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INVESTIGACIÓN BIOMÉDICA

TESIS DOCTORAL:

Effects of AMP-activated kinase modulators on intracellular Ca^{2+} signalling and *C. elegans* lifespan

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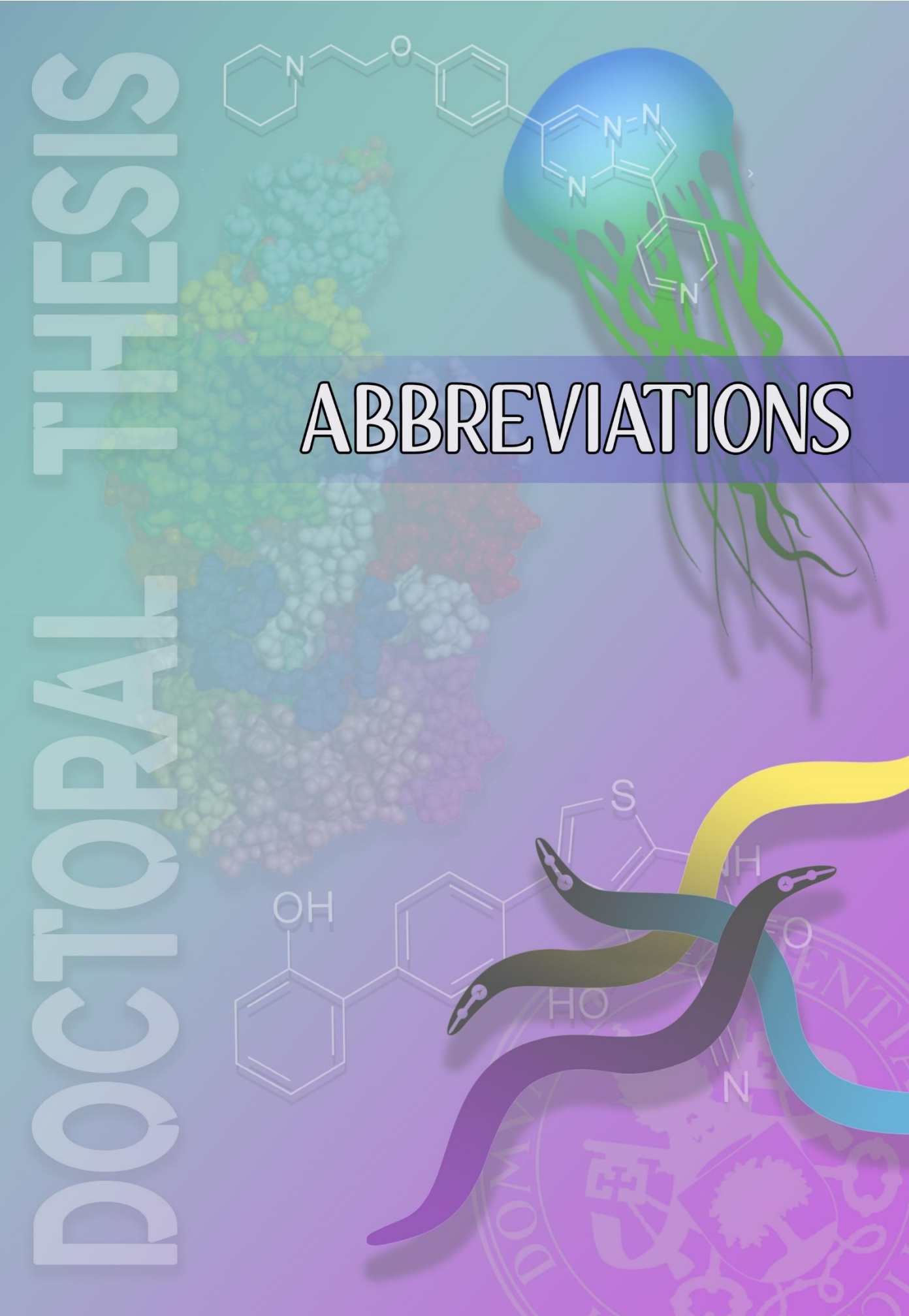
DOCTORAL THESIS

ABBREVIATIONS



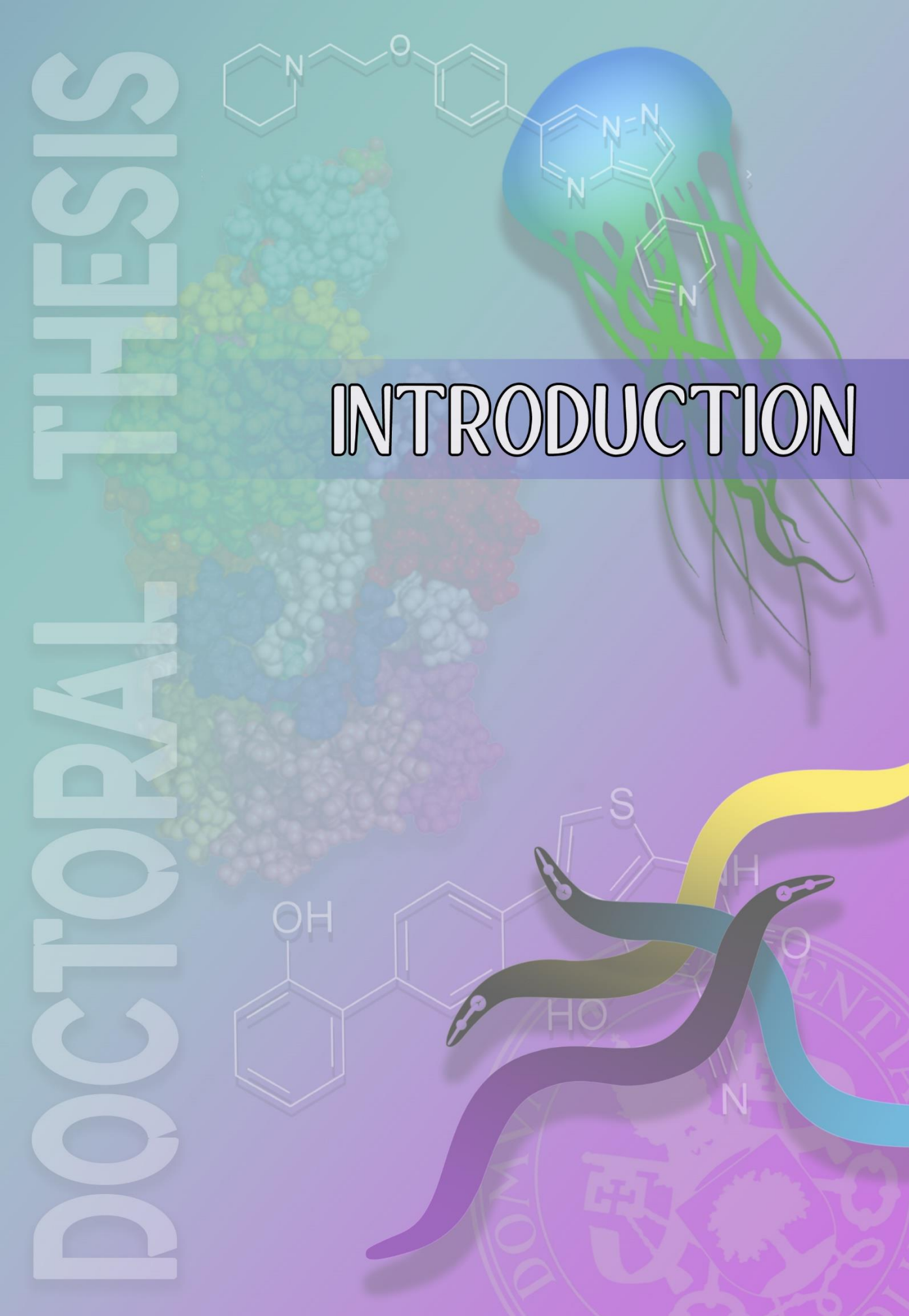
ABBREVIATIONS

AMPK: Adenosine Monophosphate (AMP)-Activated Protein Kinase.
ER: Endoplasmic Reticulum.
ETC: Electron transport chain.
FBS: Fetal Bovine Serum.
FuDR: 5-fluoro-2'-deoxyuridine
GECI: Genetic Encoded Ca^{2+} Indicator.
GFP: Green Fluorescence Protein.
GPCR: G-protein coupled receptor.
HCX: Mitochondrial H^{+}/Ca^{2+} exchanger.
HEDTA: Hydroxyethylethylenediamine triacetic acid.
HEPES: Hydroxyethyl Piperazineethanesulfonic acid.
His: Histamine.
IGF-1: (insulin-like growth factor 1).
IIS: Insulin/Insulin-Like Growth Factor 1 (IGF-1) Signalling.
IP_3: Inositol trisphosphate.
IP_3R: Inositol trisphosphate Receptor.
LAerAEQ: Double-mutated aequorin targeted to the endoplasmic reticulum.
LKB1: Liver kinase B1.
MAMs: Mitochondria-Associated Membranes.
MCU: Mitochondrial Ca^{2+} uniporter.
MitmutAEQ: Mutated aequorin targeted to mitochondria.
mTOR: mechanistic / mammalian target of rapamycin.
NAD^{+}: Nicotinamide adenine dinucleotide.
NADH: Nicotinamide Adenine Dinucleotide.
NCX: Na^{+}/Ca^{2+} exchanger.
NGM: Nematode Growth Medium.
OP50: *Escherichia coli* strain.
PBS: Phosphate Buffered Saline System.
Pi: Inorganic Phosphate.
PI3K: Phosphoinositide 3-kinase.
PKA: Protein kinase A.
PKB: Protein kinase B (AKT)
PKC: Protein kinase C.
PLC: Phospholipase C.
PMCA: Plasma Membrane Ca^{2+} ATPase.
PTP: Permeability Transition Pore.
RNA: Ribonucleic acid.
ROC: Receptor Operated Channel.
ROS: Reactive Oxigen Species.

ABBREVIATIONS

RyR: Ryanodine Receptor.
SERCA: Sarco/endoplasmic reticulum Ca^{2+} ATPase.
SIRT: Sirtuins.
SOC: Store Operated Channel.
SOCE: Store operated Calcium entry.
SPCA: Secretory Pathway Ca^{2+} ATPase.
SR: Sarcoplasmic Reticulum.
TRP: Transient Receptor Potencial.
T2DM: Type 2 diabetes mellitus.
UPR_{MT}: Mitochondrial unfolding protein response.
VDAC: Voltage Dependent Anion Channel.
VOC: Voltage Operated Channel.
γ-CD: Gamma-Cyclodextrin

DOCTORAL THESIS



INTRODUCTION

I. BIOLOGICAL AGING

Finding the cause of aging has always been a great challenge for human beings. However, there is still no universally accepted definition of the aging process, despite the fact that research on aging has undergone a rapid advance in recent years.

From a biological point of view, aging can be broadly defined as a progressive loss of physiological integrity as a consequence of the accumulation of a wide variety of molecular and cellular damage over time, leading to a gradual decline in physical and mental capacities, an increased risk of disease, and finally increased vulnerability to death (Lopez-Otin et al., 2013; Kennedy et al., 2014).

Among the common conditions associated with aging are cardiovascular disorders, cancer, diabetes, dementia or Alzheimer. In addition, it is known that as human beings get older, the likelihood of suffering from several conditions at the same time increases. Therefore, aging is thought to be the main risk factor for most human pathologies (Uno and Nishida, 2016). Thus, finding ways to delay this process, and also to make it healthier, delaying the emergence of age-related diseases, seems to be critical to improve the quality of life of many patients.

1. History of aging theories

The molecular mechanisms underlying the aging process have also attracted a great deal of attention in recent decades because it is believed that knowing those mechanisms, age-dependent diseases could be prevented or delayed. Over the last 150 years, scientific researchers have proposed different theories trying to explain the aging process, although none of them fully and satisfactorily delve into the mechanisms of age-related changes (Davidovic, 2010).

The causes of aging differ between the genetic biologists and evolutionary scientists. The first ones support that aging occurs as a consequence of programmed changes that happen in organisms, admitting that there is some genetic control of longevity though the others suggest that the aging process is the result of evolution.

Broadly speaking, the evolutionary theory of aging defends that natural selection benefits a decline related to age in survival (Kirkwood and Melov, 2011).

One of the first scientists who tried to use Darwin's theory, *On the Origin of Species,* to explain aging in a modern theoretical framework was August Weismann who proposed that aging was part of life's program which improved the species by eliminating the unfit old and worn out individuals to make room for the next generation.

Although this hypothesis was accepted for years, Weismann later rejected it, since there was evidence that natural selection occurs to ensure the reproductive success of individuals, but not the survival of individuals (Kowald and Kirkwood, 2016).

This previous idea led Peter Medawar to propose the first contemporary evolutionary theory of aging. The **Mutation Accumulation Theory**, as it was known, proposes that aging is the result of constant generation of deleterious mutations, built up in the germ line over evolutionary time, that reduce fitness late in life (Medawar, 1952). However, this concept, by itself, is not enough to account for aging (de Grey, 2015).

Few years later, in 1957, the second evolutionary theory of aging, known as **Antagonistic Pleiotropy Theory**, was formulated by George Williams to explain why certain genes have beneficial effects early in life and other genetic variants with adverse or harmful effects can emerge later in life (Sozou and Seymour, 2004; Kirkwood and Melov, 2011; Vijg and Kennedy, 2016).

In parallel to these evolutionary theories, a series of biological hypothesis in humans have also been developed and can be divided into two main categories: programmed and damage or error theories (Jin, 2010; Goldsmith, 2014).

The programmed theories assume that aging follows a biological timetable, whose regulation could depend on changes in gene expression that, in turn, affect the maintenance, repair and defense responses systems (Jin, 2010). In fact, there is evidence that aging is, at least in part, genetically regulated and it has been discovered that many mutations prolong the organism lifespan (Shmookler Reis et al., 2009).

Some of the programmed notions are included in the following theories: the **Hayflick Limit Theory** that postulates that cells have a fixed maximum mitotic capacity, known as the Hayflick Limit, which is specific to each species (Hayflick and Moorehead, 1961); the **Immunological or Autoimmune Theory** which proposes that aging is a consequence of an impaired immune function (Cornelius, 1972); the **Neuroendocrine Theory** that defends that biological clocks act through hormones to control the rhythm of aging (Dilman and Dean, 1992) and the **Programmed Longevity Theory** which defines senescence as the moment in which the deficits associated with age manifest themselves, as a consequence of a sequential switching on and off of certain genes causing the organism to rapidly degenerate and die off (Skulachev 1997, 2011).

Against the idea that aging is a programmed series of events, damage or error theories point out that aging could be a consequence of environmental assaults to

living organisms that trigger cumulative damage at different levels (Jin, 2010; Park and Yeo, 2013).

Among the theories that belong to this group, one of the most common and extended theories to explain aging is the **Free Radical Theory** which was developed by Harman (Harman, 1956). This theory suggests that aging is the result of an accumulation of molecular damage, caused in particular by free radicals derived from oxygen produced within cells, and primarily by mitochondria (Barja, 2002; Gems and de la Guardia, 2013). During respiration, oxygen is reduced producing the commonly known as reactive oxygen species (ROS) which damage the macromolecular components of the cell, such as nucleic acids, lipids, sugars, and proteins, leading to accumulated damage causing cells, and then organs, to stop functioning. Consequently, processes and antioxidant defence systems that protect against oxidative damage should attenuate free-radical-induced damage, prevent aging and increase life expectancy.

However, several authors have recently tried to test this theory directly, studying the effect of oxidative damage and antioxidants on aging and half-life in several animal species, and the results have been negative (Bokov et al., 2004). It has been found that the pharmacological or genetic neutralization of oxygen radicals or the increase of antioxidant defenses, although effective to fight against oxidative stress, are generally unable to increasing longevity (Schulz et al., 2007; Pérez et al., 2009). On the other hand, it has been suggested that chronic or complete inhibition of ROS production could be detrimental since it is believed that ROS production also has useful physiological effects in signal transduction pathways, in the control of gene expression, and in other biological functions. In fact, recent studies carried out on simple organisms, such as the nematode *Caenorhabditis elegans*, or on mice have shown that moderate oxidative damage may be positive in some cases for survival (Sohal and Orr, 2012).

Although almost all the current theories agree that the main cause of aging is the accumulation of molecular damage caused primarily by ROS, the roles of amyloid protein, the final glycation products and lipofuscin have also been recognized (Sergiev, 2015). These results have brought up new hypotheses, for which oxidative damage is only a collateral manifestation of the aging process, but not the cause (Lopez-Otin et al., 2013; Bratic and Larsson, 2013).

2. Aging as a multifactorial process

The theories and data that support both programmed and non-programmed groups continue to grow, as do many other theories that are not exclusively included in one group or another, more appropriately called hypotheses, which are shorter in scope and focus (Park and Yeo, 2013).

Age-related changes seem to be controlled not only by genetic factors but also by environmental factors, which makes it even more difficult to find a comprehensive and universal theory. Some authors agree that aging is a multifactorial phenomenon, which involves the interaction between genes, the environment, and lifestyle factors, particularly diet. In this sense, a joint work of several leading researchers in the field, which deserves special mention, refers to nine key processes related to aging in different organisms (Lopez-Otín et al., 2013), with special emphasis on aging in mammals (Figure 1). Also, it is considered that the pathological dysfunctions of some of these processes accelerate aging, and therefore, the factors involved in the regulation of these characteristics can promote aging (DiLoreto and Murphy, 2015; Martins et al., 2016).

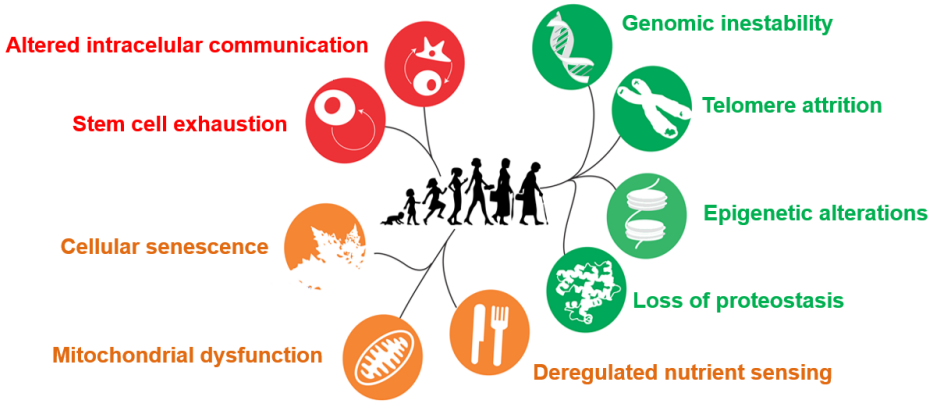


Figure 1. The cellular and molecular hallmarks of aging. Scheme that lists the nine candidate characteristics that contribute to the aging process grouped by colors in three categories according to the functional interconnections between them. In green, the characteristics considered as the main causes of cell damage are represented, in orange, those considered as part of compensatory responses to damage and finally, in red, the integrative hallmarks that are the final result of the previous two groups of characteristics and are responsible for the functional decline associated with aging. They all together determine the aging phenotype (Modified from Lopez-Otín et al., 2013).

These nine hallmarks of aging can be classified under three categories. Primary hallmarks include genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis and are those causing molecular and cellular damage. The antagonistic hallmarks including mitochondrial dysfunction, deregulated nutrient

sensing and cellular senescence refer to compensatory responses to the damage that initially reduce and protect against the damage but if chronic, they become harmful themselves. And, finally, the integrative hallmarks, that include stem cell exhaustion and altered intercellular communication, are the final result of the previous groups, that is, they appear when antagonistic homeostatic mechanisms cannot compensate for the accumulated damage and are ultimately responsible for the functional decline associated with aging (Lopez-Otín et al., 2013).

The characteristics of aging are interconnected and affect the cellular metabolism, so studying the factors that modulate the metabolism could be a promising strategy to extend human health and life expectancy. Among them, the processes related to autophagy and nutrient-sensitive signalling pathways stand out in this work, including mitochondrial dysfunction, which is related to both.

2.1. Autophagy and mitochondrial function

Autophagy widely refers to the different mechanisms that cells use to degrade and maintain organelles, in the same way that proteostasis is maintained through the degradation and resynthesis of proteins (Klionsky and Emr, 2000; Rubinsztein et al., 2011). These mechanisms include macroautophagy through the formation of the autophagosome, chaperone-mediated autophagy (CMA) and microautophagy when the cytosol is directly absorbed into the lysosome (Massey et al., 2006; Mizushima and Komatsu, 2011). In relation to longevity, it is believed that it is very important that damaged organelles are degraded and cleaned to provide new raw material for healthy cells (Kenyon, 2010b).

Aging, whether normal or pathological, has been related to a decrease in the processes of autophagy. Some findings showed that the changes associated with aging are very similar to the degenerative changes found in the tissues of mammals when inhibiting autophagy genetically. Pharmacological or genetic interventions that increase lifespan in model organisms often stimulate autophagy, whereas its inhibition compromises longevity (Rubinsztein et al., 2011). However, the mechanisms by which autophagy affects aging remain enigmatic. Several positive modulators of autophagy have been identified, including the transcription factor FOXO, the histone deacetylase SIRT1, the energy sensor AMPK and the forkhead transcription factor PHA-4/FOXA (Gelino and Hansen, 2012). Autophagy also plays a complex role in neurodegenerative diseases (Nixon, 2013) and it is not clear whether autophagy regulates Parkinson's and Alzheimer's diseases in the opposite direction (Alvarez-Erviti et al., 2010; Lipinski et al., 2010).

Among the mechanisms that modify the aging process, it is worth noting also the alteration of **mitochondrial function**. The maintenance of mitochondrial function is esencial for cellular metabolism, because these organelles are responsible for the energy production in all cell types. Mitochondria therefore regulate energy and calcium (Ca^{2+}) homeostasis, apoptosis, and the fatty acids oxidation critical for the electron transport chain (ETC). As previously mentioned, mitochondria also modulate cell signalling through the generation of ROS and it has also been described that there are complex mechanisms of mitochondrial-nuclear communication. One of them is a signal transduction pathway regulating the mitochondrial unfolding protein response (UPR_{MT}) (Haynes and Ron, 2010; Kriegenburg et al., 2012), starting by the accumulation of unfolded proteins in the mitochondria and allowing the transcription of nuclear genes involved in mitochondrial metabolism (Hill and Van Remmen, 2014).

It has been shown that longevity can be increased through a functional decline of the mitochondria. In particular, the mutation or reduction of function in nuclear genes that encode components of the electron transport chain, delays the aging process in yeasts, *C. elegans, Drosophila*, and mice (Durieux et al., 2011). Altogether, mitochondria are believed to modulate key aspects of aging, so that more recent studies focus on finding strategies that improve mitochondrial function in order to obtain beneficial effects on long-term longevity. However, the specific relationship between mitochondrial dysfunction and aging remains a major challenge for research (Sun et al., 2016).

2.2. 'Nutrient sensors' modulating aging

It has been found that several genetic pathways and biochemical processes well-preserved in evolution from yeast to the nematode *Caenorhabditis elegans* and mammals (Fontana et al., 2010b; Kenyon, 2010) modulate aging and longevity, and this is causing a rapid progress in aging research in recent years.

The signalling cascades that modulate these processes through tissue-specific physiological changes are known as “nutrient-sensitive pathways” and they are described to be biochemical pathways capable of ‘sensing’ the availability of nutrients and maintaining energy homeostasis (Rossetti, 2000; Templeman and Murphy, 2017). The key elements in these pathways are the insulin and IGF-1 (insulin-like growth factor 1) signalling pathway, known as the “IIS pathway”, the AMP kinase pathway (AMPK), the mTOR kinase pathway (mechanistic / mammalian target of rapamycin) and that of the sirtuins (Honjoh and Nishida, 2011; Davinelli et al., 2012; Bitto et al., 2015).

This thesis focuses on the pathways of nutrient sensing since it has been shown that they influence human beings aging (Bonafè, 2003; Aiello et al., 2017).

2.2.1. Insulin/Insulin-Like Growth Factor 1 (IGF-1) Signalling

IGF-1 and insulin signalling are known as the ‘‘insulin and IGF-1 signalling’’ (IIS) pathway since both are responsible for informing the cells of the presence of glucose. Altogether, the IIS system is a highly conserved signal transduction pathway, from invertebrates to mammals, that coordinates growth, differentiation and metabolism in response to changing environmental conditions and nutrient availability, and also modulates aging and longevity (Fontana et al., 2010b; Kenyon, 2010).

The first time that IIS was related to aging was when it was discovered that the loss of function mutations in the *C. elegans* phosphatidylinositol-3-kinase (PI3K) gene, *age-1*, or the insulin-like receptor gene, *daf-2*, could double the lifespan, depending on the FOXO-family transcription factor DAF-16 (Friedman and Johnson, 1988; Kenyon et al., 1993).

Few years after the role of genes *age-1*, *daf-2* and *daf-16* in the control of longevity in *C elegans* was discovered, homologies were found with mammalian proteins (Kimura et al., 1997; Ogg et al., 1997), and other components of the pathway were later identified in worms (Tissenbaum and Ruvkun, 1998) and flies (Clancy et al., 2001; Tatar et al., 2001). Definitely, the IIS system is the best-studied longevity pathway, consisting of insulin-like peptides (ILPs), an insulin/IGF-1 receptor (DAF-2), a phosphoinositide 3-kinase (PI3K), such as phosphatidylinositol 3-kinase age-1 (AGE-1) or amino acid permease 1 (AAP-1), serine/threonine kinases, namely, pyruvate dehydrogenase kinase 1 (PDK-1) or AKT serine/threonine kinase 1 and 2 (AKT-1 and AKT-2) and the pivotal downstream Forkhead Box O transcription factor or FOXO (DAF-16 in *C. elegans*) (van Heemst, 2010, Sun et al., 2017).

Aging process is known to be metabolically characterized by insulin resistance and physiological decrease of IGF-1 levels, as well as those of growth hormone (GH), a hormone that stimulates the production of IGF-1 (Schumacher et al., 2008), factors that are also considered a risk for multiple age-related diseases. In addition, it has also been shown that null mutations in PI3K and AKT kinases are lethal in mouse embryos (Renner and Carnero, 2009), suggesting that extremely low levels of IIS signalling are incompatible with life. These data contrast paradoxically with the results obtained in invertebrate model organisms, where it has been found that genetic polymorphisms or mutations reducing the functions of the IGF-1 receptor, insulin receptor or subsequent intracellular effectors such as AKT, mTOR and FOXO are directly related to longevity (Kenyon, 2010; Barzilai et al., 2012).

One possible explanation to these apparently contradictory findings is that there are differences between the aging of invertebrates and mammals. However, a longer lifespan has also been described in several insulin resistant mouse mutants (Russell and Kahn, 2007). Therefore, it seems that the role of an attenuated IIS signalling in the mechanisms of aging is evolutionarily conserved.

2.2.2. Other Nutrient-Sensing Systems

Besides the IIS pathway that participates in glucose sensing, three additional pathway systems correlated and interconnected with nutrition that affect longevity and aging are: mTOR, for the detection of high concentrations of aminoacids; AMPK, which senses low-energy states by detecting high AMP levels; and sirtuins, which sense low-energy states by detecting high NAD^+ levels (Houtkooper et al., 2010).

However, although all of these pathways seem to be critical effectors of aging, they do not act in the same direction (Figure 2). While AMPK and sirtuins signal nutrient scarcity and catabolism, IIS and mTOR, point out nutrient abundance and anabolism, that is, mTOR and IIS act on the pro-aging side and AMPK and sirtuins on the pro-longevity side (Lopez-Otin et al., 2016; Riera et al., 2016).

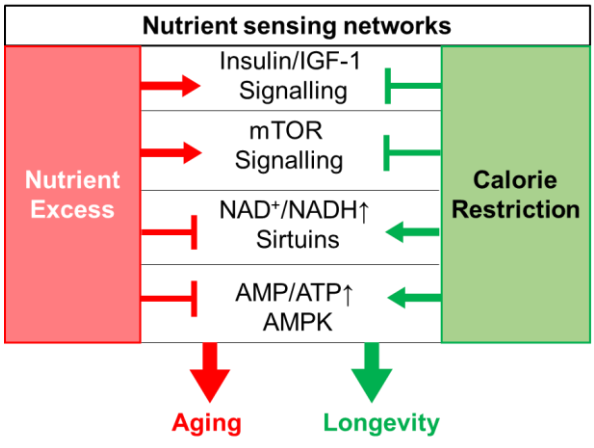


Figure 2. Nutrient-Sensing Systems modulating aging. Modified from Yokoyama et al., 2015.

2.2.2.1. TOR pathway

mTOR (mammalian/mechanistic target of rapamycin) pathway was firstly described in *C. elegans,* although later was proved to be evolutionarily conserved in other organisms (Vellai et al., 2003; Fontana et al., 2010b).

In mammals, mTOR is a serine / threonine protein kinase that belongs to the family of phosphoinositide 3-kinase (PI3K) and interacts with several proteins to form two different complexes with the name of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Huang and Fingar, 2014) that can be distinguished by their different associated proteins, “Raptor and Rictor”, respectively. The mTORC1 pathway is the best known and integrates the contributions of at least five large intracellular and extracellular signals, namely, growth factors, stress, energy status, oxygen and amino acids, to control many important processes, essentially, all aspects of anabolic metabolism (Laplante and Sabatini, 2012) such as synthesis of proteins and lipids, autophagy and mitochondrial metabolism (Kaeberlein, 2013; Johnson et al., 2015). On the contrary, the mTORC2 complex is not as well characterized and its specific activation mechanism requires more studies, although it is believed that it participates in the regulation of cell survival and spatial organization of the cytoskeleton (Zoncu et al., 2011).

It has been shown that a reduced mTORC1 signalling either by the genetic inhibition of mTOR itself or other components of the mTORC1 complex can extend the lifespan of worms (Vellai et al., 2003; Jia et al., 2004) and fruit flies (Kapahi et al., 2004). However, the first evidence that mTORC1 signalling could somehow regulate aging comes from studies where the mutation of the S6 kinase (S6K) homolog (Sch9) extended lifespan in yeast (Fabrizio et al., 2001; Kaeberlein et al., 2005). S6K is the main mTORC1 substrate and a crucial regulator of mRNA translation (Kapahi et al., 2010) that is involved in longevity in *C. elegans*. Mice deficient in S6K showed improved health span and enhanced longevity (Selman et al., 2009), evidencing that the downregulation of mTORC1/S6K1 acts as the mediator of mammalian longevity.

On the other hand, it has also been reported that mTOR is specifically related to the IIS pathway elements, playing a fundamental role in homeostasis and energy metabolism (Zoncu et al., 2011; Robida-Stubbs et al., 2012; Figure 3). The insulin/IGF pathway is known to activate mTOR kinase and thus its downstream targets, such as, eukaryotic initiation factor 4 (eIF4) and ribosomal protein S6K1, which regulate protein and ribosome biosynthesis. Consequently, that anabolic activity, signaled through the IIS or the mTORC1 pathways, could be major accelerators of aging (Blagosklonny, 2009; Węsierska-Gądek, 2010), probably because mTOR is a potent inhibitor of autophagy.

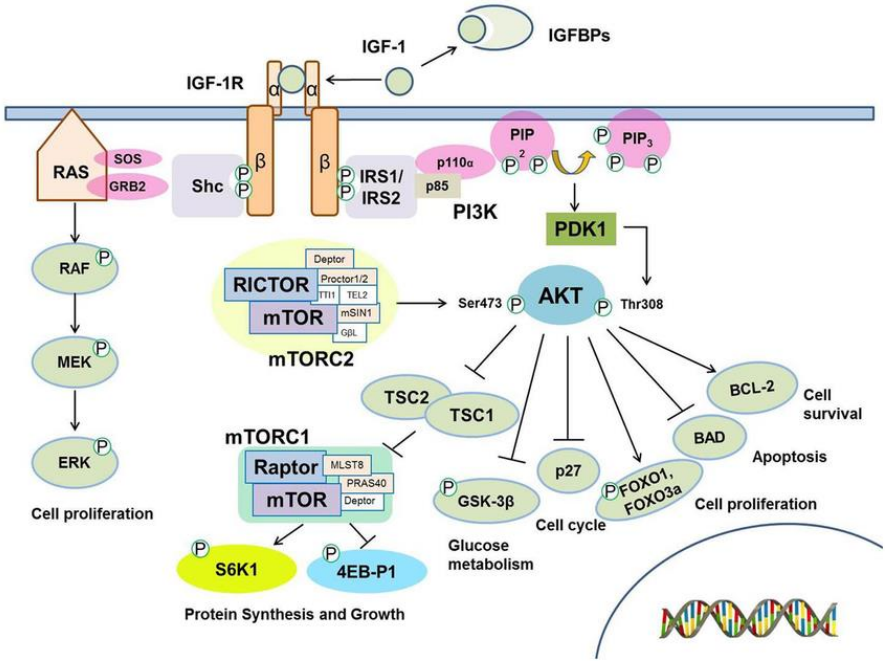


Figure 3. The relationship between IIS and mTOR signalling pathways and major downstream effects. IGF ligands are modulated by IGF binding proteins (IGFBPs) through direct binding in the extracellular space. IGF-1 interacts with IGF-1 receptor (IGF-1R) in the cellular membrane activating signalling through two pathways: PI_3K/AKT and Ras/MAPK. PI_3K/AKT produces a decrease in apoptosis, increased protein synthesis and an increase in glucose metabolism. Ras/MAPK contains a complex cascade of kinases that leads to an increase in cell proliferation (Jung and Suh, 2015). The IIS pathway also regulates mTORC1 kinase and thus its downstream targets.

2.2.2.2. Adenosine Monophosphate-Activated Kinase pathway

5' AMP-activated protein kinase (AMPK) plays a key role in cellular energy homeostasis. AMPK is a sensor of adenine nucleotides that is activated when celular energy is low and tries to restore energy balance by activating ATP-producing catabolic pathways and inhibiting ATP-consuming anabolic pathways (Figure 4). Recent studies suggest that it may be able to sense glucose levels directly by a non-canonical mechanism, that is, independently of changes in adenine nucleotides (Lin and Hardie, 2018).

At the structural level, AMPK consists of three subunits including the catalytic α-subunit and two β, γ regulatory subunits (Figure 4). AMPK activation can be triggered by phosphorylation of T172 in the α-subunit or by AMP/ADP binding to the γ-subunit. ATP competitively inhibits the binding of both AMP and ADP to the γ-subunit, and thus AMPK behaves as a sensor of AMP/ATP or ADP/ATP ratios (Hardie, 2007). Phosphorylation at T172 of the AMPK α-subunit can be carried out by several upstream kinases, such as the Ca^{2+}-/calmodulin-dependent kinase kinase 2

(CaMKK2) and liver kinase B1 (LKB1). In case of CaMKK2, the interaction with AMPK only involves the α and β subunits. Using this mechanism, AMPK becomes activated by changes in Ca^{2+} levels but not by changes in AMP/ATP or ADP/ATP ratios (Woods et al., 2005; Jeon, 2016).

Activation of AMPK leads to modulation of several metabolic pathways, which are summarized in Figure 4, including stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle fatty acid oxidation, muscle glucose uptake and mitochondrial biogenesis (Winder and Hardie, 1999; O'Neill et al., 2013; Jeon, 2016).

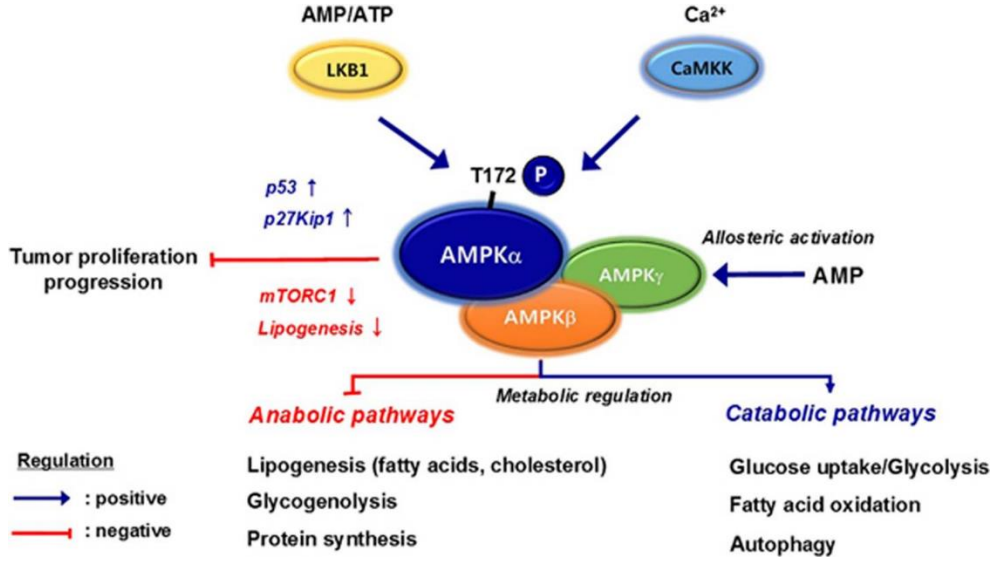


Figure 4. Physiological roles of AMP-activated protein kinase (AMPK). AMPK has a key role in maintaining the balance between anabolic and catabolic programs for cellular homeostasis in response to metabolic stress (Kim et al., 2016).

AMPK inhibits the synthesis of fatty acids by inhibiting two proteins, the acetyl-CoA carboxylase 1 (ACC1) enzyme and the transcription factor that promotes the expression of several lipogenic enzymes, the sterol regulatory element-binding protein 1c (SREBP1c) (Li et al., 2011). It is also believed that AMPK activates lipid catabolism by increasing the absorption of fatty acids and controlling the translocation of the CD36 transporter to the plasma membrane (Habets et al., 2009). In skeletal muscles, it has been described that AMPK stimulates glucose uptake by translocating intracellular vesicles containing GLUT4 to the plasma membrane (Taylor et al., 2008).

In addition to lipid and glucose metabolism, which are the first known AMPK functions, some effects of AMPK on energy metabolism are in part mediated by mTOR pathway inhibition. For instance, AMPK also regulates protein metabolism, by blocking the mTOR pathway through phosphorylation of an important protein synthesis regulator, the tuberous sclerosis complex 2 (TSC2) (Inoki et al., 2003) and Raptor (Gwinn et al., 2008). Under energy stress conditions, the synthesis of proteins is inhibited to save cellular ATP. Moreover, AMPK activation not only attenuates protein biosynthetic processes, but also induces protein degradation through autophagy and the ubiquitin-proteasome system (Polak and Hall, 2009). Thus, autophagy maintains cellular integrity during nutrient deficiency. Some studies have shown that AMPK can activate autophagy by direct phosphorylation of an autophagy-initiating regulator, the protein kinase complex ULK1, a mammalian homologue of Atg1 (Autophagy related 1) or indirectly by inhibiting mTORC1. This regulation ULK1-mTORC1 also maintains the mitochondrial integrity, since it favors the elimination of damaged mitochondria (Egan et al., 2011; Kim et al., 2011; Alers et al., 2012).

Besides, AMPK can act as a regulator of proliferative signals such as mTORC1 or the RNA polymerase I (Pol I)-associated transcription factor TIF-IA, both required for rapid cell proliferation (Hoppe et al., 2009). It has also been described that AMPK activation causes G1 cell cycle arrest, which is associated with p53 tumor suppressor gene activation (Jones et al., 2005), as well as cyclin-dependent kinase inhibitor p27kip1 phosphorilation in response to metabolic stress (Liang et al., 2007). Therefore, AMPK has shown a role in tumorigenesis, since during energy stress, expression of cell survival genes is also required, indicating that cancer cells proliferation could be modified by modulating AMPK signalling.

Current research focuses on finding activators of AMPK since it is involved in several metabolic signalling pathways where it regulates changes in the energy balance, so its deregulation is associated with numerous human pathologies. Accordingly, AMPK activation could have beneficial effects on metabolic diseases, including glycemia, type 2 diabetes and obesity, although harmful in cancer treatment because it could protect tumor cells against lack of nutrients or cytotoxic agents.

In addition, there is a great variety of physiological conditions that regulate AMPK activity. Oxidative stress (Hardie, 2004) or exercise cause metabolic stress and activate AMPK under physiological conditions (Winder and Hardie, 1996). On the contrary, overnutrition and obesity, which in turn lead to an increased risk of chronic inflammation, are critical risk factors for the previously mentioned chronic diseases. It has been suggested that the accumulation of the essential nutrients, glucose, fatty acids or amino acids, inhibits AMPK action and contributes to insulin resistance (Coughlan et al., 2013). Since an efficient regulation of energy metabolic homeostasis is considered a characteristic factor of a better healthspan and a longer lifespan,

several studies on aging have also focused on AMPK. In fact, some of them have shown that AMPK activation declines during aging (Hardie, 2004), and additionally, others with lower organisms have revealed that an increased AMPK activity can extend lifespan.

It has been shown in worms that overexpression of one of two orthologs of the AMPK-α subunit, AAK-2, lengthens lifespan, while the elimination compromises it. Likewise, in mice, chronic activation of AMPK protects against diet-induced obesity (Yang et al., 2008). Thus, the activation of AMPK has been associated with increased longevity and improved health span (Apfeld et al., 2004; Schulz et al., 2007; Greer and Brunet, 2009).

Hence AMPK activation favours healthy aging, although the specific mechanism by which it modulates aging is still not clear. It is believed that in part this effect could be mediated by its ability to inhibit mTOR signalling (Alers et al., 2012), although other authors suggest that these effects could be just part of an integrated signalling network. In mammals, for instance, AMPK activation may have an impact on FOXO, sirtuins, and mTOR pathways activities, which have been related to healthy longevity. Specifically, experiments in mammals have revealed that AMPK controls autophagy through mTOR and ULK1 signalling by increasing the quality of cellular housekeeping. In addition, stimulation by AMPK of FOXO or SIRT1 signalling pathways improves cell stress resistance, and inhibition by AMPK of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling suppresses inflammatory responses, creating an integrated signalling network through which AMPK controls energy metabolism, autophagic degradation and stress resistance and, ultimately, the aging process (Salminen and Kaarniranta 2012).

2.2.2.3. Sirtuins pathway

Sirtuins (SIRTs) are a family of nicotinamide adenine dinucleotide (NAD+)-dependent protein histone deacetylases (HDACs) present in a variety of organisms from bacteria to humans (Guarente, 2007) that catalyze posttranslational modification of proteins, not only deacetylation (Imai et al., 2000; Smith et al., 2000), but also adenosine diphosphate (ADP)-ribosylation (Haigis et al., 2006). Likewise, SIRTs constitute a group of epigenetic modifiers modulating the aging process and the age-related diseases.

The first time sirtuins were described to be involved in the aging process was when it was discovered that the activation of the Sir2 (silent information regulator 2) caused an increase in replicative lifespan in yeast (Longo and Kennedy, 2006).

Genomic stability within the ribosomal DNA is thought to be the likely mechanism by which Sir2 improves replicative longevity in yeast (Kaeberlein et al., 1999).

The mammalian orthologs of sirtuins are the SIRT proteins. Although it has not been demonstrated that overexpression of the whole body SIRT1 protein prolongs the lifespan of mice (Bordone et al., 2007), it is known to improve some metabolic parameters that may be involved in different aging-related diseases, including metabolic and neurodegenerative diseases and cancer (Herskovits and Guarente, 2014). In addition, it has been described that SIRT1 and AMPK are fed back positively, connecting both sensors in the same response (Price et al., 2012).

3. Modifying the course of aging

The mutations discovered in round worms, flies and mice that produce the most significant increases in life expectancy are those affecting one of the biochemical and metabolic pathways that influence aging, such as energy consumption, stress resistance or the regulation of the insulin / IGF-1 neuroenodrocrine pathway. However, organisms modified to increase their longevity often show inherent defects as well, such as some mutant worms having reduced fertility (Friedman and Johnson, 1988). Furthermore, modifying the course of aging in humans is still a matter of study. Meanwhile, the search for new therapeutic strategies that delay aging and make it healthier does not only include drugs or pharmacologic interventions, but also non-pharmacological approaches, such as physical exercise and low-calorie dietary intake (Mazucanti et al., 2015).

3.1. Caloric restriction as a tool to delay aging

The most studied and consensual procedure to increase longevity is **caloric restriction or dietary restriction (DR)**, which can be defined as a reduction in the availability of nutrients in the absence of malnutrition. This concept was initially proposed by Roy Walford, a pioneer in the field of caloric restriction as a method of health improvement and life extensión. He tried to demonstrate that a diet high in nutrients and low in calories could delay the process of functional aging, though, not the chronological one (Turner, 2003).

In fact, the effects of DR on the survival were seen for the first time in rats more than 70 years ago (McCay et al., 1935). Since then, hundreds of studies have shown that DR can increase life expectancy and delay the appearance of multiple phenotypes related to advanced age in a wide range of organisms, including all the major models

used in biomedical research and even non-human primates (Masoro, 2005; Colman et al., 2009; Omodei and Fontana, 2011; Fontana and Partridge, 2015).

However, knowledge of the specific composition of a diet that improves health and promotes greater human longevity as well as reducing the risk of disease-specific mortality, requires further study (Mercken et al., 2013; Micó et al., 2017). In recent years, research has also focused on separating the possible benefits of DR in humans from the adverse effects on health, as people undergoing DR often experience problems of low bone density and muscle mass, among others. It should be also noted that the benefits of DR to extend human life can be very small, since most trials begin in adulthood, while DR and longevity studies in rodents are initiated in very young animals (Speakman and Hambly, 2007).

The underlying molecular mechanisms by which DR is able to achieve these effects have been intensively studied so far. It is believed that the interaction of several mechanisms is necessary to cause the effects of DR that promote longevity. In fact, it has been reported that it may act through the direct alteration of the activities of those three key nutrient sensors mentioned above, AMPK, SIRT1 and mTOR, which affect lifespan. Therefore, the key elements by which DR acts seem to be the nutrient-sensitive pathways again (Barzilai et al., 2012).

3.2. Decreased mTOR signalling and rapamycin

The mTOR kinase pathway seems to be a key mediator in the effects on the length of life of the DR (Blagosklonny, 2010; López-Otín et al., 2013; Kaeberlein, 2013).

In response to depletion of nutrients, mTOR activity is reduced and this results in a series of events designed to promote longevity and improve resistance to stress and age-related diseases (Laplante and Sabatini, 2012). In particular, the synthesis of new proteins and lipids is inhibited, the degradation of damaged proteins and other macromolecules increases through autophagy, and the metabolism of carbohydrates and mitochondrial function are modified to adapt to the low energy situation (Markaki and Tavernarakis, 2013). Specifically, the inefficiency of cellular autophagy processes has been related to neurodegenerative diseases such as Parkinson's, Alzheimer's or Hungtington's diseases, where defective proteins that generate mitochondrial damage accumulate, leading to the accumulation of damaged mitochondria (Harris and Rubinsztein, 2011; Cai et al., 2012; Hochfeld et al., 2013).

The mTOR kinase plays an essential role in a new theory on aging, proposed by M.V. Blagosklonny and supported by several important researchers in the field,

called the **Theory of Hyperfunction** (Blagosklonny, 2010; Gems and de la Guardia, 2012; Blagosklonny, 2013). According to this theory, aging is due to the continuity in the adult state of the processes of growth and development of the initial stages of life. These processes are primarily driven by mTOR, an essential gene during development, but when it remains active thereafter, driven by the availability of nutrients, it leads to hyperfunction, which would be responsible for the loss of homeostasis, age-related diseases and death. According to this theory, the DR would act primarily by inactivating mTOR.

In this sense, this recent theory has also had great support from to the effects of rapamycin, a macrolide antibiotic that specifically inhibits mTOR kinase with high affinity. Rapamycin forms a complex with the FKBP12 protein (Laplante and Sabatini, 2012), which interacts directly with the mTOR kinase and inhibits it when it is part of mTORC1, but not when it is in mTORC2, although prolonged treatments can lead to mTORC2 inhibition as well.

Apart from DR, it is worth mentioning that rapamycin is the only non-genetic intervention that has been able to prolong life in a similar way in yeasts, nematodes, *Drosophila* flies and mice (Kaeberlein, 2013). Rapamycin has been shown to delay age-related alterations, including alterations in heart, liver, adrenal glands, endometrium, tendons, and spontaneous activity (Miller et al., 2011; Wilkinson et al., 2012; Johnson et al., 2013).

According to these data, rapamycin would be slowing the aging process and extending median and maximal life span in mice (Harrison et al., 2009), so that all these normal causes of morbidity associated with age and death would be delayed. It has been proposed therefore that rapamycin acts as a mimic of DR, which induces the same response of increased longevity, but under conditions of high nutrient level (Kaeberlein, 2010; Blagosklonny, 2013; López-Otín et al., 2013). However, other studies suggest that the rapamycin and DR pathways may not be exactly the same (Miller et al., 2013; Fok et al., 2013) and that the combination of rapamycin and DR would be more effective than either of the two isolated treatments.

The reduction of mTOR signalling in rodents has been shown to increase longevity, but further studies will be required before translating it into humans. Nevertheless, rapamycin has been clinically used for almost 25 years to prevent rejection of transplants in combination with immunosuppressants such as cyclosporin A (Bravo-San Pedro and Senovilla, 2013), and has recently been used as an antiproliferative agent in some types of cancer (Blagosklonny, 2013b; Kaeberlein, 2013) since it has shown to be a relatively well tolerated drug in prolonged treatments (Blagosklonny, 2013c). However, it is not exempt from undesirable side effects, mainly alterations of carbohydrate and lipid metabolism (Lamming et al., 2012; Wilkinson et

al., 2012; Johnson et al., 2013). Therefore, it would be interesting to understand the mechanisms involved to know if the beneficial and harmful effects of inhibiting TOR can be dissociated.

3.3. AMPK activators

Several studies have revealed that the activation capacity of AMPK decreases during the aging process (Reznick et al., 2007), so that AMPK has become a good therapeutic target. It is believed that the new AMPK activators could be useful in the therapy of metabolic and neurodegenerative human diseases (Musi, 2006; Fogarty and Hardie, 2010), since the energy balance is important in these diseases that are the main causes of morbidity or death in humans. AMPK is also known to be activated by DR, but it remains to be fully determined if the AMPK activators act as DR mimics to improve health and function.

As already mentioned, AMPK can be activated allosterically by AMP, and there is also a regulation of AMPK activity by upstream kinases, LKB1 and CaMKKβ, which phosphorylate Thr-172 of the AMPKα subunit in the cells themselves. However, since it was demonstrated that AMPK activation could be useful for the treatment of type 2 diabetes, (Winder and Hardie, 1999) numerous pharmacological activators have been developed such as mitochondrial inhibitors (biguanides, polyphenols such as resveratrol or thiazolidinediones), the adenosine analogue 5-aminoimidazole-4-carboxamide riboside (AICAR) or the thienopyridone derivative, A-769662, among many others (Figure 5).

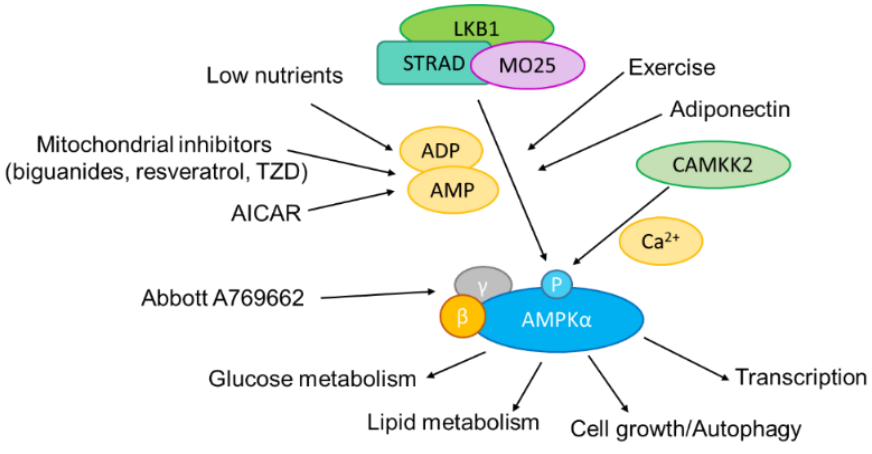


Figure 5. Major modulators of AMPK. The increase of AMP and ADP levels in the cells, due to a physiological cause or the presence of drugs, activates AMPK. LKB1 kinase activates AMPK in response to increased AMP or ADP, while CAMKK2 kinase activates AMPK in response to increased Ca^{2+}. Other pharmacological modulators activate it by direct interaction with AMPK. Activation of AMPK directly phosphorylates substrates that affect metabolism and growth, and long-term metabolic reprogramming. (Modified from Mihaylova and Shaw, 2011).

A particular class of AMPK activators are natural products of plants derived from traditional medicinal herbs. While the mechanism by which most of these AMPK activators act has not yet been addressed, many of them are believed to act by inhibiting mitochondrial function (Hardie, 2015; Kim et al., 2016).

A possible classification divides the main AMPK activators according to their mechanism of action (Hardie, 2015). In this work, some of these compounds have been grouped into two categories: the activators that directly bind to and activate AMPK without any significant change in cellular ATP, ADP or AMP levels, and those modulators that cause AMP or Ca^{2+} accumulation.

3.3.1. Indirect AMPK activators

Indirect activators do not require direct interaction with AMPK but rather cause or produce AMP or Ca^{2+} increase. This group includes some biguanides, such as metformin, as well as thiazolidinediones and polyphenols.

3.3.1.1. Biguanides

Metformin is a synthetic derivative of guanide that is a natural product of the plant *Galega officinalis*. This biguanide compound is currently the most widely clinically used medication for type 2 diabetes (T2DM) since it reduces hepatic glucose level, improves the peripheral sensitivity to insulin and is also relatively safe for human consumption. Interestingly, there is a growing belief that metformin is potentially therapeutic in a variety of multiple disorders beyond diabetes (Sui et al., 2015). It has been shown to increase longevity in *C. elegans* and in mice (Onken and Driscoll, 2010; Anisimov et al., 2011; Martin-Montalvo, 2013), and its positive effect has also been described in various age-related diseases, including cardiovascular diseases and cancer (Libby et al., 2009; López-Otín et al., 2013).

In addition, treatment with metformin in patients with T2DM has shown that this drug is well tolerated. Besides, it has been reported that metformin clearly improves life expectancy in diabetic patients, and even that diabetics who have been tretated with metformin could live longer than non-diabetics (Bannister et al., 2014). Nevertheless, there are no conclusive data supporting a clear role for metformin in human aging and it is still being studied whether metformin can diminish many factors that accelerate aging (Cameron et al., 2016; Garg et al., 2017) such as protection against DNA damage, poor mitochondrial function and chronic inflammation. In fact, a novel clinical trial, called *Targeting Aging with Metformin* (TAME), will evaluate 3000 people for six years to determine if metformin can delay conditions related to age. The

success of the study will depend on whether the medication delays the onset of typical diseases, including cancer, cardiovascular diseases and Alzheimer's disease.

These anti-aging effects are believed to be due to the fact that metformin is an activator of AMPK (Podhorecka et al., 2017). In this way metformin would also act as a mimic of DR since AMPK inhibits the action of mTOR. Then, its activation would also lead to an inhibition of mTOR, whereby rapamycin and metformin could have synergistic effects.

It has been proposed that the mechanism by which metformin activates AMPK could be indirect, inhibiting the complex I of the mitochondrial respiratory chain (El-Mir et al., 2000; Owen et al., 2000), which leads to an increase in the AMP:ATP ratio. However, it is no even clear if the effects of metformin on lifespan are direct and exclusively mediated by AMPK, since there is evidence in worms indicating that metformin may increase lifespan by altering microbial folate and methionine metabolism, suggesting that metformin could act as an anti-aging factor promoting health by modulating the microbiota (Cabreiro et al., 2013; Pryor and Cabreiro, 2015; Figure 6).

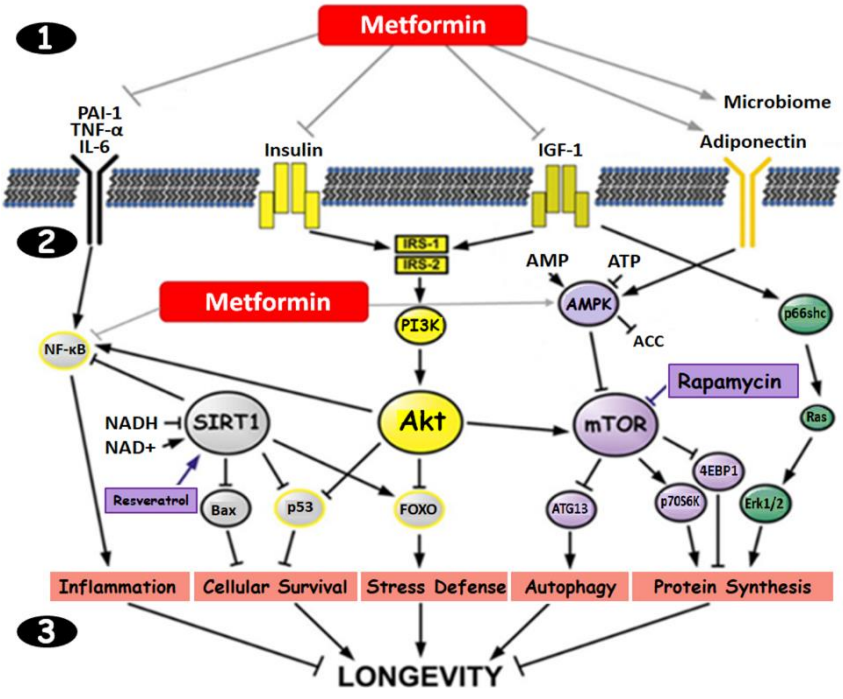


Figure 6. Effect of metformin on pathways associated with longevity. Schematic representation of the pathways that are activated during aging and the points where metformin has effect. Extracellular metformin (1) affects the receptors of cytokines, insulin, IGF-1 and adiponectin, and intracellularly (2) inhibits the inflammatory pathway and activates AMPK, increasing the inhibition of mTOR. These processes together (3) affect inflammation, cell survival, stress defense, autophagy and protein synthesis, which are the main biological results associated with longevity (Modified from Barzilai et al., 2016).

3.3.1.2. Thiazolidinediones

Thiazolidinediones (TZDs), or glitazones, are a group of anti-diabetic drugs that include troglitazone, pioglitazone and rosiglitazone and act primarily by activating the peroxisome proliferator activated receptors (PPARs).

It is also known that they exert their anti-diabetic effect, in part, through the activation of AMPK and AMP accumulation, as a result of the inhibition of the complex I of the mitochondrial respiratory chain (Brunmair et al., 2004). In skeletal muscle and liver, the treatment with TZD also promotes the expression and release of adiponectin from adipocytes (LeBrasseur et al., 2006) which, in turn, activates AMPK, increasing glucose uptake and fatty acid oxidation and decreasing hepatic glucose production.

3.3.1.3. Polyphenols

Polyphenols are a structural class of natural or synthetic chemicals products that present multiple units of phenol structure and are capable of activating AMPK and exert beneficial effects on T2DM and metabolic syndrome.

The activation of AMPK by these compounds requires the elevation of AMP levels, since many of them inhibit the mitochondrial production of ATP, either by inhibiting mitochondrial F_1F_0-ATPase/ATP synthase (namely, resveratrol, quercetin, epigallocatechin gallate and curcumin) or the complex I of the respiratory chain (such as berberine) (Zheng and Ramirez, 2000; Turner et al., 2008).

3.3.2. Direct AMPK activators

These AMPK activators bind directly and activate AMPK by inducing conformational changes in the AMPK complex, without any significant change in cellular levels of ATP, ADP or AMP.

3.3.2.1. AICAR

The 5-aminoimidazole-4-carboxamide riboside (AICAR) is an adenosine analogue recognized in cells by adenosine transporters and phosphorylated by adenosine kinase, generating AMP-mimetic, AICAR monophosphate (ZMP) (Corton et al., 1995). ZMP directly activates AMPK because it is capable of binding to site 3 on the AMPK γ-subunit.

In mice, treatment with AICAR and physical exercise clearly increased AMPKα2 activity in young rats muscles whereas in old rats these insults induced no

response in AMPKα2 activity (Narkar et al., 2008). However, there are no results on whether AICAR is able to reproduce these effects in humans.

3.3.2.2. Thienopyridone (A-769662) derivatives

The identification of A-769662 by Abbott Laboratories in 2006 provided a new insight into the development of direct AMPK activators, demonstrating that AMPK activation with non-nucleotide ligands was possible.

A-769662, is a thienopyridone compound that shows high specificity for AMPK (Cool et al., 2006) so that it activates allosterically AMPK without Thr172 phosphorylation in the AMPKα subunit, which is necessary for the activation of AMPK dependent on AMP (Scott et al., 2014). However, it requires the phosphorylation of Ser108 in the AMPKβ1 subunit. A-769662 decreases body weight, plasma glucose and hepatic triglycerides in obese mice, showing many of the metabolic effects that would be expected after the activation of AMPK *in vivo*.

Likewise, another direct AMPK activator has been identified, the compound 911 which is 5-10 times more potent than A-769662 in the allosteric activation of AMPK (Xiao et al., 2013).

3.4. Sirtuins

Resveratrol is a known activator of sirtuin SIRT1 and it has been shown that resveratrol and DR show similar anti-aging activities in SIRT1 signalling. This highlights the potential of resveratrol as a DR mimic (Li et al., 2017). However, its clinical efficiency remains controversial, probably because it also has, in turn, the ability of activating AMP-activated protein kinase (AMPK) and inhibit mTOR (Ghosh et al., 2010; Lan et al., 2017).

4. Calcium signalling and nutrient sensitive pathways

A major challenge for the future is to achieve a better understanding of the complex relationship that exists between mTOR and other signalling cascades involved in senescence and aging. Specifically, one of the signalling pathways that has been thought to be intimately related to the functioning of mTOR is the Ca^{2+} signalling pathway (Decuypere et al., 2013).

There is considerable evidence for the role of Ca^{2+} signalling in the modulation of the nutrient-sensitive pathways, although the mechanisms are not completely known (Decuypere et al., 2013). Regulation of mTOR and AMPK by Ca^{2+} may take

place at several levels. First, activation by Ca^{2+} of CaMKK2 leads to AMPK activation, which in turn, inhibits mTOR. Using this pathway, Ca^{2+} indirectly inhibits mTOR (Cárdenas and Foskett, 2012). On the other hand, there is also evidence that Ca^{2+} can directly activate mTOR. It has been described that amino acids added to nutrient-deficient cells induce an increase in cytosolic Ca^{2+} that activates mTOR through the binding of the Ca^{2+}-calmodulin complex to a class III phosphatidylinositol 3-kinase necessary for the activation of mTOR (Gulati et al., 2008). Likewise, it has been described that muscle hypertrophy induced by training is mediated by the activation of Ca^{2+} channels of the TRPV1 type, which produce Ca^{2+} entry into the cytosol. An increase in cytosolic Ca^{2+} would produce a direct and immediate activation of mTOR, responsible for initiating the hypertrophy process (Ito et al., 2013). In these cases, the increase in cytosolic Ca^{2+} would have an activating effect on mTOR.

One of the key mediators of Ca^{2+} fluxes linked to the activity of mTOR and AMPK is the inositol triphosphate receptor (IP_3R), which releases Ca^{2+} from the endoplasmic reticulum (ER) and is also responsible for transferring Ca^{2+} from the ER to the mitochondria through close contacts between the organelles. These contacts, called Mitochondrial Associated ER Membranes (MAMs), contain both IP_3R and mitochondrial Ca^{2+} uptake channels, the so-called mitochondrial Ca^{2+} uniporter (MCU). In addition to IP_3R and MCU, it has been shown that mTORC2 is also in MAM, controls its integrity and is able to activate AKT kinase, which finally phosphorylates and inhibits IP_3R (Betz et al., 2013). On the other hand, mTOR has also been reported to be able to directly phosphorylate and activate IP_3R (MacMillan et al., 2005; Fregeau et al., 2011; Regimbald-Dumas et al., 2011). The resulting increased Ca^{2+} transfer between ER and mitochondria would lead to increased ATP production and subsequent AMPK inhibition (Parys et al., 2012). In turn, variations in the activity of the IP_3R can alter the nutrient sensitive pathways. When the release of Ca^{2+} mediated by IP_3R is reduced, Ca^{2+} entry to the mitochondria decreases and this reduces the rate of ATP synthesis. This energy deficit activates AMPK, which in turn inhibits mTOR. In this way, a deficient Ca^{2+} transfer between ER and mitochondria may lead to mTOR inhibition (Cárdenas and Foskett, 2012). Finally, it has also been described that mTOR is capable of activating the store-operated Ca^{2+} entry pathway (SOCE), via an increase in the expression of the STIM1/Orai1 proteins responsible for this pathway (Ogawa et al., 2012).

To conclude this exposition it can be said that multiple interactions between Ca^{2+} homeostasis and nutrient sensitive pathways have been described in the literature, but the mechanisms and the sense of the interactions are still unclear. It has been described that the activity of mTOR depends on a correct Ca^{2+} transfer between the ER and the mitochondria, the cytosolic calcium ($[Ca^{2+}]_C$) increase can activate or inhibit mTOR by different pathways and, in turn, mTOR is capable of raising calcium levels

through activation of both SOCE and IP_3R. Therefore, any of these processes can be considered as a target of possible compounds acting on aging.

In order to understand better the results presented in this work, the Ca^{2+} signalling pathway and the generalities of the *C.elegans* model are explained below.

II. CALCIUM SIGNALLING PATHWAY

1. Cellular calcium homeostasis

Calcium ion (Ca^{2+}) is a ubiquitous intracellular second messenger involved in many cellular functions, from gene expression or cell cycle processes which last minutes or hours, to almost instantaneous processes, such as contraction or secretion (Berridge et al., 2003). Under physiological conditions, only transient increases in intracellular [Ca^{2+}] usually occur, but under pathophysiological conditions, the ability to control intracellular Ca^{2+} overload may be compromised. Consequently, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) must be precisely regulated in time and space, since alterations in Ca^{2+} signalling have been described as the possible sources of many diseases, including neurodegenerative diseases, cancer, heart disease and others (Berridge, 2012).

In addition to the functions previously quoted where this ion participates, a specific role of Ca^{2+} in brain aging has also been described (Chandran et al., 2017). In fact, the **Ca^{2+} hypothesis in aging** was proposed by Khachaturian (Khachaturian, 1994) to explain the neurophysiological mechanisms implying Ca^{2+} signalling which are related to aging and neurodegeneration. Besides, different works have also shown that any alteration of neuronal Ca^{2+} homeostasis could play a crucial role for age related changes and lead to a variety of associated diseases (Gibson and Peterson, 1987; Kirischuk and Verkhratsky, 1996). Likewise, an increased ER-mitochondrial cross-talk and diminished SOCE or deregulation of RyR have recently been associated with intracellular calcium levels imbalance leading, in turn, to increased cellular vulnerability and neuronal death (Calvo-Rodríguez et al., 2016; Abu-Omar et al., 2018).

Therefore, Ca^{2+} signalling is believed to play an important role in aging and neurological disorders associated with aging, although the specific role of altered Ca^{2+} homeostasis in mammalian aging is not entirely clear. Nevertheless, a greater knowledge of the relationship between calcium homeostasis and calcium-dependent processes during aging could help in the development of new strategies to improve longevity and quality of life. In fact, many calcium signalling proteins are already promising drug targets for many diseases related to aging (Chandran et al., 2017).

2. Characteristics of the calcium signal

The calcium signal is regulated thanks to the ion dynamic compartmentalization in different intracellular regions and through the plasma membrane.

Under resting conditions, the free [Ca^{2+}] in the extracellular space is around 1-2 mM, while in the cytosol it is maintained around 100 nM due to the Ca^{2+}-dependent ATPases that are capable of pumping this ion against its electrochemical gradient, either towards the extracellular space or towards intracellular stores, such as the ER, where the Ca^{2+} concentration is between 0.5 and 2 mM (Montero et al., 1997; de la Fuente et al., 2013). In addition, in the plasma membrane of many cell types the Na^+/Ca^{2+} or Na^+/Ca^{2+}-K^+ exchangers help in the Ca^{2+} extrusion, taking advantage of the free energy generated by transport of Na^+ or K^+ down their electrochemical gradient (Guerini et al., 2005; Krebs et al., 2015). Unlike other second messengers, Ca^{2+} cannot degrade or metabolize, so when the Ca^{2+} extrusion mechanisms from the cytosol do not work properly, $[Ca^{2+}]_C$ keeps abnormally high and maintained, and this is harmful for the cells. That is why such Ca^{2+} extrusion mechanisms are so important.

In the presence of a stimulus, the Ca^{2+} entry from the extracellular medium to the cell, or the release from intracellular stores, causes a fast increase in the $[Ca^{2+}]_C$, triggering a cellular response in a short period of time. This increase is highly modulated by several factors, including the cytosolic Ca^{2+} buffer systems and the mitochondria, that also play a fundamental role as Ca^{2+}buffer. Mitochondria are able to uptake rapidly $Ca^{2+}{}_C$ through the mitochondrial calcium uniporter (MCU), which is able to accumulate levels up to 1 mM Ca^{2+} inside the mitochondrial matrix (Montero et al., 2000). After stimulation, Ca^{2+} channels in the plasma membrane and ER-membrane close, different exchangers release Ca^{2+} from the mitochondria back to the cytosol, and exchangers and pumps extrude Ca^{2+} from the cytosol to the extracellular medium or into the Ca^{2+} stores. This allows the cell to return to its resting state. In doing so, cells are able to suitably respond to new stimuli (Berridge, 1997), without exceeding the spatial and temporal limits of the Ca^{2+} signal that can cause cell death.

The main elements involved in Ca^{2+} signalling are described in detail below.

2.1. Calcium buffers

Ca^{2+} buffers are a protein family containing "EF-hand" motifs capable of binding Ca^{2+} ions, including parvalbumins, calbindin and calretinin. Most of the proteins belonging to this family participate in the space-time regulation of Ca^{2+} signalling and

function as Ca^{2+} sensors, which change their conformation when they bind Ca^{2+} and act as regulators of specific targets (Schwaller et al., 2002; Hackney et al., 2005; Boros et al., 2009; Schwaller, 2010).

The most common example of this type of protein is calmodulin (CaM) (Chin and Means, 2000). Their expression and effect greatly varies depending on the cell type. At low concentrations, these sensors do not significantly alter $[Ca^{2+}]$. However, at high concentrations, these sensors become Ca^{2+} buffers.

The endoplasmic reticulum (ER) actively participates in Ca^{2+} signalling and therefore possesses its own Ca^{2+} buffer proteins, many of which act as chaperones, responsible for the correct protein folding and transport through the endoplasmic reticulum (Michalak et al., 2009). The most important of these proteins is calreticulin, responsible for up to 50 % of the Ca^{2+} buffering capacity of the endoplasmic reticulum (Nakamura et al., 2001) and whose presence is essential in the release of Ca^{2+} from the endoplasmic reticulum in response to a stimulus (Li et al., 2002). Other proteins also play important roles as Ca^{2+} buffers, including BiP, GRP94, and calsecuestrin in the sarcoplasmic reticulum (Murphy et al., 2009; Prins and Michalak, 2011).

2.2. Cytosolic calcium concentration increase

Cells employ a variety of Ca^{2+} channels, both from plasma membrane and from the ER, to create Ca^{2+} signals. In resting conditions, these channels are closed, but in response to an external stimulus, they are opened triggering an increase in $[Ca^{2+}]_C$.

2.2.1. Calcium entry from extracellular medium

The Ca^{2+} entry from the extracellular space is favoured, on the one hand, by a difference of concentrations between both compartments of four orders of magnitude and, on the other hand, by a membrane potential about 60mV negative inside. This generates an important electrochemical gradient that promotes Ca^{2+} entry to the intracellular space from the extracellular medium, in response to a stimulus.

Ca^{2+} entry through the plasma membrane is carried out by different channels whose names indicate their activation and deactivation properties (Figure 7): Store-Operated Calcium Channels (SOC) that are activated in response to a decrease in the Ca^{2+} level of the stores (Wu et al., 2006; Potier and Trebak 2008; Cahalan, 2009); Voltage-Operated Calcium Channels (VOC) that are activated in response to a depolarization of the plasma membrane of cells, acting as transducers of electrical signals (Catterall, 2011); Receptor-Operated Calcium Channels (ROC) that are

directly activated by binding of a ligand to a receptor (such as the nicotinic acetylcholine receptor), or indirectly, through channels associated with metabotropic receptors (such as some glutamate receptors) (Barritt, 1999) and TRP channels (Transient Receptor Potential), including some receptor operated channels along with other types of channels acting as mechanoreceptors or thermosensors, activated by a variety of factors, such as mechanical stress, osmolarity, temperature and even by the Ca^{2+} level of intracellular stores (Montell, 2005; Nilius and Owsianik, 2011).

Although these channels are opened for a very short time, the amount of Ca^{2+} entering through them is enough to cause very significant increases in the $[Ca^{2+}]_C$, especially in the close proximity of the channels, where high Ca^{2+} microdomains are produced (Rizzuto and Pozan, 2006). The type, quantity and distribution of Ca^{2+} channels depends on the cell type and its developmental stage and they can be sometimes grouped in certain regions favouring the creation of microdomains.

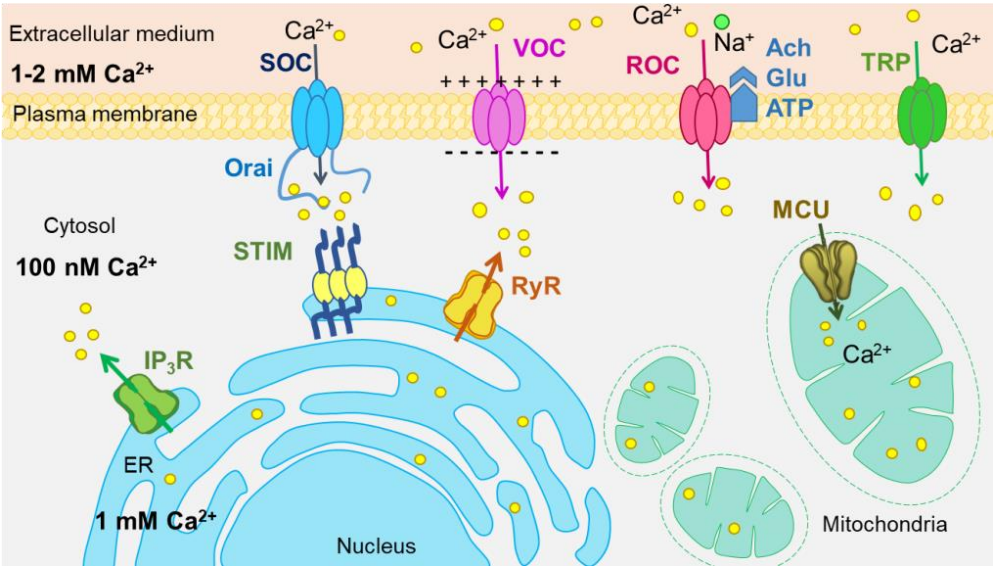


Figure 7. Calcium channels involved in cellular calcium homeostasis. Schematic representation of the Ca^{2+} channels of the plasma membrane (SOC, VOC, ROC and TRP) and of the endomembranes, inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR). The mitochondrial calcium uniporter (MCU) is also shown.

2.2.2. Calcium release from the intracellular stores

The main intracellular source of Ca^{2+} is the sarcoplasmic reticulum (SR) in striated muscle and the endoplasmic reticulum (ER) in other cell types. There are two large families of intracellular Ca^{2+} release channels located in the sarco (endo) plasmic reticulum (SR/ER), the ryanodine receptors (RyRs) and the inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Figure 8).

2.2.2.1. Ryanodine receptors (RyRs)

RyRs are the largest known ion channels formed by homotetramers that exist as three isoforms in mammals (RyR 1-3). These proteins are regulated by phosphorylation and a variety of small proteins and ions. Ca^{2+}, both cytosolic and luminal, is a physiological ligand that triggers RyRs opening, as well as caffeine, ATP or heparin. Voltage changes across the plasma membrane can also cause the RyRs opening, in the absence of extracellular Ca^{2+} during excitation-contraction coupling (Rios and Brum, 1987) in skeletal muscle, where CaV1.1 channels interact directly with the RyR1. Instead, in cardiac muscle, CaV1.2 channels opening results in entry of Ca^{2+} into the cell that activates RyR2 and initiates Ca^{2+} release from the SR (Lanner et al., 2010). This process is known as Calcium-Induced Calcium Release (CICR) (Figure 8). There are some molecules and ions, such as Mg^{2+}, ruthenium red or ryanodine that act as inhibitors of these channels (Meissener, 1986).

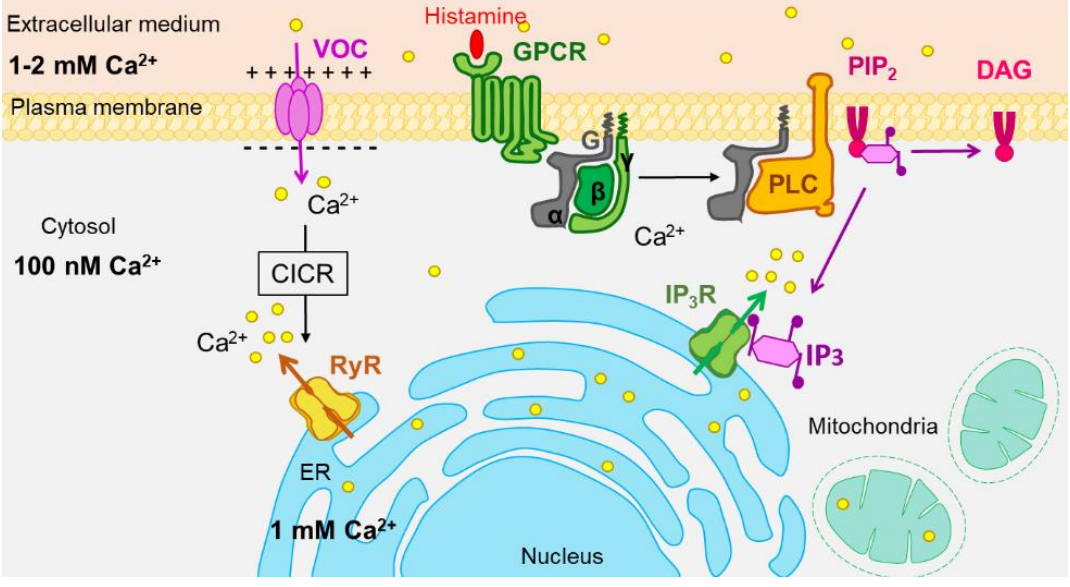


Figure 8. Ca^{2+} channels of the endomembranes: RyR and IP_3R. The calcium-induced calcium release (CICR) process and the release of Ca^{2+} from the IP_3R, as a consequence of the activation of a GPCR, are shown. PLC (phospholipase C), PIP_2 (phosphatidyl inositol-4,5-bisphosphate), DAG (diacylglycerol), GPCR (G-protein coupled receptor), G (G protein).

2.2.2.2. Inositol 1,4,5-trisphosphate receptors (IP_3Rs)

IP_3Rs are the major Ca^{2+} release channel on the ER membrane of non-excitable cells, so that they allow the release of intracellular Ca^{2+} from the stores in response to various stimuli that activate phospholipase C (PLC), normally through G-protein coupled receptors (GPCRs) or tyrosine kinases. PLC hydrolyses

phosphatidylinositol 4,5-bisphosphate lipids in the plasma membrane, generating inositol 1,4,5 trisphosphate (IP_3) (Berridge, 1993). This second messenger diffuses into the cytoplasm and binds to the IP_3R, favouring its opening and release of Ca^{2+} to the cytosol (Figure 8). The IP_3R is not only regulated by IP_3, but also by other ligands, in particular by cytoplasmic Ca^{2+}. In fact, IP_3R opening is not only induced by IP_3, but requires Ca^{2+} as coagonist (Kaftan et al., 1997). In addition, the IP_3R integrates signals from numerous additional molecules and proteins, including nucleotides, kinases, and phosphatases, as well as non-enzymatic proteins, including ATP and calmodulin (Patterson et al., 2004).

2.3. Recovery of basal calcium levels

The calcium extrusion mechanisms that maintain the basal $[Ca^{2+}]_C$ at approximately 100 nM work continuously due to their low regulation, so cells spend a large amount of the ATP they produce in these processes.

This process involves the Ca^{2+} ATPases, which pump Ca^{2+} against the concentration gradient outside the cells or inside the stores, and the exchangers, which collaborate with the pumps to extrude Ca^{2+} after a stimulus (Brini and Carafoli, 2011; Figure 9).

2.3.1. Calcium pumps

The energy to remove Ca^{2+} from the cell has its origin in the ATP hydrolysis, since the Plasma Membrane Calcium ATPase (PMCA) and the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) are primary active transport pumps that need to be phosphorylated to transport Ca^{2+} against an electrochemical gradient (Figure 9). These pumps have low transport capacity, but their affinities for Ca^{2+} are high, so they keep pumping at very low levels of Ca^{2+} in the cytosol, in order to complete the process of recovery of the resting levels.

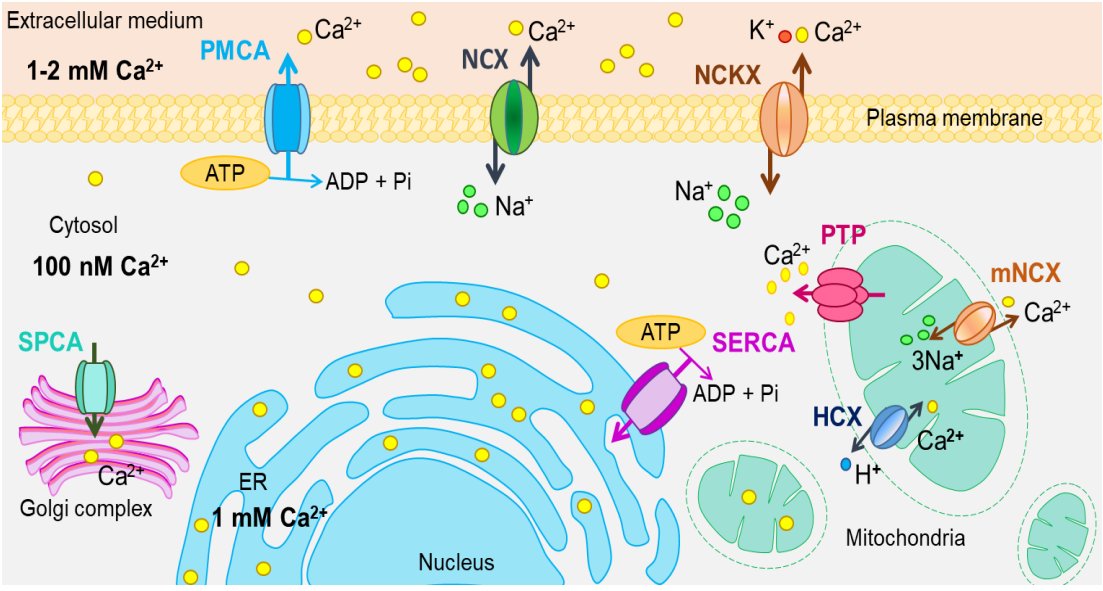


Figure 9. Structures involved in the recovery of basal Ca^{2+} levels. Ca^{2+} ATPases from the plasma membrane (PMCA), sarco/endoplasmic reticulum (SERCA) and Golgi complex (SPCA) Ca^{2+} pumps and exchangers (NCX and NCKX) are schematically represented. Mitochondrial exchangers (HCX and mNCX) and the permeability transition pore complex (PTP) are also shown.

2.3.1.1. Plasma membrane Ca^{2+} ATPase (PMCA)

The plasma membrane Ca^{2+} ATPase (PMCA) belongs to the family of P-type ATPases and contributes to the maintenance of intracellular Ca^{2+} homeostasis by transporting Ca^{2+} from the cytosol to the extracellular space. The pump operates with a 1:1 stoichiometry, transporting 1 Ca^{2+} by hydrolyzed ATP and it is electrogenic (exchanges 1 Ca^{2+} for 1 H^{+}). It has a high affinity for Ca^{2+}, with a Kd of 1 μM under conditions of maximum activation (Strehler and Treiman, 2004) although there are also many agents that modulate it, either by inhibiting it, such as vanadate, lanthanum and caloxin or by activating it, such as acid phospholipids and calmodulin (Carafoli et al., 1996). It is also regulated by proteins and tyrosine kinases.

2.3.1.2. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)

The Ca^{2+} entry from the cytosol to the ER takes place thanks to an ATP-dependent calcium pump, SERCA, which, like the PMCA, is a member of the P-type ATPases. It is located in the ER/SR membrane and pumps Ca^{2+} from the cytosol into the stores. Because of the smaller Ca^{2+} electrochemical gradient in the ER membrane, this pump is more efficient than PMCA, since for each hydrolyzed ATP it transports

two Ca^{2+} ions to the ER. It is also electrogenic (it exchanges 2 Ca^{2+} for $2H^{+}$). It is inhibited by vanadate and lanthanides, and also has some specific inhibitors: the potent and irreversible inhibitor thapsigargin (Lytton et al., 1991), and the reversible inhibitors cyclopiazonic acid (CPA) and 2,5-di-tert-butyl-benzohydroquinone (BHQ). In addition, the transmembrane proteins phospholamban and sarcolipin are well studied SERCA regulators (MacLennan et al., 2003).

2.3.2. Calcium exchangers

These proteins take advantage of the energy of the Na^{+} electrochemical gradient to extrude Ca^{2+} from the cytosol to the extracellular medium (Figure 9). The exchangers have low affinities for Ca^{2+}, but very high transport capacities, allowing them to quickly remove large amounts of Ca^{2+} from the cytosol.

2.3.2.1. Plasma membrane Na^{+}/Ca^{2+} exchanger (NCX)

The Na^{+}/Ca^{2+} exchangers of the plasma membrane (NCX) belong to SLC8 family of proteins and are part of a large superfamily of proteins known as transporters, whose main function is to control Ca^{2+} flows through the plasma membrane (Lytton, 2007). In each exchanger cycle, three Na^{+} ions enter the cell and a Ca^{2+} ion goes out against its gradient, thus being an electrogenic transport. These ions, in addition to being transported, regulate the activity of the exchanger and when intracellular Na^{+} is overloaded, the exchangers may act in reverse, allowing the entry of Ca^{2+} into the cytosol (Brini and Carafoli, 2011).

2.3.2.2. Plasma membrane $Na^{+}/Ca^{2+}/K^{+}$ exchanger (NCKX)

The SLC24 family of NCKX exchangers ($Na^{2+}/Ca^{2+}/K^{+}$) constitute the other Na^{+}/Ca^{2+} exchangers family with low affinity and high transport capacity, originally described in photoreceptors. NCKX transports $1K^{+}$ and $1Ca^{2+}$ in exchange for 4 Na^{+} ions (Lytton, 2007; Brini and Carafoli, 2011).

2.4. Role of Ca^{2+} in the mitochondria

Mitochondria are mobile and plastic organelles, which constantly change shape, fusing and forming a mitochondrial network, permanently associated with the ER membrane through physical contacts, called MAMs, where proteins such as IP_3R,

Voltage-Dependent Anion Channels (VDAC), Mitochondrial Calcium Uniporters (MCU) or RyR, participate (Rizzuto et al., 1998; Csordás et al., 2010).

Mitochondria are limited by two highly specialized membranes, defining two different compartments, the intermembrane space and the mitochondrial matrix. The outer mitochondrial membrane (OMM) allows the passage of small molecules and ions (<10 KDa) into the intermembrane space due to the abundance of aqueous channels (porins, VDAC), but most molecules and ions cannot cross the inner mitochondrial membrane (IMM). The IMM has numerous invaginations called mitochondrial crests, where the electron transport chain (ETC) is located. ETC activity generates an electrochemical gradient of protons ($\Delta\mu H^+$) that, besides driving the flow of H^+ through the ATP synthase, allows the accumulation of Ca^{2+} in the mitochondrial matrix (Rimessi et al., 2008; Rizzuto et al., 2012).

Mitochondria have almost no Ca^{2+} at rest but, during cell stimulation, they may capture large amounts of Ca^{2+} from the cytosol. When the stimulation stops, they release the accumulated Ca^{2+} to recover the resting levels (de la Fuente et al., 2012). The accumulation of Ca^{2+} into the mitochondria regulates intrinsic functions in the organelle. The main function of mitochondria is ATP production, by a process known as oxidative phosphorylation. In the matrix, Ca^{2+} regulates the activity of ATP synthase F_1F_O and three dehydrogenases of the Krebs cycle, increasing the availability of NADH, and therefore, the flow of electrons in the respiratory chain, which adjusts the synthesis of ATP to cellular requirements (McCormack and Denton, 1990; Hansford, 1994). On the other hand, the increase in $[Ca^{2+}]_M$ along with the accumulation of ROS may trigger the release of mitochondrial factors to the cytosol, such as cytochrome c and apoptosis inducing factor (AIF). Together with the effector caspases, these factors form a macromolecular machinery (apoptosome) that leads the cells to apoptotic cell death. Opening of the permeability transition pore (PTP) may be in the origin of this phenomenon, involving the collapse of the mitochondrial membrane potential ($\Delta\Psi m$), fragmentation and swelling of the organelle, release of the mitochondrial factors and finally cell death (Rizzuto et al., 2012; Patron et al., 2013). In addition, the consequences of mitochondrial calcium ($[Ca^{2+}]_M$) accumulation are not only limited to processes that occur within the organelle. Mitochondria act as a transient buffer of cytosolic Ca^{2+} microdomains by taking up large amounts of Ca^{2+} from these microdomains through MCU channels and releasing it elsewhere through the exchangers. In this way, mitochondria may control phenomena activated by high Ca^{2+} microdomains, such as neurotransmitter secretion (Montero et al., 2000), or prevent the inactivation of channels that are inhibited by Ca^{2+} in the inner mouth of the channel (Rizzuto and Pozzan, 2006; Patron et al., 2013).

2.4.1. Calcium transport in the mitochondria

Under physiological conditions, mitochondrial Ca^{2+} uptake takes place through an electrogenic process driven by the electric potential difference (-150 to -180mV) through the IMM, generated by the pumping of H^+ from the respiratory chain and mediated by a Ca^{2+}-selective ion channel, called uniporter (Bernardi, 1999). Using RNA interference studies, the mitochondrial calcium uniporter has been shown to be highly complex. The integral components of this channel have been identified, and include the pore-forming subunit or MCU (Mitochondrial Calcium Uniporter) (Baughman et al., 2011; De Stefani et al., 2011; Figure 10), the MCUb paralogue with dominant negative function (Raffaello et al., 2013) and at least five other regulatory subunits: MICU1 (Perocchi et al., 2010; Csordás et al., 2013), MICU2 and MICU3 (Plovanich et al., 2013; Patron et al., 2014; Kamer and Mootha, 2014), MCUR1 (Mallilankaraman et al., 2012) and EMRE (Sancak et al., 2013).

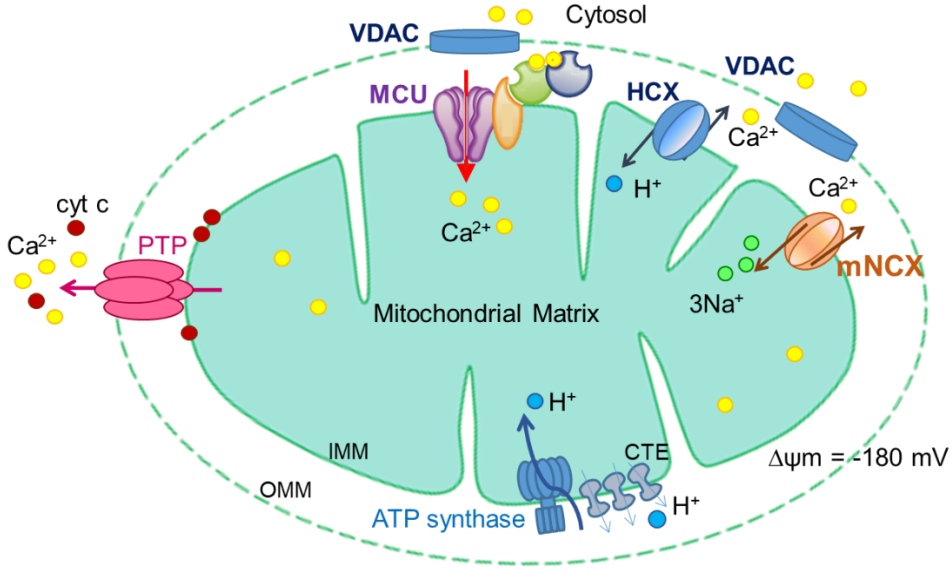


Figure 10. Mitochondrial Ca^{2+} homeostasis. Schematic representation of the channels and transporters involved in the regulation of $[Ca^{2+}]_M$. The voltage-dependent anion channel (VDAC) controls the diffusion of Ca^{2+} through the OMM. The accumulation of Ca^{2+} in the matrix occurs through the uniporter (MCU), while the exit is mediated by the H^+/Ca^{2+} (HCX) and Na^+/Ca^{2+} (mNCX) exchangers. The permeability transition pore (PTP) allows the release of Ca^{2+}, but its prolonged opening leads to cell death. OMM (Outer Mitochondrial Membrane), IMM (Inner Mitochondrial Membrane), cyt c (cytochrome c), ETC (Electron Transport Chain).

On the other hand, Ca^{2+} accumulation in the mitochondria is counteracted by the mitochondrial H^+/Ca^{2+} (HCX) and Na^+/Ca^{2+} (mNCX) exchangers (Rimessi et al., 2008; Jiang et al., 2009; Palty et al., 2010; Csordás et al., 2012; Nowikovsky et al., 2012; Figure 10).

2.4.2. Mitochondria-Associated Membranes (MAMs)

A tight interaction is established between the endoplasmic reticulum (ER) and the mitochondria of all eukaryotic cells, known as Mitochondria-Associated ER Membranes (MAMs) or Mitochondria-ER Contacts (MERCs) that play an important role in cells in physiological as well as in pathological conditions (van Vliet et al., 2014; Janikiewicz et al., 2018; Figure 11).

The scaffolding function of MAMs regulates lipid synthesis and trafficking, and the transmission of Ca^{2+} signals between the ER and the mitochondria, which is essential for intracellular Ca^{2+} homeostasis, for energy metabolism and to maintain cellular health (van Vliet et al., 2014). It has also been experimentally confirmed that it participates in processes such as the production of ROS, fundamental for the modulation of ER stress, autophagy and inflammasome signalling.

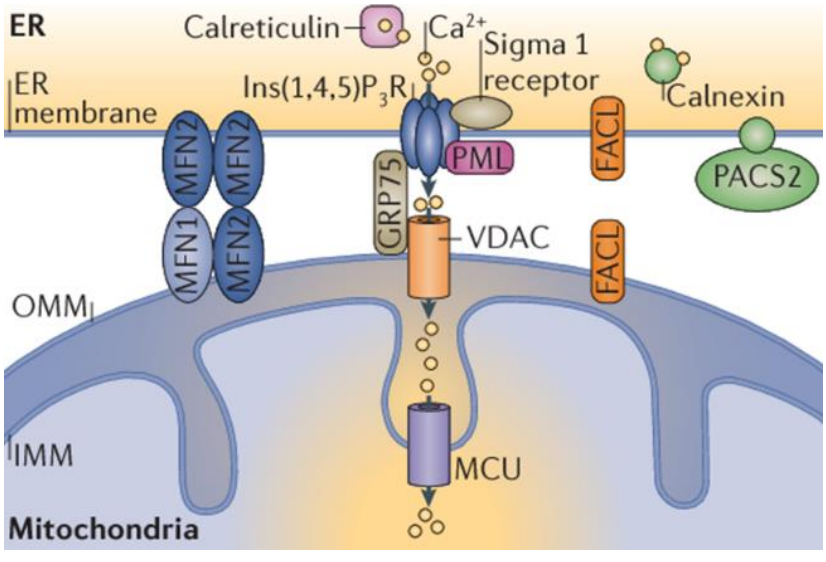


Figure 11. The ER–mitochondria junctions. Diagram representing the main proteins associated with MAMs that are essential for Ca^{2+} uptake by mitochondria (Rizzuto et al., 2012).

MAMs are considered a dynamic structure since more than one thousand proteins associated with MAMs have been discovered. Among them, we can mention BiP/GRP78, calnexin, calreticulin, ERP44, MFN1, PDIA3, VDAC1, VDAC2, and VDAC3 (Zhang et al., 2011; Poston et al., 2013). Alterations in the ER-mitochondria architecture are related to pathological conditions and human diseases (Missiroli et al., 2018), including neurodegenerative or inflammatory diseases, type 2 diabetes mellitus and some authors even associate MAMs with aging or senescence (Gonzalez-Freire et al., 2015; Bernhardt et al., 2015; Veitia, 2017).

III. *CAENORHABDITIS ELEGANS*

Caenorhabditis elegans is a tiny, transparent, free-living and non-parasitic round organism belonging to the nematode phylum that lives on the soil and can be found worldwide (Andersen et al., 2012). It reaches an adult length of about 1 mm (Riddle et al., 1997) and 80 µm in diameter and feeds mainly on bacteria, although it can survive in periods when food is scarce. *C. elegans* was the first multicellular organism where the genome (97 Mb) was completely sequenced (*C. elegans* Sequencing Consortium, 1998) and today it has provided a large variety of tools to help new discoveries, such as WormBase, WormBook and WormAtlas.

This nematode has become an important model organism for studies in molecular genetics, developmental biology, cell biology, and neuroscience, among other research areas since Sydney Brenner decided to adopt this species as a genetic model in the mid-1960s (Brenner S, 1974). Thanks to this contribution Brenner was awarded in 2002 the Nobel Prize along with John Sulston and Robert Horvitz. In addition, a second Nobel Prize related to this worm was awarded to Craig Mello and Andrew Fire for describing the mechanism of RNA interference, and a third, to Martin Chalfie along with Roger Y. Tsien and Osamu Shimomura for the discovery and development of the green fluorescent protein (GFP).

1. *C. elegans* as a suitable experimental model for laboratory

C. elegans has several characteristics that identify it as the best candidate to comply with laboratory conditions. These features are the following.

First, because of its small size, *C. elegans* has a relatively inexpensive growing cost. C elegans can be brought up and maintained easily in the laboratory on cholesterol-supplemented agar Petri dishes, with Escherichia coli bacteria as a food source, what allows to handle large quantities of animals at the same time over several generations (Strange, 2006). It is also transparent throughout its life, facilitating the observation under the microscope and the use of fluorescent proteins to study different biological processes, isolate cells or characterize protein interaction *in vivo* (Chalfie et al., 1994; Boulin et al., 2006; Feinberg et al., 2008).

Second, its short lifespan (18-23 days) and its short embryonic development (about three days in optimal laboratory conditions) (Luo et al., 2009) make *C. elegans* an organism with a large storage capacity. It can remain at rest for long periods of time thanks to the natural ability of dauer larvae to live for several months, feature that also offers a convenient option for short-term storage. Besides, it is extremely useful as

species for genotype storage and experimental design because *C. elegans* survives freezing, which also allows its long-term storage at -80°C or in liquid nitrogen.

Third, its breeding system is ideal for genetic studies. *C. elegans* has two sexes: hermaphrodite and male. An adult hermaphrodite produces near 200-300 progeny in a few days by self-fertilization. By isolating the hermaphrodites, it is possible to get pure genetic crosses or create new genetic material by crossing the males. In addition, the high number of offspring makes it a high performance model with short-term results. Besides, its eggs are highly resistant to many environmental stresses, which allows to bleach the worms in order to synchronize the population and eliminate microbial contaminants, since only eggs will survive bleaching.

Finally, another feature that has made *C. elegans* a very useful model in the area of genetic research is its easy manipulation. The function of specific genes can be interrupted using interfering RNA (RNAi) (Grishok, 2013) and thus silencing their expression allows determining its functions and interactions (Kamath et al., 2003; Zhuang and Hunter, 2011).

2. *C. elegans* life cycle

C. elegans has a short lifespan, with a 19 days-half-life when kept at 20°C. Two alternative life cycles have been described in the laboratory, depending on environmental conditions (temperature, food availability). When the animals have free access to food, the embryogenesis lasts approximately 10h and the newly hatched eggs go through four larval developmental stages of different duration (L1, L2, L3, L4), reaching the adult stage in around 3 days (Félix and Braendle, 2010) (Figure 12).

During larval development, different processes of cell division and apoptosis occur, as well as a size increase. The transition from one stage to another involves a change of cuticle (molt), previously undergoing an inactivity period that is known as lethargy (Raizen et al., 2008). The young adult hermaphrodites begin to lay eggs approximately 8 hours after the molting of L4 (approximately 200-300) with a maximum period of activity of 2 to 3 days, until the spermatheca is exhausted.

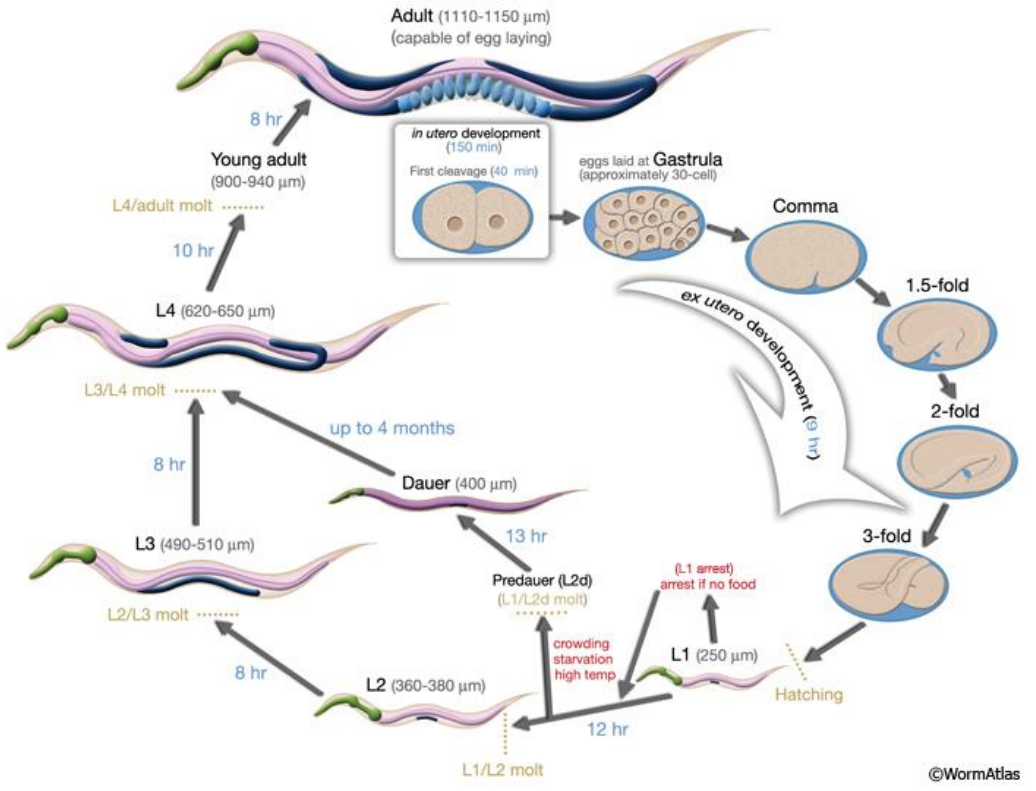


Figure 12. Life cycle of *C. elegans*. Diagram including the duration of each stage of the nematode development (Altun and Hall, 2009).

Under stressful conditions, the organisms can stop development during stage L1 and enter an alternative larval stage or predauer stage (L2d), followed by a stage of diapause without food called dauer. Dauer larvae are resistant to several kinds of stress and can survive for some months without food since during "dauer" stage, the cuticle completely surrounds the larva, including the mouth, so preventing it from eating and continuing its development, which, in turn, leads to a change in the metabolism (Barrière and Félix, 2007; Hu, 2007; Figure 12). When the conditions become favorable, these "dauer" larvae feed again and resume the development towards L4. It is particularly interesting to point out that genes that regulate the entry to dauer phase are highly related to life and aging (Larsen et al., 1995).

3. *C. elegans* anatomy

The relative simplicity of *C elegans*, consisting of 959 cells and 302 neurons in an adult hermaphrodite nematode, and the knowledge of its biology has allowed the characterization of how each cell lineage is generated throughout the development (Sulston et al., 1983; Kipreos, 2005). This reduced number of cells remains unchanged all adult life through. *C. elegans* has a non-segmented cylindrical body that tapers at

the ends. Despite its small size, the anatomy of *C elegans* has well-defined tissues that can be grouped into two concentric tubes separated by the pseudocoelomic space, full of fluid that exerts an internal hydrostatic pressure, regulated by an osmoregulation system, which helps maintaining the shape (Riddle et al., 1997; Figure 13).

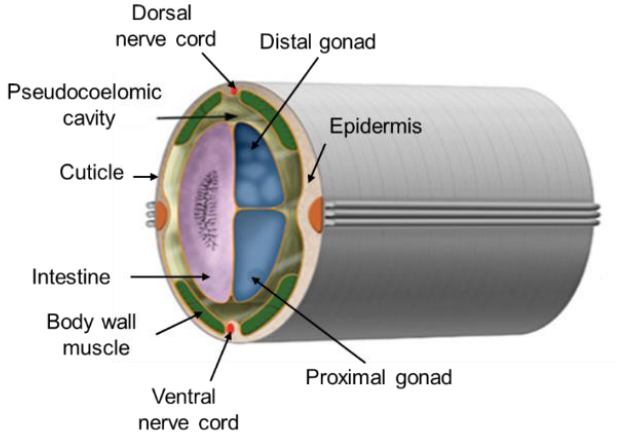


Figure 13. Nematode posterior body region. Cross section of the posterior body region of the adult nematode. The body wall (outer tube) is separated from the inner tube (alimentary and reproductive system) by a pseudocoelomic cavity (Modified from ©WormAtlas, 2002-2012).

The outer tube comprises the **body wall**, including the cuticle, the epidermis, and the excretory, nervous and muscular systems.

The outer epithelial layer, the **epidermis** is the outermost tissue in which cell fusions take place during embryogenesis to form pluri-nucleated cells, forming a long epidermal syncytium. These cells secrete the cuticle, a specialized protective layer of extracellular matrix (ECM) that consists mainly of collagen, lipids and glycoproteins (Chisholm and Hardin, 2005; Page and Johnstone, 2007). The **cuticle** is, therefore, a flexible and durable outer shell whose function is to protect animals from any environmental risk as well as maintaining the shape of the animal's body and allow motility acting as an external skeleton through connections or anchor points between the epidermis and the muscle, so allowing muscle contraction (©WormAtlas, 2002-2012).

The **nervous system** consists of 302 cells in an adult hermaphrodite, 282 neurons form the somatic nervous system and another 20 make up the pharyngeal nervous system. Most neuronal bodies are arranged in small ganglia in the head, ventral cord and tail (Sulston and Horvitz, 1977). In addition to neurons, *C. elegans* has several glia-like support cells, which are primarily associated with sensory neurons (Oikonomou and Shaham, 2011). Regarding nerve conduction, it is still unknown whether it takes place through graduated potentials or passive conduction, because

C. elegans cannot generate typical action potentials since it lacks voltage-gated Na^+ channels (Lockery et al., 2009). But it is known that neurons express voltage-gated potassium and calcium channels (Hobert, 2013) and, in fact, Ca^{2+} action potentials have been described in body wall muscle (Gao and Zhen, 2011). Likewise, neurotransmitters such as acetylcholine, glutamate, γ-aminobutyric acid (GABA), dopamine and serotonin have been identified and receptors have been found for them (Hobert, 2013).

The **muscular system,** consisting of 95 cells, is divided into 4 quadrants throughout its body, and is located just below the epidermis where they have an intimate connection. These cells are somatic cells obliquely striated and mononuclear with multiple sarcomeres per cell (Moerman and Fire, 1997), whose contraction and relaxation produces the sinusoidal movement of the nematode. In addition to the body wall muscle, *C. elegans* has muscle cells that control food intake (pharyngeal muscle), egg-laying (the vulva and uterus muscles), mating (tail specific muscle of the male) and excretion (enteric muscles). It is important to point out that muscle cells send extensions ("muscular arms") to the ventral and dorsal medulla to receive the synapses of motor neurons, instead of receiving axonal projections of these neurons (White et al., 1986).

Finally, the **excretory system** formed basically by four cells located on the ventral side of the posterior head, opens to the outside through the excretory pore and participates fundamentally in osmoregulation and waste disposal.

On the other hand, the inner tube, or internal system, comprises the digestive and reproductive systems. The pseudocoelomic cavity also contains three pairs of coelomocytes or scavenger cells that make up **the coelomocyte system** and endocytose the fluid from the pseudocoelom, which possibly acts a primitive immune system in *C. elegans*.

C. elegans has two sexual forms, hermaphrodite (XX) and male (X0) that shares the same autosomes but lacks a sex chromosome (Herman, 2005). It can reproduce either sexually by breeding hermaphrodites with males or by self-fertilization of hermaphrodites. In nature, the percentage of males, which appear spontaneously by self-fertilization, is very low, approximately 1 %, due to the loss of X chromosome (Riddle et al., 1997). The hermaphrodites mated with males produce a uniform sex ratio. However, under environmental stress situations, such as an increase in temperature, the proportion of males increases. In fact, in the laboratory, the number of males can be increased by raising the temperature at the beginning of sexual maturity (Nicholas, 1975).

The **reproductive system** of the hermaphrodite consists of the gonad, the germ line and the vulva (Figure 14). The hermaphrodite has reproductive organs that produce gametes associated with male and female sexes, and therefore the gonad contains oocytes and spermatheca (Hubbard and Greenstein, 2005).

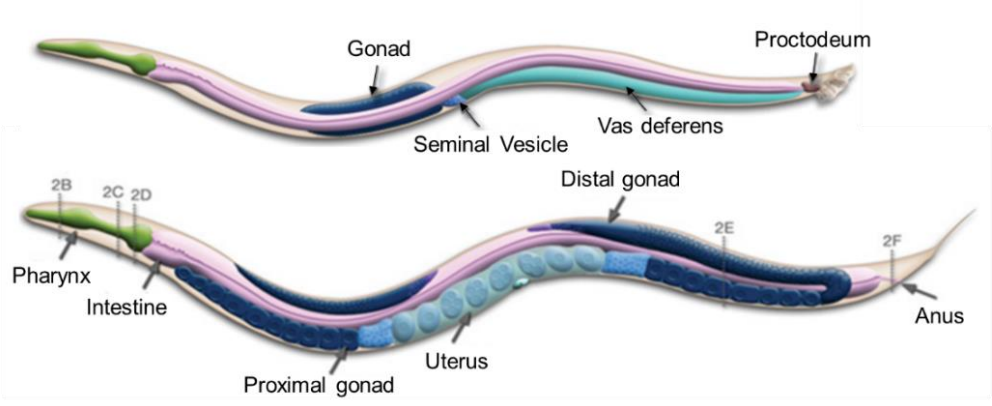


Figure 14. *C. elegans* adult, male and hermaphrodite. Schematic drawing of an adult male (above) and another hermaphrodite (below) showing the main and different anatomical structures (Modified from ©WormAtlas, http://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introfig5leg.htm).

The hermaphroditic germline produces the male gametes in stage L4 (150 sperm per gonadal arm) and the female ones become oocytes later. The sperm cells are stored in the same area of the gonad as the oocytes until the first oocyte pushes the sperm to the spermathecal and along the oviduct, the path to the uterus where the fertilization and eggs development take place. The fertilized eggs are released through the vulva after internal fertilization. There is also the possibility that males fertilize hermaphrodites, which increases the number of eggs laid (Nayak et al., 2005).

Anatomically both sexes are very similar, but they differ in the sexual structures. Hermaphrodite has the vulva in the posterior third, while the males are smaller, thinner and only produce sperm (Herman, 2006). From the L2 stage the male develops a brush-shaped tuft at the end of the tail, through which he will introduce the sperm into the hermaphrodite´s vulva (Figure 15; ©WormAtlas).

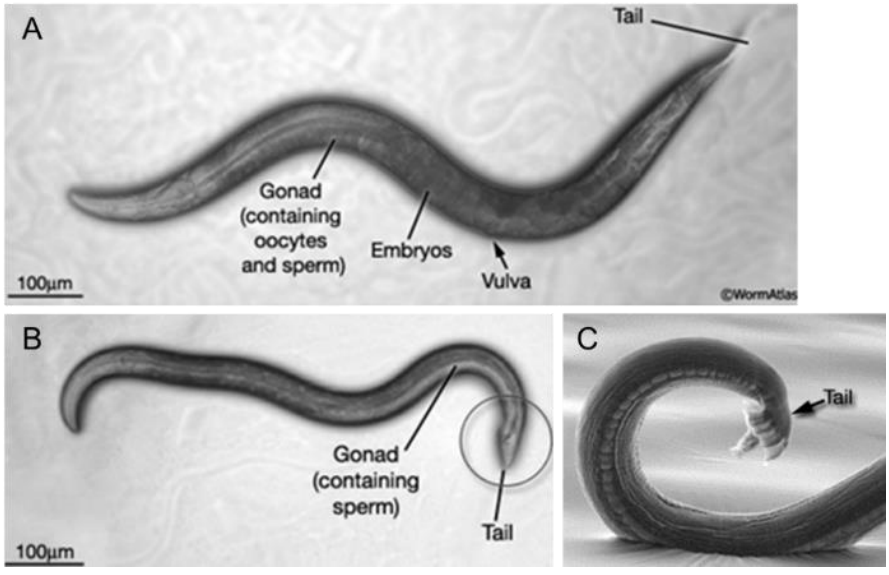


Figure 15. Hermaphrodite and male anatomy. A. Light microscopy image of the hermaphrodite. B. Light microscopy image of the male. C. Magnified view of the tail male region by Nomarski DIC (Modified from ©WormAtlas, http://www.wormatlas.org/male/introduction/Introframeset.html).

The **digestive system** composed of the pharynx, intestine, rectum and anus, is the main organ of *C. elegans*, whose function is to ingest, digest and excrete nutrients (Figure 16).

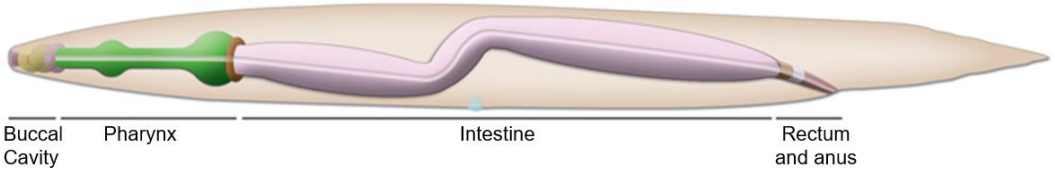


Figure 16. The digestive system in *C. elegans*. Schematic drawing of the alimentary system of *C. elegans,* consisting of the buccal cavity, pharynx (green), intestine and rectum (©WormAtlas, http://www.wormatlas.org/hermaphrodite/alimentary/Alimframeset.html).

C. elegans feeds on bacteria in liquid suspension that are absorbed by the mouth and then concentrated, milled and transported to the intestine by a neuromuscular pump, called “pharynx”. The anterior pharynx is connected to the mouth, while the posterior one connects to the intestine, the latter being composed of 20 elongated polyploid epithelial cells arranged in pairs forming a tube along the animal and ending in the anus. The pharynx sucks and crushes the food before it passes into the intestine where it is digested (McGhee, 2007; Figure 16).

4. *C. elegans* as aging model

The biological aging and the molecular bases underlying this phenomenon have been studied deeply during the last decades thanks to the availability of some model organisms such as the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse *Mus musculus* (Bitto et al., 2015). However, of these four models, the worm presents a series of characteristics over the other models, making it an ideal model for longevity studies and for the pharmacological investigation of aging and age-related phenotypes as well as the characterization of new drug targets.

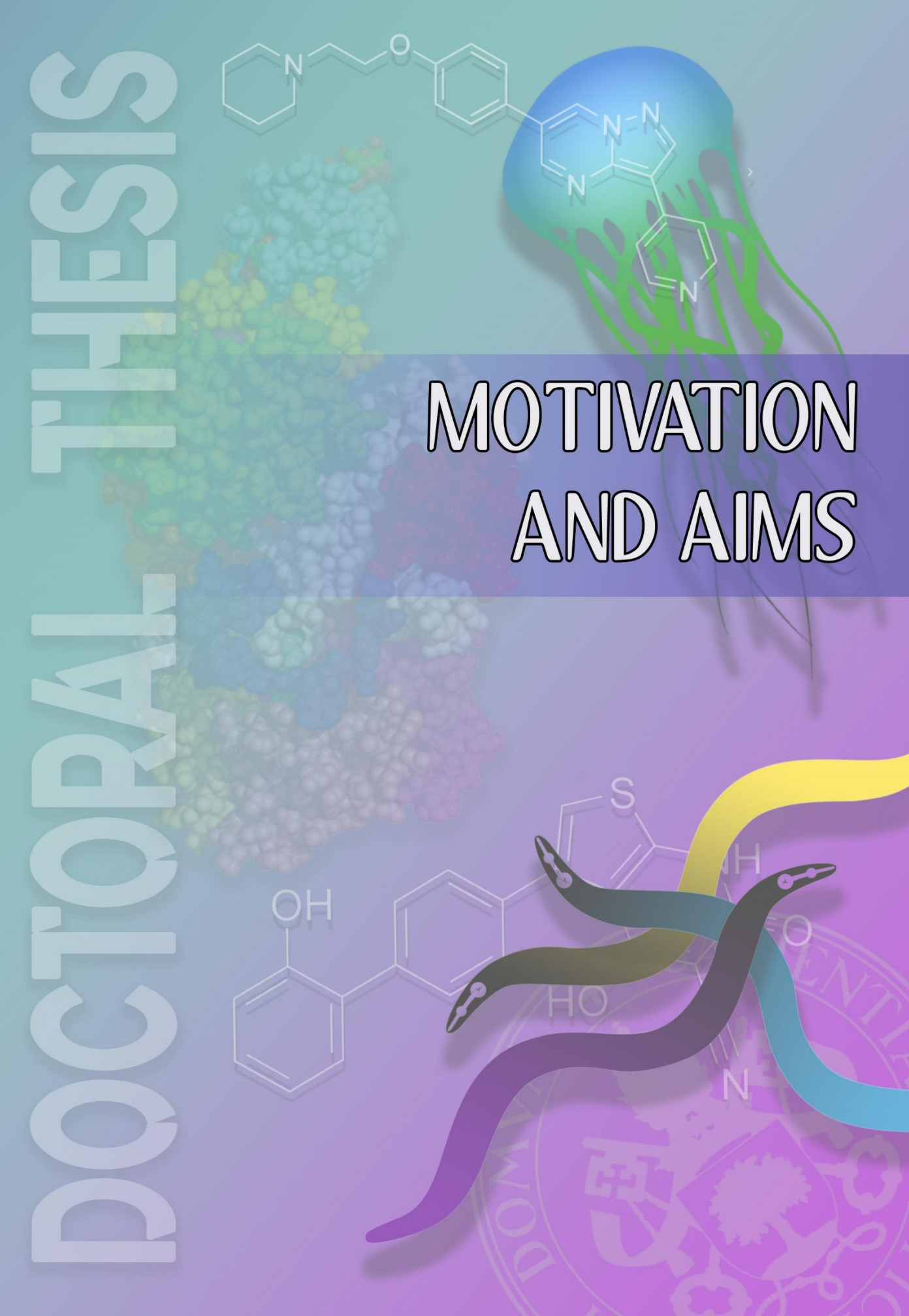
C. elegans has been specifically used in human aging research because there are several evidences of parallel phenomena during aging in nematodes and humans, for example the accumulation of pigments with age (Collins et al., 2008), or the identification of several biochemical pathways that have been evolutionarily conserved and are related to aging. In fact, this nematode has approximately 60-80 % homologous genes with humans. This has already allowed to study functions of orthologous genes related to aging and disease, to test new drugs and obtain more information about their mechanism of action (López-Bigas and Ouzounis, 2004; Luo et al., 2009).

Some of these pathways have been previously cited in this work, as the ones related with nutrient sensitive signalling cascades or mitochondrial signalling. There are already numerous mutants of these genes with prolonged life expectancy (Antebi, 2007). However, mutations in some components of these pathways sometimes not only prolonged life expectancy under laboratory conditions, but could also cause other harmful or unexpected effects, because if these signalling pathways have other functions in the organism its effect in aging could only be a by-product.

In addition to the above, several model strains of various human diseases related to aging are also available. Besides helping in the search for new drugs, these model strains also allow the study of their metabolism and mechanisms of action in a complex eukaryotic organism (Hashmi et al., 2013). A variety of anti-aging compounds have already been tested in *C. elegans*, such as plant extracts (Ibe et al*.,* 2013), polyphenols (Wood et al., 2004), vitamins, antibiotics and many others (Petrascheck et al., 2009).

Finally, we should mention that some compounds as resveratrol or metformin, which prolong the life of the worm (Wilson et al., 2006), have already undergone clinical trials where their effects are being tested in several diseases associated with aging such as Alzheimer's disease, diabetes or cancer.

DOCTORAL THESIS



MOTIVATION AND AIMS

Healthy aging in human beings is one of the key objectives of modern science because the risk of developing disease increases drastically with age. In this way, scientific studies on aging are intended to find ways to delay this process, and also to make it healthier by delaying the onset of diseases associated with old age.

Although there is as yet no clear consensus on the molecular mechanisms of aging, new and well-founded hypotheses have emerged in recent years pointing out to the key role played by nutrient-sensitive signalling pathways: the insulin/IGF-1 pathway, the AMP kinase (AMPK) pathway, the mTOR kinase pathway and the sirtuins one. These pathways have complex interactions among them at several levels, being one of them Ca^{2+} signalling, a key messenger in cellular activation and metabolism.

AMPK is a key energy homeostasis regulator that coordinates metabolic pathways by balancing the supply of nutrients with energy demand. The concepts of energy balance and nutrient-sensitive pathways are considered nowadays key determinants of lifespan. As Ca^{2+} signalling is tightly related to both of them, the main goal of this thesis is to investigate the relationship between the activity of the AMPK signalling pathway, intracellular Ca^{2+} signalling and aging or longevity. To address this general goal, this thesis is structured on two fundamental axes:

Aim 1. Study of the effects of AMPK pathway modulators on Ca^{2+} signalling in HeLa cells.

To achieve this objective, the following studies have been carried out in the presence or absence of the AMPK activator A769662 or the AMPK inhibitor dorsomorphin.

- $[Ca^{2+}]_C$ and $[Ca^{2+}]_M$ measurements using cytosolic aequorin or mitochondrially targeted mutated aequorin in HeLa cells stimulated with histamine.

- $[Ca^{2+}]_{ER}$ measurements using ER-targeted double mutated aequorin, in orden to monitor histamine (IP_3)-induced Ca^{2+} release.

- Changes in the protein phosphorylation status generated by the treatment with AMPK modulators using mass spectrometry.

Aim 2. Study of the effects of AMPK pathway modulators on *C. elegans* longevity.

- Survival assays made on wild-type worms to study if AMPK pathway-modulating compounds, such as those mentioned above, modify their longevity.

- Survival assays in several *C. elegans* mutants that have greater or lesser longevity due to affectation of some of these pathways, such as:

    - *aak-2* (AMPK pathway mutant model)
    - *daf-2* (IGF-1 pathway mutant model)
    - *eat-2* (caloric restriction model)
    - *nuo-6* (mitochondrial dysfunction model)

DOCTORAL THESIS

METHODS AND MATERIALS

1. CELL CULTURE

1.1. Cell line

In this work, the immortal HeLa cell line, available in the laboratory, was used to perform different experiments, both calcium concentration measurements in cell populations and phospho-proteomic experiments.

This cell line comes originally from a sample of human cervical cancer, belonging to the patient Henrietta Lacks, hence its acronym. In the 1950s, George Gey managed to maintain successfully these human tumour cells under *in vitro* culture, and ever since, this line has been widely used in laboratories all around the world, as it has proved to be particularly effective in both growth rate and in temporal durability. These cells are able to proliferate in an unlimited and rapid way due to the activation of telomerase during cell division. Gene horizontal transference of the human papillomavirus (HPV 18) has also transformed the karyotype of this cell line, which is very different from the usual human karyotype.

However, this line is widely used as a study model, so its behaviour and management is well known (Macville et al., 1999). In particular, they have been widely used in the last 30 years by many groups working in Ca^{2+} signalling. For example, the first [Ca^{2+}] measurements obtained inside mitochondria, nucleus or endoplasmic reticulum were obtained in HeLa cells stimulated with histamine (Rizzuto R et al., 1992; Brini et al., 1993; Montero et al., 1997). Regarding the stimulus, HeLa cells express endogenous histamine H1 G-protein coupled receptors that elicit a very strong response of IP_3 production followed by IP_3R activation and Ca^{2+} release from the ER. In fact, most of the studies of Ca^{2+} signalling made in HeLa cells in the last 30 years use histamine to trigger IP_3 production and Ca^{2+} release from the ER.

1.2. Cell maintenance

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM GlutaMAX I) supplemented with 5 % fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin (all materials from Gibco), creating adherent monolayers on culture flasks inside an incubator. These culture flasks have a surface area of 25 cm^2 and a 0.2 µm pore size filter cover (Nunc). The *Heracell* (Heraeus) incubator maintains a constant temperature at 37 ºC and a 10 % CO_2 saturation.

To maintain the cultures, cells were trypsinized when the monolayers reached confluence. To do so, the culture medium was removed and the monolayers were washed with phosphate-buffered saline (PBS). Next, cells were incubated in 1 ml of a

0.05 % trypsin-EDTA solution and when they were detached from the culture flask surface, 6ml of DMEM medium were added to inactivate the trypsin. Then, cells were centrifuged and the cell pellet was re-suspended in 1 ml of medium.

A small aliquot was used to count the total number of cells using a *Neubauer* chamber and the vital blue dye trypan. With this value, the necessary volume of freshly re-suspended cells was taken and about 100,000 cells were left grown on the flask, so that the culture was kept under optimal conditions. Finally, 5 ml of culture medium were added and the flask was placed in the incubator to continue the culture.

Culture cells with more than 10 passes were discarded.

1.3. Cell seeding

To carry out experiments in the measuring equipment (aequorinometer, a customized luminometer), HeLa cells were cultured on round coverslips of 13 mm diameter, previously sterilized. These coverslips were placed in the bottom of each one of the wells of 24-well plates before seeding. When the cells were confluent in the flask, they were trypsinized and counted. Then, cells were resuspended in DMEM and seeded at a final concentration of 100,000 cells per well in the 24-well plates. Plates were then placed in the incubator for at least 24 hours before transfection, under the same conditions as the flasks.

Dorsomorphin was always added to the culture medium 48 hours prior to the experiments. In the case of A769662, it was either added to the culture medium 48 hours prior to the experiments or incubated with the cells for 2 h at 37 ºC just before the experiments. This latest method proved to be more effective to reach the maximum effect.

1.4. Transfection and gene expression

In this work, a chemical method, known as lipofection, was used to perform transfection, which is based on the lipid-cation interaction.

Transfections were carried out using Metafectene Pro. This method consists of mixing the DNA of interest with the transfection reagent. In order to obtain optimal transfection efficiency, the DNA/Metafectene ratio used was of 0.5 µg DNA / 2 µl Metafectene per coverslip, as indicated in the Protocol provided by *Biontex*.

In order to do aequorin experiments, wild type HeLa cells were cultured on sterile 13 mm round coverslips with 10^5 cells/well density. Cells were maintained with

DMEM culture medium (with or without a modulator) for 24 hours to adhere to the coverslips, as indicated in the previous section (at 37 ºC and 10 % of CO_2 saturation). When the cells reached visual confluence, they were transfected with one of the plasmids of the calcium sensitive protein aequorin. The constructs used were: cytosolic native aequorin, mitochondrially targeted mutated aequorin and ER-targeted double mutated aequorin (see point 3.2.).

2. MOLECULAR BIOLOGY

2.1. Plasmids used

In this work, different plasmids containing sequences of genetically encoded Ca^{2+} indicators (GECIs) cloned in the vector pcDNA 3.1 (+) have been used.

Plasmid pcDNA 3.1 is a 5.4 kb vector designed for gene expression in mammalian cells. The vector contains the human Cytomegalovirus promoter (pCMV), providing a high expression level and also the T7 promoter site, which allows *in vitro* transcription, the gene conferring ampicillin resistance, which allows selection in prokaryotic organisms, and a multiple cloning sequence (MCS), that allows the insertion of different sequences, in our case the different aequorins. All the genetic sequences of the different aequorins were cloned in this vector, specifically, by using the EcoRI restriction site (Figure 17).

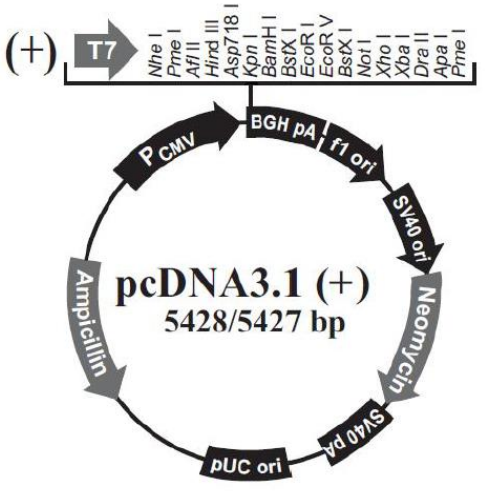


Figure 17: **Diagram of pcDNA3.1 plasmid**. The cleavage sites for restriction enzymes located in the multiple cloning sequence (MCS) are shown.

2.2. Transformation, amplification and purification of aequorin plasmids.

To amplify and subsequently transfect these plasmids in eukaryotic cells, competent bacteria *Escherichia coli* DH5α (Invitrogen) were transformed with such plasmids by thermal shock, as indicated by the manufacturer.

Transformed bacteria were seeded on LB-agar plates supplemented with 125 µg/ml ampicillin and incubated overnight in a stove at 37 °C. Once grown, several isolated bacteria colonies (containing the plasmids of interest) were selected using a sowing handle and grown in 2 ml of LB-broth liquid medium supplemented with 125 µg/ml ampicillin. The bacteria were grown overnight at 37 °C (250 rpm in an Innova 4000 orbital shaker) and the presence of the plasmid of interest was verified by plasmid purification, cut with restriction enzymes and gel electrophoresis. Only those colonies including the plasmids of interest were amplified.

For the amplification, the remaining bacterial culture was transferred to 40 ml of LB-broth fresh medium, again supplemented with 125 µg/ml of ampicillin. The culture was grown for 24 hours under agitation at 37 °C. Extraction of the plasmid DNA contained in these bacteria was carried out using the commercial kit "Quantum Prep® Plasmid Midiprep" (Bio-Rad), following the protocol indicated by the manufacturer. The plasmid DNA obtained was dissolved in sterile deionized water and stored at -20 °C until its use. An aliquot was used to determine the DNA purity and concentration obtained, using Nanodrop (Thermo Scientific).

For long-term preservation of the bacteria, 500 µl of the same bacteria culture were taken and mixed with another 500 µl of bacteria freezing medium (60 % glycerol in sterile H_2O). This mixture was frozen in 2 ml cryotubes at -80 ºC for its preservation and subsequent use.

3. [Ca^{2+}] MEASUREMENTS IN CELL POPULATIONS WITH THE PHOTOPROTEIN AEQUORIN

The [Ca^{2+}] measurements in cell population were performed using the genetically encoded Ca^{2+} indicator (GECI) aequorin (AEQ).

3.1. The probe

Aequorin is a calcium-sensitive photoprotein isolated from the hydrozoan *Aequorea Victoria* (Shimomura et al., 1962) that has the property of emitting photons in the blue zone of the spectrum in the presence of Ca^{2+}.

At the structural level, aequorin is a holoprotein consisting of an apoprotein called apo-aequorin which has 189 aminoacids, with an approximate molecular weight of 21 kD. Its prosthetic group, coelenterazine, is an imidazopyrazine compound with a molecular weight of 423 Da (Kendall and Badminton, 1998). Aequorin contains four binding sites for Ca^{2+} ions, but only three of them are functional (Charbonneau et al., 1985; Shimomura, 1995). These helix-alpha-helix domains, called EF-hands motifs, are capable of interacting with Ca^{2+} ions (Inouye et al., 1985; Vysotski and Lee, 2004) (Figure 18).

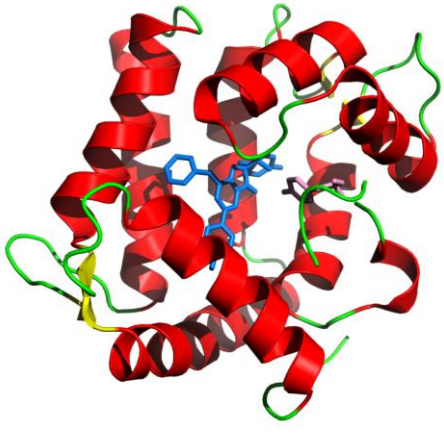


Figure 18. Aequorin structural representation, highlighting the three EF-Hand functional domains (helix-loop-helix motif) in red and its prosthetic group, celenterazine, in blue.

Purification of aequorin from the natural source allowed carrying out the first experiments to monitor the physiological calcium changes in the cytosol of several cell types. For that, the aequorin protein was injected into live animal tissues, such as the muscle fibers of the giant barnacle (balanus nubilus) or giant squid axon (loligo forbesi) (Ridgway and Ashley, 1967; Baker et al., 1971). Subsequently, the cDNA coding for

aequorin was cloned in 1985 (Inouye et al., 1985; Prasher et al., 1985), and this allowed expressing it in many model biological systems, including zebrafish, rats, mice (Sheu et al., 1993) and different cell types. In addition to that, it allowed developing recombinant aequorins specifically targeted to different organelles of interest for calcium homeostasis study. Such organelles are the mitochondria (Rizzuto et al., 1992), the nucleus (Brini et al., 1993) the endoplasmic reticulum (Montero et al., 1995) or the cytosol (Brini et al., 1995).

Cultured cells expressing the aequorin gene can only synthesize apoaequorin. Therefore it is necessary to add coelenterazine, the prosthetic group of aequorin, to the culture medium to obtain a functional protein. Coelenterazine is a hydrophobic molecule easily absorbed through the plasma membrane of higher eukaryotes and can bind to aequorin stably, making this probe suitable as Ca^{2+} reporter in mammalian cells (Montero et al., 1995). The process of non-covalent interaction between the apo-protein and the coelenterazine is known as “reconstitution”. The bioluminescence reaction requires, in addition, oxygen. Then, in the presence of Ca^{2+}, the protein undergoes a conformational change and through oxidation, transforms its prosthetic group, coelenterazine, into coelenteramide and CO_2 (Figure 19). When the coelenteramide goes back to the basal state, it emits blue light (465 nm) (Shimomura et al., 1990, Inouye and Sasaki, 2006; Figure 20).

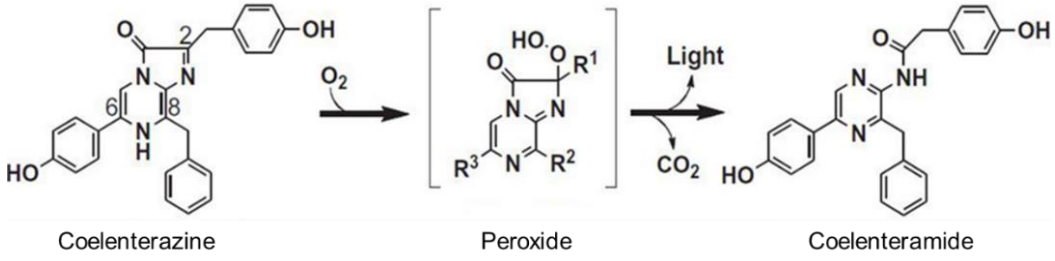


Figure 19. Representation of the reaction carried out by celenterazine after oxidative decarboxylation

The amount of light photons emitted will be proportional to the amount of free Ca^{2+} in the medium, so for each [Ca^{2+}], a certain fraction of aequorin molecules emits their photon. Given that aequorin reconstitution is quite slow, each aequorin molecule can only emit one photon during a typical experiment. Thus, photon emission by aequorin is generally considered irreversible during experiments, and this implies that the probe (aequorin) is progressively consumed during each experiment.

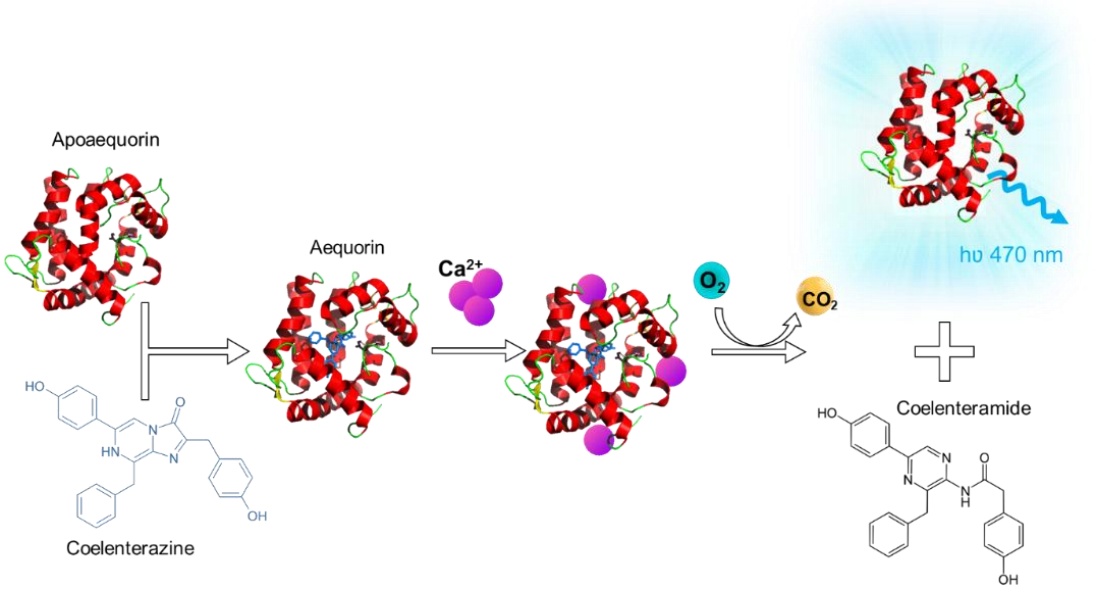


Figure 20. Bioluminescence reaction catalyzed by aequorin. After reconstitution in the presence of Ca^{2+} ions and O_2, the reaction takes place by an oxidative decarboxylation of the coelenterazine. When Ca^{2+} binds to aequorin, the coelenterazine is oxidized and at the end of the reaction, the oxidized prosthetic group, coelenteramide and CO_2 are produced and a blue light photon (470 nm) is emitted.

3.2. Aequorin constructs

In this work, aequorins targeted to cytosol, mitochondria and endoplasmic reticulum were used, all of them inserted in the pcDNA 3.1 plasmid.

All the constructs used, except that of cytosol, have a targeting sequence that sends them to the organelle where the Ca^{2+} measurements are to be made. To direct aequorin to mitochondria the targeting sequence is a portion of the cDNA coding for subunit VIII of human cytochrome c oxidase (COX) (Rizzuto et al., 1992). To target the protein to the sarco-endoplasmic reticulum, a portion of murine immunoglobulin heavy-chain was cloned. Specifically, the L sequence targets the protein to the endoplasmic reticulum and the CH1 domain binds it to the BiP chaperone, retaining the protein in the sarco-endoplasmic reticulum (Montero et al., 1995) (Figure 21).

Moreover, prior to the aequorin sequence, nine amino acids of the HA1 epitope derived from hemagglutinin were introduced. This antibody recognition domain allows detecting the protein localization by immunocytochemistry (Brini et al., 1995).

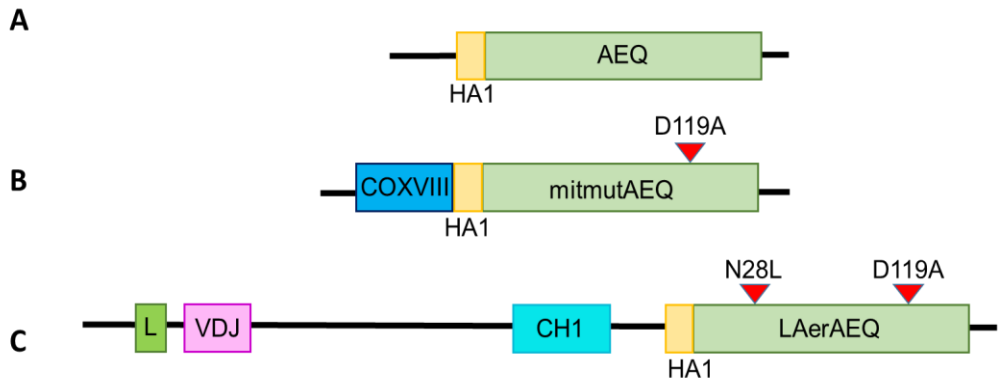


Figure 21. Different aequorin inserts diagrams used. A. Aequorin targeted to the cytosol (Brini et al., 1995). B. Mutated aequorin targeted to the mitochondria (Rizzuto et al., 1992). C. Double-mutated aequorin targeted to the endoplasmic reticulum (Montero et al., 2000; de la Fuente et al., 2013).

In addition, the calcium affinity of the protein was also modified to measure higher concentration ranges. The native aequorin (AEQ wt) has a high affinity for Ca^{2+} and only measures [Ca^{2+}] reliably between 0.1 and 5 μM. At higher [Ca^{2+}], aequorin would be consumed in a few seconds and the measurements would be erroneous.

To avoid this consumption problem and measure [Ca^{2+}] in the endoplasmic reticulum or in the mitochondria, it is necessary to decrease the aequorin affinity for Ca^{2+}. To do so, a punctual mutation was introduced in the second calcium binding domain, in position 119, changing aspartate by alanine (119-Asp/Ala). This new aequorin, called mutAEQ, reduced the protein affinity by an order of magnitude, so that it was able to do calcium measurements up to 100 μM (Kendall et al., 1992). To further reduce the affinity, another punctual mutation was introduced, in this case at position 28 in the first calcium binding domain, replacing an asparagine residue with a leucine one (28-Asn/Leu). The doubly mutated aequorin (LAAEQ) has an affinity 10 times lower than that with only one mutation (de la Fuente et al., 2013), and allows measuring [Ca^{2+}] up to near the millimolar range.

Nevertheless, to achieve wider ranges of calcium measurement, the different aequorins can be combined with semi-synthetic coelenterazines (Table 1), which may reduce the affinity for calcium even more. In this work, the native (w) and a semisynthetic coelenterazine (i) were used.

	Calcium concentration ranges	
Aequorin	**Coelenterazine w**	**Coelenterazine i**
AEQ wt	0.1 - 5 µM	-
mutAEQ	2 - 100 µM	-
LAAEQ	20 - 500 µM	100 µM – 1 mM

Table 1. Calcium concentration ranges measured for each of the combinations of aequorin and coelenterazine.

3.3. The equipment

The photons emitted when calcium binds to aequorin were captured by a luminometer built for this purpose. The equipment was specially designed to optimize the capture of such photons and then be able to transform this amount of light into [Ca^{2+}] data.

The luminometer consists of a perfusion chamber of 15 mm in diameter and 2 mm deep, thermostated by a water jacket, and built entirely of black methacrylate to prevent photon loss and light entry from the outside. The base of the perfusion chamber is composed of a reflective aluminum surface that allows all the emitted photons to be directed towards the photomultiplier located in the perfusion chamber upper part. The 13 mm diameter coverslips with HeLa cells attached and expressing the corresponding aequorin were placed in this perfusion chamber sealed at the top using a square 24x24 mm coverslip adhered to it by a silicone grease thin film (Figure 22).

The chamber has an inlet and an outlet hole to perfuse different solutions throughout the experiment. Such solutions were placed in a thermostated bath and the passage of the desired solution was controlled by an electrovalve system. The difference in height between the bath and the perfusion chamber allowed a flow of approximately 1 ml/min. The chamber was thermostated by a water jacket continuously renewed by a pump placed in a bath external to the equipment. In addition, to ensure that the temperature was correct, an "on-line" heater from Harvard Apparatus was placed at the entrance of the different solutions.

The perfusion chamber was located immediately below a high gain photomultiplier EMI 9789a from Electron Tubes, collecting the photons emitted by a 10 mm photocathode, placed just above the perfusion chamber containing the adhered cells. The camera and the photomultiplier were placed inside a refrigerator at

4 ºC, to avoid the electron thermal emission from the photocathode and significantly reduce the basal noise of the sample (Figure 23).

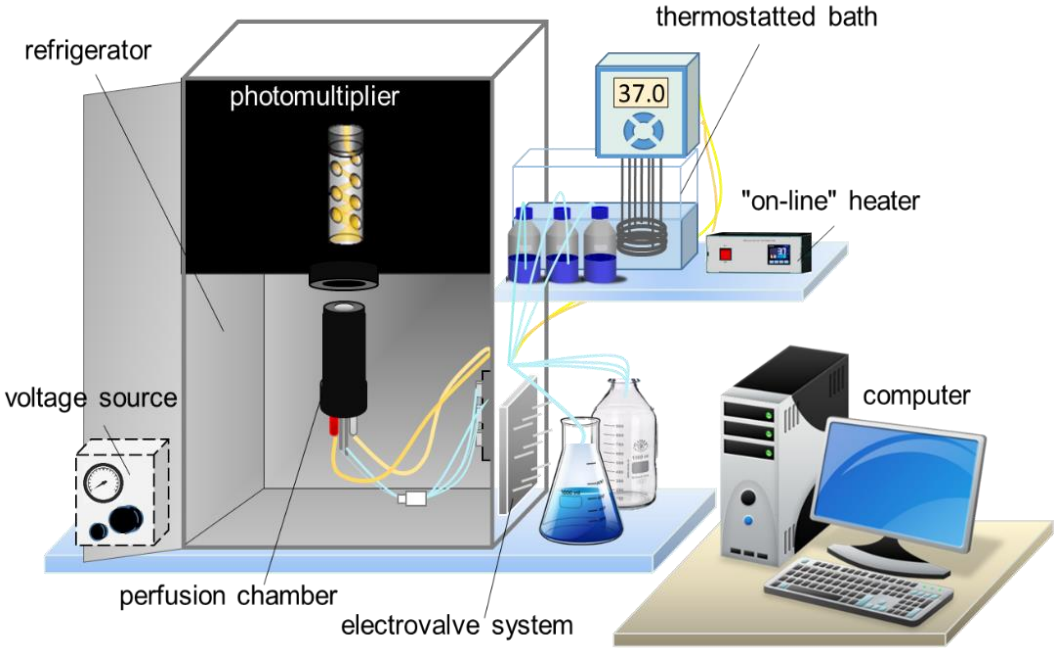


Figure 22. Calcium measurement system by bioluminescence. Diagram of the luminometer used in aequorin experiments, showing each one of the component parts.

The difference in potential between the anode and the cathode of the photomultiplier was generated by a high voltage source, Thorn EMI type and PM28B model. The signal emitted by the photomultiplier was sent through an EMI AD2 discriminator-amplifier to a CT2 Counter Timer from Electron Tubes, from which the data were input to the computer.

The luminescence data were collected every second and displayed on the computer through the Electron Tubes EM6 counter software. These stored data were then transformed into $[Ca^{2+}]$ data using a previously designed calibration program (Álvarez and Montero, 2002).

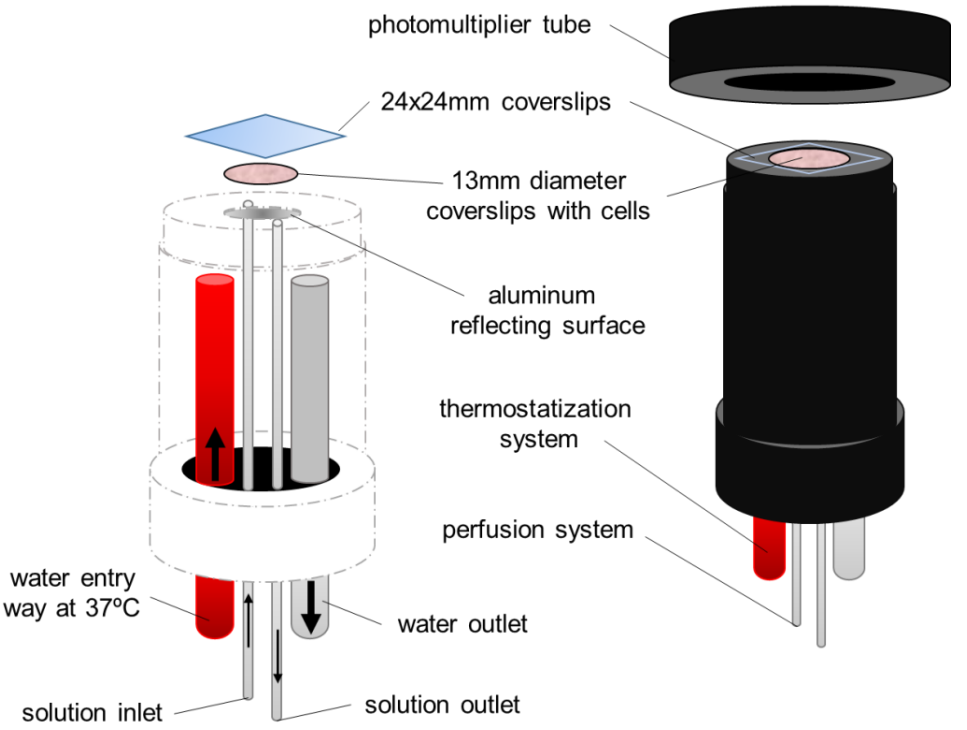


Figure 23. Perfusion chamber of bioluminescence equipment. The diagram shows the main components of the perfusion chamber where the cells are placed.

3.4. Solutions

To perform the [Ca^{2+}] measurement experiments in the different compartments, several mediums were used, depending on the experiment.

For the experiments in intact cells, two media were used. One medium with 1 mM Ca^{2+} and the other without Ca^{2+}, using the EGTA compound as Ca^{2+} chelator. Both media were similar in their composition and osmolarity to the extracellular medium of the cells under physiological conditions (Table 2), so its pH was adjusted to 7.4 with NaOH.

Compound	1 mM Calcium External Media	0.5 mM EGTA External Media
NaCl	145 mM	145 mM
KCl	5 mM	5 mM
$MgCl_2$	1 mM	1 mM
Glucose	10 mM	10 mM
HEPES	10 mM	10 mM
$CaCl_2$	1 mM	-
EGTA	-	0.5 mM

Table 2. External media composition used in intact cells (pH 7.4 NaOH buffered). In the case of intact-cell experiments, basically two external media were used, one with calcium and the other with a calcium chelator, EGTA. These media have a composition that mimics extracellular conditions.

In the case of permeabilized cell experiments, media with a similar composition and osmolarity to the intracellular medium under physiological conditions were used, either calcium free media (in the presence of EGTA) or with known [Ca^{2+}], adjusting the pH to 7 with KOH (Table 3).

To prepare media with a specific [Ca^{2+}], mixes of the 1 mM EGTA internal media with and without calcium were used, so that the proportions of both media resulted in the desired calcium concentration. In this work, we use a medium with a 100 nM calcium concentration that was prepared as follows (Table 4).

Compound	EGTA 0.5 mM Internal media	EGTA 1 mM Internal media	EGTA 1 mM Ca^{2+} 1 mM Internal media
KCl	130 mM	130 mM	130 mM
NaCl	10 mM	10 mM	10 mM
$MgCl_2$	1 mM	1 mM	1 mM
H_2KPO_4	1 mM	1 mM	1 mM
HEPES	20 mM	20 mM	20 mM
Succinate	5 mM	5 mM	5 mM
Malate	5 mM	5 mM	5 mM
Glutamate	5 mM	5 mM	5 mM
ATP K^+	1 mM	1 mM	1 mM
ADP	20 µM	20 µM	20 µM
EGTA	0.5 mM	1 mM	1 mM
$CaCl_2$	-	-	1 mM

Table 3. Internal media composition used in permeabilized cells (pH 7 buffered with KOH). When the experiments were performed in permeabilized cells, media were used recreating the internal physiological conditions of the cells. These media were calcium free prepared, or with known concentrations of the ion and the chelator. The internal medium 0.5 mM EGTA is used as calcium free medium.

$[Ca^{2+}]$	EGTA 1 mM Ca^{2+} 1 mM Internal media	EGTA 1 mM Internal media
100 nM	22.4 %	77.6 %

Table 4. Composition of 100 nM calcium internal medium used in permeabilized cell experiments (pH 7 buffered with KOH). The calcium concentrations indicated in the table correspond to free calcium at 37 ºC and pH 7.

To permeabilize the cells and measure $[Ca^{2+}]$ in the endoplasmic reticulum, an internal medium of 0.5 mM EGTA (without ADP or ATP) supplemented with Digitonin 50 µM, was perfused for 1 minute. This detergent produces pores in the cellular membrane leaving the cellular interior accessible to all the media that are perfused later.

3.5. Experimental determination

To measure [Ca^{2+}], the plasmid containing the corresponding type of targeted aequorin was transfected, as mentioned above. Measurements were carried out 24 hours after transfection.

HeLa cells were plated onto 13 mm round coverslips and transfected with the corresponding plasmid. For aequorin reconstitution, HeLa cells were incubated for 1-2 h at room temperature in standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) with 1 µM of wild-type coelenterazine for $[Ca^{2+}]_C$ and $[Ca^{2+}]_M$ measurements, or 1 µM of coelenterazine i for endoplasmic reticulum calcium concentrations ($[Ca^{2+}]_{ER}$) measurements. After reconstitution, cells were placed in the perfusion chamber of the purpose-built luminometer and perfused with external medium prior to the stimulus.

For the ER [Ca^{2+}] measurements in permeabilized cells, $[Ca^{2+}]_{ER}$ was reduced before reconstitution by incubating the cells for 10 min with the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase inhibitor 2,5-di-tert-buthyl-benzohydroquinone (BHQ) 10 µM in standard medium supplemented with 0,5 mM EGTA. Cells were then washed and incubated for 1 hour at room temperature in the same medium with 1 µM of wild-type coelenterazine. Then, the coverslip was placed in the perfusion chamber of the luminometer and standard medium containing 0,5 mM EGTA was perfused for 5 min, followed by 1 minute of intracellular medium containing 50 µM digitonin. Intracellular medium without digitonin was later perfused for 5 min, followed by a 100 nM [Ca^{2+}] buffer prepared in intracellular medium to refill the ER with Ca^{2+}. Temperature was set at 37 ºC during the experiments. Calibration of the luminescence data into [Ca^{2+}] was made using an algorithm adjusted to the calibration of each aequorin form, as previously described (Montero et al., 2002). Statistical data are given as mean±S.E.M.

At the end of the experiments it is necessary to measure all the remaining aequorin luminescence in the sample, in order to obtain the total luminescence value (L_{max}), which is required to calibrate the experiment in [Ca^{2+}]. To do so, a 10 mM calcium solution with 100 µM digitonin was perfused in the intact cells. In the case of the experiments in permeabilized, cells, only the solution with 10 mM calcium was used here.

3.6. Transformation of bioluminescence data in [Ca^{2+}]

To transform the luminescence data obtained during each experiment into free Ca^{2+} concentration data, it was necessary to calculate a calibration curve for each of the possible aequorin and coelenterazine combinations at 37 °C. These curves were obtained previously (Montero et al., 1997, de la Fuente et al., 2012, de la Fuente et al., 2013).

In the calibration experiments, L/L_{max} (the fraction of aequorin molecules that emit light in a second) values were obtained for several [Ca^{2+}] and a calibration curve was constructed. Then after each experiment, a computer program calculates the fractions of L/L_{max} at each point throughout the experiment, where L is the value of the luminescence (counts / second) and L_{max} is the total remaining luminescence from that point to the end of the experiment, subtracting in both cases the basal luminescence. The L/L_{max} values obtained are then transformed into [Ca^{2+}] values using the following mathematical algorithm.

$$[Ca^{2+}](M) = \frac{ratio + (ratio\ x\ K_{TR}) - 1}{K_R - (ratio\ x\ K_R)}$$

$$ratio = \left(\frac{L}{L_{max}\ x\ \lambda}\right)^{\frac{1}{n}}$$

This equation comes from the mathematical model proposed to explain the aequorin light emission dependence with respect to the concentration of Ca^{2+} (Allen et al., 1977). The values of the different parameters for the combinations of aequorin and coelenterazine used in this work are indicated in Table 5.

Aequorin	AEQwt	mitmutAEQ	LAerAEQ	LAerAEQ
Coelenterazine	**w**	**w**	**w**	**i**
K_R	4.18×10^7	1.61×10^7	4.922×10^6	56700
K_{TR}	601	22000	25000	31480
n	2,3	1,43	1,08	1,089
λ	1	1	1	0,4

Table 5. Calibration parameters of the different aequorins for 37 ºC.

3.7. Statistical analysis

The statistical analysis and graphs were carried out with the Origin 8.5 software (Origin Lab). The data were calculated from a minimum number of three similar experiments (mean ± standard error). To verify differences between the groups, a variance analysis (ANOVA) was performed. The significance level is shown as * $p<0.05$, ** $p<0.01$ and *** $p<0.005$.

4. PROTEIN ASSAYS

To carry out the protein assays, cells were seeded in 25 cm^2 culture flasks at 4×10^5 cells/flask and the total protein extraction was performed when they reached 80-90 % confluence.

4.1. Protein extraction

After the treatment (with A769662 or Dorsomorphin) and when the cellular confluence was between 80 and 90 %, the cultures were removed from the incubator and placed directly on ice (at 4 ºC) where the extraction process was fully carried out. To do so, the culture medium was removed from the culture flasks and cells were washed twice with PBS before lysis. Then, PBS was carefully aspired and lysis was performed by adding 500 µl of RIPA buffer containing the inhibitor cocktail (see Table 6). After 2 minutes incubation, cells were scratched and each lysate was transferred to a properly labeled Eppendorf tube and vortexed several seconds.

The lysates were incubated on ice for 15 minutes, vortexing for 20 seconds every 5 minutes. The lysates were finally clarified by centrifugation at 8000 xg for 10 minutes at 4 ºC to pellet the cell debris, and the supernatants were transferred to a new Eppendorf on ice. A small volume from each lysate was taken to quantify the proteins using BCA Protein Assay.

RIPA Buffer		Inhibitor Cocktail	
Tris-HCl, pH 8.0	50 mM	Complete protease inhibitors cocktail	1 X
Sodium Chloride	150 mM	PhosSTOP phosphatase inhibitors cocktail	1 X
Igepal CA-630 (NP-40)	1.0 %	NaF	10 mM
Sodiumdeoxycholate	0.5 %	Na-ortovanadate	2 mM
Sodium Dodecyl Sulfate (SDS)	0.1 %	PMSF	0.1 µM

Table 6. Protein Material for Cell lysis. A769662, dorsomorphin and non-treated HeLa cell lysis was carried out for 20 minutes at 4 °C in RIPA buffer containing broad spectrum kinase and phosphatase inhibitors.

4.2. Protein quantification

The total protein concentration was quantified using the Pierce BCA Protein Assay Kit (in 96-well plates) that measures (562 nm) total protein concentration compared to a protein standard. This method combines the "Biuret" reaction with the high sensitivity of the cuprous cation colorimetric detection (Cu^{+1}) using bicinchoninic acid.

A 20 µl-aliquot of total protein was taken from each sample and five serial dilutions of 50 % in PBS were made. To obtain a standard curve, known BSA (bovine serum albumin) concentrations were prepared and added in triplicate to wells of a 96-well plate. 200 µl of BCA Working Solution were added and incubated for 30 minutes at 37 ºC in a stove. The absorbance at 562 nm was read using the VERSAmax plate reader (Molecular Device) and the protein concentration of each sample was determined from the equation of the line corresponding to the BSA standard curve.

4.3. Phosphoproteomics

Hela cells were grown in 25 cm^2 culture flasks. Some of them were treated with the AMPK activator A769662 for 2 hours and others with the AMPK inhibitor Dorsomorphin, for 48 hours before the protein extractions. Five culture flasks were prepared in duplicate for each condition.

After protein extraction these samples were quantified, frozen and stored at -80 ºC for 24 hours and sent on dry ice to Nestlé Institute of Health Science (Lausanne, Switzerland) for the phosphoproteomic analysis.

Briefly, after reduction and alkylation, proteins were precipitated, digested with an enzyme cocktail of trypsin/LysC (Promega, WI, USA) and the resulting peptides were isobarically labelled with Tandem Mass Tag 10plex™ (TMT-10-plex from Thermo Scientific, IL, USA). The differentially labeled samples were then pooled after reaction quenching with hydroxylamine and cleaned-up (Oasis HLB cartridges from Waters, MA, USA). The samples were finally dried and enriched for the phosphorylated peptides with TiO2 Mag Sepharose magnetic beads (GE Healthcare, Glattbrugg, Switzerland) according to the manufacturer's instruction and analyzed with reversed phase liquid chromatography tandem mass spectrometry (RP-LC MS/MS). In parallel, non-phospho-enriched fractions (1/10 of the samples) were kept for complementary RP-LC MS/MS analysis.

5. *IN VIVO C. ELEGANS* ASSAYS

5.1. *C. elegans* strains

5.1.1. Control strain

In this work, the **AQ2038 strain** kindly provided by Drs. Robyn Branicky and W. Schafer, (MRC Laboratory of Molecular Biology, Cambridge, UK) was used to perform lifespan assays in *C.elegans*. This integrated strain expresses the cytosolic cameleon 2.1. (YC2.1) protein derived from GFP in pharynx cells under the myo-2 promoter (pmyo2::YC2.1) (Miyawaki et al., 1997, Nagai et al., 2004, Alvarez-Illera et al., 2016). This worm strain, whose lifespan was not different from that of N2 wild type strain, was used here as a control strain to study the relationship between the activity of the AMPK signalling pathway, intracellular calcium signalling and aging in *C. elegans.*

5.1.2. Mutant strains

***aak-2* mutants** show accelerated aging and shortened lifespan (Apfeld et al., 2004), as well as oxidative stress hypersensitivity (Lee et al., 2008) as a consequence of a mutation in the aak-2 gene that codes for the 5'-AMP-activated protein kinase catalytic subunit alpha-2 (*ok524*).

***daf-2* worms** have a mutation in *daf-2* (*e1370*) gene, which codes for the insulin-like growth factor receptor (IGFR-1). This gene is related to aging regulation and it has also been found to regulate development, oxidative stress resistance, temperature or hypoxia (Kenyon, 2011).

***eat-2* mutants** have a mutation that affects a subunit of the nicotinic acetylcholine receptor (*ad1113*) of the pharyngeal muscle (McKay et al., 2004). Consequently, these worms have reduced pumping in the pharynx which in turn, leads to a caloric restriction and increased life expectancy (Lakowski and Hekimi, 1998).

***nuo-6* worms** have a low oxygen consumption, slow growth, slower mobility and an increased life expectancy (Yang and Hekimi, 2010) as a consequence of a mutation in a subunit of the mitochondrial complex I (*qm200*).

All mutant strains were provided by Caenorhabditis Genetics Center.

5.2. *C. elegans* growth and maintenance

Worms were maintained and handled as previously described by Stiernagle (Stiernagle, 2006). Hardened agar was seeded with *Escherichia coli* OP50 and worms were maintained at 20 ºC. Calcium imaging and survival experiments were also conducted at 20 ºC on synchronized worms.

5.2.1. Preparation of bacterial food source *E. coli* OP50

Bacterial strain *E. coli* OP50 was used as the food source for the worms (Brenner, 1974). From a starter culture, single colonies were obtained on a streak plate prepared with LB agar medium. A single colony from the streak plate was aseptically inoculated in a 100ml rich liquid microbial growth medium 2xYT, previously autoclaved in 250 ml screw-cap bottles.

The OP50 was allowed to grow in 2xYT at saturation, overnight at room temperature or at 37 °C for 8 hours. Both the *E. coli* OP50 streak plate and liquid culture were stored at 4 °C. However the streak plate was maintained for several months and the liquid culture was renewed every fortnight.

5.2.2. Preparation of growth media and NGM petri agar plates

C. elegans was maintained and grown on agar plates with Nematode Growth Medium (NGM) (Brenner, 1974), placed in plastic boxes inside an incubator. Briefly, the NGM agar was prepared by adding in a 500 ml screw-cap bottle, NaCl, agar and peptone dissolved in sterile water. Then, it was autoclaved for 20 minutes at 121 °C and tempered in a hot water bath at 55 °C over 20 minutes. Subsequently, under the flame of a Bunsen burner, cholesterol, $CaCl_2$, $MgSO_4$ and KPO_4 buffer were added and the NGM agar bottle was stirred avoiding formation of precipitates (Table 7). Finally, 4, 10 or 20 ml per plate were distributed in 35, 60 or 100 mm diameter Petri plates, respectively, filling all equally and avoiding bubbles formation. When needed, drugs (FuDR, ML-347, SB-421543, DMH-1, Dorsomorphin or A769662) were added at the desired concentration to the NGM solution just prior to be distributed in the plates (Caldicott et al., 1994).

NGM Agar	Amount		500 ml NGM	Amount
NaCl	1.5 g	121 ºC 20 min	Cholesterol (5 mg/ml) in ethanol	500 µl
Agar	8.5 g		$CaCl_2$ (1 M)*	500 µl
Peptone	1.25 g		$MgSO_4$ (1 M)*	500 µl
Sterile Water	500 ml		KPO_4 (1 M) pH6.00**	12,5 ml

Table 7. Growth Medium Nematode Preparation. Compounds and amounts needed to prepare the agar media where the nematodes were grown and kept. * These solutions were prepared and autoclaved before being added ** 1M KPO_4 buffer was prepared by adding 108.3 g KH_2PO_4, 35.6 g K_2HPO_4, and H_2O to 1 litre and adjusting to pH 6.0 before being autoclaved.

The NGM agar medium was aseptically distributed into petri plates using a pipette controller ensuring a constant volume of agar in the plates, to avoid refocusing the microscope when switched from one plate to another.

Several sizes of petri plates were used for different assays. Smaller plates (35 mm diameter) were used for survival assays or when using expensive drugs. Medium size plates (60 mm diameter) were used for general maintenance, and larger plates (100 mm diameter) were employed for preparing worms for calcium imaging assays.

5.2.3. NGM agar plates with FuDR (15 µM)

5-fluoro-2'-deoxyuridine (FuDR) is a thymidylate synthase inhibitor, which affects cell division. Therefore, in an adult worm, FuDR only affects the development of the eggs, since when the worm reaches the adult stage, its cells will not divide again (Mitchell et al., 1979). Using FuDR, egg-laying of the adult worms was interrupted and plates were not contaminated with progeny.

NGM plates with FuDR were used in survival experiments, where it was necessary to have synchronized worms that did not lay eggs. Otherwise, the new generation would have been mixed with the previous one, making impossible to distinguish the first generation from the second.

NGM plates with 15 µM FuDR were prepared by adding 250 µl of 30 mM FuDR solution to the 500 ml of NGM agar before pouring it into the plates.

5.2.4. Seeding NGM plates

Once agar was poured, plates were allowed to solidify at room temperature. When the agar was cold and hardened, the small and medium plates were seeded with 250 µl of the *E. coli* OP50 strain, and the larger ones with 500 µl.

Worms tend to spend most of the time in the bacteria. Therefore, OP50 was placed in the center of the plates so that the worms remained inside the food. Otherwise, if the lawn was extended near the edge, the worms crawled up the edges of the plate, drying out and dying (Stiernagle, 2006).

5.3. Preparation of the γ-cyclodextrin inclusion compounds

To test the effect of some lipophilic modulatory compounds (ML-347, SB-421543, DMH-1, Dorsomorphin or A769662) on survival, it was necessary to administer them orally. To do that, we used γ-cyclodextrin inclusion compounds.

Gamma-cyclodextrin (γ-CD) is a cyclic oligosaccharide able to include lipophilic molecules on its cavity, thus increasing its water solubility (Figure 24). This property allowed to administer the drugs dissolved in the OP50 bacterial food culture. The γ-CD inclusion compounds were prepared as described before (Kashima et al., 2012).

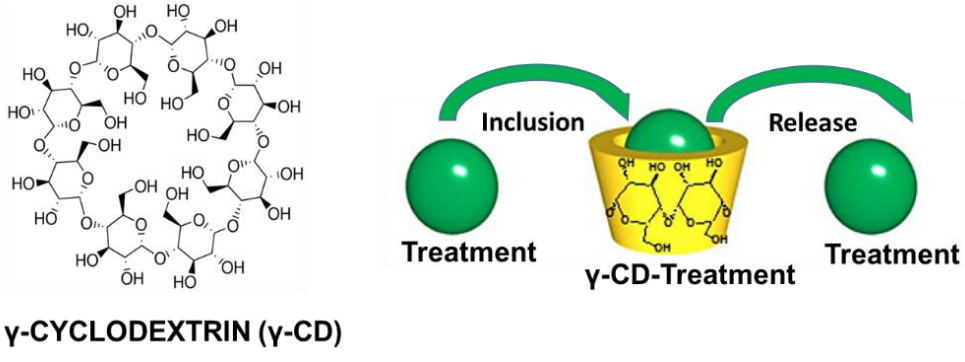


Figure 24. **γ-CD molecular structure**. Cyclic oligosaccharide able to include lipophilic molecules on its cavity and transport them to their place of action.

All the drugs (A769662, Dorsomorphin and the BMP inhibitors ML-347, SB-431542, DMH-1) were dissolved in 20 mM DMSO prior to preparation of the inclusion compounds (Figure 25). Briefly, a 230 mg/ml water solution of γ-CD was mixed 10:1 with the 20 mM DMSO solution of each compound, stirred in a shaker at

1200 rpm during 20 hours and centrifuged at 12,500 rpm for 10 min. The supernatant was carefully discarded and the resulting inclusion compound was dried in the hood and dissolved in filtered M9 buffer (for the composition of M9 buffer see Table 8). The inclusion compounds containing either ML-347, SB-431542, DMH-1, Dorsomorphin or A769662 were added directly to the plates (along with OP50) in the amounts indicated before. Then, synchronized young adult worms (day 1) were transferred to the plates to start the lifespan assay. In the case of DMH-1, Dorsomorphin or A769662, in some assays they were dissolved directly in the NGM agar by strong stirring at the indicated concentrations (assuming homogeneous distribution).

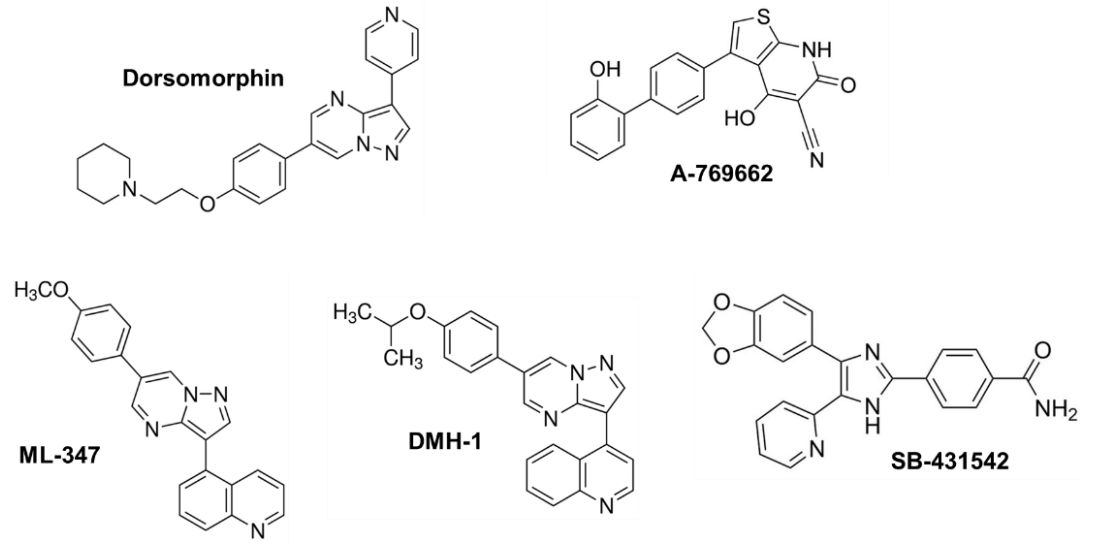


Figure 25. **Molecular structure of AMPK modulators and BMPR inhibitors**. Chemical structure representation of the compounds used. The inhibitor and activator of AMPK (above) and dorsomorphin analogues, which are in turn BMPR inhibitors (below) are shown.

5.4. Worms's synchronization to obtain eggs

To synchronize the worms, a large population of adult worms with many eggs inside is needed. It is known that, when the worms do not have food they retain the eggs inside until the environmental conditions improve and they have nutrients available again. For this reason, plates with worms at day 2 or 3 of the adult stage were used for the synchronization. In some cases those left over from the previous synchronization were used at this moment. Otherwise, 50 adult worms were transferred to an OP50-seeded NGM agar plate and synchronization was carried out two days later, when the food ran out, as previously described (Stiernagle, 2006).

The synchronization process, that was essential for the aging experiments, consisted of applying a chemical treatment (commercial bleach solution-NaOH) to break the worms and obtain the eggs free. The eggs are resistant to this treatment, so their viability is not affected.

For the synchronization, worms were collected with a glass Pasteur pipette with 1 ml of deionized water, transferred to a sterile eppendorf and centrifuged 2 minutes at 2000 rpm. The supernatant was removed and 150 µl of a 2:1 mixture of 5N NaOH and bleach, and 250 µl of M9 buffer were added (see Table 8). Worms were then vortexed 10 seconds every 2 minutes for a maximum of 10 minutes (under microscopic magnifying glass it was observed that the worms were fragmented). After ten minutes, worms were centrifuged over 1 minute at 8000 rpm. The supernatant was removed and 1ml of M9 buffer was added to wash the bleach, followed by 1 min centrifugation at 8000 rpm.

Compound	Amount
KH_2PO_4	3 g
Na_2HPO_4	6 g
NaCl	5 g
1M $MgSO_4$	1 mL
H_2O	to 1 L

Table 8. Composition of M9 Buffer for *C.elegans*. Compounds and amounts needed to prepare buffer for worms.

The supernatant was discarded, and approximately 100 µl of eggs in M9 buffer were resuspended with a glass Pasteur pipette and transferred to an OP50-seeded NGM plate. One drop of the suspension was added to each plate. When the M9 was reabsorbed in the agar, the plates with the eggs were introduced in the incubator at 20 °C (Stiernagle, 2006; Figure 26).

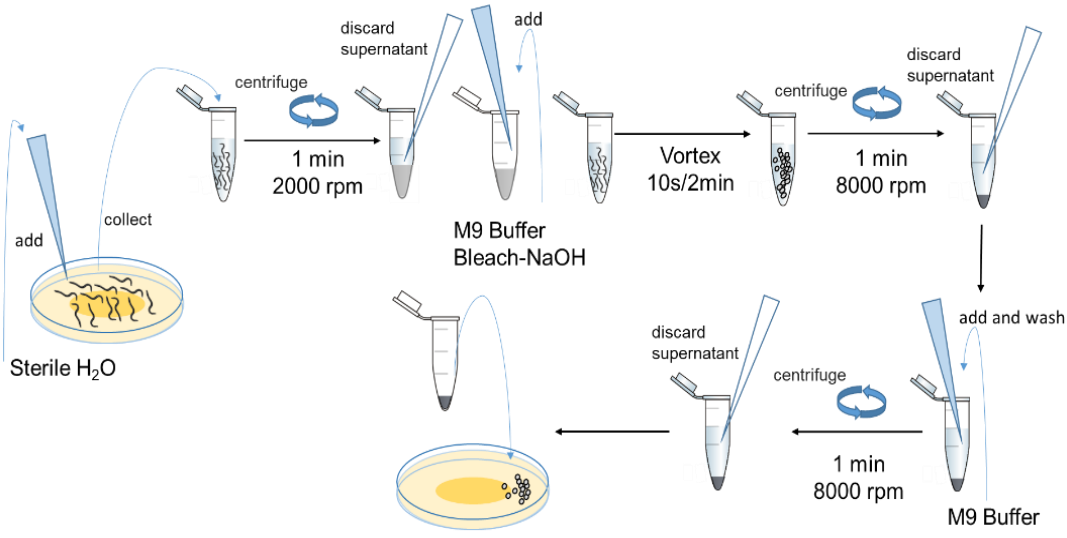


Figure 26. Worms synchronization. Representative scheme of the process followed to obtain synchronized eggs.

Once the worms developed and reached the young adult stage (day 1), they were transferred to *E. coli* OP50 seeded NGM agar plates (containing 15 µM FuDR to avoid progeny) to carry out the longevity assays.

5.5. *C. elegans* lifespan assay

Eggs were obtained and transferred to *E. coli* (OP50) seeded NGM plates, either control plates or plates prepared in the presence of the required drug (both dissolved in the agar or included in γ-CD and dissolved in the OP50). For each assay, around 100 synchronized young adults (day 1) were transferred to E. coli (OP50) seeded NGM plates (35 mm plates, 10 worms/plate) containing 15 µM FUdR. Age refers to days following adulthood. Control and drug-containing assays were always carried out in parallel and control and drug-containing plates were kept close together in a temperature controlled incubator set at 20 ºC. Plates were scored for dead worms every day. Worms that did not respond to touch with a platinum wire were considered dead. Plates with fungal contamination during the first 10 days of the assay were excluded from the study. Missing worms, individuals with extruded gonad or desiccated by crawling in the edge of the plate were censored, as well as plates with fungal contamination after the first 10 days. These exclusion criteria were established before starting the experiments. Statistics was made with the SPSS software using the Kaplan-Meier estimator and the log-rank routine for significance.

6. MATERIALS

Culture media DMEM, FBS, antibiotics and PBS were supplied by GIBCO, Paisley, UK.

Metafectene Pro was by Biontex, Munich, Germany.

Culture flasks were purchased to Thermo Fisher, Roskilde, Denmark.

All the necessary reagents for molecular biology were obtained from Bio-Rad, Hercules, Ca, U.S.A.

The competent DH5α bacteria were purchased to Invitrogen.

Coelenterazine wild-type and coelenterazine i were obtained from Biotium Inc., Hayward, Ca, U.S.A.

A769662, Dorsomorphin, ML-347, SB-431542 and DMH-1 were obtained from Tocris, Bristol, U.K.

Inositol 1,4,5-trisphosphate, digitonin, ethylene glycol tetraacetic acid (EGTA), hydroxyethyl ethylenediamine (HEDTA), histamine, cholesterol, sodium phosphate dibasic (Na_2HPO_4), magnesium sulphate ($MgSO_4$) and dimethylsulphoxide (DMSO) were supplied by Sigma, Madrid, Spain.

Calcium chloride, sodium chloride, dipottasium hydrogenophosphate and pottasium dihydrogenophosphate and other salts and substrates were supplied by Merck, Darmstadt, Germany.

RIPA buffer containing broad spectrum kinase and phosphatase inhibitors and other reagents for protein extraction were supplied by Roche, Madrid, Spain.

Agar was obtained from Calbiochem, MA, U.S.A.

Bacteriological Peptone was obtained from Pronadisa, Madrid, Spain.

Plates 55 and 100mm were supplied by Deltalab, Barcelona, Spain.

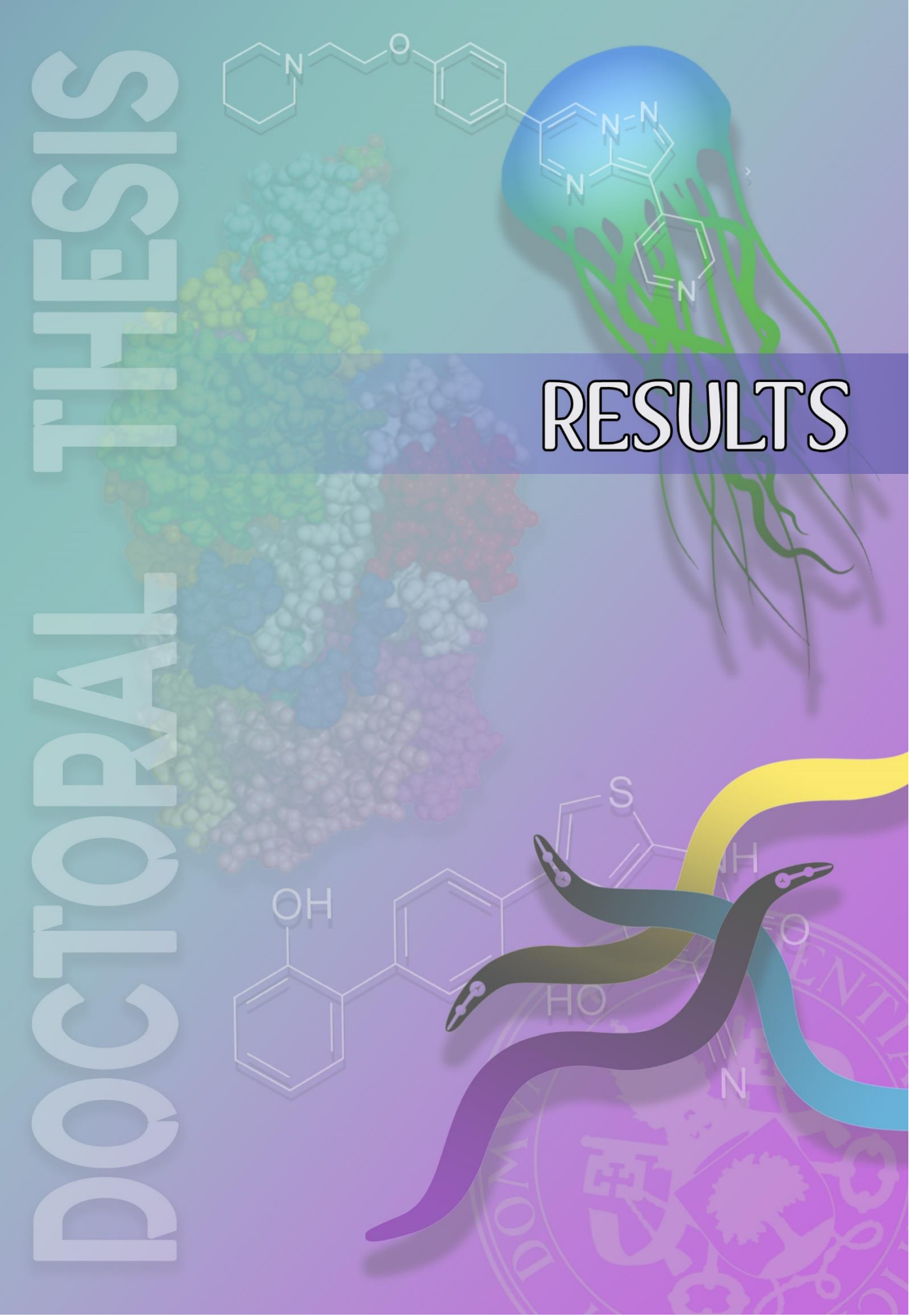
The smaller plates, Plates 35mm, were supplied by Thermo Fisher Scientific, Madrid, Spain.

2xYT (LB broth) and Pasteur Pipete 230-250 were purchased to FisherBrand, Geel, Belgium.

FuDR 5'-fluoro-2'-deoxiuridina was obtained from Alfa-Aesar, Karlsruhe, Germany.

C.elegans worm strains: *aak-2 (ok524)* RB754; *daf-2* (e1370) CB1370; *eat-2* (ad1113) DA1113 and *nuo-6 (qm200)* MQ1333, were obtained from Caenorhabditis Genetics Center (CGC), University of Minnesota, Twin Cities.

DOCTORAL THESIS



RESULTS

As explained in the introduction, several nutrient-sensitive signalling pathways, including the AMPK pathway, have gained importance in recent years because they are believed to be essential to determine longevity in a large number of species. Some data also suggest that their activation or inhibition is mediated at least in part by Ca^{2+}. At the same time those pathways are known to be capable of modulating intracellular signalling by Ca^{2+}. Therefore, studying this interrelation could provide important clues to understand the aging process and perhaps to find new compounds able to act on it.

Based on these well known facts, this work focuses the research on two fundamental axes. The first one is linked to the studies at the cellular level and the other to studies in a living organism, in this case the nematode *C. elegans*.

1. EFFECTS OF THE AMPK PATHWAY MODULATORS ON Ca^{2+} SIGNALLING AT CELLULAR LEVEL

The cytosolic Ca^{2+} signal acts on cascades of signal transduction through the direct activation of ion channels or as a second messenger in the indirect signal transduction pathways, such as G protein-coupled receptors. In this case, cells were stimulated with histamine 100 µM, which evokes a release of Ca^{2+} from the ER through the activation of the IP_3R. Specifically, histamine exerts its biological actions by binding to specific cellular receptors, located on the surface of the cell membrane. In this case, the H1 Histamine receptors that are present in HeLa cells are coupled to Gq proteins, thus activating the PLC-IP_3 pathway. Activation of phospholipase C (PLC) generates inositol triphosphate (IP_3), that activates IP_3R and releases Ca^{2+} from the ER, and diacil glycerol (DAG), that activates protein kinase C (PKC) (Berridge et al., 2000).

1.1. Effect of AMPK modulators on histamine-induced cytosolic and mitochondrial [Ca^{2+}] peaks.

The effects of the AMPK activator A769662 and the AMPK inhibitor dorsomorphin on intracellular Ca^{2+} signalling in HeLa cells were first adressed. A769662 is capable of activating AMPK by direct binding to the enzyme (Kurumbail and Calabrese, 2016; Guigas and Viollet, 2016). On the other hand, dorsomorphin (originally called Compound C) is a potent inhibitor of AMPK (Zhou et al., 2001), although it has also been found to inhibit potently several bone morphogenetic protein type I receptors (BMPRs) (Yu et al., 2008).

The effects of these compounds on the cytosolic [Ca^{2+}] peak induced by histamine can be observed in Figure 27. They induce opposite effects so that A769662 reduces the height of the peak while dorsomorphin enhances it. In addition, both effects were highly significant.

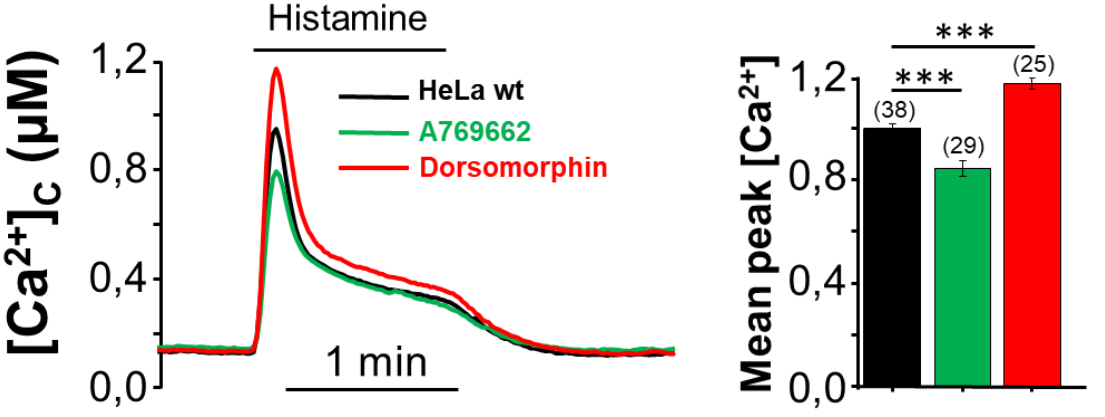


Figure 27. Effects of A769662 and dorsomorphin on the cytosolic [Ca^{2+}] peak induced by histamine. HeLa cells were transfected with cytosolic aequorin and then cultured during 48 hours with either none, 50 µM A769662 or 1 µM dorsomorphin. Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100 µM histamine as shown in the figure. The right part of the panel shows the statistics of the [Ca^{2+}] peaks. The numbers on top of the bars are the number of experiments of each type. ***, p<0,005.

During stimulated ER-Ca^{2+} release, mitochondria takes up Ca^{2+} from local high-Ca^{2+} microdomains generated in the cytosolic mouth of the IP_3R channels. This ER-mitochondria Ca^{2+} transfer occurs at MAMs, where both ER-IP_3R and mitochondrial MCU channels are placed (Filadi et al., 2017). This preferential Ca^{2+} pathway between both organelles allows mitochondria to sense and amplify the cytosolic [Ca^{2+}] signalling induced by Ca^{2+} release from the ER.

The second approach was aimed to verify if the effects observed in the cytosol in the presence of AMPK modulators were maintained in mitochondria. The effects of these compounds on the mitochondrial Ca^{2+} uptake induced by histamine are shown in Figure 28.

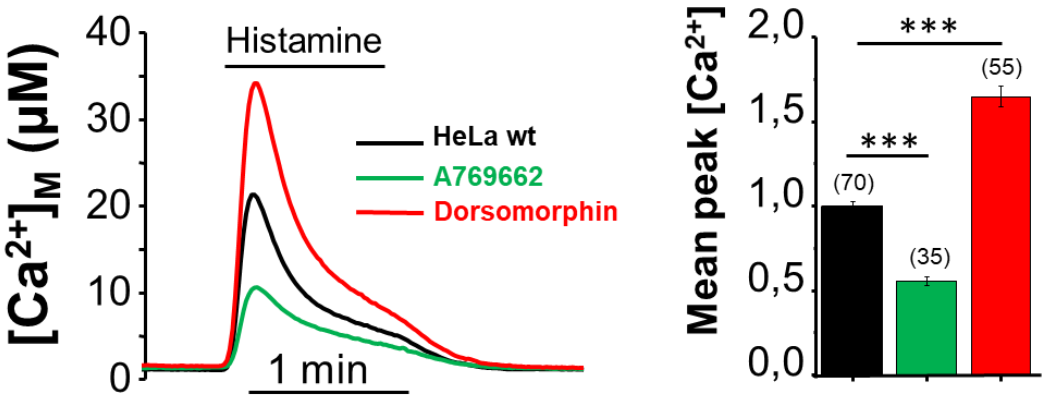


Figure 28. Effects of A769662 and dorsomorphin on the mitochondrial [Ca^{2+}] peak induced by histamine. HeLa cells were transfected with mitochondrially targeted mutated aequorin and then cultured during 48 hours with either none, 50μM A769662 or 1μM dorsomorphin. Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100μM histamine as shown in the figure. The right part of the panel shows the statistics of the [Ca^{2+}] peaks. The numbers on top of the bars are the number of experiments of each type. ***, p<0,005.

It can be observed that the mitochondrial [Ca^{2+}] peak induced by histamine in control cells is much larger than the cytosolic one, around 20μM as previously reported (Fonteriz et al., 2010). Moreover, as a result of the amplification, the compounds A769662 and dorsomorphin produced also much larger effects, inhibition in the case of A769662 and activation in the case of dorsomorphin. A769662 inhibited the histamine induced cytosolic [Ca^{2+}] peak by 16±3 %, but reduced the mitochondrial [Ca^{2+}] peak by 45±3 %. Similarly, dorsomorphin increased the histamine-induced cytosolic [Ca^{2+}] peak by 17±2 % and the mitochondrial one by 65±6 %. Therefore, both compounds induce the same effect on both cytosolic and mitochondrial [Ca^{2+}] peaks elicited by histamine (decrease for A769662 and increase for dorsomorphin).

The amplification of the effects of these compounds on histamine-induced mitochondrial Ca^{2+} uptake provides a much better resolution of the effects at different drug concentrations. Figure 29 shows the dose-response curves for the effects of A769662 and dorsomorphin on the histamine-induced mitochondrial Ca^{2+} uptake.

The upper panels (A and B) show the effects at different concentrations of both compounds in a typical experiment. The middle panels (C and D) show the statistics of a series of experiments performed at each concentration, and the lower panels (E and F) show the dose response curves obtained. Half-maximal inhibition of the histamine induced mitochondrial Ca^{2+} peak was obtained at 29 μM A769662, and half-maximal activation was obtained at 0.4 μM dorsomorphin.

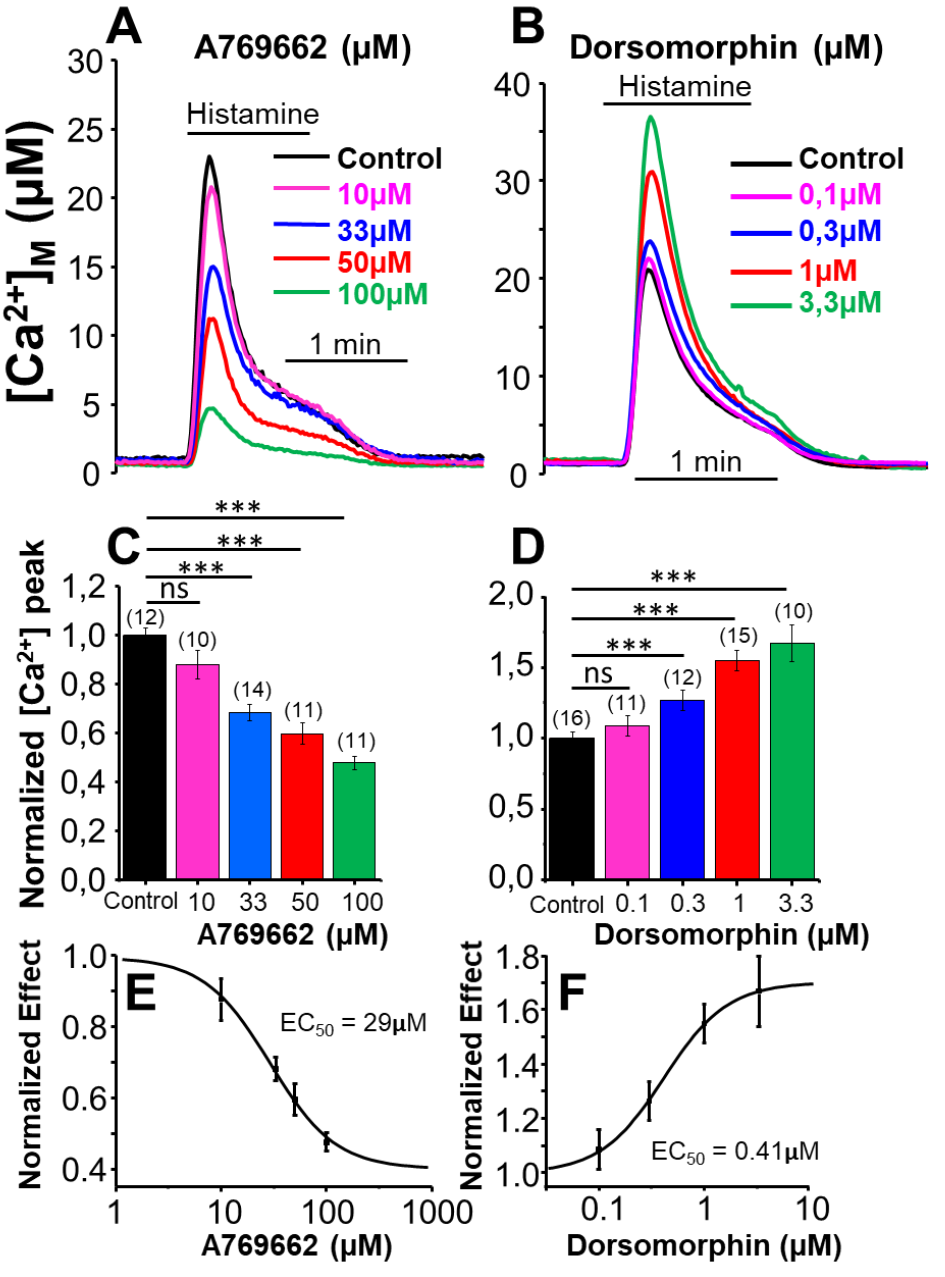


Figure 29. Dose-response relationship of the effects of A769662 and dorsomorphin on the mitochondrial [Ca^{2+}] peaks induced by histamine. HeLa cells were transfected with mitochondrially targeted mutated aequorin and then cultured during 48 hours with either none or different concentrations of dorsomorphin. In the case of A769662, cells were incubated with different concentrations of the compound for 2 h at 37 ºC prior to the experiment. Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100 μM histamine as shown in panels A and B. Panels C and D show the statistics of the effects obtained in a series of experiments similar to those of panels A and B. The numbers on top of the bars are the number of experiments of each type. Panels E and F show the dose-response curve fitted to the experimental data and the EC_{50} obtained for each of the compounds. ***, p<0,005.

It was also found that the effects of the AMPK activator A769662 could be observed also after a shorter preincubation with the drug. In fact, the larger reduction in the mitochondrial [Ca^{2+}] peak induced by histamine was obtained by incubating the cells with 100 µM A76966 for 2 hours.

We then studied if the effects of AMPK modulators on Ca^{2+} dynamics could be mediated by store-operated calcium entry mechanisms. Accordingly, the same experiments but in the absence of extracellular Ca^{2+} were performed. To do that, we used an external media containing no Ca^{2+} and EGTA as a chelating agent.

Figure 30 shows that the same effects were obtained in the absence of extracellular Ca^{2+}, that is, in the presence of EGTA in the extracellular medium. This means that these compounds act on IP_3-induced Ca^{2+}-release triggered by histamine and not on the store operated Ca^{2+} entry activated as a consequence of ER Ca^{2+} depletion.

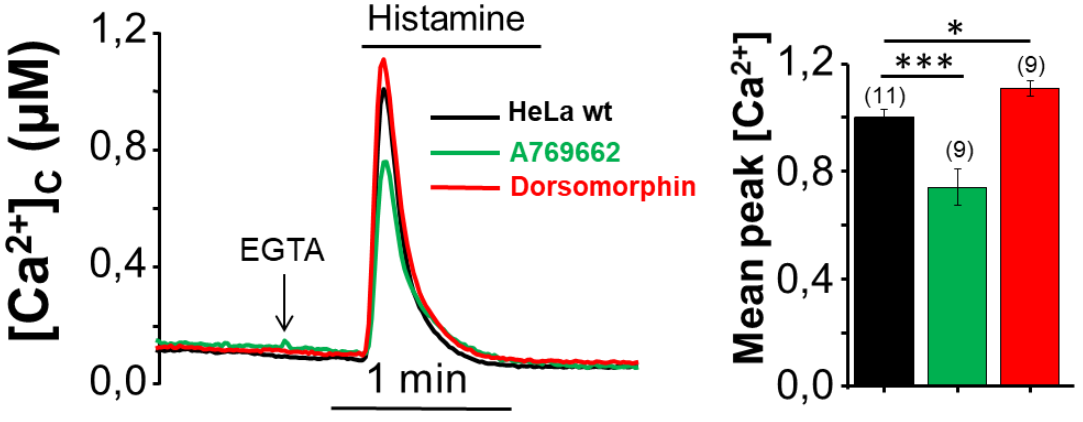


Figure 30. A769662 and dorsomorphin do not act on the store operated Ca^{2+} entry. HeLa cells were transfected with cytosolic aequorin and then cultured during 48 hours with either none, 50 µM A769662 or 1 µM dorsomorphin. Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100 µM histamine as shown in the figure. Medium containing 0,5 mM EGTA instead of 1 mM Ca^{2+} was perfused when indicated. The right part of the panel shows the statistics of the [Ca^{2+}] peaks. The numbers on top of the bars are the number of experiments of each type. *, p<0,05; ***, p<0,005.

1.2. Effect of AMPK modulators on ER-Ca^{2+} release.

The data of Figures 27 and 28 show that A769662 and dorsomorphin induce the same effect (decrease for A769662 and increase for dorsomorphin) on both cytosolic and mitochondrial [Ca^{2+}] peaks induced by histamine. This suggests that both AMPK regulators influence calcium signalling at the level of ER Ca^{2+} release induced by histamine.

For this reason, we then studied the effect of AMPK modulators on ER-Calcium release induced by histamine. In fact, Figure 31A shows that A769662 strongly inhibited the ER Ca^{2+} release induced by histamine, while dorsomorphin enhanced it. The statistics shows the mean values of the minimum $[Ca^{2+}]_{ER}$ reached after histamine addition. A769662 largely increased that value, as a result of the inhibition of Ca^{2+} release. Dorsomorphin produced the opposite effect, increased $[Ca^{2+}]$ release and therefore reduced the minimum $[Ca^{2+}]_{ER}$ level.

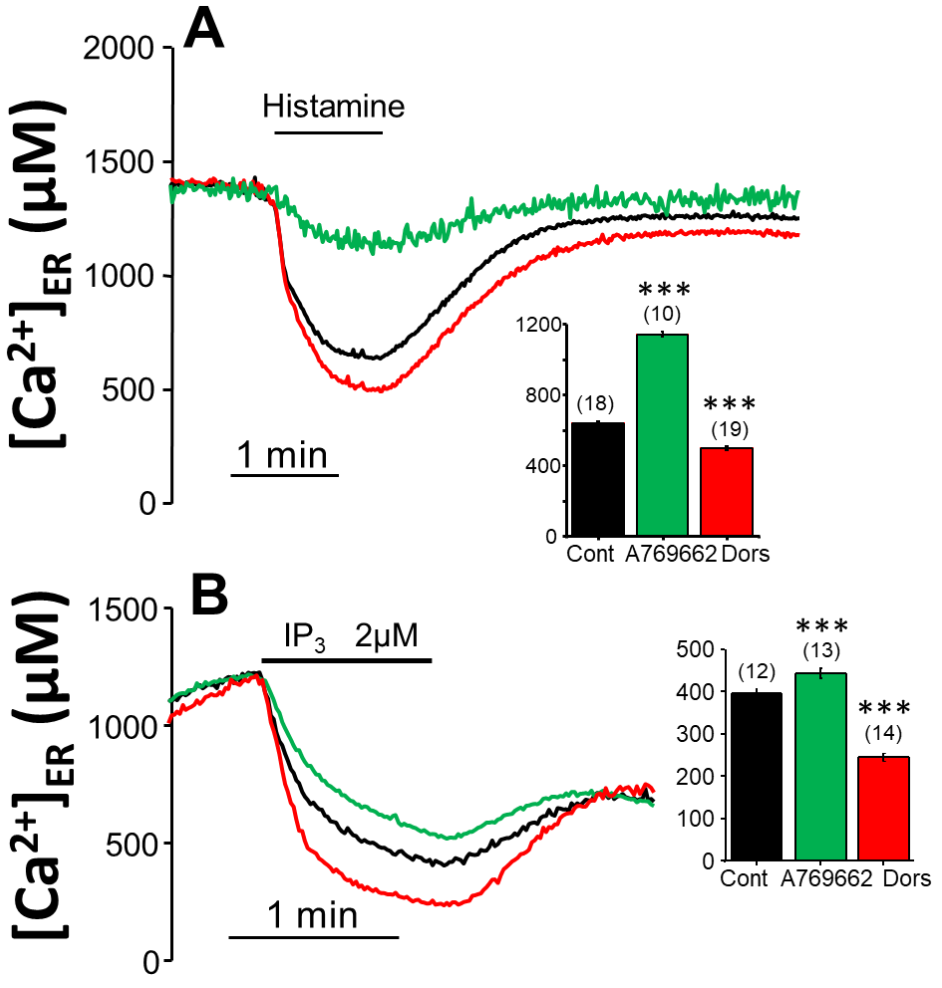


Figure 31. Effects of A769662 and dorsomorphin on ER-Ca^{2+} release induced by histamine. HeLa cells were transfected with ER-targeted double-mutated aequorin and then cultured during 48 hours with either none or 1 µM dorsomorphin. In the case of A769662, cells were incubated with 100 µM of the compound for 2 h at 37 ºC prior to the experiment. In panel A, cells were reconstituted with coelenterazine i and then stimulated with 100 µM histamine, as shown in the figure. In panel B, cells were permeabilized as described in Methods, reconstituted with wild-type coelenterazine and stimulated with 2 µM IP_3. The insets show the statistics of the effects obtained in a series of experiments similar to those of panels A and B. The numbers on top of the bars are the number of experiments of each type. ***, p<0,005. The effect of each compound is compared with the control.

The effects of A769662 and dorsomorphin on histamine-induced ER Ca^{2+}-release suggest that these compounds influence IP_3R function, which is responsible for ER Ca^{2+} release triggered by histamine. However, we still cannot exclude an effect of these compounds on the histamine receptor or the pathway responsible for IP_3 production after histamine action.

To investigate this point, we tested the effect of A769662 and dorsomorphin on ER Ca^{2+}-release induced directly by IP_3 in permeabilized cells. Figure 31B shows that the same modulation can be obtained under these conditions. A769662 reduced IP_3-induced Ca^{2+} release and dorsomorphin enhanced it. In conclusion, A769662 and dorsomorphin produce an opposite modulation of IP_3R activity, inhibition in the case of A769662 and activation in the case of dorsomorphin. In addition, any effect of the pharmacological reagents, A769662 and dorsomorphin, on the activity of the histamine receptor and/or the pathways leading to the generation of IP_3 can be completely ruled out.

1.3. Effect of BMPR inhibitors on histamine-induced cytosolic and mitochondrial [Ca^{2+}] peaks.

Dorsomorphin has been widely used as a selective AMPK inhibitor. However, it is also a potent inhibitor of bone morphogenetic protein type I receptors (BMPRs) (Hong and Yu, 2009). These receptors are on the cell surface and are specific of a group of signalling molecules or Bone Morphogenetic Proteins (BMPs), that belong to the Transforming Growth Factor-β (TGF-β) superfamily of proteins (Miyazono et al., 2005).

To exclude that the effect of dorsomorphin could be mediated by inhibition of BMPRs, two different inhibitors of BMPR which have no activity on AMPK (ML-347 and DMH-1) were tested. These compounds are even more potent inhibitors of BMPRs than dorsomorphin (Engers et al., 2013), and were thus assayed at the same concentration. Figure 32, shows their effect on the cytosolic and mitochondrial [Ca^{2+}] peak induced by histamine. None of them increased the cytosolic or the mitochondrial [Ca^{2+}] peak. In fact, both produced significant reductions in the height of the cytosolic [Ca^{2+}] peak, in contrast with the very significant increase induced by dorsomorphin.

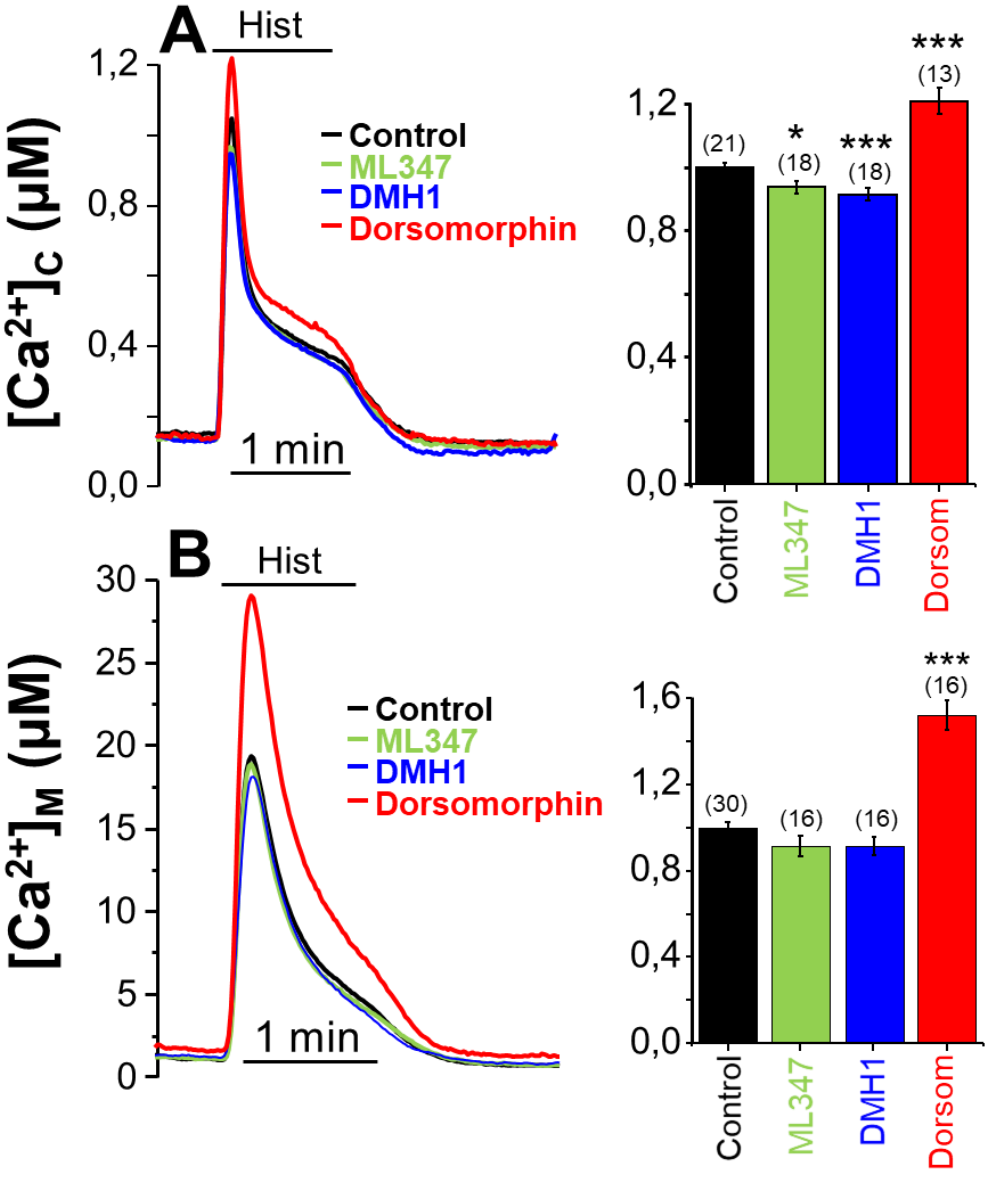


Figure 32. Effects of the BMPR-inhibitors ML-347 and DMH-1 and of the AMPK/BMPR inhibitor dorsomorphin on the cytosolic and mitochondrial [Ca^{2+}] peaks induced by histamine. HeLa cells were transfected with either cytosolic aequorin (panel A) or mitochondrially targeted mutated aequorin (panel B) and then cultured during 48 hours with either none, 1 μM of ML-347, DMH-1 or dorsomorphin. Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100 μM histamine as shown in the figure. The right part of each panel shows the statistics of the [Ca^{2+}] peaks. The numbers on top of the bars are the number of experiments of each type. *, p<0,05; ***, p<0,005. The effect of each compound is compared with the control.

Moreover, to obtain further evidence that the effect of these drugs on the IP_3R was mediated by AMPK and not by BMP signalling or other possible mechanisms, we tested if dorsomorphin was able to reverse the effect of A769662 when administered together. We found that dorsomorphin partially reversed the inhibition of the histamine induced-mitochondrial [Ca^{2+}] peak induced by the AMPK activator A769662 (Figure 33). In conclusion, the effect of dorsomorphin on the IP_3R is not mediated by BMPR inhibition, but rather by AMPK inhibition.

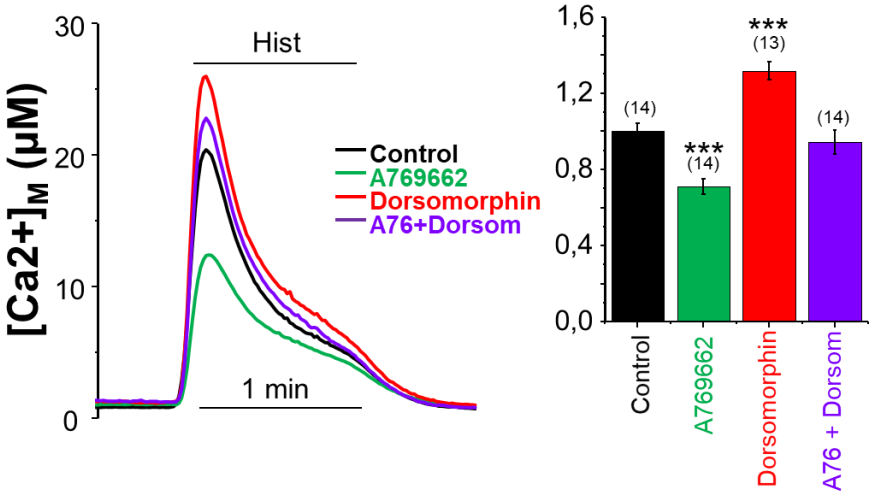


Figure 33. Dorsomorphin reverses the inhibition induced by the AMPK activator A769662. HeLa cells were transfected with mitochondrially targeted mutated aequorin and then cultured during 48 hours with either none, 1 μM of dorsomorphin, 50 μM of A769662 or both (A76 + Dorsom). Then, cells were reconstituted with wild-type coelenterazine and stimulated with 100 μM histamine as shown in the figure. The right part of the panel shows the statistics of the [Ca^{2+}] peaks. The numbers at the top of the bars are the number of experiments of each type. ***, p<0,005. The effect of each compound is compared with the control.

1.4. Phosphoproteomic study

To obtain further insight on the mechanism of the effect of A769662 and dorsomorphin on the IP_3R activity, a Phosphoproteomic study was carried out.

1.4.1. Effect of AMPK modulators on the IP_3R expression levels

Although three subtypes of inositol 1,4,5-trisphosphate receptor exist, HeLa cells highly express only two of them, IP_3R1 and IP_3R3, in comparable amounts (Zhang et al., 2011).

First, we took advantage of mass spectrometry to determine if the treatment with the activator A769662 or the inhibitor dorsomorphin, produced changes in the expression levels of the IP_3R isoforms in HeLa cells, that could explain the changes observed in Ca^{2+} signalling in the ER after histamine stimulation.

Figure 34 shows the protein levels of ITPR1 (Inositol 1,4,5-Trisphosphate Receptor Type 1) and ITPR3 (Inositol 1,4,5-Trisphosphate Receptor Type 3) in HeLa cells with and without treatments with AMPK modulators, obtained by mass spectrometry. None of the tested modulators changed the expression levels of either of the two IP_3R isoforms present in HeLa cells. These data show that the effects observed in ER-Ca^{2+} signalling in HeLa cells treated with AMPK modulators were not due to changes in the IP_3R expression levels.

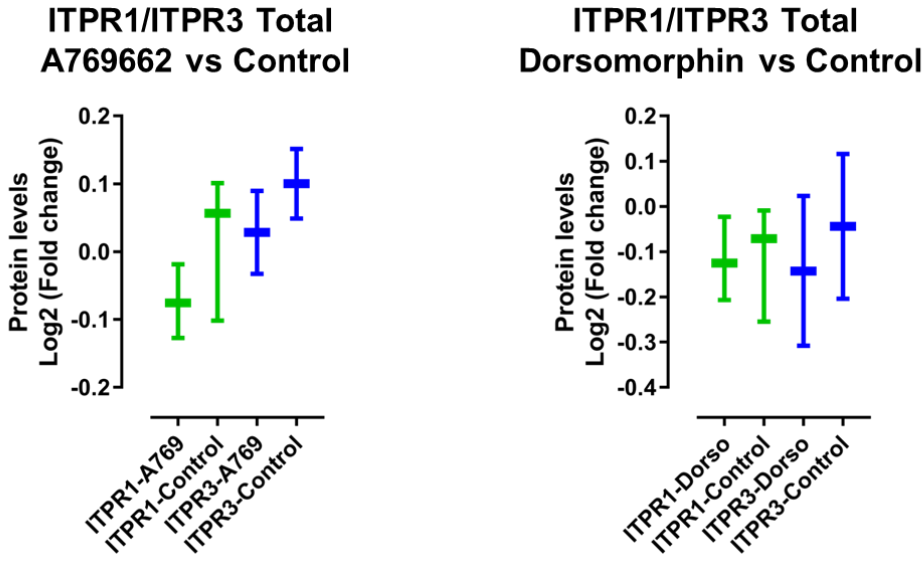


Figure 34. IP_3R expression levels. Mean level of the peptides obtained in the phosphoproteomic study from the two IP_3R isoforms present in HeLa cells (3 peptides for ITPR1 and 2 peptides for ITPR3).

1.4.2. Effects of AMPK modulators on HeLa cells phospho-proteome

In order to obtain additional information on the mechanism of the effect of these compounds on the IP_3R activity, we looked extensively for changes in the proteins phosphorylation state generated by the treatment with A769662 or dorsomorphin, and specifically, changes in the phosphorylation state of the IP_3R.

The comparative phosphoproteomic study revealed 304 P-sites with significant changes in phosphorylation induced by A769662 and 257 P-sites having significant changes in phosphorylation induced by dorsomorphin (Figure 35).

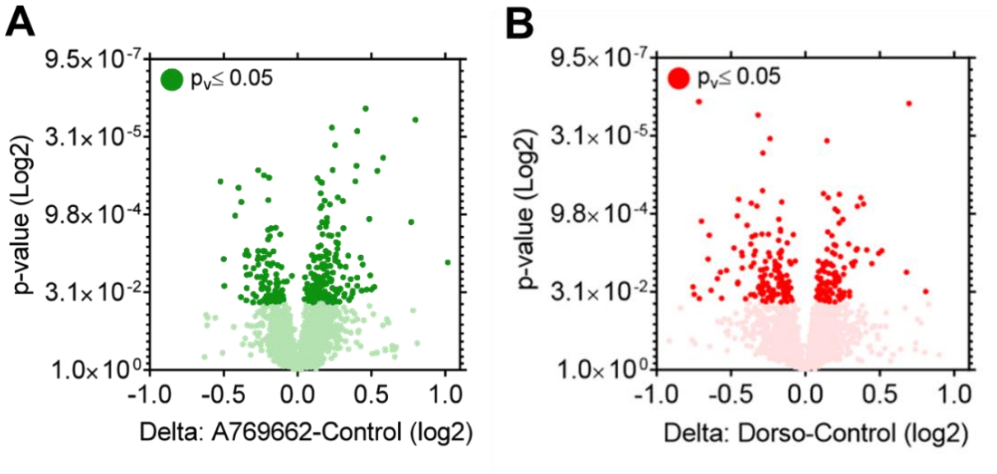
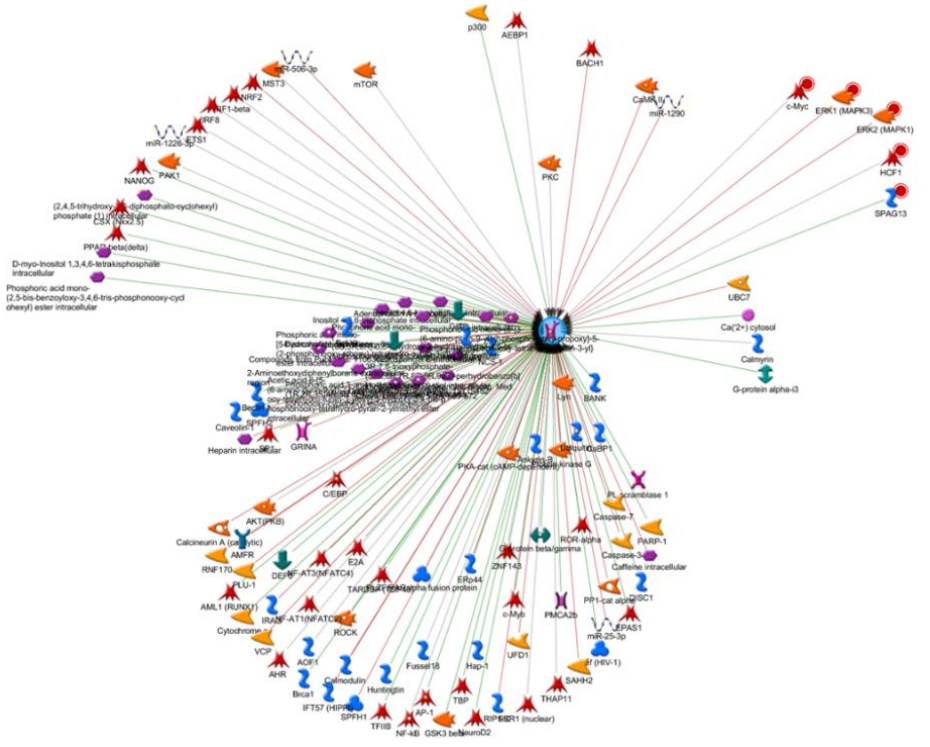


Figure 35. Effects of AMPK modulators on Hela cells phospho-proteome. A) Volcano plot displaying significant regulated P-sites in the presence of A769662. B) Volcano plot displaying significant regulated P-sites in the presence of dorsomorphin.

Although no significant changes in phosphorylation of the IP_3R itself could be detected, the range of proteins whose phosphorylation status was modified by A769662 and dorsomorphin included several proteins belonging to the IP_3R interactome (Figures 36, 37 and 38).

This group included transcription factors such as c-Myc and several kinases, such as ERK1/2 (MAPK1/3), whose phosphorylation was increased by A769662 (1.214 fold, T185/Y187) (Figure 36B and 38), and PKCα and GSK3B, whose phosphorylation was decreased in the presence of dorsomorphin (0.883 fold, S226; 0.899 fold, S9) (Figure 37B and 38).

A

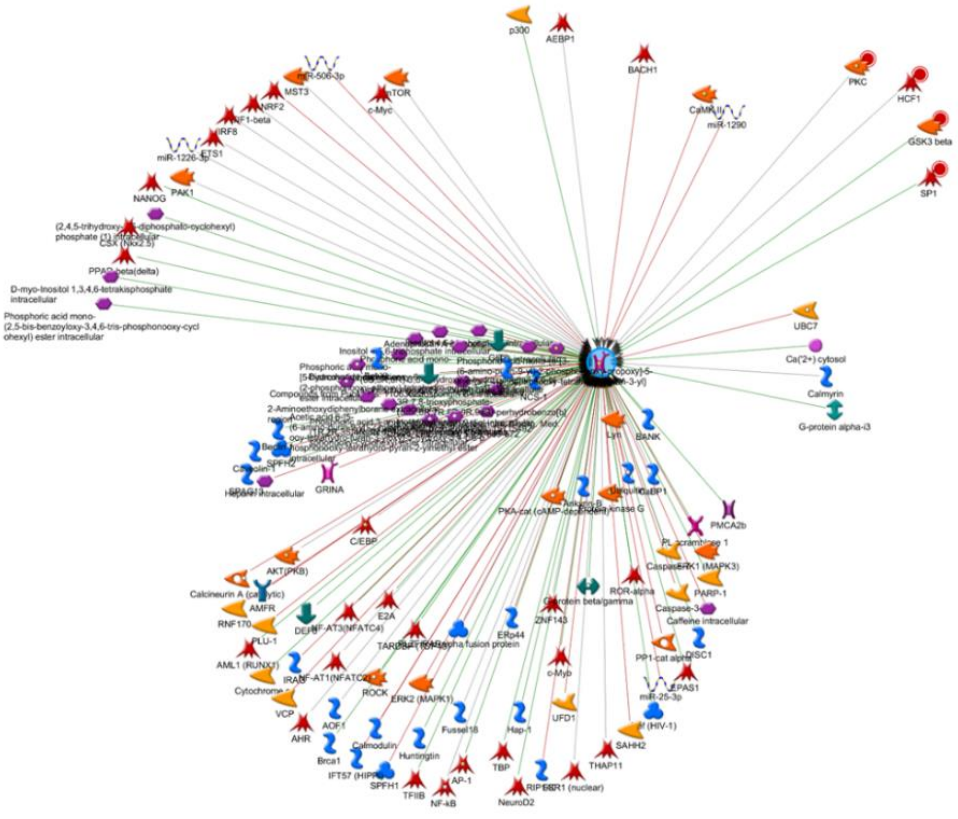


B

Protein (Gene name)	Fold Change A769662	P-Site	Interaction mechanisms	Effect	Reference (PMID Number)
C-Myc	0.888	S72/T58	Transcription	Inhibition	17093053
ERK1/2 (MAPK1/3)	1.214	T185/ Y187	Phosphorylation	Inhibition	16925983; 16925983; 16979595, 16925983; 16925983; 16979595
HCF1	1.105	S1507	Transcription	Unspecified	20581084
SPAG13	1.187	S746	Binding	Activation	21420385; 21501587; 22992961

Figure 36. Network showing direct upstream IP_3R interactors significantly phospho-regulated in the presence of AMPK modulators. The central node (object) of the network corresponds to the IP_3R. The rest of the object represents direct upstream interactors previously curated in the Metacore® database. Significant regulated objects at the phosphorylation level in presence of A669662 are indicated with red spots on the upper right corner. Panel B lists the significant regulated interactors, indicating the phosphorylation fold change, the P-site involved, the mechanism of interaction, the effect and some references.

A



B

Protein (Gene Name)	Fold Change Dorsomorphin	P-Site	Interaction mechanisms	Effect	Reference (PMID Number)
SP1	1.075	S59	Transcription	Activation	19666470; 20096353
PKCα	0.883	S226	Phosphorylation	Activation/ Inhibition	17332533; 17332533; 17320950; 15184066
HCFC1	1.056	S1507	Transcription	Unspecified	20581084
GSK3B	0.899	S9	Phosphorylation	Activation	26206086

Figure 37. Network showing direct upstream IP_3R interactors significantly phospho-regulated in the presence of AMPK modulators. The central node (object) of the network corresponds to the IP_3R. The rest of the object represents direct upstream interactors previously curated in the Metacore® database. Significant regulated objects at the phosphorylation level in presence of dorsomorphin are indicated with red spots on the upper right corner. Panel B lists the significant regulated interactors, indicating the phosphorylation fold change, the P-site involved, the mechanism of interaction, the effect and some references.

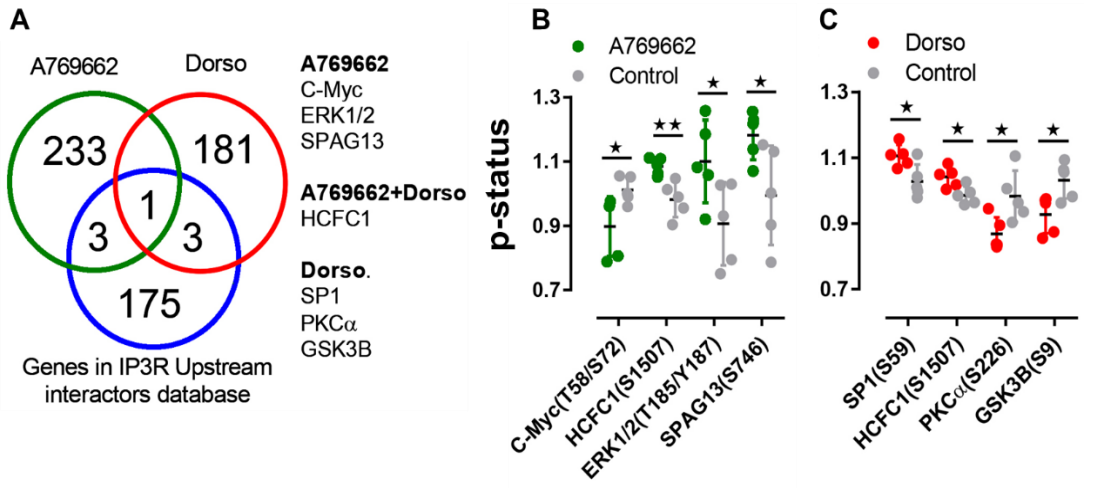


Figure 38. Identification of upstream IP$_3$R interactors significantly regulated in the presence of A769662 and Dorsomorphin. A) Identification of upstream IP$_3$R interactors significantly regulated in the presence of A769662 and Dorsomorphin (Venn diagram). The genes corresponding to the intersections are mentioned on the right, and panel B and C show the magnitude of the changes in those genes. B) Effect of A769662 on the phosphorylation status of c-Myc, HCF1, Erk1/2 and SPAG13. C) Effect of dorsomorphin on the phosphorylation status of SP1, HCFC1, PKCα and GSK.

An additional Gene Ontology study was performed applying Metacore (Thompson Reuters) to reveal proteins related to Ca^{2+} signalling that become phospho-regulated in the presence of A769662 or dorsomorphin. Some of them are directly related with the regulation of Ca^{2+} release from the endoplasmic/sarcoplasmic reticulum (Figure 39A). Among them, it is worth mentioning the phospho-regulation of phosphodiesterase 3A (PDE3A) (Maurice et al., 2014), which becomes phosphorylated in the presence of A769662 (1.2534 fold, S570) and dephosphorylates in the presence of dorsomorphin (0.84 fold, S408) (Figure 39B).

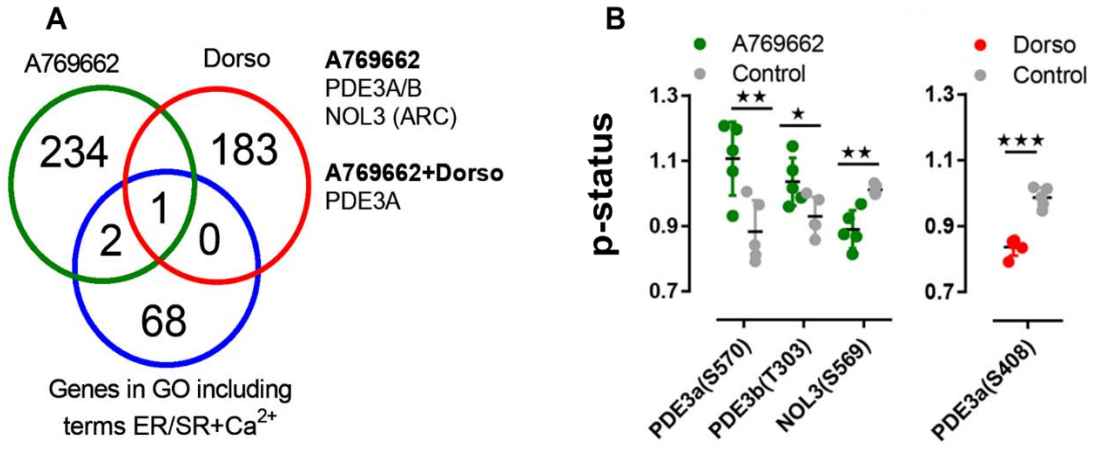


Figure 39. Identification of Dorsomorphin and A769662 phospho-regulated proteins in specific gene ontologies containing the words "Endoplasmic Reticulum + Calcium" (Metacore®). A) Identification of genes belonging to gene ontologies including the terms: Endoplasmic reticulum / Sarcoplasmic reticulum and calcium, and significantly regulated in presence of A769662 and Dorsomorphin (Venn diagram). B) Effect of A769662 and dorsomorphin on the phosphorylation status of PDE3.

2. EFFECT OF AMPK MODULATORS ON THE LONGEVITY OF THE NEMATODE *C. ELEGANS*

Many researchers have proposed the use of invertebrate model organisms as a way to connect the research made in cell cultures and in mammals (Kaletta and Hengartner, 2006). Experiments with these invertebrate models provide more complex data than those obtained from human cell cultures, in a relatively short time and at a lower price compared to experiments with mammalian models.

The *C. elegans* model has been used in this work to study the effects of the compounds modulators of the AMPK pathway on *C. elegans* longevity. The term longevity comprises two different concepts: maximal and mean longevity. Mean longevity refers to the mean life expectancy of individuals of a given species while maximal longevity is the maximum age of any individual of a given species that can be recorded (Viña et al., 2007). The objective of this work was to advance in the research on whether AMPK modulators may affect the mean longevity of the worm *C. elegans.*

2.1. AMPK inhibitor dorsomorphin slightly extends *C. elegans* lifespan at very low concentrations

AMPK is a key regulator of energy homeostasis almost universally expressed in eukaryotic cells where it coordinates the metabolic pathways by balancing the supply of nutrients with energy demand.

In mammalians, AMPK is composed of two isoforms α, two β and three γ, and it has been shown that α1 knockout mice shows no phenotype, whereas in α2 knockout mice, insulin secretion is decreased (Viollet et al., 2006). In *C. elegans*, AMPK is encoded by two catalytic subunits, *aak-1* and *aak-2*, which are 52 % and 40 % identical to human AMPKα1, respectively. In addition, the kinase domains of AAK-1 and AAK-2 share 80 % and 71 % amino acid identity, with the AMPKα1 human subunit, respectively. It has also been shown that an *aak-2* mutation reduces the life of the worm by almost 12 % and produces hypersensitivity to heat shock and to a mitochondrial venom. However, a phenotype for aak-1 has not yet been reported (Apfeld et al., 2004).

The AMPK is activated by a great variety of conditions that diminish the concentrations of ATP and increase the AMP ones. This is the case of oxidative stress (Hardie, 2004) or exercise, which cause metabolic stress and activate AMPK under physiological conditions (Winder and Hardie, 1996). However, current research has

focused mainly on the search for AMPK activators that improve the healthspan and lifespan. We have used here both the activator A769662 and the inhibitor dorsomorphin to test their effect on the lifespan of the *C. elegans* worms.

Due to the poor water solubility of these compounds, γ-CD inclusion compounds were used for the administration of drugs to worms. The drugs were enclosed in a chemical cover of γ-CD, forming an inclusion compound (Kashima et al., 2012), which was then added to the plates together with the *E. coli OP50* (see Methods and Materials). Young adults (day 1) were then transferred to these plates to begin the lifespan assay. In this way, worms ingest the inclusion compound through the pharynx together with the food, and the active inhibitor or activator are then released directly into the digestive tube.

Table 9 shows the results of a series of lifespan assays performed with four amounts of dorsomorphin γ-CD inclusion compounds. Figure 40 shows representative lifespan assays performed in each of the conditions. The plots correspond to the assays labelled in bold in Table 9. Panels A-D show the effect of four different amounts of dorsomorphin: 2, 10, 20 and 50 µg of the inclusion compound. Panel E shows a comparison of the mean increase in survival obtained in each condition.

The maximum effect was reached when 10 µg of the dorsomorphin inclusion compound was added to the plate, and for this concentration all the lifespan assays were highly significant and produced increases in survival between 6.34 and 24.09 % (mean 11.83 ± 2.2 %). The effects were similar when 20 µg of the dorsomorphin inclusion compound were used, but concentrations below or above those produced a much smaller effect.

Treatment with dorsomorphin γ-CD inclusion compounds

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
Dorsomorphin 2 µg	22,4	74/102	22,0	84/101	2,1	0,434	3,2 ± 2,0
	23,0	**74/110**	**22,4**	**80/100**	**2,4**	**0,96**	
	21,5	88/104	21,6	94/107	-0,6	0,233	
	24,7	89/101	22,7	96/100	8,9	<0,0001	
Dorsomorphin 10 µg	23,3	82/98	20,1	75/96	15,9	<0,0001	11,8 ± 2,2
	22,4	78/101	20,8	80/99	7,8	<0,016	
	22,8	72/98	19,8	78/99	15,4	<0,0001	
	21,3	82/102	20,0	82/100	6,3	<0,007	
	23,9	51/60	19,3	113/124	24,1	<0,0001	
	26,1	77/83	24,6	79/80	6,1	<0,048	
	23,7	**56/58**	**21,2**	**120/120**	**11,6**	**<0,0001**	
	24,1	106/112	22,5	119/128	7,5	<0,0001	
Dorsomorphin 20 µg	23,4	56/79	20,1	75/96	16,5	<0,0001	11,5 ± 2,4
	22,1	74/97	20,8	80/99	6,1	0,174	
	20,7	63/99	19,8	78/99	4,8	0,203	
	21,7	**72/97**	**20,0**	**82/100**	**8,1**	**<0,004**	
	24,6	82/99	20,8	91/100	18,4	<0,0001	
	23,7	94/99	20,5	100/101	15,1	<0,0001	
Dorsomorphin 50 µg	**21,0**	**75/100**	**22,0**	**84/101**	**-4,5**	**0,393**	1,0 ± 3,7
	23,9	74/87	22,4	80/100	6,7	<0,035	
	20,3	95/106	21,6	94/107	-6,2	<0,004	
	24,5	90/103	22,7	96/100	7,9	<0,0001	

Table 9. Lifespan assays performed with dorsomorphin γ-CD inclusion compounds in wild-type worms. The table shows the amount of drug used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug amount (in µg for γ-CD compounds). In bold, series shown in the survival plots of Figure 40.

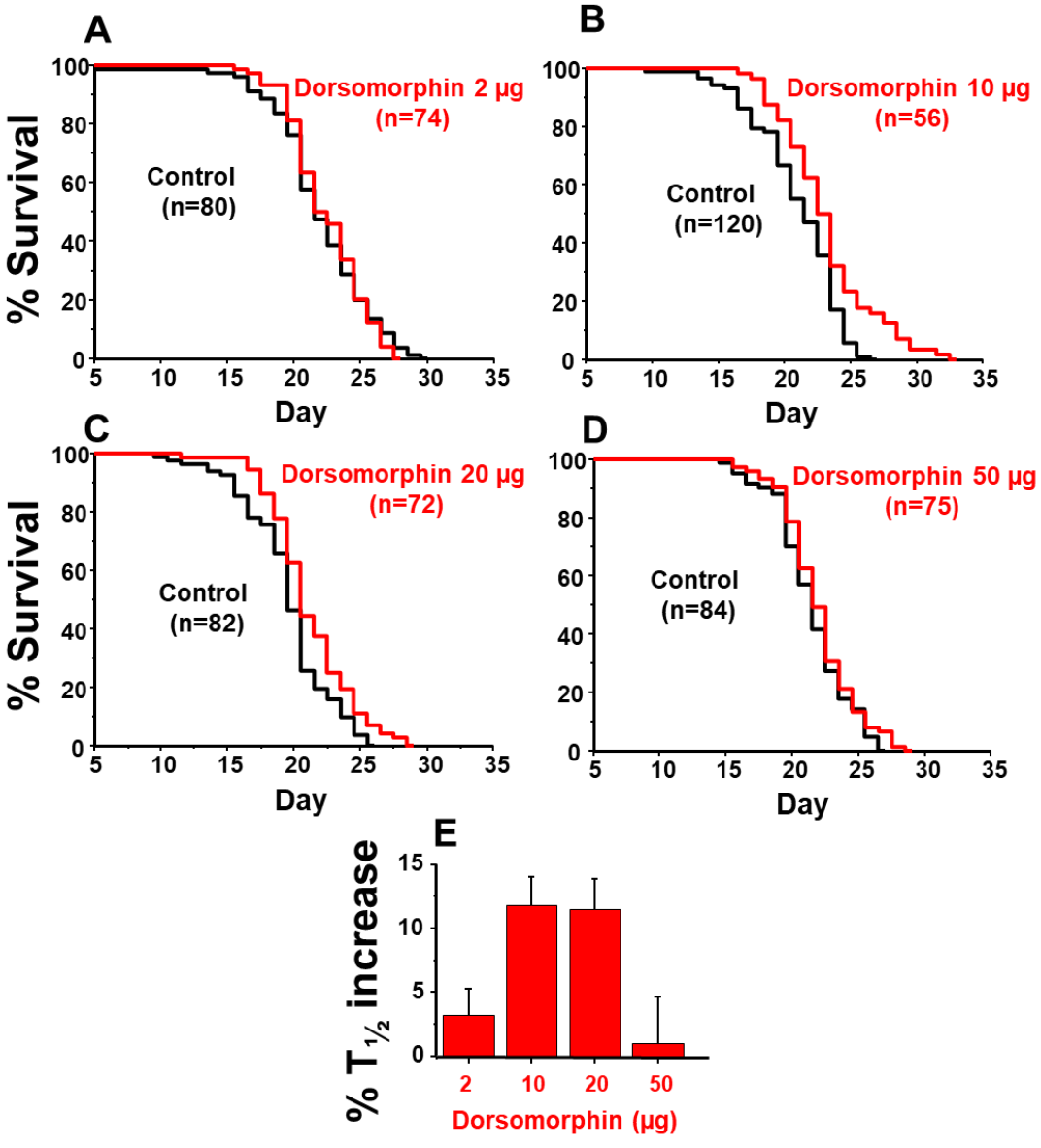


Figure 40. Effects of dorsomorphin γ-CD inclusion compounds on survival in *C. elegans*. Panels A-D show representative survival plots corresponding to parallel lifespan assays performed using γ-CD compounds in the following conditions: Control/dorsomorphin (A: 2 µg; B: 10 µg; C: 20 µg; D: 50 µg). The assays shown correspond to those marked in bold in Table 9. Panel E shows the mean increase in survival obtained in several similar lifespan assays of each kind (more details of all the assays in Table 9).

Despite the low water solubility of this compound, we have also tested the effect of different concentrations of dorsomorphin directly dissolved in the NGM agar after strong stirring: 2, 5, 10, 50, 100 and 250 µM (Table 10 and Figure 41).

Treatment with dorsomorphin

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
Dorsomorphin 2 µM	18,3	82/105	16,0	97/101	14,6	<0,0001	7,1 ± 3,06
	16,5	96/110	16,6	92/102	-0,4	0,867	
	19,3	77/103	18,0	82/100	7,1	0,008	
	18,3	**82/105**	**17,1**	**76/86**	**7,0**	**<0,018**	
Dorsomorphin 5 µM	19,1	88/102	16,0	97/101	19,4	<0,0001	12,9 ± 2,2
	18,3	83/101	16,6	92/102	10,5	<0,001	
	19,9	93/106	18,0	82/100	10,2	<0,0001	
	19,1	**88/102**	**17,1**	**76/86**	**11,5**	**<0,0001**	
Dorsomorphin 10 µM	24,2	81/100	21,7	100/110	11,9	<0,0001	12,2 ± 1,6
	17,2	85/104	16,0	97/101	7,8	0,086	
	19,9	97/104	16,6	92/102	20,1	<0,0001	
	20,2	87/107	18,0	82/100	11,8	<0,0001	
	19,3	**78/100**	**17,1**	**76/86**	**12,9**	**<0,0001**	
	20,3	100/111	17,9	116/128	13,3	<0,0001	
	20,1	92/113	18,8	100/114	7,3	<0,0001	
Dorsomorphin 50 µM	**19,6**	**96/100**	**21,7**	**100/110**	**-9,7**	**<0,0001**	-6,5 ± 1,8
	17,3	99/101	17,9	116/128	-3,3	<0,003	
	17,5	142/144	18,8	100/114	-6,5	<0,0001	
Dorsomorphin 100 µM	17,8	95/100	20,1	97/100	-11,6	<0,0001	-17,4 ± 2,3
	16,8	103/103	21,7	100/110	-22,4	<0,0001	
	14,9	117/118	17,9	116/128	-16,8	<0,0001	
	15,2	**116/116**	**18,8**	**100/114**	**-19,0**	**<0,0001**	
Dorsomorphin 250 µM	16,8	77/100	20,1	97/100	-16,4	<0,0001	-23,3 ± 3,7
	13,5	**113/113**	**17,9**	**116/128**	**-24,4**	**<0,0001**	
	13,3	125/125	18,8	100/114	-29,0	<0,0001	

Table 10. Lifespan assays performed with dorsomorphin in wild-type worms. The table shows the drug concentration used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug concentration. Concentrations are in µM for compound dorsomorphin dissolved in NGM agar. In bold, series shown in the survival plots of Figure 41.

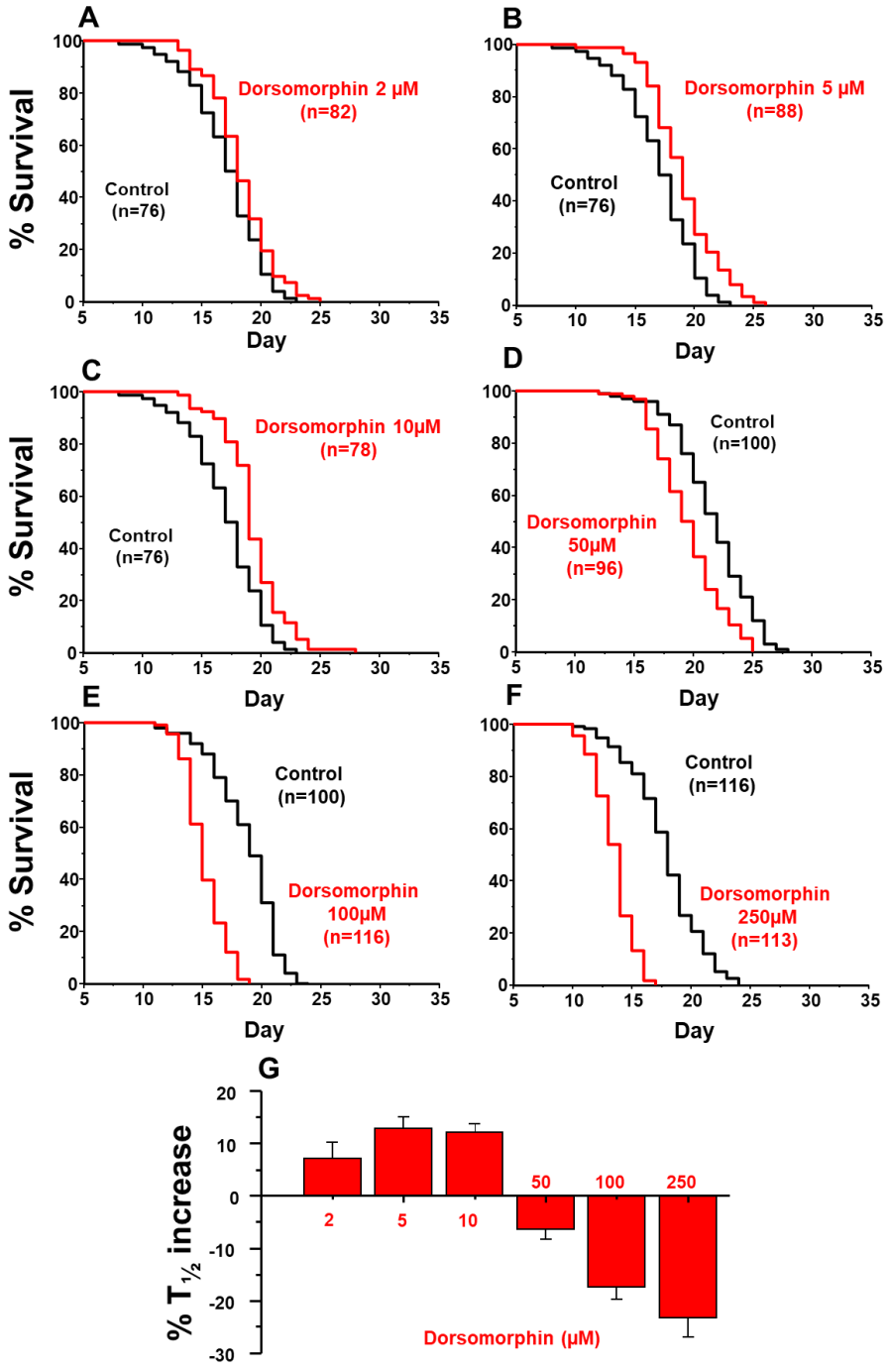


Figure 41. Effects of dorsomorphin on survival in *C. elegans*. Panels A-F show representative survival plots corresponding to parallel lifespan assays in which dorsomorphin was dissolved in the NGM agar in the following conditions: Control/dorsomorphin (A: 2 µM; B: 5 µM; C: 10 µM; D: 50 µM; E: 100 µM; F: 250 µM). The assays shown correspond to those marked in bold in Table 10. Panel G shows the mean increase in survival obtained in a series of lifespan assays of each kind as those shown in the figure (more details of each assay in Table 10).

We found that feeding worms from day 1 of adult until death with dorsomorphin at a concentration of 2, 5 and 10 μM extended lifespan by 7.08, 12.89 and 12.17 %, respectively with high statistical significance. However, higher concentrations, such as 50, 100 or 250 μM, reduced the mean lifespan of the worms in a dose-dependent manner (Figure 41).

Small concentrations of dorsomorphin (5-10 μM) dissolved in the NGM agar produced an increase in the *C. elegans* lifespan of similar magnitude to that induced by 10 or 20 μg of the dorsomorphin γ-CD compounds, demonstrating that the effect is not dependent on the method of drug administration (Figure 40 and Figure 41). Panel G shows a comparison of the mean increase in survival obtained in each condition when dorsomorphin was dissolved in the agar.

When the concentration of dorsomorphin in the agar was further increased, we observed an increase in the death rate and a consequent decrease in worm lifespan, in a dose-dependent manner. It can be observed that the mortality curve shifts to the left, the more the bigger it is the concentration of dorsomorphin. These results suggest that inhibition of AMPK by dorsomorphin negatively affects longevity in *C. elegans* worms, as might be expected. Nevertheless, dorsomorphin was able to extend the lifespan of *C. elegans* in a certain lower concentration range, but this effect is likely to be independent of the inhibition of AMPK.

2.2. AMPK activator A769662 does not extend *C. elegans* lifespan

Compound A769662 was developed as a potent activator of AMPK, and we have then tested whether it was capable of extending the lifespan of *C. elegans* worms.

Table 11 shows the results of a series of lifespan assays performed with several concentrations of the AMPK activator A769662. Figure 42 shows representative lifespan assays performed in each of the conditions. The plots correspond to the assays labelled in bold in Table 11. Panels A-C show the effect of three different amounts of A769662-γCD inclusion compound: 100 μg, 500 μg and 1 mg. Panel D shows a comparison of the mean increase in survival obtained in every condition.

Treatment with A769662 γ-CD inclusion compounds

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
A769662 100 µg	**22,4**	**74/103**	**21,8**	**84/101**	**3,1**	**0,317**	**1,9 ± 2,0**
	23,4	84/104	22,4	80/100	4,2	0,167	
	20,5	89/111	21,6	94/107	-5,1	0,602	
	23,9	84/100	22,7	96/100	5,4	<0,014	
A769662 500 µg	21,7	110/118	24,6	79/80	-11,6	<0,0001	**-4,2 ± 3,8**
	21,5	87/90	21,2	120/120	1,5	0,853	
	21,9	**97/106**	**22,5**	**119/128**	**-2,4**	**0,226**	
A769662 1 mg	**20,5**	**81/101**	**20,8**	**91/100**	**-1,5**	**0,079**	**4,8 ± 6,3**
	22,8	85/93	20,5	100/101	11,1	0,066	

Table 11. Lifespan assays performed with A769662 γ-CD inclusion compounds in wild-type worms. The table shows the drug amount used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug amount (in µg for γ-CD compounds). In bold, series shown in the survival plots of Figure 42.

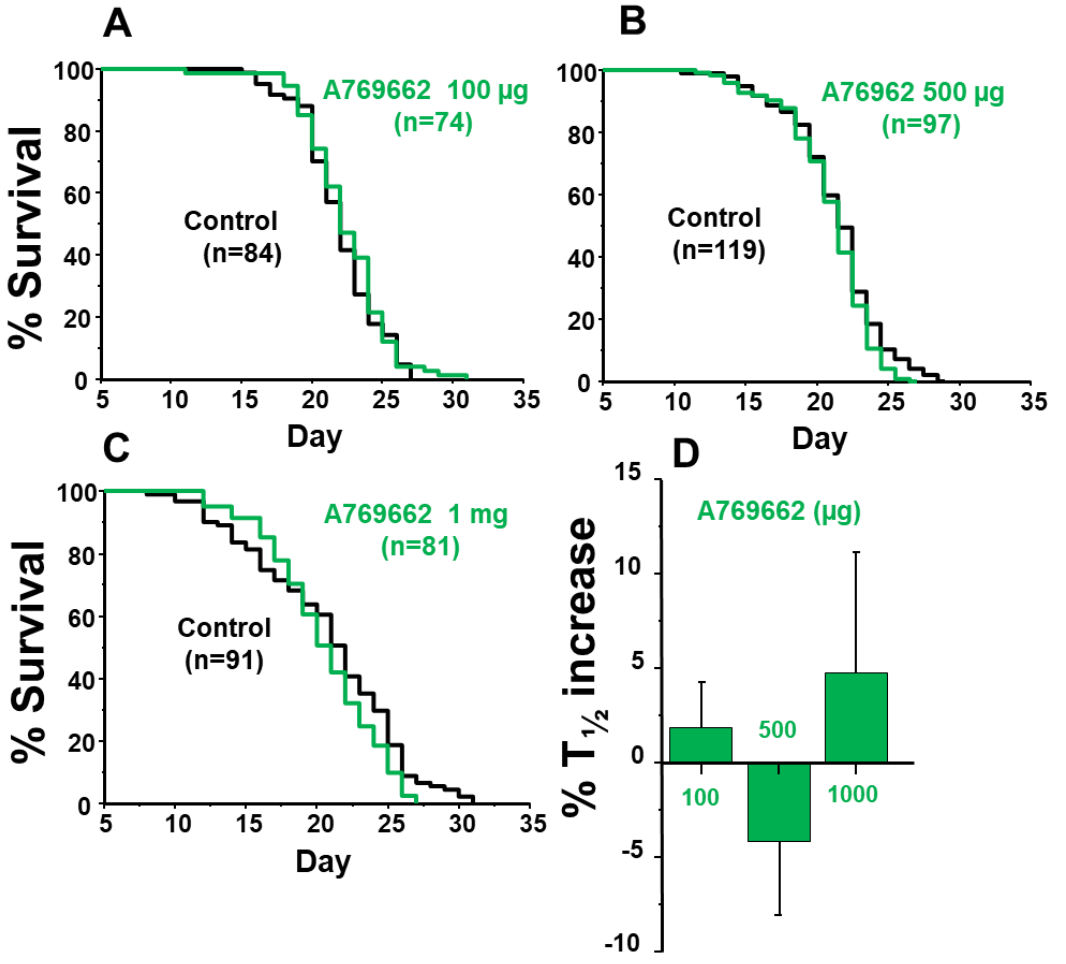


Figure 42. Effects of A769662 γ-CD inclusion compounds on survival in *C. elegans*. Panels A-C show representative survival plots corresponding to parallel lifespan assays performed using γ-CD compounds in the following conditions: Control/A769662 (A: 100 µg; B: 500 µg; C: 1 mg). The assays shown correspond to those marked in bold in Table 11. Panel D shows the mean increase in survival obtained in several similar lifespan assays of each kind (more details of all the assays in Table 11).

Contrary to expectations, the AMPK activator, A769662, did not produce an increase in the lifespan of the worms at any of the concentrations tested.

In order to exclude that the method of drug administration could be influencing the expected results on the longevity of *C. elegans*, the activator was directly dissolved in the agar, just like the dorsomorphin.

However, we found that treating worms from day 1 of adult until death with A769662 γ-CD compounds at concentrations of 100 µM, 250 µM and 500 µM did not significantly modify the life expectancy of the worms (Table 12, Figure 43).

Treatment with A769662

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
A769662 100 µM	20,1	86/99	20,1	97/100	0,2	0,143	**-3,0 ± 5,1**
	15,6	109/114	17,9	116/128	-13,0	<0,0001	
	19,5	**102/111**	**18,8**	**100/114**	**3,7**	**<0,021**	
A769662 250 µM	20,9	91/100	20,1	97/100	4,1	0,234	**3,0 ± 1,0**
	18,1	**112/112**	**17,9**	**116/128**	**1,0**	**0,258**	
	19,5	113/113	18,8	100/114	3,9	0,074	
A769662 500 µM	22,2	89/100	20,1	97/100	10,5	<0,004	**-0,6 ± 5,6**
	16,7	112/113	17,9	116/128	-6,5	<0,0001	
	17,6	**118/120**	**18,8**	**100/114**	**-5,9**	**<0,01**	

Table 12. Lifespan assays performed with A769662 in wild-type worms. The table shows the drug concentration used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug concentration. Concentrations are in µM for compound A769662 dissolved in NGM agar. In bold, series shown in the survival plots of Figure 43.

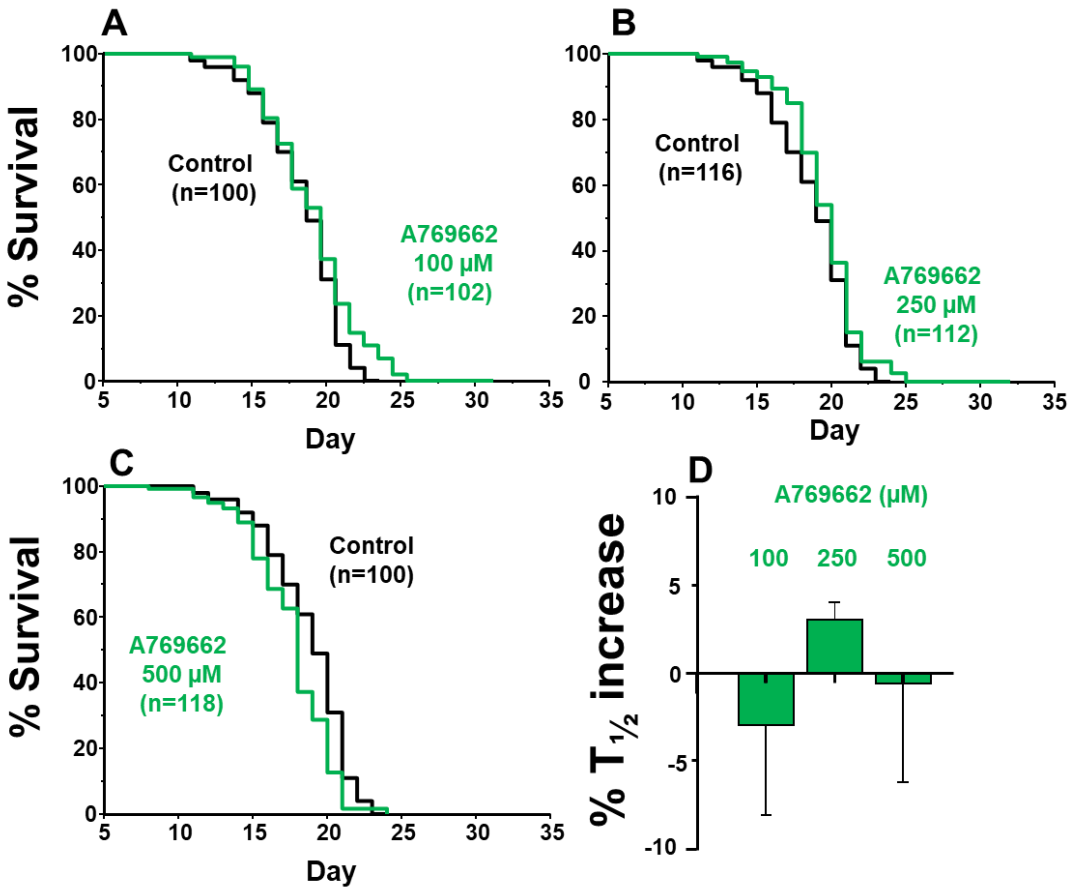


Figure 43. Effects of A769662 on survival in *C. elegans*. Panels A-C show representative survival plots corresponding to parallel lifespan assays in which A769662 was dissolved in the NGM agar in the following conditions: Control/A769662 (A: 100 µM; B: 250 µM; C: 500 µM). The assays shown correspond to those marked in bold in Table 12. Panel D shows the mean increase in survival obtained in a series of lifespan assays of each kind as those shown in the figure (more details of each assay in Table 12).

These results indicate that, contrary to expectations, the AMPK activator A769662 does not extend *C. elegans* lifespan. The lack of effect of this AMPK activator may be due to the fact that, in contrast to other pharmacological activators of AMPK, A769662 directly activates AMPK through AMPK β1 subunit phosphorylation (Scott et al., 2014). Therefore, although A769662 has been described as a potent activator of AMPK, its lack of effect on *C. elegans* longevity may be due to the fact that these nematodes only have a catalytic α-subunit and not the β regulatory subunit, which is the target of this compound.

2.3. BMPR inhibitors extend *C. elegans* lifespan

Although we did not found positive effects of treatment or feeding with AMPK activator on the longevity of *C. elegans*, we decided to continue with a deeper investigation of the effect of low concentrations of dorsomorphin on *C. elegans* longevity.

To do so, some dorsomorphin analogues and selective inhibitors of bone morphogenic protein type-I receptors (BMPRs) were chosen to verify whether the small but significant increase in *C. elegans* longevity mediated by dorsomorphin treatment could be related to the activity of BMPRs.

In addition to the BMPRs inhibitors DMH-1 and ML-347, previously used in the Ca^{2+} measurement experiments, another selective inhibitor of TGF-β signalling that has no effect on BMPs signalling, SB-431542, was used (Inman et al., 2002). Regarding the concentrations, we tested those that proved to be more effective for dorsomorphin, 10 µg and 20 µg of compound.

All three compounds increased the lifespan of the worms at the two concentrations tested. Table 13 shows the results of a series of lifespan assays performed with two amounts (10µg and 20µg) of γCD-enclosed selective BMPRs/TGF-β inhibitors.

Treatment with BMPR inhibitors

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
ML-347 10 µg	23,5	91/101	19,3	113/124	21,9	<0,0001	9,7 ± 4,1
	25,6	64/67	24,6	79/80	4,1	0,505	
	22,9	**127/128**	**21,2**	**120/120**	**8,1**	**<0,003**	
	23,5	84/86	22,5	119/128	4,7	<0,028	
ML-347 20 µg	23,4	85/99	20,8	91/100	12,5	<0,004	15,1 ± 2,6
	24,2	**95/100**	**20,5**	**100/101**	**17,8**	**<0,0001**	
SB-431542 10 µg	21,3	81/82	19,3	113/124	10,2	0,296	4,2 ± 3,6
	25,2	98/101	24,6	79/80	2,5	0,995	
	23,3	**91/94**	**21,2**	**120/120**	**9,6**	**<0,001**	
	21,3	81/82	22,5	119/128	-5,4	<0,001	
SB-431542 20 µg	22,4	76/100	20,8	91/100	7,7	<0,041	9,7 ± 2,0
	23,0	**88/102**	**20,5**	**100/101**	**11,8**	**<0,0001**	
DMH-1 10 µg	25,3	76/80	19,3	113/124	31,0	<0,0001	11,7 ± 6,8
	25,8	120/128	24,6	79/80	4,8	0,341	
	23,6	**118/121**	**21,2**	**120/120**	**11,0**	**<0,0001**	
	22,5	83/84	22,5	119/128	0,1	0,392	
DMH-1 20 µg	23,1	94/99	20,8	91/100	11,4	<0,011	13,1 ± 1,7
	23,6	**94/98**	**20,5**	**100/101**	**14,9**	**<0,0001**	

Table 13. Lifespan assays performed with BMPR inhibitors in wild-type worms. The table shows the drug amount used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug amount (in µg for γ-cyclodextrin-inclusion compounds). In bold, series shown in the survival plots of Figure 44.

Figure 44 shows plots of typical lifespan assays obtained for each condition. The plots correspond to the assays labelled in bold in Table 13. Panels A and B show the effect of two different amounts of ML-347 γ-CD inclusion compound at 10 µg and 20 µg and the Panels C and D and E and F, the same amounts of the γ-CD inclusion compounds of SB-431542 and DMH-1, respectively. Panel G shows a comparison of the mean increase in survival obtained in every condition.

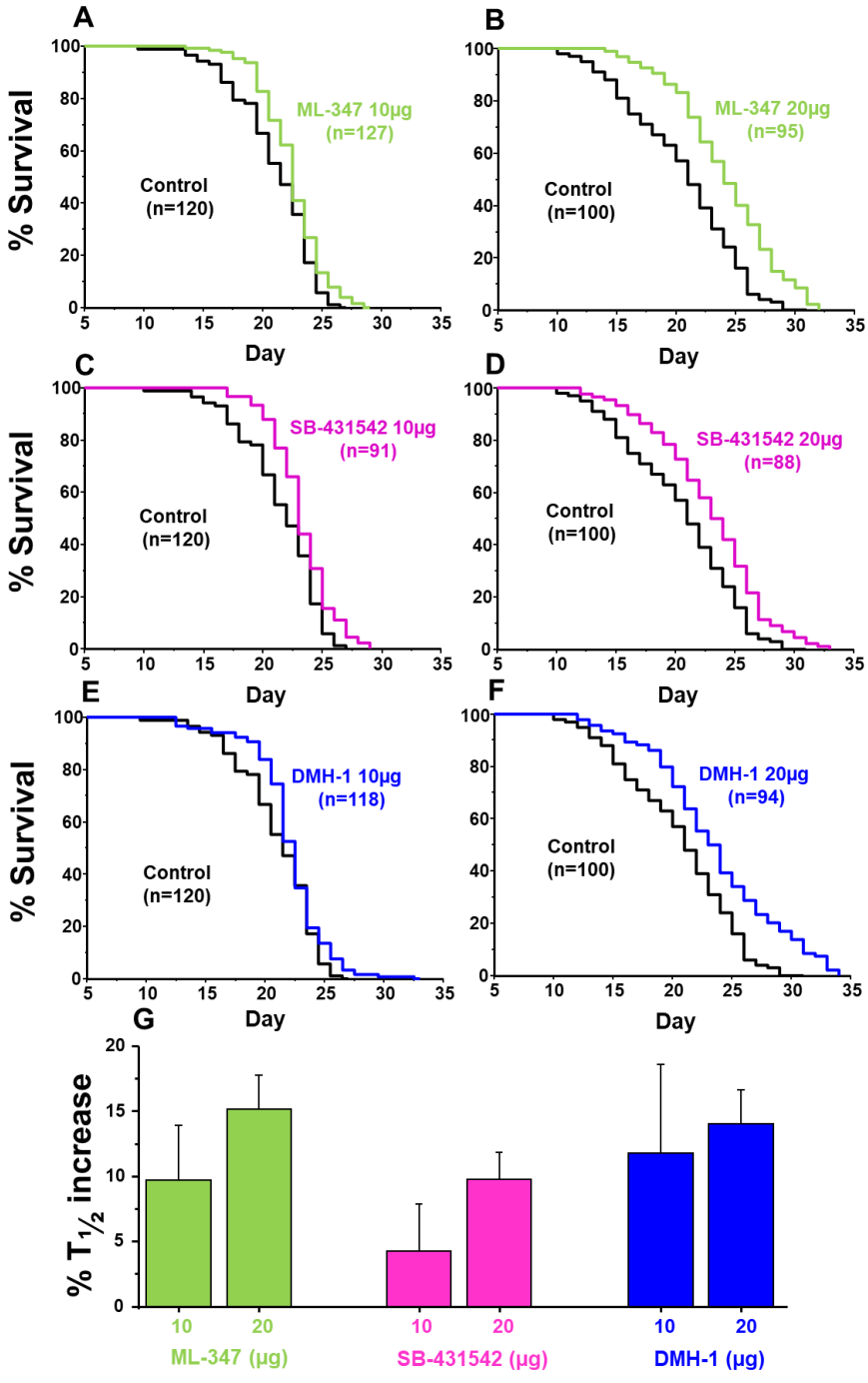


Figure 44. Effects of BMPR inhibitors on survival in *C. elegans*. Panels A-F show representative survival plots corresponding to parallel lifespan assays performed using γ-CD compounds in the following conditions: Control/ML-347 (A: 10 µg; B: 20 µg), Control/SB-431542 (C: 10 µg; D: 20 µg) and Control/DMH-1 (E: 10 µg; F: 20 µg). The assays shown correspond to those marked in bold in Table 13. Panel G shows the mean increase in survival obtained in several similar lifespan assays of each kind (more details of all the assays in Table 13).

The maximum lifespan extension was obtained at 20 µg, with all the three γ-CD compounds, and the increase in lifespan was about 10-15 %. The lower amount (10 µg) produced a much smaller effect in all the cases. Therefore, all the BMPRs inhibitors were able to extend the *C. elegans* lifespan similarly to dorsomorphin, suggesting that the increase in longevity due to treatment or feeding with dorsomorphin could be mediated by BMPRs inhibition.

Dorsomorphin has been used in a large number of works as an AMPK inhibitor and is also well known as a BMPR inhibitor. However, other studies have shown that dorsomorphin may also have additional effects. For example, it has been shown that dorsomorphin was able to induce autophagy in cancer cells through Akt inhibition (Vucicevic et al., 2011). Interestingly, one of the analogues used, DMH-1 (dorsomorphin homologue 1), initially developed as a selective BMPRs inhibitor, has also been shown to act on AKT in L6 cells, but in this case through its activation (Xie et al., 2014).

To investigate the mechanism of the effects of dorsomorphin and the BMPR inhibitors on the worm longevity, we first studied the effect of DMH-1 directly dissolved in NGM agar on *C. elegans* longevity.

2.4. The selective BMPR inhibitor DMH-1 extends *C. elegans* lifespan

We decided to use DMH-1 because it has been described to block BMPR signalling by targeting the intracellular kinase domain of BMP type I receptors (Hao et al., 2010). As a consequence, it reduces lung cancer cell proliferation and promotes cell death, offering a promising novel strategy for lung cancer treatment (Hao et al., 2014). However, we have to take into account that DMH-1 has been reported to have other effects. For example it has been shown to promote neurogenesis of human-induced pluripotent stem cells (hiPSCs) (Neely et al., 2012), to increase the number of cardiomyocyte progenitors (Ao et al., 2012) or to increase glucose metabolism by AKT activation mediated by the inhibition of PP2A activity in rat L6 cells (Xie et al., 2014).

Although DMH-1 and dorsomorphin have a similar chemical structure (Hao et al., 2010), these compounds exert a different action on AMPK and AKT. Therefore, we wanted to study and compare the effects on *C. elegans* longevity of DMH-1 and dorsomorphin.

Table 14 shows the results of a series of lifespan assays performed with several concentrations of DMH-1 dissolved in the agar. Figure 45 shows plots of typical lifespan assays obtained for each condition. The plots correspond to the assays labelled in bold in Table 14. Panels A-F show the effect of six concentrations of DMH-1 (2, 5, 10, 50, 100 and 250 µM) directly dissolved in NGM agar. Panel G shows a comparison of the mean increase in survival obtained in each condition.

Treatment with DMH-1

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
DMH-1 2 µM	18,2	96/103	16,0	97/101	13,9	<0,0001	17,8 ± 4,3
	17,8	98/110	16,6	92/102	7,6	0,09	
	22,2	**84/104**	**18,0**	**82/100**	**23,0**	**<0,0001**	
	21,7	63/70	17,1	76/86	26,6	<0,0001	
DMH-1 5 µM	17,6	91/103	16,0	97/101	10,2	<0,002	17,8 ± 3,1
	20,7	87/109	16,6	92/102	24,7	<0,0001	
	21,7	**81/102**	**18,0**	**82/100**	**20,0**	**<0,0001**	
	19,9	88/102	17,1	76/86	16,4	<0,0001	
DMH-1 10 µM	24,6	87/101	21,7	100/110	13,4	<0,0001	18,3 ± 1,9
	21,8	**91/117**	**17,9**	**116/128**	**21,8**	**<0,0001**	
	21,3	63/69	18,8	100/114	13,4	<0,0001	
	17,7	93/103	16,0	97/101	10,7	<0,001	
	21,6	81/102	16,6	92/102	30,0	<0,0001	
	21,1	84/107	18,0	82/100	16,8	<0,0001	
	20,4	88/104	17,1	76/86	19,0	<0,0001	
	19,4	94/103	15,7	90/106	23,7	<0,0001	
	19,5	105/106	16,1	93/106	21,3	<0,0001	
	18,2	105/112	16,2	94/101	12,5	<0,0001	
DMH-1 50 µM	23,8	93/94	21,7	100/110	9,8	<0,0001	8,9 ± 1,5
	19,0	93/107	17,9	116/128	6,0	0,077	
	20,8	**63/72**	**18,8**	**100/114**	**11,0**	**<0,0001**	
DMH-1 100 µM	24,3	107/108	21,7	100/110	12,1	<0,0001	6,1 ± 3,2
	18,8	121/125	17,9	116/128	5,2	0,07	
	19,0	**76/79**	**18,8**	**100/114**	**1,1**	**0,283**	
DMH-1 250 µM	19,0	109/111	17,9	116/128	6,2	0,052	1,9 ± 4,3
	18,3	**115/115**	**18,8**	**100/114**	**-2,3**	**0,027**	

Table 14. Lifespan assays performed with selective inhibitor DMH-1 in wild-type worms. The table shows the drug concentration used in each series of assays, the half-life (T½) of the worms incubated with the drug obtained from the Kaplan-Meier analysis, the number of worms in the drug-containing assay (final/total), the half-life (T½) of the control worms, the number of worms in the control assay (final/total), the % increase in the half-life, the statistical significance of the difference between control and treated worms, obtained from the log-rank test, and the mean±s.e. increase in half-life from all the series made with the same drug concentration. Concentrations in µM for compound dissolved in NGM agar. In bold, series shown in the survival plots of Figure 45.

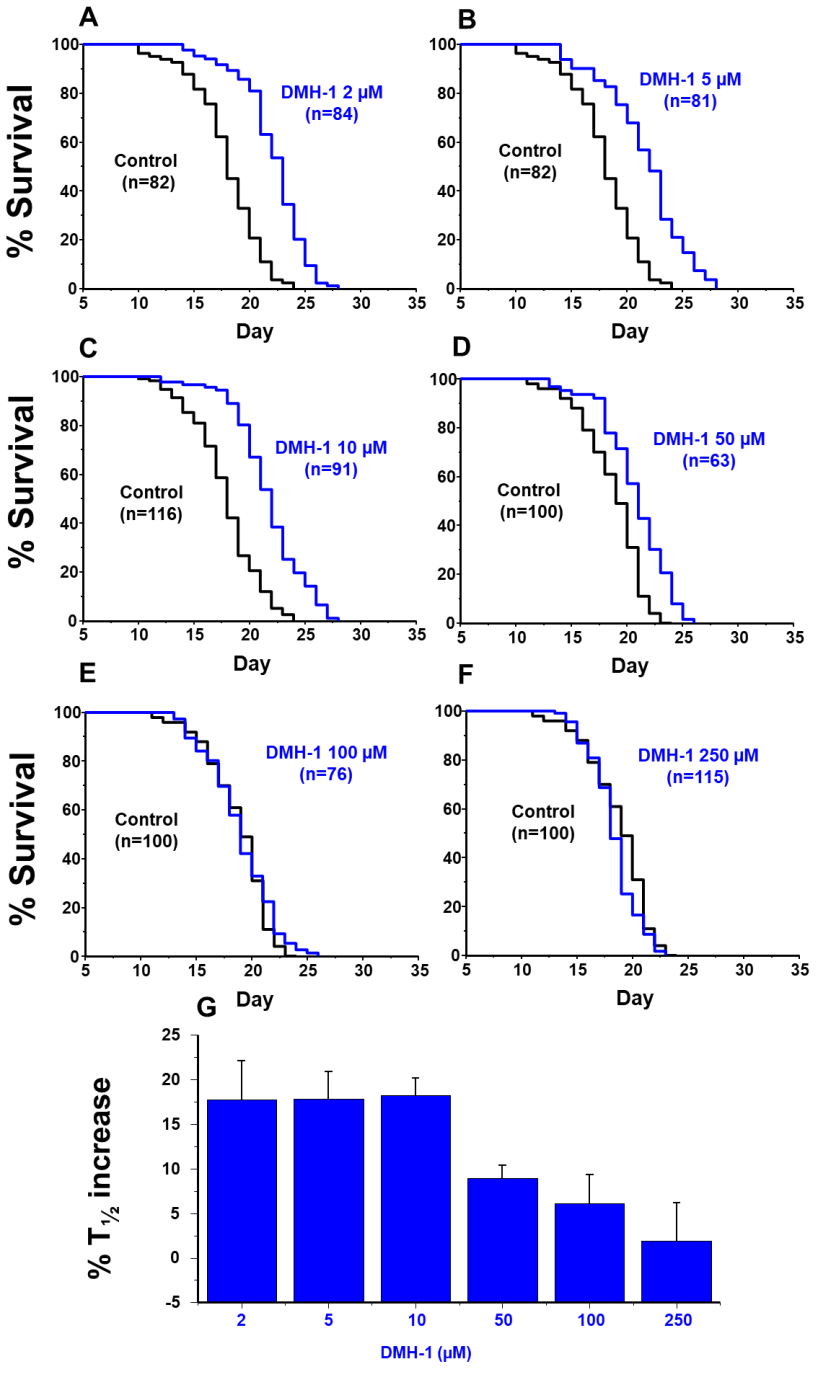


Figure 45. Effects of DMH-1 on survival in *C. elegans*. Panels A-F show representative survival plots corresponding to parallel lifespan assays in which DMH-1 was dissolved in the NGM agar in the following conditions: Control/DMH-1 (A: 2 µM; B: 5 µM; C: 10 µM; D: 50 µM; E: 100 µM; F: 250 µM). The assays shown correspond to those marked in bold in Table 14. Panel G shows the mean increase in survival obtained in a series of lifespan assays of each kind as those shown in the figure (more details of each assay in Table 14).

Our data show that concentrations of 2, 5 and 10 µM extended lifespan by 18 %. DMH-1 therefore produced a larger increase in lifespan than dorsomorphin at the same concentrations, although the effect was also maximum when 10 µM of the compound was dissolved in the agar. At this concentration (10 µM) all the lifespan assays were highly significant and produced increases in survival between 10.66 and 30.02 % (mean 18.3 ± 1.9 %). These effects of DMH-1 were also independent on the administration pathway, because similar effects were obtained when DMH-1 was directly added to the NGM agar or when the inclusion compound was used (see Figure 44 and Figure 45).

Concentrations of DMH-1 above 10 µM produced a progressively smaller effect or even no effect. Therefore, the effects on the lifespan were smaller at concentrations of DMH-1 above 10 µM but, unlike dorsomorphin, these concentrations (50, 100 and 250 mM) did not have negative effects on survival. Therefore, DMH-1 produced remarkable increases in survival of about 20 % in the 2-10 µM concentration range, and concentrations above these values produced a smaller, but not negative, effect on survival.

2.5. DMH-1 extends the lifespan in *aak-2* and *daf-2* mutants

The use of mutants is particularly interesting when testing compounds with potential medical use in order to determine their mechanism of action (Strange, 2006). Today, many mutant strains are available in the Caenorhabditis Genetics Center (CGC). To obtain more information about the possible mechanism of action of DMH-1 on *C. elegans* longevity, some mutant lines have been used.

First, to exclude that the mechanism of the increased lifespan in the presence of DMH-1 could be related to the AMPK pathway, we have studied the effects of DMH-1 on the survival of *aak-2* (*ok524*) mutants. The *aak-2* gene codes for the 5'-AMP-activated protein kinase catalytic subunit alpha-2. *aak-2* worms have a mutation in this gene and show reduced life span and a phenotype often correlated with oxidative stress (Apfeld et al., 2004).

A representative plot of such assays is shown in Figure 46A. The plot corresponds to the assay labelled in bold in Table 15. The results show that DMH-1 increased also the lifespan of *aak-2* worms with a similar magnitude to that found in wild-type worms. Specifically, 10 µM DMH-1 extended lifespan by 17 % with high statistical significance. Therefore, this suggests that the AMPK pathway is not involved in the mechanism of the lifespan increase induced by this compound.

Next, the *daf-2* mutant was used to discard that the effect of the DMH-1 on *C. elegans* longevity could be mediated by IGF-1 signalling. *daf-2* (*e1370*) mutants live more than twice as long as normal worms (Kenyon et al., 1993) as a consequence of a mutation in the insulin-like growth factor receptor (IGFR-1).

Treatment of *aak-2* and *daf-2* worms with DMH-1

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
aak-2							
DMH-1 10µM	**16,2**	**92/101**	**12,9**	**81/100**	**25,3**	**<0,0001**	**16,7 ± 4,2**
	15,7	81/104	14,0	80/104	11,9	<0,0001	
	14,2	56/100	12,6	73/100	13,1	<0,0001	
daf-2							
DMH-1 10µM	26,9	82/101	23,4	64/102	15,2	<0,0001	**11,8 ± 1,7**
	27,1	104/114	23,9	85/102	13,5	<0,0001	
	32,7	**67/101**	**29,4**	**67/91**	**11,4**	**<0,0001**	
	27,6	74/139	25,7	75/146	7,1	<0,004	

Table 15. Lifespan assays performed with CGP37157 in mutant *aak-2* or *daf-2 C. elegans* strains. The table shows a series of lifespan assays performed in either *aak-2* or *daf-2* mutant worms in the presence or in the absence of 10 µM DMH-1. In bold, series shown in the survival plots of Figure 46.

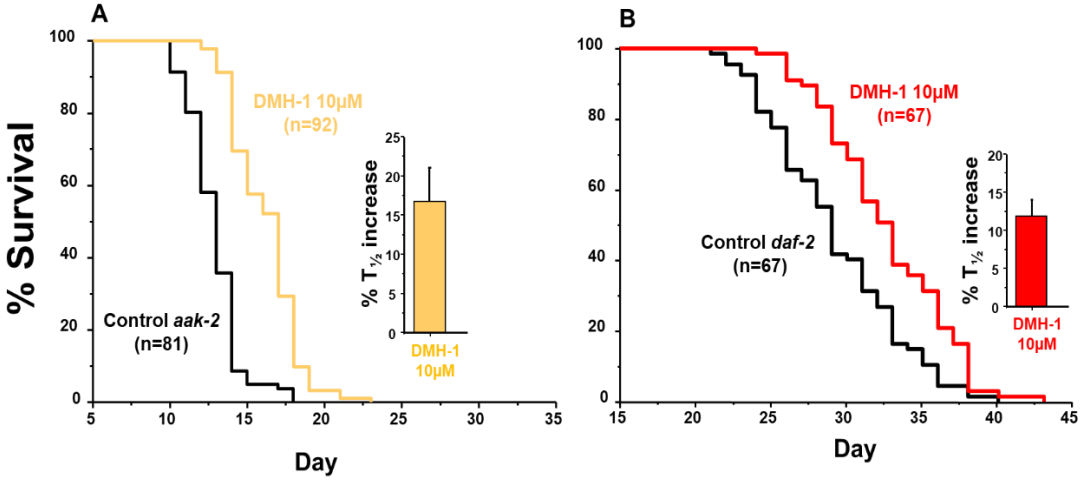


Figure 46. Effects of DMH-1 on survival in *aak-6* and *daf-2 C. elegans* mutants. Panels A and B show representative survival plots corresponding to parallel lifespan assays performed in *aak-2* (A) and *daf-2* (B) mutants in which 10 µM DMH-1 was dissolved in the NGM agar The assays shown correspond to those marked in bold in Table 15. The inserts show the mean increase in survival obtained in a series of lifespan assays of each kind as those shown in the figure (more details of each assay in Table 15).

Table 15 shows the results of a series of lifespan assays performed in *daf-2* mutants with the concentration of DMH-1 that was most effective in the control worms (10 µM). Then, if the effects of DMH-1 were mediated by the IIS pathway, we would expect it to produce little or no effect in *daf-2* mutants.

Figure 46B shows a plot of a typical lifespan assay obtained by treating *daf-2* mutants with 10 µM DMH-1. The plot corresponds to the assay labelled in bold in Table 15. Therefore, DMH-1 also extended the *C. elegans* lifespan in *daf-2* mutants, suggesting that the mechanism by which DMH-1 lengthens longevity is independent of IGF-1 signalling.

2.6. DMH-1 does not extend the lifespan in *eat-2* and *nuo-6* mutants

To investigate whether the mechanism of the increased lifespan in the presence of DMH-1 could be related to caloric restriction or mitochondrial dysfunction, we have studied the effects of DMH-1 on the survival of *eat-2* and *nuo-6* mutants.

eat-2 mutants have a reduced rate of pharyngeal pumping and an increased lifespan that is generally considered to be due to caloric restriction (Lakowski and Hekimi, 1998). So, if the effects of DMH-1 on *C. elegans* survival were mediated by caloric restriction, we would expect it to produce little or no effect in *eat-2* mutants. Table 16 shows the results of a series of lifespan assays performed in *eat-2 (ad1113)* mutants with the concentration of DMH-1 that was most effective in the controls (10 µM).

A representative plot of such assays is shown in Figure 47A. The plot corresponds to the assay labelled in bold in Table 16. The results show that 10 µM DMH-1 had a very small effect on lifespan in the *eat-2 (ad1113)* mutant worms. Therefore, this suggests that caloric restriction could be involved in the mechanism by which this compound increases lifespan in *C. elegans* nematodes.

Treatment of *nuo-6* and *eat-2* worms with DMH-1

DRUG	T½ Drug (days)	N Drug	T½ Control (days)	N Control	% T½ increase	p value Drug vs Control	Mean % T½ increase
eat-2							
DMH-1 10µM	23,0	100/106	22,0	88/98	4,7	0,121	**2,6 ± 1,0**
	21,7	**102/114**	**21,2**	**95/105**	**2,6**	**0,241**	
	21,4	113/115	20,7	111/115	3,4	0,071	
	21,3	105/111	21,3	100/101	0,0	0,806	
nuo-6							
DMH-1 10µM	22,5	54/100	22,0	62/110	2,4	0,258	**1,7 ± 2,3**
	24,4	65/108	22,7	66/112	7,6	<0,019	
	20,9	71/109	20,8	53/106	0,6	0,898	
	23,5	**81/104**	**24,4**	**92/101**	**-3,6**	**0,451**	

Table 16. Lifespan assays performed with CGP37157 in mutant *eat-2* or *nuo-6* C. elegans strains. The table shows a series of lifespan assays performed in either *eat-2* or *nuo-6* mutant worms in the presence or in the absence of 10 µM DMH-1. In bold, series shown in the survival plots of Figure 47.

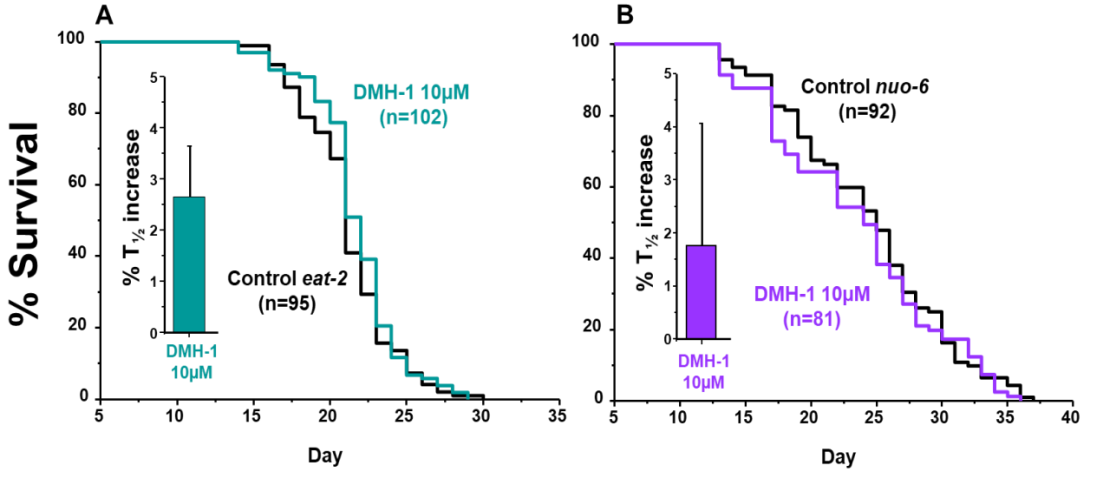


Figure 47. Effects of DMH-1 on survival in *eat-2* and *nuo-6 C. elegans* mutants. Panels A and B show representative survival plots corresponding to parallel lifespan assays performed in *eat-2* (A) and *nuo-6* (B) mutants in which 10 µM DMH-1 was dissolved in the NGM agar The assays shown correspond to those marked in bold in Table 16. The inserts show the mean increase in survival obtained in a series of lifespan assays of each kind as those shown in the figure (more details of each assay in Table 16).

Finally, we tested the effect of the most efficient DMH-1 concentration, 10 µM, on *nuo-6 (qm200)* mutants. These mutants have a defect in a subunit of complex I of the mitochondrial respiratory chain, and have reduced mitochondrial function, lower oxygen consumption, slow growth and movement (Yang and Hekimi, 2010) and decreased ATP levels (Yee et al., 2014). This is accompanied by a significant lifespan extension, underscoring the importance of mitochondrial metabolism in survival (Yang and Hekimi, 2010).

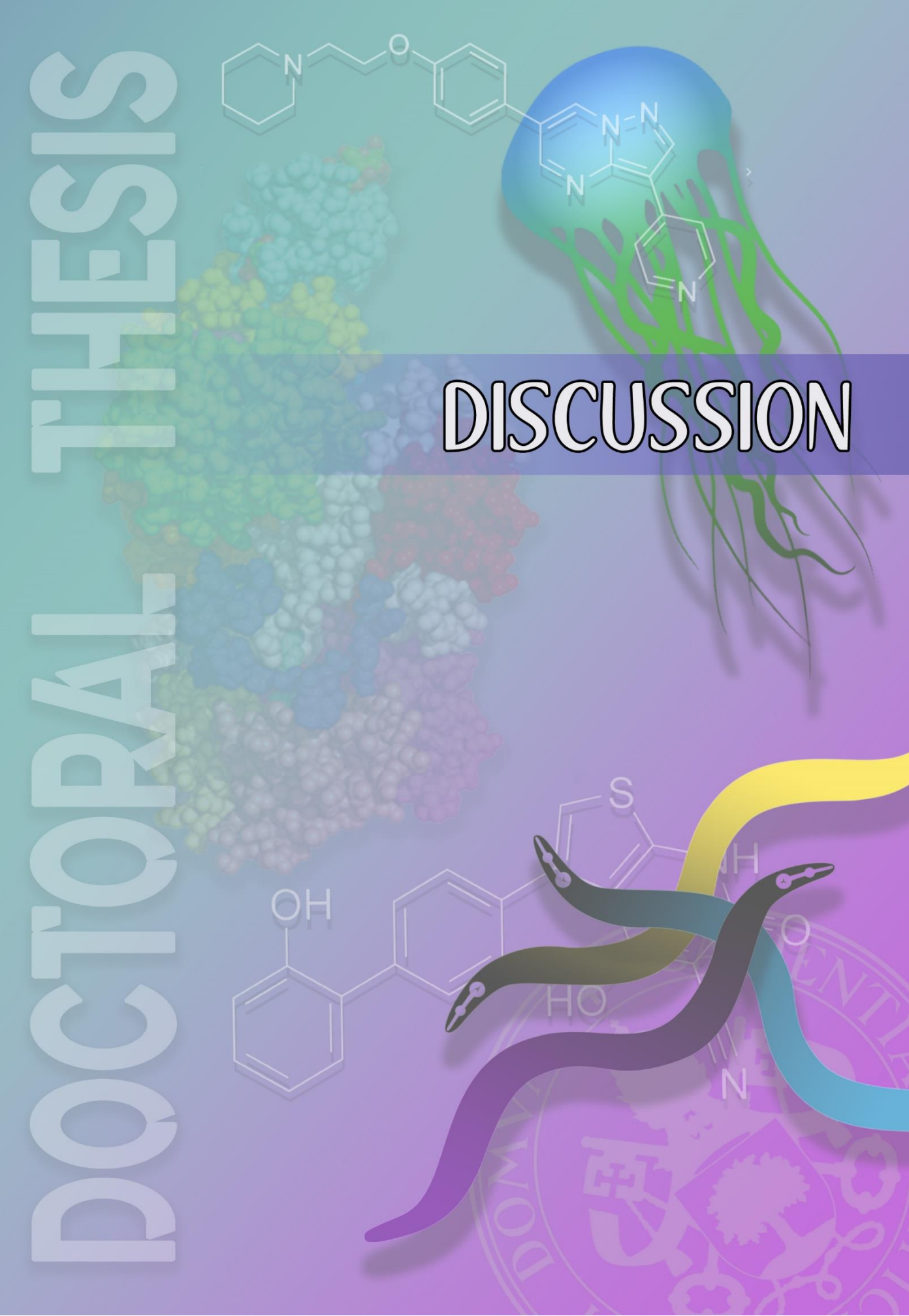
Table 16 shows the results of a series of lifespan assays performed in *nuo-6* mutants with the concentration of DMH-1 that was more effective in the control worms (10 µM). Then, if the effects of DMH-1 were related to mitochondrial dysfunction, we would expect it to produce little or no effect in *nuo-6* mutants.

Figure 47B shows a plot of a typical lifespan assay comparing *nuo-6* mutants in the presence and in the absence of 10 µM DMH-1. The plot corresponds to the assay labelled in bold in Table 16. We can see that DMH-1 had very little effect on the *C. elegans* lifespan in *nuo-6* mutants, suggesting that the mechanism by which DMH-1 lengthens longevity is somehow dependent of mitochondrial function.

Altogether, these data suggest that the lifespan extension induced by DMH-1 could be related to caloric restriction and mitochondrial dysfunction. This is consistent with the role that DMH-1 might have over AKT (Xie et al., 2014), a serine / threonine protein kinase involved in glucose metabolism and cell proliferation among other multiple cellular processes, such as render cells more sensitive to metabolic stress (Los et al., 2009; Coloff et al., 2011). The limited availability of glucose and metabolic stress would activate survival pathways, as occurs in *eat-2* and *nuo-6* mutants, responsible for the *C. elegans* lifespan extension.

DOCTORAL THESIS

DISCUSSION



AMPK pathway and Ca^{2+} signalling

The AMP-activated protein kinase (AMPK) has a key role as a nutrient sensor capable of regulating cellular energy homeostasis and whole-body metabolism (Cantó and Auwerx, 2011). In the last decades, several molecular mechanisms and physiological conditions that regulate AMPK activity have been identified, and it has been found that AMPK is deregulated in the main chronic diseases, such as obesity, diabetes and cancer. Thus, AMPK has become one of the most promising targets for the prevention and treatment of these diseases (Jeon, 2016). In this sense, it is believed that understanding the AMPK metabolism and physiological regulation could provide important keys to better understand the aging process, and perhaps also to find new compounds capable of acting on it, making it slower and healthier.

AMPK is activated by an increase in the AMP/ATP ratio, which indicates the cell energy state. ATP competitively inhibits the binding of both AMP and ADP to the γ-subunit, and thus AMPK behaves as a sensor of AMP/ATP or ADP/ATP ratios. In addition, starvation, altered mitochondrial respiration or hypoxia are cellular stresses that activate AMPK by affecting the AMP/ATP ratio. Another mechanism of AMPK activation is phosphorylation in the α-subunit T172 by protein kinases such as LKB1, CaMKK2 or TGFβ-activated kinase 1 (TAK1), or dephosphorylation by protein phosphatases, such as protein phosphatase 2A (PP2A), protein phosphatase 2C (PP2C) and Mg^{2+}/Mn^{2+}-dependent protein phosphatase 1E (PPM1E) (Hardie, 2007; Hardie et al., 2012).

Intracellular Ca^{2+} is a key messenger in cellular signalling, which activates AMPK through CaMKK2-mediated phosphorylation (Hawley et al., 2005). However, the relationship between AMPK regulation and Ca^{2+} signalling is still unclear. In order to delve into this relationship, the effects of the AMPK activator A769662 and the AMPK inhibitor dorsomorphin on subcellular Ca^{2+} signalling in HeLa cells have been investigated in this work.

Our results show that A769662 and dorsomorphin have a clear and opposite effect on IP_3-induced ER Ca^{2+} release. The AMPK activator (A769662) inhibited IP_3-induced ER Ca^{2+} release and the AMPK inhibitor (dorsomorphin) activated it. In both cases, the effects were large enough to produce highly significant changes in the histamine-induced cytosolic and mitochondrial [Ca^{2+}] peaks. In particular, the Ca^{2+} transfer between ER and mitochondria was specially affected, as the AMPK modulators dramatically modified agonist-induced mitochondrial [Ca^{2+}] increase. Our data indicate that the AMPK modulators directly influence IP_3R activity, as they produced similar effects on histamine-induced Ca^{2+} release in intact cells and on IP_3-induced Ca^{2+} release in permeabilized cells.

This effect adds complexity to the role that IP_3R could have in Ca^{2+} signalling, linking the activity of mTOR, AMPK and other nutrient-sensitive pathways. The IP_3R releases Ca^{2+} from ER and also transfers Ca^{2+} from the ER to the mitochondria through MAMs, where it has been described that mTORC2 is also an important component, able to activate AKT kinase, which may then phosphorylate and inhibit IP_3R (Betz et al., 2013). On the other hand, it has also been reported that mTOR is able to directly phosphorylate and activate IP_3R (MacMillan et al., 2005; Frégeau et al., 2011; Régimbald-Dumas et al., 2011). In turn, IP_3R activity may also modulate the nutrient sensitive pathways. When ER Ca^{2+}-release mediated by IP_3R is reduced, both Ca^{2+}-entry and ATP synthesis in mitochondria decrease. The resulting energy depletion activates AMPK, which then inhibits mTOR. The activity of the IP_3R and the Ca^{2+}-transfer between ER and mitochondria may therefore control mTOR. Finally, resveratrol treatment decreases ER calcium storage and store-operated Ca^{2+}-entry, inducing ER stress, activating AMPK and inhibiting the mTOR pathway (Selvaraj et al., 2016).

A769662 is a direct activator of AMPK that stimulates its activity even in the absence of AMPK phosphorylation (Scott et al., 2014). The EC_{50} for inhibition by A769662 of IP_3- induced Ca^{2+} release was 29 μM, a concentration in the same range as that previously determined to activate AMPK in HeLa cells (Goransson et al., 2007; Konagaya et al., 2017). Regarding dorsomorphin/Compound C, it was originally identified as an inhibitor of AMPK and it has been widely used as an AMPK inhibitor in HeLa cells (Ting et al., 2010; Lee et al., 2015; Law et al., 2017). However, it has been later found that it is also a potent inhibitor of several BMPRs (Engers et al., 2013). In our work, the EC_{50} for stimulation by dorsomorphin of the histamine-induced $[Ca^{2+}]_M$ peak was very low, only 0.4 μM, compatible with a specific effect either on AMPK or BMPRs. To distinguish between these two possibilities, we have assayed the effect on Ca^{2+} homeostasis of two different BMPRs inhibitors which have no effect on AMPK. Our data show that these inhibitors did not reproduce at all the effects of dorsomorphin on the cytosolic and mitochondrial $[Ca^{2+}]$ peaks induced by histamine, suggesting that the effect of dorsomorphin shown here is not due to BMPRs inhibition. Moreover, dorsomorphin reversed the effects of the AMPK activator A769662 on the histamine-induced $[Ca^{2+}]_M$ peaks, suggesting that its effects are actually due to AMPK inhibition.

The observed effect of the modulators is consistent with the well-known effects of AMPK on metabolism and with the general antagonism between AMPK and mTOR activation. We found that the AMPK activator, A769662, inhibited IP_3-induced Ca^{2+} release, while the AMPK inhibitor activated it. As Ca^{2+} is a key second messenger for cell activation, inhibition of Ca^{2+} release should slow down energy consumption for different Ca^{2+}-dependent activities such as contraction, secretion, proliferation, and others. In addition, less energy would be required to restore the Ca^{2+} gradients via ATP dependent Ca^{2+} pumps. Therefore, inhibition of IP_3R may reduce energy

expenditure. Furthermore, inhibition of IP_3R should also reduce Ca^{2+} transfer from ER to mitochondria, and this should slow down ATP production. This apparent contradiction adds to some other uncertainties that remain on the relationship between nutrient sensitive pathways and Ca^{2+} signalling. As it has been mentioned before, mTOR has been reported to induce both activation (MacMillan et al., 2005; Fregeau et al., 2011; Regimbald-Dumas et al., 2011) and inhibition of IP_3R (Betz et al., 2013).

We would like to highlight that IP_3R-induced Ca^{2+} transfer from ER to mitochondria has been shown to be essential to maintain mitochondrial function and cellular energy balance not only in normal cells, but especially in cancer cells. Genetic or pharmacological inhibition of IP_3R produces cell death with much greater potency in cancer cells than in normal cells, and IP_3R seems to play an important role in cancer progression and metastasis (Cárdenas et al., 2016; Bustos et al., 2017). Under this perspective, the regulation of IP_3R by AMPK described in this work may be of interest for cancer research.

The molecular mechanism linking AMPK regulation to ER Ca^{2+} release will require further study. In order to obtain some additional information on the possible mechanisms, we performed a phosphoproteomic study looking for changes in protein phosphorylation state induced by treatment with these compounds. This study showed that A769662 and dorsomorphin generate a large amount of changes in the protein phosphorylation pattern. In the case of A769662, the phosphorylated proteins include several targets of AMPK, such as acetyl-CoA carboxylase or Raf, consistent with activation of AMPK in the presence of this compound. However, the large number of protein phosphorylation changes makes it difficult to define clearly the mechanisms involved. Treatment with the modulators did not affect the IP_3R expression levels, nor its phosphorylation status, suggesting that AMPK modulators influence IP_3R activity through changes in the activity of mediators present in the IP_3R upstream signalling pathway.

Analysis of the phosphoproteomic data suggests several possible candidates to mediate the effects. First, several kinases that are upstream of the IP_3R in the interactome become phosphorylated or dephosphorylated in the presence of these compounds. They are ERK1/2 (MAPK1/3), whose phosphorylation was increased by A769662, and PKCα and GSK3B, whose phosphorylation was decreased in the presence of dorsomorphin. Both ERK1/2 and PKC have been shown to phosphorylate IP_3R (Vanderheyden et al., 2009). In addition, we should mention the phosphodiesterase PDE3A, which becomes phosphorylated in the presence of A769662 and dephosphorylates in the presence of dorsomorphin. PDE3A (as well as PDE3B, which also becomes phosphorylated in the presence of A769662) has been found associated with the endoplasmic reticulum (Maurice et al., 2014) and cAMP is an important activator of the IP_3R, both directly and via PKA-mediated phosphorylation

(Taylor, 2017). Activation of PDE in the presence of A769662 could therefore reduce the cAMP concentration (from the resting levels, see Borner et al., 2011; Harada K *et al.,* 2017) and thereby inhibit the IP_3R. In fact, it has been shown that a closely related phosphodiesterase, PDE4B, is phosphorylated by AMPK at three sites (Johanns et al., 2016). The Ca^{2+}-dependent apoptosis inhibitor NOL3 (ARC), whose phosphorylation decreases in the presence of A769662, may also be an interesting candidate, as it has been shown to regulate Ca^{2+}-release from sarcoplasmic reticulum (Lu et al, 2013).

In conclusion, our data suggest that AMPK negatively regulates IP_3-induced Ca^{2+} release from the ER. This effect constitutes a very important link between Ca^{2+} signalling and the AMPK pathway. As the IP_3R is also regulated by mTOR, this ER Ca^{2+} channel appears to be a key signalling hub for these two major nutrient-sensitive pathways. In fact, it has been reported that submaximal SERCA inhibition has a pro-longevity effect in *C. elegans*, which is explained on the basis that it decreases $[Ca^{2+}]_{ER}$, leading to a reduced Ca^{2+} release from the ER and a smaller Ca^{2+} transfer from ER to mitochondria, which in turn, should activate AMPK and inhibit mTOR (García-Casas et al., 2018).

Nutrient-sensitive pathways modulators and *C. elegans* longevity

As mentioned above, AMPK is a key energy sensor. When AMPK senses that the energy levels are low, tries to restore the energy balance by activating ATP-producing catabolic pathways and inhibiting ATP-consuming anabolic pathways. In the short term, it promotes glycolysis and fatty acid oxidation, and in the long term, it uses mitochondrial substrates as an energy source and increases the mitochondrial content (Cantó and Auwerx, 2010). Current evidence strongly supports the idea that anabolic signalling accelerates aging while a decrease in nutrient signalling extends longevity (Fontana et al., 2010). Therefore, a pharmacological intervention that mimics a limited availability of nutrients or activates the catabolic pathways could extend the longevity. In fact, both dietary restriction and compounds such as rapamycin, mTOR inhibitor, or metformin, AMPK activator, have already shown their ability to increase longevity in a large number of species, including mammals (Harrison et al., 2009).

Likewise, it is believed that the positive regulation of AMPK would favor a healthy aging, since the activation of AMPK has multiple effects on the metabolism that in turn would have positive effects on the survival, in part mediated by inhibition of the mTOR pathway (Alers et al., 2012).

Intracellular Ca^{2+} dynamics is also a key controller of cellular metabolism. The rate of mitochondrial ATP synthesis depends on the $[Ca^{2+}]$ inside mitochondria

($[Ca^{2+}]_M$), which stimulates several key dehydrogenases responsible for NADH production (McCormack et al., 1990). As a consequence of this, an increase in $[Ca^{2+}]_M$ immediately leads to an increase in ATP production (Robb-Gaspers et al., 1998; Jouaville et al., 1999). The increase in $[Ca^{2+}]_M$ is generally a consequence of the stimulation of Ca^{2+}-release from the ER that occurs during cell activation. This mechanism constitutes a basic homeostatic response that associates cell activation to energy production, in order to cover the energy requirements of the response to the stimulus.

Given that Ca^{2+} dynamics strongly interplays with energy metabolism and nutrient-sensitive pathways, both of them involved in the aging process, we have studied the effect of AMPK modulators on *C. elegans* survival.

On the first place, we have investigated the effect of the inhibition or activation of AMPK, on *C. elegans* longevity. For this study, we have used the same modulators used in the study with HeLa cells, the AMPK activator A769662 and the AMPK inhibitor dorsomorphin. In theory, AMPK activation should increase *C. elegans* lifespan while, on the contrary, AMPK inhibition could reduce nematodes survival.

The results, however, were somewhat surprising. The AMPK activator A769662 did not increase the lifespan of *C. elegans* worms at any of the amounts or concentrations tested, regardless of the administration method. The best explanation we have for this phenomenon relies on the fact that these nematodes only have a catalytic AMPK α subunit, and lack the β regulatory subunit, which has been previously described to be the target of compound A769662 (Scott et al., 2014). This would explain why this compound is effective in HeLa cells, but not in *C. elegans* worms.

On the other hand, the AMPK inhibitor dorsomorphin produced a significant increase in the lifespan of *C. elegans* worms. This effect was not dependent on the drug administration method, because similar or even larger effects were obtained when dorsomorphin was directly added to the NGM agar, than when the worms were fed with a dorsomorphin-containing inclusion compound. These results appear initially contradictory with the effect of AMPK on lifespan. Thus, we tried to check whether the effect of dorsomorphin was actually mediated by AMPK. As dorsomorphin is also a potent inhibitor of BMPRs, we tested several compounds that have been described as inhibitors of BMPR signalling, but not of AMPK. We used here the same BMPR inhibitors used in the study with HeLa cells, ML-347 and DMH-1, and also compound SB-431542, an inhibitor of the related family of the TGF-β type I receptor. Our data showed that all of these compounds, which are not AMPK inhibitors, increased the longevity of the worms similarly to dorsomorphin. Among them, ML-347 and DMH-1 had similar activity, and were more effective than SB-431542. Therefore, these results

suggest that the positive effect of dorsomorphin on *C. elegans* survival could be mediated by the inhibition of the BMPR pathway and not by AMPK inhibition.

However, BMPR signalling is not the only target of these inhibitors. One of these compounds, DMH-1, which was initially developed as a BMPR selective inhibitor, also activates AKT through the inhibition of PP2A activity in L6 cells, which in turn, increases glucose metabolism (Xie et al., 2014). Interestingly, DMH-1 is a very close dorsomorphin analogue with only two small structural modifications (Hao et al., 2010). Despite this structural homology, dorsomorphin has been reported to inhibit AKT in cancer cells (Vucicevic et al., 2011). Protein kinase B (AKT) is an anti-apoptotic factor for a variety of stimuli such as hypoxia or radiation, which is activated in multiple cancers, acting as a proto-oncogene that inhibits cell death. However, other studies have shown that this anti-apoptotic capacity is coupled to glucose metabolism leading the cells to be more sensitive to metabolic stress after AKT activation (Los et al., 2009; Coloff et al., 2011; Fruman and Rommel, 2014). In addition, AMPK and AKT have been described as two primary effectors with antagonistic roles in response to metabolic stress (Zhao et al., 2017). In addition to the independent regulation of these kinases, there is also a mutual negative phosphorylation regulation of AMPK and AKT. When AKT is activated, it can phosphorylate AMPKα, preventing LKB1 or CaMKK2 from accessing the active Thr-172 site. On the other hand, AMPK phosphorylates insulin receptor substrate 1 (IRS1), inhibiting AKT signalling. Besides, some AMPK activators, such as AICAR or phenformin, activate AMPK, which in turn, dephosphorylates and inhibits AKT, suggesting that these drugs cause a coordinated inverse regulation of these kinases (King et al., 2006; Zhao et al., 2017).

DMH-1 and dorsomorphin have therefore a very similar chemical structure, but some of their effects are quite different. Both of them inhibit BMPR. However, dorsomorphin inhibits AMPK and DMH-1 does not, and dorsomorphin inhibits AKT while DMH-1 activates it. We have therefore compared the pharmacological effects of DMH-1 and dorsomorphin on nematode longevity, with the purpose of obtaining clues about the mechanism of the lifespan extension induced by these drugs.

Our data show in fact that treatment with dorsomorphin or DMH-1 produces a significant increase in the *C. elegans* lifespan. However, the concentration-dependence of the effect was very different. The effect of dorsomorphin increasing survival reached a maximum at a concentration of 10 µM, but concentrations of 50 µM and above had a negative effect on survival, and the higher the concentrations, the more negative the effects. Instead, the effect of DMH-1 increasing survival was maximum between 2 and 10 µM and then the effect decayed at 50 µM and above, but it never had a negative effect on survival, even at very high concentrations. These results suggest that the increase in survival induced by low concentrations of both compounds may be mediated by their common target, the BMPRs, and by some

reason the effect is larger under conditions of submaximal BMPR inhibition. Instead, the negative effect on survival of high concentrations of dorsomorphin may be attributed either to AMPK or AKT inhibition, which are produced by dorsomorphin but not by DMH-1.

To investigate further the mechanism of the lifespan extension induced by DMH-1, we have studied its effects on four *C. elegans* mutants, *aak-2, daf-2, eat-2* and *nuo-6*.

We have first used the *aak-2* mutant to completely rule out that DMH-1, having a very similar structure to dorsomorphin, could also be modulating AMPK in some way. *aak-2* (ok524) mutants have shorter lifespan than wild-type worms as a consequence of a mutation in the *aak-2* gene (coding for the AMPKα2 subunit) that accelerates aging (Apfeld et al., 2004). Our data show that DMH-1 also produced an increase in *aak-2* survival by nearly 17 %. The effect of DMH-1 at the maximum effective dose was similar in *aak-2* mutants compared to wild-type worms, suggesting that AMPK signalling is clearly not involved in the increased lifespan. This is consistent with the lack of inhibitory activity DMH-1 has on AMPK (Xie et al., 2014), and provides strong evidence in favor that lifespan extension mediated by DMH-1 does not involve AMPK.

AKT is activated by extracellular signals, mostly growth factors through PI3K signalling. In mammalian, AKT participates in insulin receptor signalling (Kohn et al., 1996) and regulates glycogen synthesis by direct GSK3 phosphorylation (Cross et al., 1995). Similarly, in *C. elegans* it acts in insulin receptor-like signalling (Ogg et al. 1997) and in the insulin/IGF-I anti-apoptotic pathway (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kulik et al., 1997). Many mutations within genes in the insulin/IGF-1 pathway have been shown to significantly extend lifespan in C. elegans (Dorman et al., 1995). Specifically, we have used here *daf-2* mutants, which have an altered IGF-1R and a much longer lifespan. We have studied the effect of DMH-1 on *daf-2* mutants to investigate if its effects on *C. elegans* longevity were mediated by the IGF-1 signalling pathway. Our results show that DMH-1 also increased the lifespan of the *daf-2* mutants, suggesting that the lifespan extension induced by DMH-1 is not due to IGF-1 signalling. Although this result may be interpreted to suggest that AKT is not involved in the increase in lifespan extension induced by DMH-1, we have to take into account that the PI3K/AKT signalling pathway may be activated by other pathways in *daf-2* mutants.

Once it was found that the AMPK and IGF-1 pathways were not directly involved in *C. elegans* lifespan extension mediated by DMH-1, we decided to study the relationship of this effect with dietary restriction (DR), which is also strongly related with the nutrient sensitive pathways (Barzilai et al., 2012). In fact, it has been reported that DR can induce *C. elegans* lifespan extension through a mechanism involving

AMPK-FOXO pathway (Greer et al., 2007). Likewise, DR could also activate SIRT1 and inhibit mTOR and S6K1 in senile mice (Ma et al., 2015). In particular, it has been found that DR in humans inhibits PI3K/AKT pathway and induces changes in transcriptional skeletal muscle profile that resembles those of younger people (Mercken et al., 2013). In order to explore whether DMH-1 could mediate lifespan extension by DR, we used the *eat-2* mutant, which has a defect in pharynx pumping that reduces the rate of feeding. This produces an increase in survival of the mutant worms, and is considered to be a model for the effects of caloric restriction (Lakowski and Hekimi, 1998). In these mutants, we did not find a significant effect of DMH-1 on lifespan, suggesting that *C. elegans* lifespan extension induced by DMH-1 is related in some way to DR. However, the molecular mechanisms responsible for the lifespan extension induced by DR are not completely clear, and therefore from this result we still cannot draw any detailed conclusions about the mechanism.

Finally, we tested the effect of the maximally effective DMH-1 concentration, 10 µM, on *nuo-6* mutants. These mutants have a defect in a subunit of complex I of the mitochondrial respiratory chain, and have reduced mitochondrial function (Yang and Hekimi, 2010) and decreased ATP levels (Yee et al., 2014) which lead to a significant lifespan extension, underscoring the importance of mitochondrial metabolism in survival (Yang and Hekimi, 2010). In these mutants, however, we also could not find any significant effect of DMH-1 on lifespan, suggesting that the lifespan extension induced by DMH-1 is related to mitochondrial dysfunction, or at least requires a functional mitochondria to operate. These results are consistent with those obtained in L6 cells, where it was demonstrated that treatment with DMH-1 inhibited mitochondrial function and reduced ATP levels in a dose-dependent manner (Xie et al., 2014). Under these conditions, DMH-1 could actually activate AMPK indirectly, because AMPK activation is usually a consequence of a decrease in ATP production. Besides, a relationship between AKT activation and mitochondrial stress has also been reported (Bijur and Jope, 2003; Guha et al., 2010; Yang et al., 2017).

Mitochondria are cellular organelles essential for cellular metabolism and apoptosis. They are also an important source of ROS which promote cells, tissues and complete organism deterioration. For a long time, it was believed that build-up of mitochondrial ROS was the main cause of aging. Nowadays, RNAi studies have revealed that most genes that promote life extension are involved in mitochondrial metabolic pathways and ETC components (Lee et al., 2003), suggesting that mitochondria have a key role in aging. In addition, partial mitochondrial uncoupling attenuates age-dependent neurodegeneration and increases survival in *C. elegans* (Lemire et al., 2009; Cho et al., 2017), and mitochondrial respiratory chain mutants such as *nuo-6* also show a large increase in survival (Yang and Hekimi, 2010). On the other hand, as mitochondria are fundamental for cellular energy production, it would be also possible that mitochondria could regulate lifespan in response to dietary

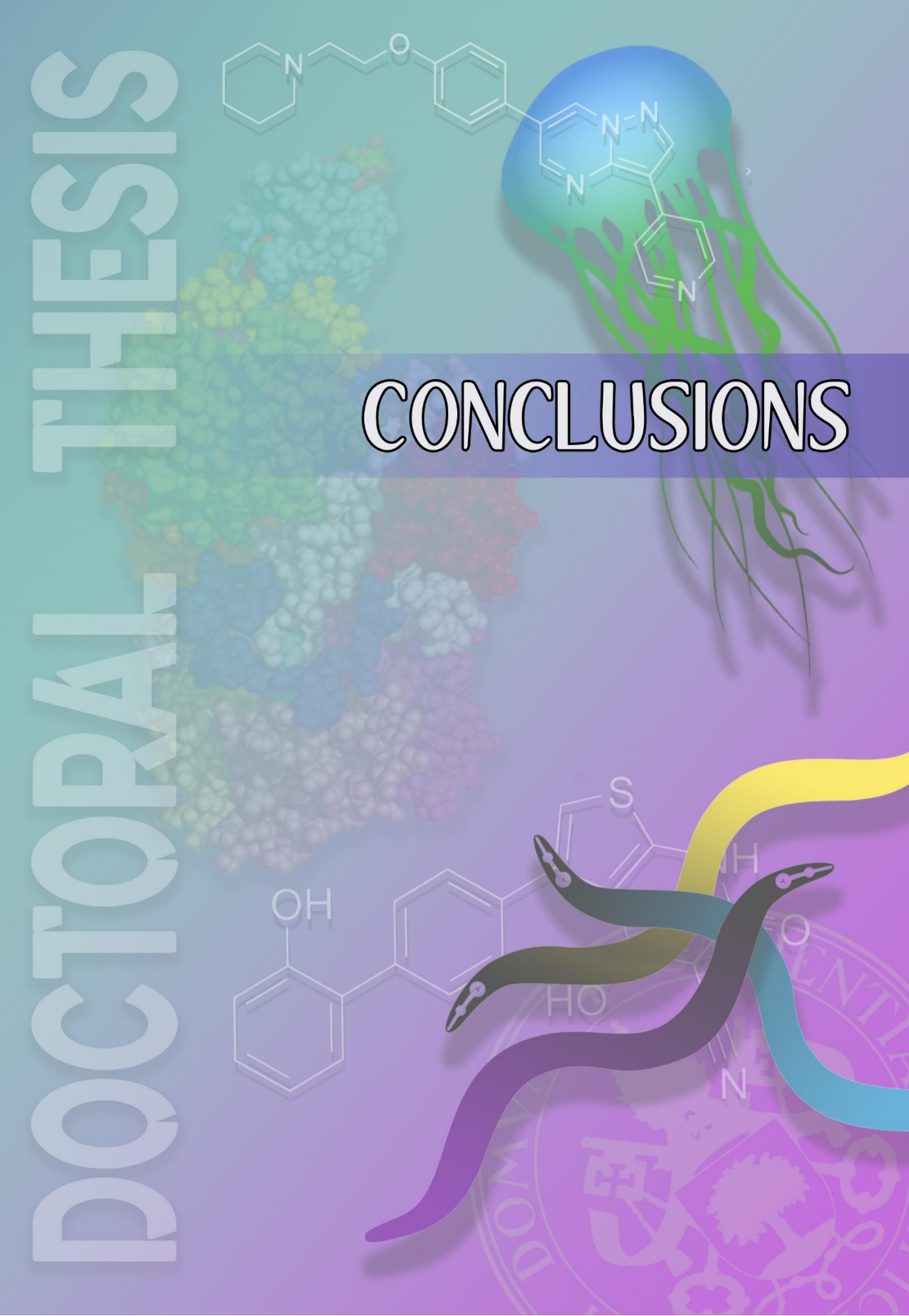
restriction (Hansen et al., 2005). This would explain why DMH-1 does not produce effects in both DR and mitochondrial mutants. In addition, it is also believed that life expectancy extension by glucose restriction may be mediated by mitohormesis, a coordinated response to mild mitochondrial stress that includes some cytosolic signalling pathways and appears to protect cells from subsequent perturbations (Schulz et al., 2007; Haigis and Yankner, 2010; Yun and Finkel, 2014).

Since it is known that energy levels decrease with age and predict life expectancy in *C. elegans* (Hardie and Hawley 2001), many studies have been devoted to understanding the mechanism by which AMPK detects energy levels to regulate life expectancy. Our data show the AMPK inhibitor dorsomorphin increases the life expectancy at low concentrations by an AMPK-independent mechanism, probably mediated by BMPRs inhibition. Instead, at high concentrations decreases the lifespan, and this effect is probably mediated by AMPK inhibition, because it is absent in the analogous DMH-1. The dorsomorphin homologue DMH-1 has a very similar chemical structure and increases the lifespan at the same concentrations, suggesting that the mechanism also involves BMPRs. In addition, we have also seen that the mechanism of lifespan extension by DMH-1 requires functional mitochondria to operate and has something in common with DR. Further studies will be necessary to completely clarify the molecular mechanism of this effect.

In summary, there is little doubt that Ca^{2+} dynamics modulates AMPK and *viceversa*, although a complete understanding of the mechanisms involved requires much further work. We think therefore that Ca^{2+} signalling is a pathway that is worth exploring for the modulation of nutrient-sensitive pathways and aging. We also believe that the results of this thesis can provide important keys to better understand the aging process, and to find new approaches to slow it.

DOCTORAL THESIS

CONCLUSIONS



1. AMPK modulators have an opposite effect on IP_3-induced ER Ca^{2+} release. The AMPK activator A769662 inhibits IP_3-induced ER Ca^{2+} release, while the AMPK inhibitor dorsomorphin activates it.

2. These effects are large enough to produce highly significant changes in the histamine-induced cytosolic and mitochondrial [Ca^{2+}] peaks. In particular, AMPK modulators dramatically modified agonist-induced mitochondrial [Ca^{2+}] increase, suggesting that Ca^{2+} transfer between ER and mitochondria is specially affected.

3. The AMPK modulators directly influence the activity of the IP_3R, but without modifying their expression or phosphorylation status. The phosphoproteomic analysis suggested several possible candidates in the IP_3R upstream signalling pathway that could mediate the effects, although much further work is required to test it.

4. A769662 does not extend *C. elegans* lifespan. Instead, dorsomorphin and several dorsomorphin analogues (ML-347 and DMH-1) lacking AMPK inhibitory activity, produced a large increase in *C. elegans* lifespan (nearly 20 %) with a “bell-shaped” concentration dependence.

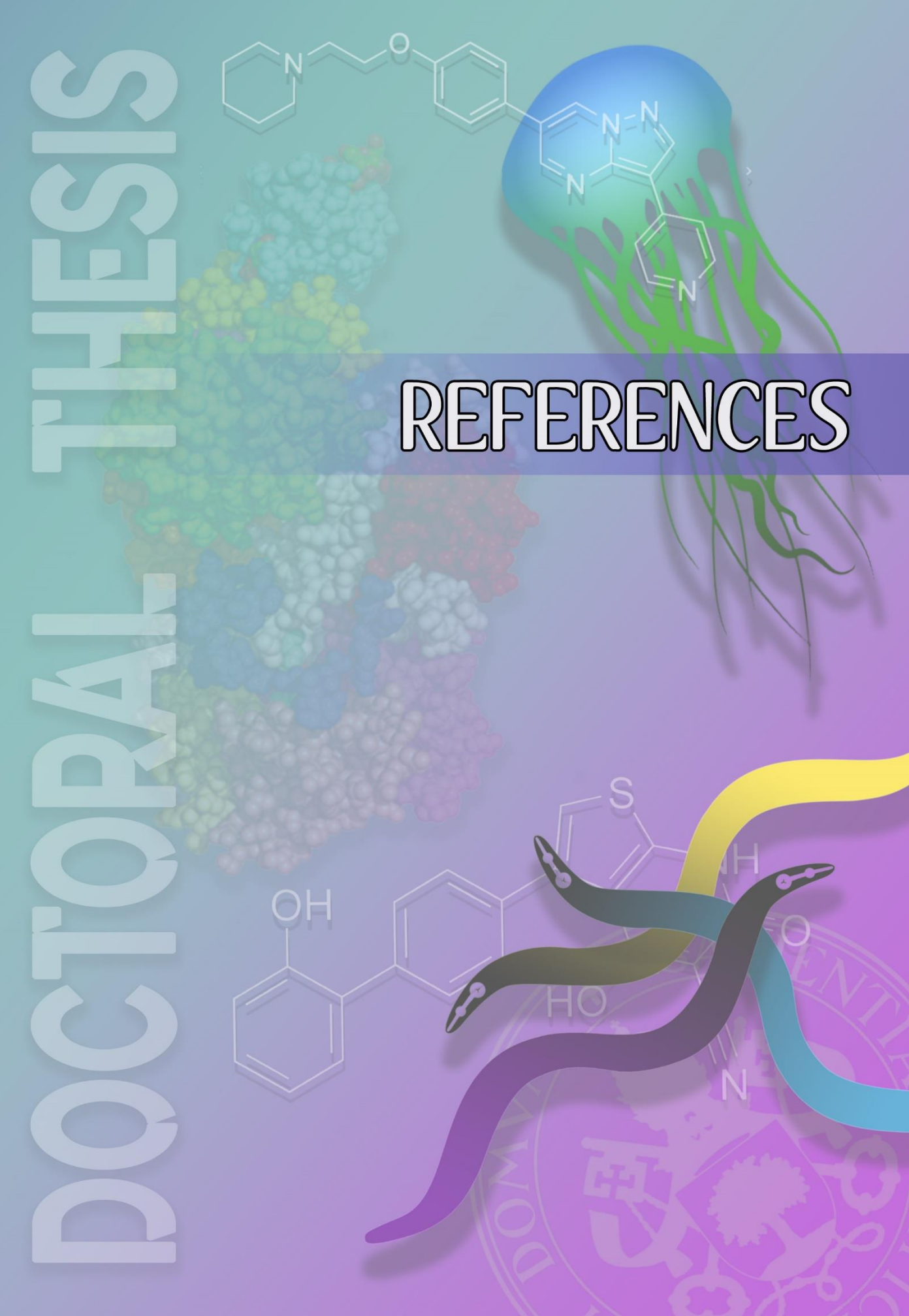
5. The increase in lifespan induced by dorsomorphin and dorsomorphin analogues does not depend on AMPK, but is most likely due to a submaximum inhibition of an alternative target of these compounds, the bone morphogenetic protein receptors (BMPRs).

6. The effects of these compounds on *C. elegans* longevity are independent of the drug administration method, either dissolved in the NGM agar or added in the form of an inclusion compound with γ-cyclodextrin.

7. The pro-longevity effect of DMH-1 was maintained in *aak-2* (AMPKα2 subunit) and *daf-2* (IGF1 receptor) mutants, suggesting that AMPK and IGF-1 pathways are not involved in the effect. Instead, it disappeared in *eat-2* (Dietary Restriction) and *nuo-6* (respiratory chain complex I) mutants, suggesting that the effect requires functional mitochondria and shares components with DR.

8. In summary, we conclude that AMPK negatively regulates IP_3-induced Ca^{2+} release from the ER and this effect constitutes a very important link between Ca^{2+} signalling and the AMPK pathway. Ca^{2+} signalling could therefore be a promising novel target pathway to act on aging. Moreover, we have found BMPRs as novel targets for anti-aging drugs, as submaximal BMPR inhibition produced a large increase in lifespan in *C. elegans* worms.

DOCTORAL THESIS



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