90A silencing do not affect significantly the other MCU complex components function

CCDC90A also known as Mitochondrial Calcium Uptake Regulator 1 (MCUR1) has been originally identified as an important regulator of MCU activity. It was showed that MCUR1 biochemically interacts with MCU (Mallilankaraman, K. et al. Nat. Cel Biol (2012)), and on one hand this suppose a possible functional interaction between these two proteins, or CCDC90A and the other components of the MCU complex. On the other hand, given the latest results, CCDC90B also may play a role on mitochondrial Ca^{2+} uptake activity. Thus, it was decided to measure the mitochondrial Ca^{2+} Uptake when either proteins, CCDC90A or CCDC90B, were silenced whilst each one of MCU complex components were overexpressed. The purpose is to find out any functional interaction between both the CCDC90 proteins and the mitochondrial Calcium Uptake machinery. The tests were performed in HeLa cells transfected using Ca^{2+}-Phosphate technique for 72 hours with the specific siRNAs (Sigma-Aldrich) to silencing respectively MCUR1 or CCDC90B. 24 hours after, it was carried out news 48 hours Ca^{2+}-Phosphate transfections with MCU, MCUb,

MICU1 and MICU1 DNA plasmids, one-by-one. The significance of the data (*p-value*) is calculated using *Student's t-test* method. The couples of data set compared for the statistical analysis are: CONTROL Vs Overexpressed, CONTROL Vs silenced, Overexpressed Vs Silenced *plus* Overexpression. In this manner we obtained as first conditions the CCDC90A silencing *plus* MCU overexpression (**Fig.9A-B**) and CCDC90B silencing *plus* MCU overexpression (**Fig.10A-B**. In this experiment CCDC90A (MCUR1) silencing do not show significant changes on its activity. CCDC90A silencing decreases mitochondrial Ca^{2+} responses with no significant functional changes correlated with the MCU overexpression. Similarly, also MCU overexpression does not affect significantly the reduction on mitochondrial calcium uptake triggered by CCDC90B silencing.

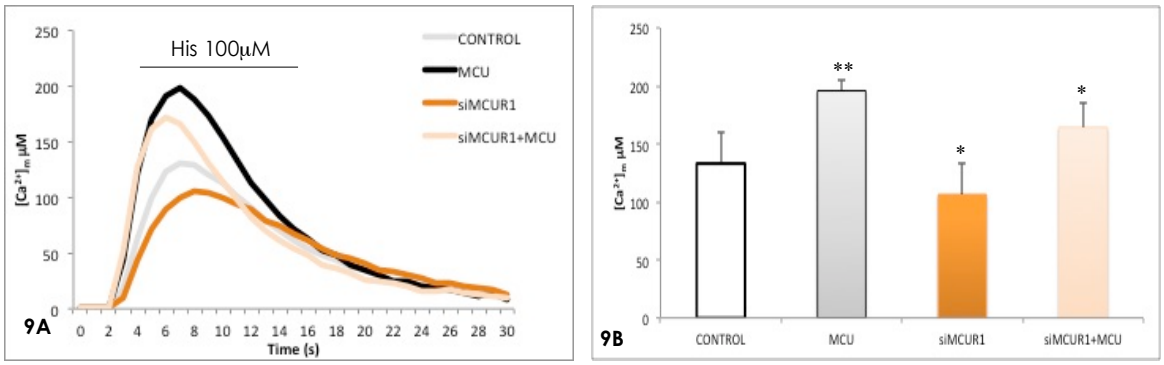

Fig.9A-B: Functional interaction between CCDC90A (MCUR1) and MCU. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MCU and siRNA against CCDC90A (MCUR1), to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05, **p<0.01).

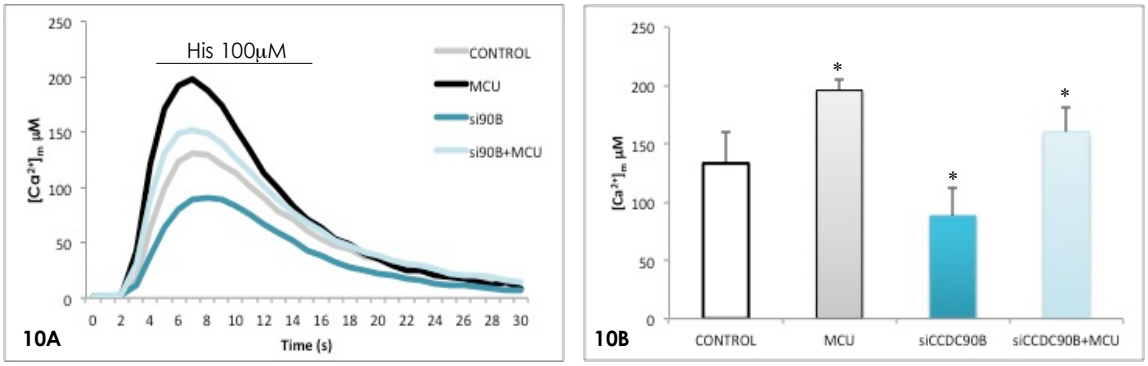

Fig.10A-B: Functional interaction between CCDC90B and MCU. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MCU and siRNA against CCDC90B to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05, **p<0.01).

The second condition tested is the CCDC90A silencing *plus* MCUb overexpression (**Fig.11A-B**) and CCDC90B silencing *plus* MCUb overexpression (**Fig.12A-B**). In these conditions, nothing is changed in the trend of both proteins. MCUb overexpression does not produce any effect on the mitochondrial decrease caused by CCDC90A and CCDC90B down regulation. The effect is simply additive, thus suggesting this two proteins impinge on different pathways.

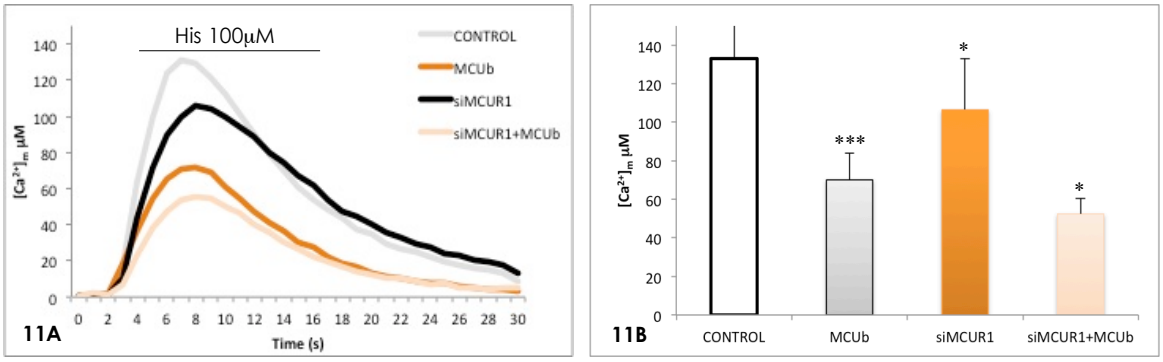

Fig.11A-B: Functional interaction between CCDC90A (MCUR1) and MCUb. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MCUb and siRNA against CCDC90A (MCUR1), to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05, **p<0.01, ***p<0.001).

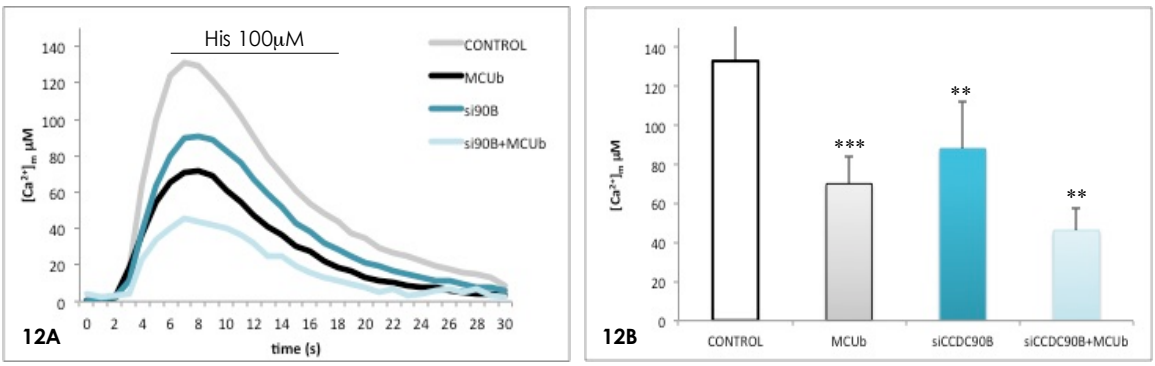

Fig.12A-B: Functional interaction between CCDC90B and MCUb. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MCUb and siRNA against CCDC90B, to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05, **p<0.01,***p<0.001).

Then as third experiments, we performed CCDC90A (MCUR1) silencing *plus* MICU1 overexpression (**Fig.13A-B**) and CCDC90B silencing *plus* MICU1 overexpression (**Fig.14A-B**). MICU1 overexpression causes a recovery on the mitochondrial $[Ca^{2+}]$ decrease

obtained from the CCDC90A (MCUR1) downregulation, while for CCDC90B silencing, no change is observed on the protein activity. The hypothesis of a functional interaction between MICU1 and CCDC90A (MCUR1) is quite unexpected, because it is reported that MCU may exists in a complex with either MICU1 or MCUR1, but not both simultaneously (Mallilankaraman, K. et al. Nat. Cel Biol (2012)), suggesting an unlikely possibility of interaction between MCUR1 and MICU1. This is another important point to investigate more, because it could suggest an alternative mechanism whereby Ca^{2+} may have access to the mitochondrial matrix.

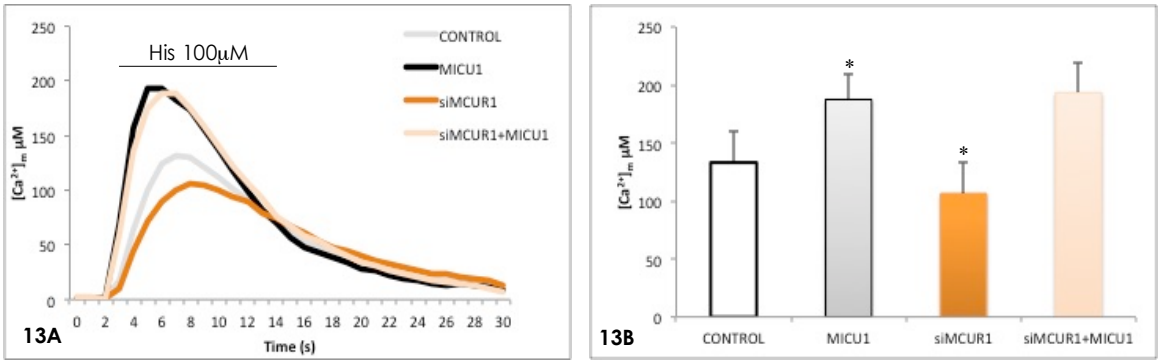

Fig.13A-B: Functional interaction between CCDC90A (MCUR1) and MICU1. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MICU1 and siRNA against CCDC90A, to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05).

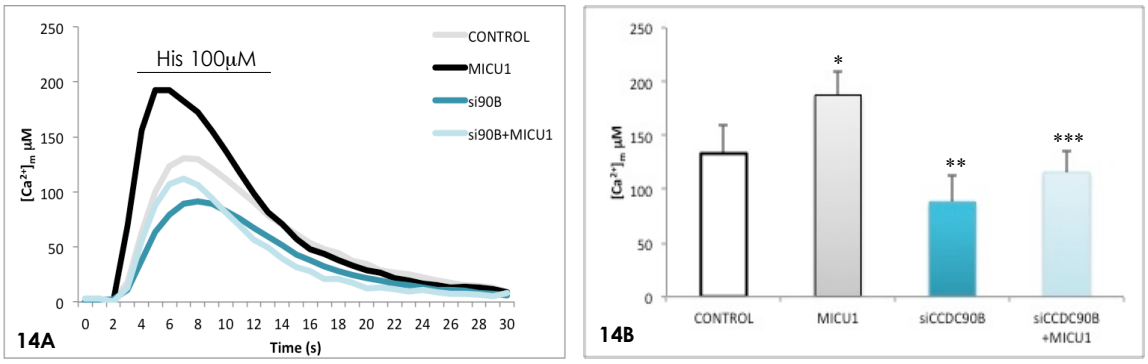

Fig.14A-B: Functional interaction between CCDC90B and MICU1. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MICU1 and siRNA against CCDC90B, to obtain over-expression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* p<0.05,**p<0.01,***p<0.001).

The last experiments show CCDC90A (MCUR1) silencing *plus* MICU2 overexpression (**Fig.15A-B**) and CCDC90B silencing *plus* MICU2 overexpression (**Fig.16A-B**). Both proteins silencing maintained the same trends, with a stable decrease of Ca^{2+} amount, without changes associated to MICU2 overexpression.

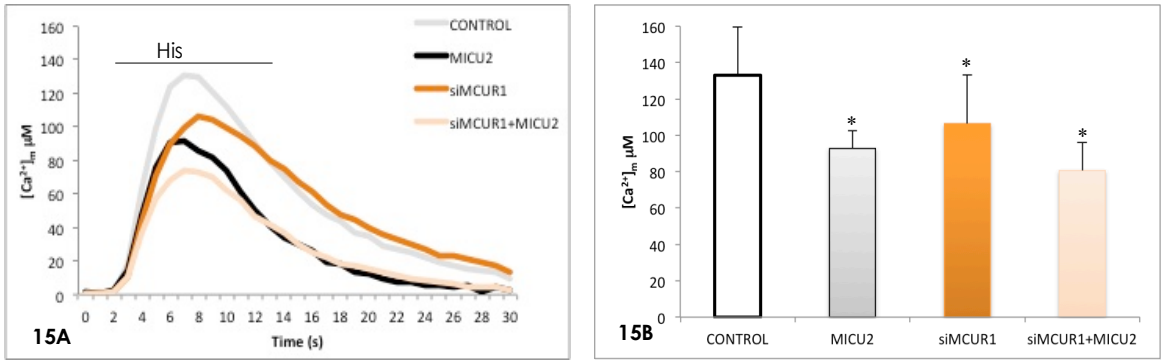

Fig.15A-B: Functional interaction between CCDC90A (MCUR1) and MICU1. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MICU2 and siRNA against CCDC90A, to obtain overexpression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 µM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* *p<0.05).

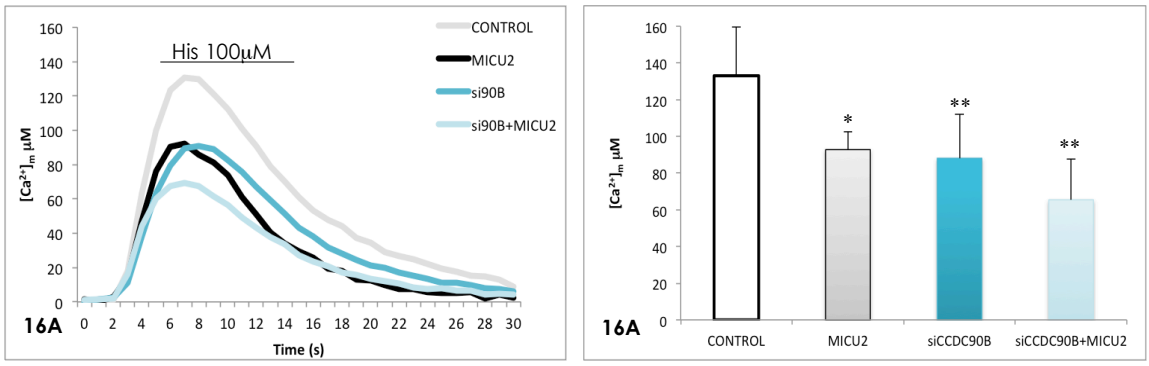

Fig.16A-B: Functional interaction between CCDC90B and MICU2. A) Representative traces from HeLa cells with mt[AEQ]mut, siRNA co-transfected with MICU2 and siRNA against CCDC90B, to obtain over-expression of the first protein and silencing of the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 µM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* p<0.05,**p<0.01).

CCDC90B overexpression shows a recovery on mitochondrial Ca^{2+} uptake decreased by MCUb

Is not possible to take for granted the absence of a functional interaction only by down-regulation of the protein expression level. Protein up-regulation can modify protein

functions as much as much silencing. In this experiment we focused our attention on CCDC90B and three of the main components of MCU complex: MCU, MCUb and MICU1. As in previous test, HeLa cells were co-transfected for 48 hours, using Ca^{2+}-Phosphate technique, with Mt(AEQ)mut *plus* CCDC90B together with MCU, MCUb or MICU1 depending on the interaction that was investigated. The significance of the data (*p-value*) is calculated, as previously, using *Student's t-test* method. The couples of data set compared for the statistical analysis are: CONTROL Vs Overexpressed, Overexpressed protein from the MCU complex Vs Double protein overexpression. The experiment starts with CCDC90B overexpression together with MCU overexpression. CCDC90B overexpression do not alter the $[Ca^{2+}]_m$. Also when the co-overexpression is performed with MCU and CCDC90B, it is possible to observe the same increase in $[Ca^{2+}]_m$ as MCU overexpressed alone, with no significant change. (**Fig.17A-B**).

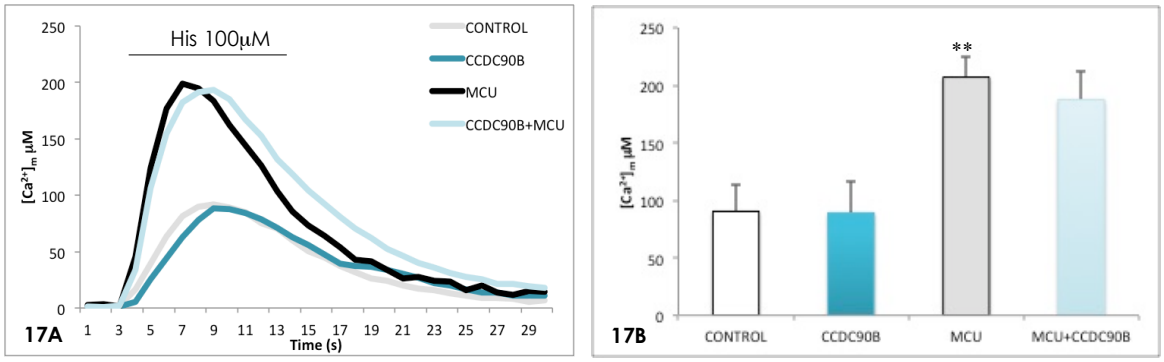

Fig.17A-B: Functional interaction between CCDC90B and MCU. A) Representative traces from HeLa cells with mt[AEQ]mut, co-transfected with MCU and CCDC90B, to obtain over-expression of the first protein and the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* ***p<0.001).

The second experiment performed with CCDC90B overexpressed together with MICU1, again did not shows significant changes on the proteins behavior (**Fig.18A-B**). MICU1 overexpression is not sensitive to CCDC90B overexpression. The $[Ca^{2+}]_m$ remains stable.

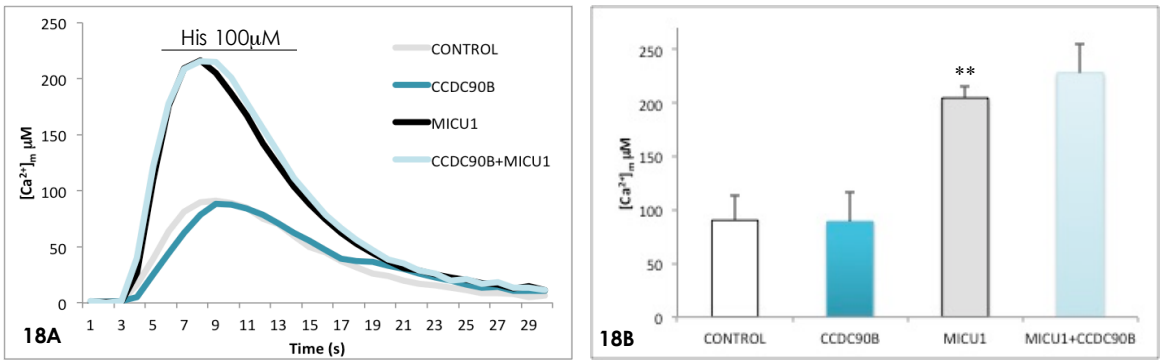

Fig.18A-B: Functional interaction between CCDC90B and MICU1. A) Representative traces from HeLa cells with mt[AEQ]mut, co-transfected with MICU1 and CCDC90B, to obtain over-expression of the first protein and the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* ***p<0.001).

Unexpectedly, in the third experiment is possible to observe a total recovery on mitochondrial Ca^{2+} when CCDC90B and MCUb are co-overexpressed (**Fig.19A-B**). While the Control does not undergo changes when CCDC90B is overexpressed (as in the previously tests), when MCUb and CCDC90B are co-overexpression, is observed a total recover of the mitochondrial Ca^{2+} amount lost by MCUb overexpression, suggesting a possible functional change on one or both proteins activity.

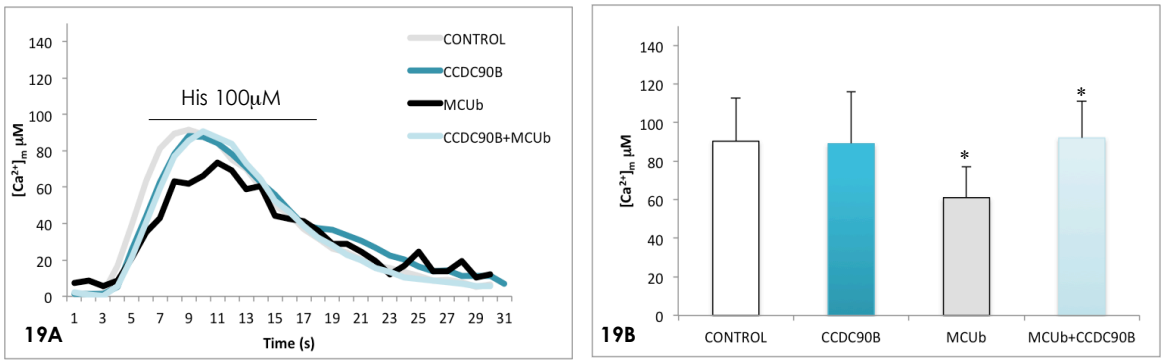

Fig.19A-B: Functional interaction between CCDC90B and MCUb. A) Representative traces from HeLa cells with mt[AEQ]mut, co-transfected with MCUb and CCDC90B, to obtain over-expression of the first protein and the second one at the same time. $[Ca^{2+}]_m$ was measured under Histamine 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_m$. Mean ± S.D. of n=8 values are shown. (*t-test* ***p<0.001).

Silencing of MCUR1 and CCDC90B cause the mitochondrial membrane depolarization

The oxidative phosphorylation (OXPHOS) system consists of five multimeric complexes embedded in the mitochondrial inner membrane. The mitochondrial respiratory chain (I-IV) transfers electrons from reducing equivalent to water, creating an electrochemical gradient across the inner mitochondrial membrane which is used by a fifth complex, the F_1-F_0 ATPase, to drive synthesis of ATP. CCDC90A (MCUR1) was described as a regulator of the mitochondrial calcium uniporter (MCU) and its suppression in human cells lines resulted in decreased mitochondrial Ca^{2+} uptake (Mallilankaraman, K. et al. Nat. Cel. Biol. (2012)) and defects in mitochondrial respiration, whose molecular basis were not determined (Paupe,V., et al., Cell Metabolism (2015)). Respiratory chain defects influence mitochondrial membrane potential, few studies have investigated their direct consequence on mitochondrial Ca^{2+} uptake (Brini, M. et al., Nat. Med. (1999)). Recently was reported that CCDC90A (MCUR1) primarily functions as a complex IV (cytochrome c oxidase or COX) assembly factor, suggesting that the mitochondrial Ca^{2+} uptake defect, that was described to modulate MCU function, is in fact secondary to the mitochondrial depolarization caused by the respiratory chain defect. (Paupe, V. et al., Cell Metabolism (2015)).

In order to understand the origin of the observed decrease in mitochondrial Ca2+ uptake CCDC90A and CCDC90B silenced cells, we decided to analyze a the mitochondrial membrane potential, with the purpose of excluding a possible respirator chain defect.

HeLa cells transfected for 72 hours with the specific siRNAs were used to perform measurements of mitochondrial membrane potential using tetramethylrhodamine methyl ester (TMRM) as fluorescent probe. TMRM is a cell-permeant, cationic, red fluorescent dye. Incubation was conducted at 37°C in saline solution for 30 minutes. The fluorescence emission of TMRM was acquired at 565 nm. The mitochondrial membrane potential (Ψ_m) was monitored over time, before and after treatment with Olygomicin 10 μM, that is known to inhibit mitochondrial H^+-ATP-synthase blocking the flow of protons

through the F_0 subunit. To measure specifically the mitochondrial membrane potential (Ψ_m) we quantified the fluorescence intensity at the resting levels, and after applying the ionophore Carbonyl Cyanide m-CloroPhenylhydrazone 10μM (CCCP), that acts on inner membrane destroying the proton gradient by uncoupling proton pumping from ATP synthesis, obtaining the full mitochondrial depolarization.

TMRM traces analysis showed no significant changes when Olygomicin was added to inhibit the phosphorylation. This is possible because the ultimate acceptor of electron in the mitochondrial Electron Transfer Chain (ETC_m) is oxygen that is converted to water at Complex IV (Cytocrome c Oxidase). The mitochondrial proton leak across inner membrane, allows adjustment of the coupling efficiency, and a slow respiration persists. Mitochondria still consume oxygen, even in the presence of the ATP synthase inhibitor Oligomycin (Chance, B., Williams, G.R. J Biol. Chem (1955)).

Here it is reported the representative traces about the quantification of the basal TMRM signals registered. It was strongly reduced from 36% to 55% and 68% respectively when CCDC90A (MCUR1), CCDC90B or both together were down regulated (**Fig.20**). These data indicate that silencing of CCDC90A and CCDC90B causes mitochondrial depolarization. The mitochondrial Ca^{2+} uptake is al electrogenic process driven by mitochondrial transmembrane potential ($\Delta\Psi_m$) which is generated by proton pumping in the respiratory chain and mediated by a Ca^{2+} selective ion channel MCU (Kirichok, Y. et al., Nature (2004)). Considering this, our results may suggest the hypothesis that the reduction of mitochondrial Ca^{2+} uptake caused by CCDC90A and CCDC90B silencing could be correlated with a defect on mETC, that causes a reduction on Ψ_m and consequently a decrease in the driving force that allow Ca^{2+} to entry into mitochondria.

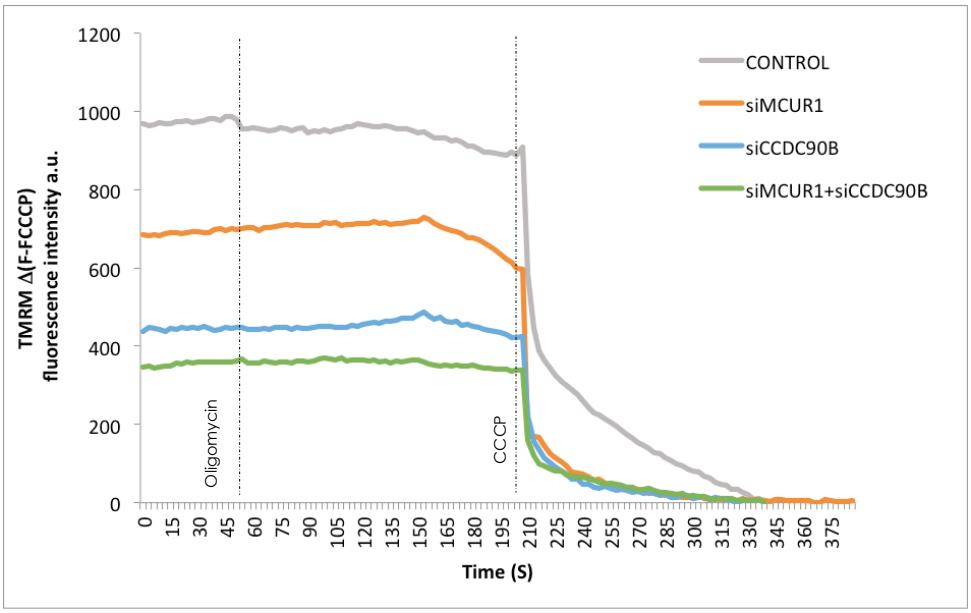

Fig.20: Effect of CCDC90A (MCUR1) and CCDC90B silencing on mitochondrial membrane potential. Living HeLa cells were incubated with 20nM TMRM for 30 min. Signal was acquired at 565nm with addiction of Oligomycin at 30th frame and then CCCP at 70h frame (1Frame/3sec.).There is a significant mitochondrial depolarization at basal level when CCDC90A (MCUR1) and CCDC90B are singly or both together down regulated.

CRISPR/Cas9 Lentiviral CCDC90B-Knockout shows a decrease in mitochondrial Ca^{2+} uptake and in mitochondrial membrane potential (Ψ_m)

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) consists of two components: a "guide" RNA and a non-specific CRISPR-associated endonuclease (Cas9). CRISPR/Cas9 can be used to generate knockout cells by co-expressing a guide RNA specific to the gene to be targeted and the endonuclease Cas9. Cas9 will only cleave the target if sufficient homology exists between the guide RNA spacer and target sequences. (Shalem, O., et al. Science (2014)). While shRNA results in a wide range of expression values for the target gene, using CRISPR the population of cells are expected to provide higher screening sensitivity, especially in case where reduced expression retains gene function. For the CCDC90B knockout the one vector system with both Cas9 and guide RNA in a single vector was chosen (Sanjana, N.E. et al., Nat Meth (2014)). For lentivirus production, transfer plasmid was co-transfected into HEK293-T cells with the packaging plasmids pVSVg (AddGene #8454) and pMDLg (AddGene #12251). The

identification of suitable target sites for the Cas9 is made computationally, calculating most likely off-targets within the genome. The access to Cas9 target design tool is www.genome-engineering.org. Lentiviral CRISPR/Cas9 infected HeLa cells and after 24 hours started the Puromycin selection for 1 week. At the end of the Puromycin selection, single-cell clones were obtained through infinite dilution. Western Blot analysis showed that we obtained CCDC90B knockout in all the clones generated. (**Fig.21**).

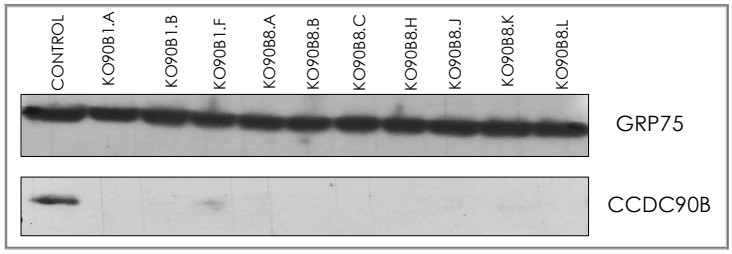

Fig.21: CCDC90B CRISPR/Cas9 Lentiviral gene-Knockout. A) Ten Clones' colonies, generated from single cell resulted positive to CCDC90B Knockout, CCDC90B protein level was measured by Western blot.

These clones were transfected by Ca^{2+}-Phosphate technique, using cytosolic and mutated-mitochondrial aequorin (cyt[AEQ] and Mt[AEQ]mut) for 48 hours. Cells were stimulated by ATP 100 μM as agonist. While cytosolic Ca^{2+} concentration did not show any significant change between control and CCDC90B Knockout clones (**Fig.22A-B**), the mitochondrial Ca^{2+} concentration shows a significant decrease, approximately of 65% (**Fig.23A-B**

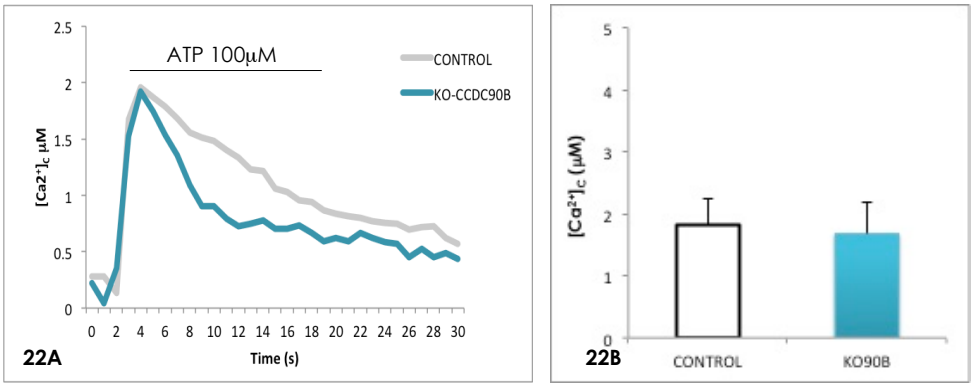

Fig.22A-B: **A)** Representative $[Ca^{2+}]$ traces from HeLa cells CCDC90B knockout transfected with cyt[AEQ]. $[Ca^{2+}]_C$ was measured under ATP 100 μM stimulation. **B)** Histogram show the amount of $[Ca^{2+}]_c$. Mean ± S.D. of n=8 values are shown.

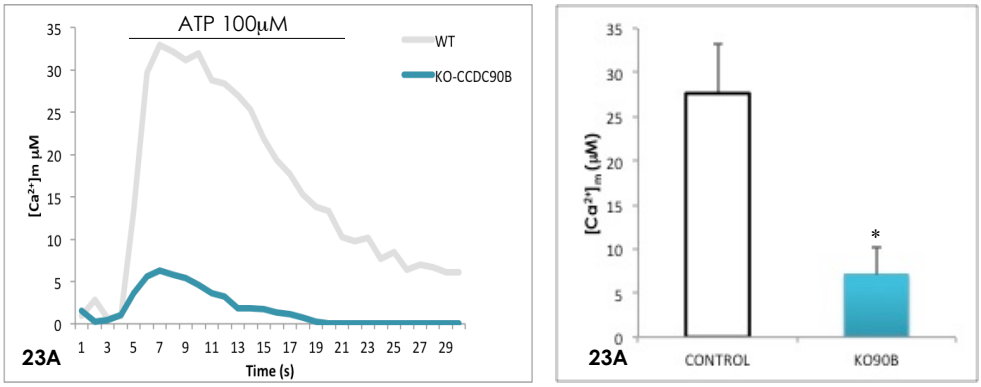

Fig.23A-B: **A)** Representative [Ca^{2+}] traces from HeLa cells CCDC90B knockout transfected with Mt[AEQ]mut. [Ca^{2+}]$_m$ was measured under ATP 100 μM stimulation. **B)** Histogram show the amount of [Ca^{2+}]$_m$. Mean ± S.D. of n=8 values are shown. (t-test *p<0.05).

CCDC90B knockout clones were also used to perform mitochondrial membrane potential ($\Delta\Psi_m$) measurement using TMRM 20nM as red fluorescent dye. Control and CCDC90B-Knockout cells were incubated at 37°C for 30 minutes in 1mL of saline solution containing TMRM 20nM and Cyclosporine-H 2μM. Cyclosporine-H blocks the multi-drugs-resistance pump (MDR), which ejects many types of lipophilic cations from cell, causing a mitochondrial slow dye loading and avoiding the achievement of an equilibrium state (Rottemberg, H. et al. Biochim Biophys Acta (1998)). TMRM excitation was carried out a 565 nm, after 2 minutes was added Oligomycin 10μM and then, 10 minutes later, CCCP 10μM. When CCDC90B is Knockout, significant changes in mitochondrial membrane potential were observed. TMRM lost 50% of its intensity and this means that mitochondria are losing their membrane gradient. Less positive charge inside mitochondria avoided the probe accumulation. TMRM traces were normalized to initial fluorescence value (F0) to better evaluate the traces trend when Oligomycin inhibited ATP-synthase proton flow (**Fig.24A-B**). In CCDC90B knockout cells, the ATP-synthase (Complex V) inhibition by Oligomycin causes a significant decrease on mitochondrial membrane potential. This indicates that ATP-synthase is working in reverse mode, consuming ATP in order to generate membrane potential, thus suggesting impairment in ETCm. This reinforces the hypothesis about the existence of a correlation between CCDC90B lack and an ETC_m defect. As previously proposed for CCDC90A (MCUR1) knockout cells (Paupe, V. et al.,

Cell Metabolism (2015)), the decrease of mitochondrial Ca^{2+} uptake detected in CCDC90B knockout cells could be a secondary effect to a mitochondrial depolarization caused by the respiratory chain defect.

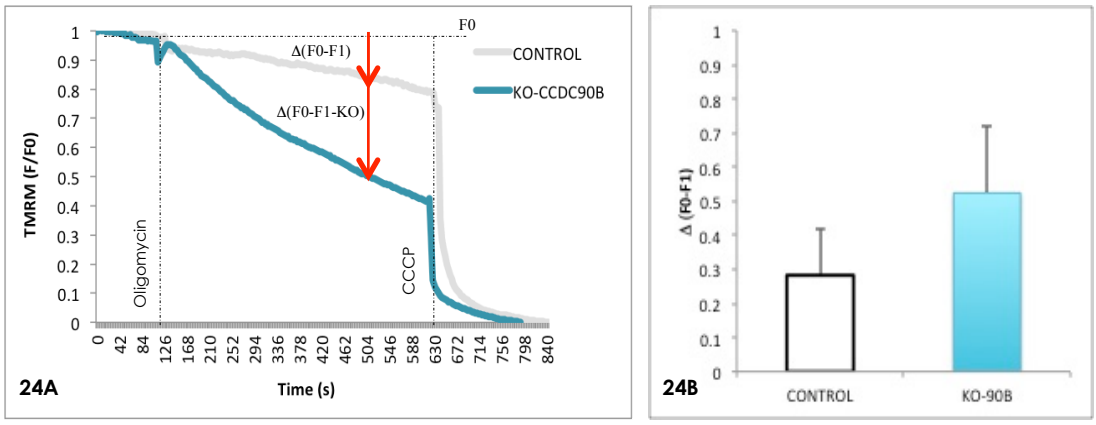

Fig.24A-B: Effect of CCDC90B knockout on mitochondrial membrane potential ($\Delta\Psi_m$). A) Living CCDC90B-knockout cells were incubated with 20nM TMRM and 2 μM Cyclosporine-H for 30 min. Then TMRM signal was acquired at 565nm with addiction of Oligomycin 10μM after 90 second and then Cccp 10μM after 10 minutes. **B)** Histogram shows the difference between Control and CCDC90B knockout cells (in terms of TMRM signal intensity lost), after 8 minutes from the Oligomycin addiction. For control mean ± S.D. of n=28 values are shown. For CCDC90B mean ± S.D. of n=16 values are shown.

CCDC90B knockout clones show alterations of the OXPHOS expression levels

Assembly of the oxidative phosphorylation (OXPHOS) system in the mitochondrial inner membrane is an intricate process in which many factors must interact. OXPHOS is a metabolic process that provides most of the energy usable by cells. The OXPHOS system consists of five multiprotein complexes and two mobile electron carriers embedded in the lipid bilayer of the mitochondrial inner membrane. It couples two set of reaction, respiration and ATP synthesis. Respiration consists of the sequential transfer of electrons extracted from nutrient compounds through a chain of oxidoreductase reaction, leading to reduction of molecular oxygen to water. The energy liberated during respiration sustains the formation of an electrochemical gradient across the inner mitochondrial membrane that is used by complex V (F_1F_0ATPase) to catalyze the condensation of ADP

and inorganic Phosphate into ATP (Fernàndez-Vizarra, E. et al., BBA (2009)). It is quite obvious that a lack of oxidative phosphorylation could make mitochondrial Ca^{2+} uptake machinery susceptible by a low mitochondrial membrane potential and therefore a barrier to Ca^{2+} access through MCU channel. OXPHOS system were analyzed in HeLa cells CCDC90B silenced by siRNA and clones CCDC90B Knockout, using MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (MitoSciences #MS604). Unexpectedly, the OXPHOS system do not shows any alteration when CCDC90B is silenced. Only in the CCDC90B Knockout condition is possible notice a slight decrease in Complex I and Complex III protein expression levels (**Fig.25A**). This result is in line with our hypothesis about the possible involvement of CCDC90B in ETC_m function. However, when all the other clones resulting from the CRISPR/Cas9 gene knockouts were tested, using the same antibody for the total OXPHOS system, different conditions about the OXPHOS complexes were found (**Fig.25B**). Only 50% of CCDC90B knockout clones show a decrease in Complex IV expression level, and this data could be in line with the results reported for MCUR1 as a Cytochrome c Oxidase assembly factor and not a regulator of the mitochondrial Calcium Uniporter (Paupe, V. et al., Cell Metabolism (2015)). The remaining 50% did not show any significant changes in OXPHOS system, and they appear similar with the control. Investigation of these defects has not only been useful in understanding the molecular pathogenesis of several mitochondrial disorders, but has also been helping to unravel the mechanistic aspects of respiratory chain biogenesis in a mammalian system.

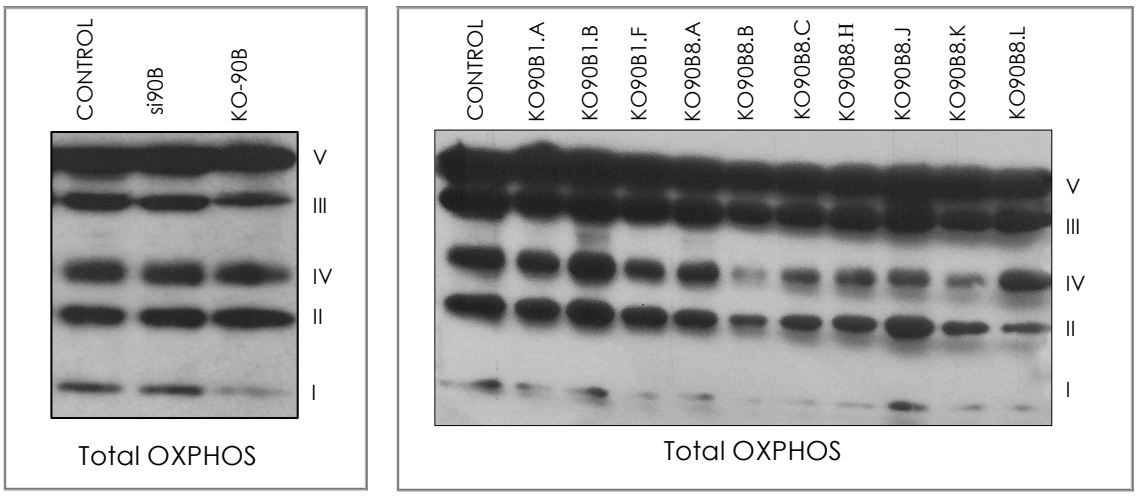

Fig.25A-B: CCDC90B Knockout and OXPHOS system. A) OXPHOS system complexes protein expression levels measured by western blot in CCDC90B silencing and Knockout conditions. **B)** OXPHOS system complexes protein expression levels, measured by western blot in CCDC90B different clones with CCDC90B Knockout.

MATERIAL and METHODS

Cell Cultures

The experiment were performed in HeLa (ATCC-CCL-2) and HEK293T (ATCC-CRL) grown in Dulbecco Modified Eagle's Medium (DMEM, LifeTechnologies) with high D-Glucose and L-Glutamine supplemented, 10% Fetal Bovine Serum (FBS-LifeTechnologies), 100 UmL^{-1} Penicillin and Streptomycin Sulfate (EuroClone) and cells were maintained in culture at 37°C and 5%CO_2.

Transient transfection

For transient transfection, cells were transfected with Ca^{2+}-Phosphate procedure using prepared stock solutions: $CaCl_2$ 2.5 M ($CaCl_2 \bullet 2H_2O$, Sigma-Aldrich) and HEPES Buffered Solution 2x (HBS) (280 mM NaCl (Sigma-Aldrich), 50 mM HEPES (Sigma-Aldrich), 1.5 mM Na_2HPO_4 (Sigma-Aldrich); pH 7.18) sterilized by filtration through 0.22 μM filter and conserved at -20°C and until used. The day prior to transfection, $5x10^4$ cells per well were plated into a 24 well plate in Complete DMEM. Confluence was approximately 60-70% on the day of transfection. For each 24-well plate, 5μL of 2.5 M $CaCl_2$ and 2μg of DNA or 0.4 μL siRNA were dissolved in 45μL of DNase/RNase-Free Distilled water (LifeTechnologies) and added to 50 μL HBS under vigorous agitation. Transfection mix was incubated for 20 minutes at room temperature. For 6 well plates, the amount of solutions was doubled, while for 10cmØ dish it was multiplied ten times. The transfection mix was directly added drop-by-drop to the cells monolayer and shacked gently. After 16 hours from the DNA addition, cells were washed with PBS until the precipitate was completely removed. Measurements were performed from 36 to 72 hours after transfection depending on experimental conditions.

To silence CCDC90A (MCUR1) and CCDC90B specific human siRNA sequences were used:

siRNA MCUR1 # SASI_Hs01_00037402 (Sigma-Aldrich)

siRNA CCDC90B # SASI_Hs01_00113312 (Sigma-Aldrich)

CCDC90B overexpression was performed with pCMV6-CCDC90B C-terminal Myc-DDK(Flag) Human cDNA Open Reading Frame (ORF) clones, purchased by Origene (Origene Cat.No.#RC213680). pCMV6-CCDC90B-myc-Flag, as all the other plasmids used in this work, were transformed into One Shot TOP10 Chemically Competent E.coli (Invitrogen), following a standard protocol for Bacterial Transformation (www.addgene.org) and transfected using the standard Ca^{2+}-Phosphate technique previously descripted.

Plasmids:

pCMV6-CCDC90B-myc-Flag was purchased by Origen

pcDNA3.1/MCU-Flag was gently provided by De Stefani D.

pcDNA3.1/MCUb-6xHis was gently provided by Raffaello A.

pcDNA3.1/MICU1-HA was gently provided by Patron M.

pcDNA3.1/MICU2-Flag was gently provided by Patron M.

CRISPR/Cas9 mediated generation of stable CCDC90B knockout cell lines

The Clustered Regulary Short Palindromic Repeat (CRISPR) technology employing the RNA-guided nuclease Cas9 has rapidly dominated the genome engineering fields as unique and powerful gene editing tool. CRISPR-associated protein-9 nuclease (Cas9) genes are essential in adaptive immunity in select bacteria and archaea, enabling

microbial host organisms to defend and eliminate invading genetic material as phages and plasmids (Barrangou, R. et al., *Nature* Biotechnology (2012)). The lentiviral production started with the one vector lentiviral system (lentiCRISPRv2) containing S. pyogenes Cas9 (SpCas9) and the chimeric guide RNA. The vector was digested using BsmBI, and a pair of annealed oligos was cloned into the single guide RNA scaffold. The oligos were designed based on the target site sequence (20bp) and were flanked on the 3' end by a 3bpn NGG PAM sequence according to Target Guide Sequence Cloning Protocol (www.genome-engineering.org).

The Guide sequences adopted for hsCCDC90B (Human, chromosome 11, exon1):

Guide #1: GCCGGCGAGAAATGCCCGCGGGG

Guide #8: CCAAGGCAGAGGAGATCGTTGGG

The oligos sequences designed:

hsCCDC90B-g1-s: CACCGGCCGGCGAGAAATGCCCGCG

hsCCDC90B-g1-as: AAACCGCGGGCATTTCTCGCCGGCC

hsCCDC90b-g8-s: CACCGCCAAGGCAGAGGAGATCGTT

hsCCDC90B-g8-as: AAACAACGATCTCCTCTGCCTTGGC

Then, 20μg of lentiCRIPRv2 (with sgRNA cloned) was co-transfected in 10⦸plate into HEK293T ≈ 80% confluent cells with the packaging plasmids: 10μg pMDLg/pRRE (Addgene#12251), 6μg pMD2.g (Addgene#12259) and 5μg pRSV-Rev (Addgene#12253). As positive control for viral production, was used CMV-EGFP lentiviral transfer plasmid (Addgene#19319). The precipitate was left at room temperature for 20 minutes and then added to the cell drop wise 1 mL/dish, mixed gently until the medium

has recovered a uniformly red color and then incubate overnight at 37°C, 5% CO_2. After 48 hours from transfection, medium was aspirated and stored at -80°C. The day before transduction, HeLa cells were splitted at proper density in a 6-well tissue culture plate in 2mL of DMEM containing 10%FBS and 1% Pen-Strep, incubated in a 37°C incubator at 5% CO_2 overnight. The day after, frozen lentivirus was removed from -80°C and thaw at room temperature. 1mL of viral preparation was transferred in a fresh tube and 2μL of Polybrene (at a stock of 4 μg μL^{-1}) was added, for a final concentration of 8μg mL^{-1}. Then, this mixture was added to the HeLa cells, mixed gently and placed in the incubator at 37°C and 5%CO_2. One day after transduction the virus media was removed and replaced with normal DMEM media supplemented with 10%FBS and 1%PenStrep. The plate was placed back in the incubator at 37°C and 5%CO_2. 48 hours later, the media was removed and replaced with 2mL of DMEM supplemented with 10%FBS, 1%, Pen-Strep and Puromycin 2μg mL^{-1} for the selection. Puromycin selection was considered concluded when in the well containing the control not infected, cells were completely dead.

Western Blot and Antibodies

Cells were re-suspended in RIPA lysis buffer (150 mM NaCl, 50mM Tris, 1mM EGTA, 1% Titon X-100, 0,1% SDS), incubated on ice for 30 minutes, spinned for 15 minutes at 15000 g and proteins in the supernatant were quantified by BCA assay (Perce™ ThermoFisher).

40 μg of protein were separated by SDS-PAGE, in commercial4-12% acrylamide gels (Life Techologies) and transferred into nitrocellulose membranes (Life Techologies) by wet electrophoretic transfer. Blots were blocked 1 hour with 5% fat-free dry milk (BioRad) in TBS-Tween solution: 0.5M Trizma, 1.5M NaCl, 0.001% Tween (Sigma-Aldrich) at room temperature and incubated at 4°C with primary antibodies overnight. The day, after 3x 10 minutes washes on orbital shaker with TBS-Tween solution, membranes were incubate with fresh 5% fat-free dry milk and secondary antibodies for 1 hour at room temperature.

Then, after antibodies incubation, 3x 10 minutes washes with TBS-Tween solution were done on orbital shaker.

Antibodies:

- Primary: anti-MCU (1:1000, Sigma-Aldrich #HPA016480), anti-β-tubulin (1:7500, Santa Cruz #sc-9104), anti-CCDC90B (1:1000, Sigma-Aldrich #HPA011931), anti-CCDC90A(1:1000, Sigma-Aldrich#HPA055189),anti-GRP75(H-155) (0.5:1000, Santa Cruz#sc-13967) , MitoProfile®Total OXPHOS Rodent WB Antibody Cocktail (1:1000, MitoSciences® #MS604).

- Secondary: Goat anti-Rabbit IgG (H+L)- HRP Conjugated antibodies (1:5000, Bio-Rad#1721011); Goat anti-Mouse IgG (H+L)-HRPConjugated antibodies (1:10000, Bio-Rad#1706515).

Immunofluorescence

HeLa cells were grown on 24mm coverslips and co-transfected by $Ca2^{+}$-Phosphate standard method, with CCDC90B-Myc-DDK and mtRFP encoding plasmid. After 24 hours, cells were washed gently with Phosphate-Buffered Saline (PBS) for few seconds, twice. 1 mL/well of 4% formaldehyde solution in PBS was added and put on orbital shaker for 10 minutes. Quenching solution 0.1M Glycine in PBS was used for 10 minutes after washing with fresh PBS for 2 minutes. Cell were permeabilized for 10 minutes with 1% Triton X-100 in PBS and blocked in 2% BSA in PBS for 1 hour. Incubation with primary antibody anti-FLAG in blocking solution lasted 3hours at room temperature and then washed with PBS 3 times for 5 minutes with gentle shaking. The Goat anti-Rabbit Alexa Fluor®488 conjugated secondary antibody (LifeTechologies #A-11008) was used and coverslips were mounted with ProLong Gold Antifade reagent (LifeTechnologies #P10144).

Measurement of cytoplasmic and mitochondrial [Ca^{2+}] using Aequorin

Aequorin is a monomeric Ca^{2+} binding protein isolated from jellyfish *Aequorea Victoria* that emits blue light in presence of Ca^{2+}. The protein has three Ca^{2+} binding site, three cysteine residues., that confer to the protein a particular global structure, forming the hydrophobic core cavity that accommodates the ligand coelenterazine . When Ca2+ bind to the three high affinity EF hand sites, coelenterazine is oxidized to coeleteramide, with a concomitant release of CO2 and emission of light. (Head, J.F., et al. Nature (2000)). 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 logarithm of L/L_{max}, where L is the instant rate of light emission while L_{max} is the maximal values of light emission measured in saturated conditions.

Aequorin allows the possibility of molecular engineering on its sequence; introducing specific targeting sequences and thus directing the Ca^{2+} probe to a specific subcellular compartment. (Brini M., Methods (2008)). Cytoplasmic (cytAEQ) is 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. Mitochondrial (mtAEQ) Aequorin was generated to measure the mitochondrial matrix [Ca^{2+}]. This construct includes the targeting pre-sequence 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.

Cytoplasmic [Ca^{2+}] and mitochondrial [Ca^{2+}] were measured in HeLa cells using respectively the cytAEQ and mt(AEQ)mut chimeras (Pinton P., et al., Methods Cell Biol. (2007)). Cells were seeded 24 hours before transfection into sterile 24-wells cell culture plate and allowed to grow to 50% confluence. Then cells were co-transfected by Ca^{2+}-Phosphate standard method with cytAEQ or mtAEQmut probe together with siRNA or

plasmids depending on the experiment conditions. Mission® siRNA Universal Negative Control (Sigma-Aldrich), pcDNA3.1 or WT HeLa Cells were used respectively as control, depending on the experiment condition. One day before to perform the [Ca^{2+}] measurement, 200μL of cell culture (i.e. 0.03 x 10^6 cells) were plated into the wells of sterile 96-well cell culture plate and incubated for 18 hours at 37°C. Then, when cells result attached to the well, they were incubated with 5 μM coelenterazine for 1 hours in Krebs-Ringer modified buffer (KRB: 135 mM NaCl, % mM KCl, 1mM $MgSO_4$, 0.4 mM $K2HPO_4$, 20 mM HEPES, pH 7.4) supplemented with 1mM $CaCl_2$, and 25mM Glucose. All Aequorin measurements were carried out in KRB saline solution. Agonists were added to the same solution. The agonist stimuli used for maximal stimulation were: 100 μM histamine or 100 μM ATP, depending on the experiment conditions. Measurements were performed using EnVision ® Multilabel Reader (PerkinElmer #2104-0010). The experiments terminated by lysing cells with 100μM digitonin in a hypotonic Ca^{2+}- rich solution (10 mM $CaCl_2$ in H_2O), discharging the unbound Aequorin pool. The light signal was collected and calibrated into free [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 P., et al., Methods Cell Biol (2007)).

Measurement of mitochondrial membrane potential (ΔΨ)

The measurement of mitochondrial membrane potential is based on the distribution of TetraMethylrhodamine Methyl Ester (TMRM, Life Techologies), a cell-permeant, cationic, red-orange fluorescent dye that accumulates in active mitochondria with intact membrane potential. Its distribution into intracellular compartments is triggered by electrochemical gradients. Hence, at low concentrations TMRM accumulation into mitochondria it is demonstrated driven by mitochondrial membrane potential (approximately -180mV). In order to self-quencing of the probe, HeLa cells were loaded with the dye-solution at very low concentration (20 nM). Changes in mitochondrial membrane potential cause a redistribution of the dye between mitochondria and

cytoplasmic environment. Cells were loaded with TMRM 20nM (in KRB saline solution) and Cyclosporine-H 2 μM for 30 minutes at 37°C. The probe was excited at 560 nm and the emission light was recorded in the 590-650 nm range. Oligomycin 10μM, an inhibitor of ATP synthase, was added after 2minutes, while CCCP 10 μM (carbonyl cyanide 3-chlorophenylhydrazone,), an uncoupler of oxidative phosphorylation, was added after 10 minutes to completely collapse the electrochemical gradient established by the respiratory chain ($\Delta\Psi$). Data are expressed as difference between the TMRM fluorescence before and after CCCP depolarization, normalized to 1 value. TMRM imaging8 were performed on an inverted Zeiss Axiovert 200 objective 40X/1.3, using a spinning disk confocal head (BD CarvII). Probe was exited by a 300W Xenon arc lamp (Sutter Lambda LS) with a 543/22 nm filter and collected through a 593/40 nm emission filter.

DISCUSSION and CONCLUSION

The existence of a pathway that allows Ca^{2+} accumulation into mitochondria has been established for about 50 years. Nevertheless, only few years ago, the revolutionary discovery of the Mitochondrial Ca^{2+} Uniporter (MCU) (De Stefani, D., et al., Nature (2011); Baughman, J.M.,et al., Nature (2011)) arrived into the sort of "Pandora's box", leaded one by one to the identification of MCU isoform named MCUb (Raffaello, A., et al, EMBO J.(2013)) and many interactors: the Mitochondrial Calcium Uptake protein 1 (MICU1) (Perocchi, F., et al., Nature (2010)), the Mitochondrial Calcium Uptake protein 2 (MICU2) (Plovanich, M., et al., PLos One(2013)), the Essential Mitochondrial calcium uniporter Regulator (EMRE) (Sancak, Y., et al., Science (2013)) clarified the molecular property of the mitochondrial Ca^{2+} uptake mechanism. A variety of cellular systems recognized MCU as the gateway to allow the Ca^{2+} accumulation into mitochondria, including liver (Baughman, J.M.,et al., Nature (2011)), cancer cells (Marchi, S., et al., Curr. Biol.(2013); Curry, M.C., et al., Biochem. Biophys. Res. Commun. (2013)), cardiomyocytes (Drago, I., et al., Proc. Natl. Acad. Sci. U.S.A. (2012); Joiner, M.L., et al., Nature (2012)) neurons (Qui, J., et al.,Nat.Commun. (2013)), and pancreatic β-cells (Tarasov A.I. et al. Plos One (2012; Alam, M.R. et al., J Biol Chem (2012)). MCU is part of a higher complex that migrates around 480 kDa in blue native gel electrophoresis (Sancak, Y., et al., Science (2013)), and during the years the list of interactors is growing rapidly. This work is focused on the last protein discovered as part of the MCU complex: CCDC90A, renamed Mitochondrial Uniporter Regulator 1 (MCUR1) (Mallilankaraman, K.,et al., Nat Cell Biol (2012)), and its isoform CCDC90B, which function is totally unknown yet.

It was demonstrated that MCUR1 is physically associated with MCU and necessary for MCU-mediated mitochondrial Ca^{2+} uptake. It has been showed that MCUR1 knockdown strongly inhibits mitochondrial Ca^{2+}uptake (Mallilankaraman, K.,et al., Nat Cell Biol (2012)). However, few years later, CCDC90A (MCUR1) has been reported to be also involved with

COX assembly, affecting the turnover on newly synthesized COX subunits. This COX deficiency causes a decrease in mitochondrial membrane potential, impairing the mitochondrial Ca^{2+} uptake activity. This observation opened the possibility that CCDC90A could be not directly involved in MCU function, and the Ca^{2+} uptake defect associated with loss of CCDC90A function may be secondary to the respiratory chain deficiency (Paupe, V. et al., Cell Metab (2015)). In spite of that, Ca^{2+} currents recording in cells with MCUR1 (CCDC90A) knock down, showed a correlation between the level of MCUR1 expression and the magnitude of MCU-mediated Ca^{2+} currents. Considering that applying the patch-clamp technique is possible the comparison of protein activity under tightly controlled condition of $\Delta\Psi m$ and H^+ gradients across IMM (Kirichok, Y. et al., Nature (2004)), these last data suggesting again that MCUR1 functions as a direct regulator of the mitochondrial Ca^{2+} uniporter (Vais, H., et al., Cell Metab, (2015)). We decided to deal with these discrepancies by extending the analysis to other genes closely related to CCDC90A. Indeed, proteins of similar sequence are usually homologous and share similar function (Reeck, G.R. et al. Nature (1987)), even if exceptions exist (Whisstock, J.C., Quart. Rev Biophysics (2003)). CCDC90A has a closely related isoform named CCDC90B, with 67 of similarity, and it is predicted to be a mitochondrial protein. Starting with the assumption of a possible correlation between similarity and function in protein isoforms, CCDC90B and CCDC90A were found both ubiquitous expressed in all human tissues. Density gradient subcellular fractionation together with immunofluorescence confirmed that CCDC90B is a mitochondrial protein as CCDC90A. Short interfering RNA successfully down regulates both proteins expression levels, without any change on MCU protein expression level. As expected, CCDC90A silencing shows lower agonist-induced mitochondrial Ca2+ transients. CCDC90B silencing not only shows the same effect on mitochondrial Ca^{2+} uptake activity, but it produces even a stronger inhibition of mitochondrial Ca^{2+} Uptake, almost doubled in comparison with CCDC90A down regulation. Anyhow, just finding a defect in $[Ca^{2+}]_m$ uptake that occurs with the loss of CCDC90A or CCDC90B it is not sufficient to affirm that these two protein have a direct

effect on the MCU complex activity. As a matter of fact, an unexpected scenario opened when CCDC90A and CCDC90 were silenced along with the overexpression of the components of the core MCU complex (MCU, MCUb, MICU1 and MICU2). It was reported that Immunoprecipitation of MCUR1, MCU or MICU1 suggest the possibility that MCU exists in a complex with either MICU1 or MCUR1, but not both simultaneously (Mallilankaraman, K.,et al., Nat Cell Biol, (2012)). However, when CCDC90A (MCUR1) was silenced and MICU1 overexpressed at the same time, was observed a total rescue in $[Ca^{2+}]_m$, suggesting a functional interaction between this two protein. If it is true that CCDC90A and MICU1 are not part of the same MCU complex, how is it possible that they show a functional interaction? No other similar effects were observed between CCDC90A and the other components of the MCU complex. This unexpected result was obtained only with MICU1 overexpression, a regulator of MCU Ca^{2+} uptake. Overexpressing a regulator of MCU Ca^{2+} uptake activity could induces an imbalance in mitochondrial Ca^{2+} accumulation. The high amount of Ca^{2+} accumulated into the matrix could surpass the upper limit detectable by Mt[AEQ]mt, causing saturation of the probe and therefore a false Ca^{2+} quantification. On the other hand, CCDC90B silencing does not show any functional interaction with any of the MCU complex components, with the exception of a slight recovery in mitochondrial calcium uptake activity in presence of MCU overexpression. However, co-overexpressing CCDC90B together with MCUb, was observed a substantial recovery in the mitochondrial Ca^{2+}. uptake. These results do not encourage us to conclude that CCDC90A and CCDC90B have a role on mitochondrial Ca^{2+} uptake activity. It is possible to identify just marginal protein functional interactions between CCDC90A, CCDC90B and MCU complex components, with few exceptions, quantitatively not sufficient to affirm the possible role of both in the MCU Ca^{2+} uptake machinery. It is also impossible to not consider that MCU Ca^{2+} uptake depends on the electrochemical gradient across the inner mitochondrial membrane. When CCDC90A and CCDC90B are silenced the mitochondrial membrane potential (Ψ_m) shows a significant decrease, suggesting a possible defect on the mitochondrial respiratory chain.

The decrease in Ψ_m registered by TMRM measurements, was directly proportional to the loss of Ca^{2+} found with $[Ca^{2+}]_m$ measurements. In cells where CCDC90B is abolished by gene knockout, it is possible to observe a strong mitochondrial depolarization by Olygomicin addition. ATPase (complex V) inhibition shows a fast mitochondrial depolarization, strengthening the hypothesis of a possible defect in the OXPHOS system and relative impairment in mitochondrial Ca^{2+} uptake driven force. CCDC90B knockdown cells show heterogeneous results on the OXPHOS system protein expression level. A significant percentage of clones show defect in complex IV expression level, in line with previous results reported for CCDC90A (Paupe, V. et al., Cell Metab (2015)). The results reported in this work, touch different aspects, which involved the same protagonist: the mitochondrial Ca^{2+} concentration, underling the complexity of the MCU Ca^{2+} uptake machinery. It is impossible to exclude a role of CCDC90A and/or CCDC90B in mitochondrial calcium dynamics or in OXPHOS system. It is known that proteins may have more than one functional role, and it is premature make conclusions when you are working with a complex system with countless variables, as the MCU complex and OXPHOS are.

BIBLIOGRAFY

Alam, M.R., Groschner, L.N., Parichatikanond, W., Kuo, L., Bondarenko, A.I., Rost, R., Waldeck-Weiermair, M., Malli, R., and Graier, W.F. (2012). Mitochondrial Ca2+ uptake 1 (MICU1) and mitochondrial ca2+ uniporter (MCU) contribute to metabolism-secretion coupling in clonal pancreatic β-cells. J. Biol. Chem. *287*, 34445–34454.

Barrangou, R. (2012). RNA-mediated programmable DNA cleavage. Nat. Biotechnol. *30*, 836–838.

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., et al. (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature *476*, 341–345.

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. *1*, 11–21.

Bick, A.G., Calvo, S.E., and Mootha, V.K. (2012). Evolutionary diversity of the mitochondrial calcium uniporter. Science *336*, 886.

Brini, M., Pinton, P., King, M.P., Davidson, M., Schon, E.A., and Rizzuto, R. (1999). A calcium signaling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency. Nat. Med. *5*, 951–954.

Calcraft, P.J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Rietdorf, K., Teboul, L., Chuang, K.-T., et al. (2009). NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature *459*, 596–600.

Carafoli, E. (2002). Calcium signaling: a tale for all seasons. Proc. Natl. Acad. Sci. U.S.A. 99, 1115–1122.

Cárdenas, C., Miller, R.A., Smith, I., Bui, T., Molgó, J., Müller, M., Vais, H., Cheung, K.-H., Yang, J., Parker, I., et al. (2010). Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell *142*, 270–283.

Chance, B., and Williams, G.R. (1955). Respiratory enzymes in oxidative phosphorylation. III. The steady state. J. Biol. Chem. *217*, 409–427.

Clapham, D.E. (2007). Calcium signaling. Cell *131*, 1047–1058.

Csordás, G., Golenár, T., Seifert, E.L., Kamer, K.J., Sancak, Y., Perocchi, F., Moffat, C., Weaver, D., de la Fuente Perez, S., Bogorad, R., et al. (2013). MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca^{2+} uniporter. Cell Metab. *17*, 976–987.

Cully, T.R., Edwards, J.N., and Launikonis, B.S. (2014). Activation and propagation of Ca2+ release from inside the sarcoplasmic reticulum network of mammalian skeletal muscle. J. Physiol. (Lond.) *592*, 3727–3746.

Curry, M.C., Peters, A.A., Kenny, P.A., Roberts-Thomson, S.J., and Monteith, G.R. (2013). Mitochondrial calcium uniporter silencing potentiates caspase-independent cell death in MDA-MB-231 breast cancer cells. Biochem. Biophys. Res. Commun. *434*, 695–700.

Denton, R.M., Randle, P.J., and Martin, B.R. (1972). Stimulation by calcium ions of pyruvate dehydrogenase phosphate phosphatase. Biochem. J. *128*, 161–163.

De Stefani, D., and Rizzuto, R. (2014). Molecular control of mitochondrial calcium uptake. Biochem. Biophys. Res. Commun. *449*, 373–376.

De Stefani, D., Raffaello, A., Teardo, E., Szabò, I., and Rizzuto, R. (2011). A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature *476*, 336–340.

Drago, I., De Stefani, D., Rizzuto, R., and Pozzan, T. (2012). Mitochondrial Ca2+ uptake contributes to buffering cytoplasmic Ca2+ peaks in cardiomyocytes. Proc. Natl. Acad. Sci. U.S.A. *109*, 12986–12991.

Duarte, J.M.N., Schuck, P.F., Wenk, G.L., and Ferreira, G.C. (2014). Metabolic disturbances in diseases with neurological involvement. Aging Dis *5*, 238–255.

Duchen, M.R., and Szabadkai, G. (2010). Roles of mitochondria in human disease. Essays Biochem. *47*, 115–137.

Ebashi, S., and Lipmann, F. (2008). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. Biochem. Biophys. Res. Commun. *369*, 1–12.

Fatt, P., and Katz, B. (1953). The electrical properties of crustacean muscle fibres. J. Physiol. (Lond.) *120*, 171–204.

Sancak, Y., Markhard, A.L., Kitami, T., Kovács-Bogdán, E., Kamer, K.J., Udeshi, N.D., Carr, S.A., Chaudhuri, D., Clapham, D.E., Li, A.A., et al. (2013). EMRE is an essential component of the mitochondrial calcium uniporter complex. Science *342*, 1379–1382.

Fieni, F., Lee, S.B., Jan, Y.N., and Kirichok, Y. (2012). Activity of the mitochondrial calcium uniporter varies greatly between tissues. Nat Commun *3*, 1317.

Gaude, E., and Frezza, C. (2014). Defects in mitochondrial metabolism and cancer. Cancer Metab *2*, 10.

Giacomello, M., Drago, I., Pizzo, P., and Pozzan, T. (2007). Mitochondrial Ca2+ as a key regulator of cell life and death. Cell Death Differ. *14*, 1267–1274.

Hajnóczky, G., Robb-Gaspers, L.D., Seitz, M.B., and Thomas, A.P. (1995). Decoding of cytosolic calcium oscillations in the mitochondria. Cell *82*, 415–424.

Head, J.F., Inouye, S., Teranishi, K., and Shimomura, O. (2000). The crystal structure of the photoprotein aequorin at 2.3 A resolution. Nature *405*, 372–376.

Hoeflich, K.P., and Ikura, M. (2002). Calmodulin in action: diversity in target recognition and activation mechanisms. Cell *108*, 739–742.

Joiner, M.-L.A., Koval, O.M., Li, J., He, B.J., Allamargot, C., Gao, Z., Luczak, E.D., Hall, D.D., Fink, B.D., Chen, B., et al. (2012). CaMKII determines mitochondrial stress responses in heart. Nature *491*, 269–273.

Kamer, K.J., and Mootha, V.K. (2014). MICU1 and MICU2 play nonredundant roles in the regulation of the mitochondrial calcium uniporter. EMBO Rep. *15*, 299–307.

Kirichok, Y., Krapivinsky, G., and Clapham, D.E. (2004). The mitochondrial calcium uniporter is a highly selective ion channel. Nature *427*, 360–364.

Logan, C.V., Szabadkai, G., Sharpe, J.A., Parry, D.A., Torelli, S., Childs, A.-M., Kriek, M., Phadke, R., Johnson, C.A., Roberts, N.Y., et al. (2014). Loss-of-function mutations in MICU1 cause a brain and muscle disorder linked to primary alterations in mitochondrial calcium signaling. Nat. Genet. *46*, 188–193.

Lipskaia, L., Chemaly, E.R., Hadri, L., Lompre, A.-M., and Hajjar, R.J. (2010). Sarcoplasmic reticulum Ca(2+) ATPase as a therapeutic target for heart failure. Expert Opin Biol Ther *10*, 29–41.

Mallilankaraman, K., Cárdenas, C., Doonan, P.J., Chandramoorthy, H.C., Irrinki, K.M., Golenár, T., Csordás, G., Madireddi, P., Yang, J., Müller, M., et al. (2012a). MCUR1 is an essential component of mitochondrial Ca2+ uptake that regulates cellular metabolism. Nat. Cell Biol. *14*, 1336–1343.

Mallilankaraman, K., Doonan, P., Cárdenas, C., Chandramoorthy, H.C., Müller, M., Miller, R., Hoffman, N.E., Gandhirajan, R.K., Molgó, J., Birnbaum, M.J., et al. (2012b). MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. Cell *151*, 630–644.

Marchi, S., Lupini, L., Patergnani, S., Rimessi, A., Missiroli, S., Bonora, M., Bononi, A., Corrà, F., Giorgi, C., De Marchi, E., et al. (2013). Downregulation of the mitochondrial calcium uniporter by cancer-related miR-25. Curr. Biol. *23*, 58–63.

Marchi, S., and Pinton, P. (2014). The mitochondrial calcium uniporter complex: molecular components, structure and physiopathological implications. J. Physiol. (Lond.) *592*, 829–839.

Martell, J.D., Deerinck, T.J., Sancak, Y., Poulos, T.L., Mootha, V.K., Sosinsky, G.E., Ellisman, M.H., and Ting, A.Y. (2012). Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat. Biotechnol. *30*, 1143–1148.

Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.-E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K., et al. (2008). A mitochondrial protein compendium elucidates complex I disease biology. Cell *134*, 112–123.

Patron, M., Checchetto, V., Raffaello, A., Teardo, E., Vecellio Reane, D., Mantoan, M., Granatiero, V., Szabò, I., De Stefani, D., and Rizzuto, R. (2014). MICU1 and MICU2 finely tune the mitochondrial Ca2+ uniporter by exerting opposite effects on MCU activity. Mol. Cell *53*, 726–737.

Paupe, V., Prudent, J., Dassa, E.P., Rendon, O.Z., and Shoubridge, E.A. (2015a). CCDC90A (MCUR1) is a cytochrome c oxidase assembly factor and not a regulator of the mitochondrial calcium uniporter. Cell Metab. *21*, 109–116.

Paupe, V., Prudent, J., Dassa, E.P., Rendon, O.Z., and Shoubridge, E.A. (2015b). CCDC90A (MCUR1) is a cytochrome c oxidase assembly factor and not a regulator of the mitochondrial calcium uniporter. Cell Metab. *21*, 109–116.

Perocchi, F., Gohil, V.M., Girgis, H.S., Bao, X.R., McCombs, J.E., Palmer, A.E., and Mootha, V.K. (2010). MICU1 encodes a mitochondrial EF hand protein required for Ca(2+) uptake. Nature *467*, 291–296.

Pinton, P., Pozzan, T., and Rizzuto, R. (1998). The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J. *17*, 5298–5308.

Plovanich, M., Bogorad, R.L., Sancak, Y., Kamer, K.J., Strittmatter, L., Li, A.A., Girgis, H.S., Kuchimanchi, S., De Groot, J., Speciner, L., et al. (2013). MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. PLoS ONE *8*, e55785.

Qiu, J., Tan, Y.-W., Hagenston, A.M., Martel, M.-A., Kneisel, N., Skehel, P.A., Wyllie, D.J.A., Bading, H., and Hardingham, G.E. (2013). Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. Nat Commun *4*.

Raffaello, A., De Stefani, D., Sabbadin, D., Teardo, E., Merli, G., Picard, A., Checchetto, V., Moro, S., Szabò, I., and Rizzuto, R. (2013). The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. EMBO J. *32*, 2362–2376.

Reeck, G.R., de Haën, C., Teller, D.C., Doolittle, R.F., Fitch, W.M., Dickerson, R.E., Chambon, P., McLachlan, A.D., Margoliash, E., and Jukes, T.H. (1987). "Homology" in proteins and nucleic acids: a terminology muddle and a way out of it. Cell *50*, 667.

Rimessi, A., Giorgi, C., Pinton, P., and Rizzuto, R. (2008). The versatility of mitochondrial calcium signals: from stimulation of cell metabolism to induction of cell death. Biochim. Biophys. Acta *1777*, 808–816.

Ringer, S. (1883). A further Contribution regarding the influence of the different Constituents of the Blood on the Contraction of the Heart. J. Physiol. (Lond.) *4*, 29–42.3.

Rizzuto, R. (2003). Calcium mobilization from mitochondria in synaptic transmitter release. J. Cell Biol. *163*, 441–443.

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nat. Rev. Mol. Cell Biol. *13*, 566–578.

Rottenberg, H., and Wu, S. (1998). Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. Biochim. Biophys. Acta *1404*, 393–404.

Sancak, Y., Markhard, A.L., Kitami, T., Kovács-Bogdán, E., Kamer, K.J., Udeshi, N.D., Carr, S.A., Chaudhuri, D., Clapham, D.E., Li, A.A., et al. (2013). EMRE is an essential component of the mitochondrial calcium uniporter complex. Science *342*, 1379–1382.

Stahl, W.L., and Swanson, P.D. (1975). Effects of freezing and storage on subcellular fractionation of guinea pig and human brain. Neurobiology *5*, 393–400.

Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J., and Zeman, J. (2005). Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. Biochem. J. *392*, 625–632.

Stokes, D.L., and Wagenknecht, T. (2000). Calcium transport across the sarcoplasmic reticulum: structure and function of Ca2+-ATPase and the ryanodine receptor. Eur. J. Biochem. *267*, 5274–5279.

Tarasov, A.I., Semplici, F., Ravier, M.A., Bellomo, E.A., Pullen, T.J., Gilon, P., Sekler, I., Rizzuto, R., and Rutter, G.A. (2012). The mitochondrial Ca2+ uniporter MCU is essential for glucose-induced ATP increases in pancreatic β-cells. PLoS ONE *7*, e39722.

Vais, H., Tanis, J.E., Müller, M., Payne, R., Mallilankaraman, K., and Foskett, J.K. (2015). MCUR1, CCDC90A, Is a Regulator of the Mitochondrial Calcium Uniporter. Cell Metab. *22*, 533–535.

Wan, B., LaNoue, K.F., Cheung, J.Y., and Scaduto, R.C. (1989). Regulation of citric acid cycle by calcium. J. Biol. Chem. *264*, 13430–13439.

Wang, C.-H., Tsai, T.-F., and Wei, Y.-H. (2015). Role of mitochondrial dysfunction and dysregulation of Ca(2+) homeostasis in insulin insensitivity of mammalian cells. Ann. N. Y. Acad. Sci. *1350*, 66–76.

Weber, A. (1959). On the role of calcium in the activity of adenosine 5'-triphosphate hydrolysis by actomyosin. J. Biol. Chem. *234*, 2764–2769.

Whisstock, J.C., and Lesk, A.M. (2003). Prediction of protein function from protein sequence and structure. Q. Rev. Biophys. *36*, 307–340.