Study of the contribution of ORMDL3 to the cannabinoid antitumoral action

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Abstract

Genome-wide association studies have been very useful tools in the last years to relate genes and diseases. Thereby, single nucleotide polymorphisms have been linked to increased *ORMDL3* expression and to different pathologies including gliobastoma. This thesis has aimed to elucidate the possible implication of ORMDL3 in tumor generation and development taking in consideration its role as negative regulators of sphingolipid synthesis. Moreover, we have explored the possibility that the expression levels of ORMDL3 would alter antitumoral treatments like cannabinoids whose mechanism of action imply ceramide synthesis.

In the first block of this thesis, we show that THC as antitumoral drug in glioblastoma cells downregulates ORMDLs expression to release the serine palmitoyltransferase activity and induce ceramide synthesis. In addition, we demonstrate that the expression levels of ORMDL3 alter antitumoral activity of THC by reducing the ceramide synthesis and endoplasmic reticulum stress and, on the other hand, inducing anti-apoptotic proteins. Furthermore, in the second block we show that ORMDL3 expression has an important impact in the ER-mitochondria network structure and in the transduction of signals between both organelles affecting cell fate decision like autophagy and apoptosis. Altogether, this thesis highlights the relevance of ORMDL3 in the field of cancer and antitumoral treatments.

Resumen

Los estudios de asociación genómica han sido una importante herramienta en las últimas décadas para relacionar genes y enfermedades. Debido a ello, polimorfismos de un solo nucleótido han permitido asociar el incremento en la expresión de ORMDL3 a diferentes patologías como el glioblastoma. Esta tesis tiene como objectivo dilucidar la posible implicación de la proteína ORMDL3 en la generación tumoral y su desarrollo, considerando una de sus principales funciones, la regulación de forma negativa de la síntesis de esfingolípidos.

En el primer bloque de esta tesis se muestra que el THC como tratamiento antitumoral en células de glioblastoma es capaz de downregular la expresión de las proteínas ORMDL permitiendo la actividad de la serina palmitoil transferasa e induciendo de esta forma la síntesis de ceramidas. Por otra parte, demostramos que los niveles de expresión de ORMDL3 modifican la actividad antitumoral del THC, reduciendo la síntesis de ceramidas y el estrés reticular y, por otra parte, induciendo proteínas anti-apoptoticas. Además, en el segundo bloque mostramos que la expresión de ORMDL3 tiene un papel importante en la red estructural retículo-mitocondria y en la transducción de señales entre ambos orgánulos, afectando en el desarrollo celular como puede ser la alteración en mecanismos de autofagia y apoptosis. En conjunto, esta tesis destaca la relevancia de la proteína ORMDL3 en el campo del cáncer y en los tratamientos antitumorales.

Prologue

Genome-wide association studies are a useful tool to reveal the genetic component associated to different pathologies. in certain diseases. In this context, the SNP rs7216389 located at the region 17q12-q21 has been associated to develop asthma, inflammatory diseases and increase the risk of certain tumors as glioblastoma. This association implies increased expression levels of the *ORMDL3* gene. In this thesis we wanted to study the underlying mechanism of the connection between ORMDL3 expression levels and cancer. As starting point we took in consideration the role of this protein inhibiting ceramide synthesis because many apoptotic signals of antitumoral agents are triggered by an initial rise in ceramide production. In this thesis we focus our attention on one of the novel antitumoral treatments in the last decade, the Δ^9-tetrahidrocannabinol (THC). This stimulus increases ceramide synthesis in the cell by *de novo* pathway, increasing ER stress promoting autophagy and posterior cell apoptosis. Being ORMDLs a family of proteins that inhibits the first enzyme implicated in ceramide synthesis, the seril palmitoiltransferase (SPT), we wanted to explore the role of ORMDL3 expression levels in the antitumoral pathway of THC and how the expression levels of this protein would affect its effectiveness.

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1.INTRODUCTION

1.1 ORMDL family

The gene family encoding for oromucoid-like (ORMDL) proteins was mentionedfor the first time by Børsting and colleagues in 1997(Børsting C et al.,1997). In this study, analyzing open reading frames in yeast, the ORM1 sequence was identified. Few years later, analysis in the human genome revealed the existence of three ORMDL genes. The human genes are mapped in different chromosomes: *ORMDL1* in at 2q31-q33., ORMDL2 in 12q13.2 and ORMDL3 in 17q21.1 chromosomic region. All three genes are ubiquitously expressed in adult and fetal tissues, but the individualy they have a differential expression patern (Hjelmqvist L et al., 2002).

Each human ORMDL gene has a conserved counterpart in vertebrates. Moreover, they have homologues in plants and yeast (figure 1). For example, in yeast two orthologs for this family have been described, orm1 and orm2, that present a sequence identity of about 35% compared to the human orthologs (Hjelmqvist L et al., 2002).

Human ORMDL proteins contain 153 amino-acid residues and share around 80- 84% sequence identity. Immunofluorescence studies using heterologous expression system and specific antibodies against endogenous ORMDLs, allowed to determinate the subcellular location of ORMDLs in the endoplasmic reticulum (ER) (Cantero-

Recasens G et al.,2010; Miller M et al.,2012, Miller M et al., 2014; Ha SG et al., 2013; McGovern DP et al., 2010).

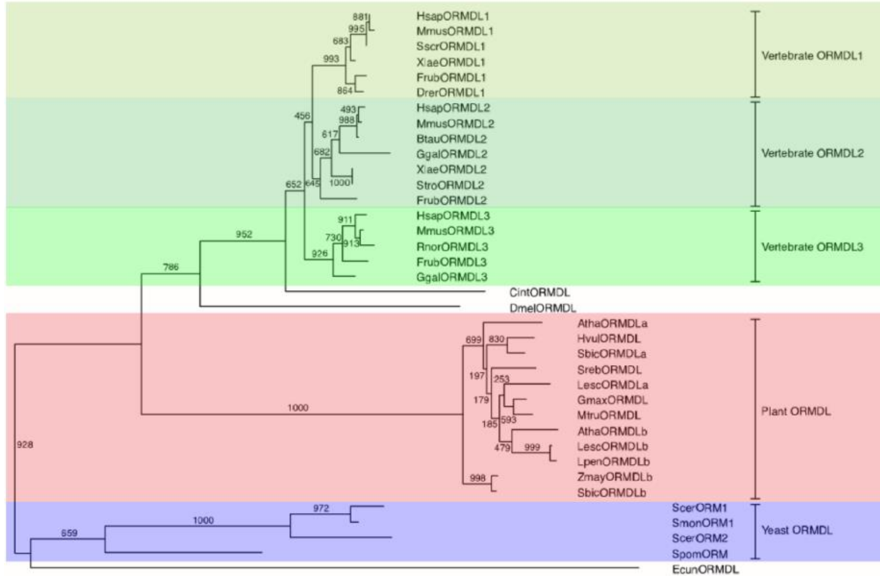


Figure 1. Phylogenetic tree of relationship between ORMDL sequence. Different ORMDL vertebrate are highlighted in green tones, plant ORMDL in red and yeast ORMDL in purple. Species abbreviations are: Hsap, human; Mmus, mouse; Rnor, rat; Sscr, pig; Btau, cow; Ggal, chicken; Xlae, *Xenopus laevis*; Stro, *Silurana tropicalis*; Frub, *Takifugu ribripes* (pufferfish); Drer, *Danio rerio* (zebrafish); Cint, *Ciona intestinalis*; Dmel, *Drosophila melanogaster*; Atha, Arabidopsis thaliana; Hvul, *Hordenum vulgare* (barley); Sbic, Sorghum vulgare; Sreb, *Stevia rebaudiana*; Lesc, *Lycopersicon esculentum* (tomato); Gmax, *Glycine max* (soybean); Mtru, *Medicago truncatula*; Lpen, *Lycopersicon* pennellii; Zmay, *Zea mays* (maize); Scer, *Saccharomyces* cerevisiae, Smon, *Saccharomyces monacensis*, Spom, *Schizosaccharomyces pombe*; Ecun, *Encephalitozoon cuniculi*, . Adapted from Hjelmqvist *et al* 2002.

1.1.1 Structure of ORMDL family

The ORMDL family has been described as transmembrane (TM) ER resident proteins. The number of TM domains has been subject of discussion. In the beggining, four hydrophobic domains were predicted (Hjelmqvist L et al., 2002), and recognized as putative TM sequences, but different alignment studies have predicted between one to four TM domains (Hjelmqvist L et al., 2002, McGovern DP et al., 2010, Araki W et al., 2008). Interestingly, the TM domains are conserved in their relative position but the sequence conservation at the residue level is much lesser than their flanking sequences conservation. In our laboratory, a model for ORMDL3 was predicted based in a fluorescence protease protection assay. This approach allowed us to determine that both, C- and N-terminus were facing the cytoplasm, whereas the middle fragment of the protein localized on the ER lumen (Cantero-Recasens G et al.,2010, Carreras-Sureda A et al., 2013). In this respect, both N- and C- terminus have been proved to be essential for the protein function (Carreras-Sureda A et al., 2013). Besides, in yeast the N-terminus contains a regulatory domain with different putative phosphorylation sites that is lost in mammals (Breslow DK et al., 2010; Roelants FM et al., 2011; Kiefer K et al., 2015)

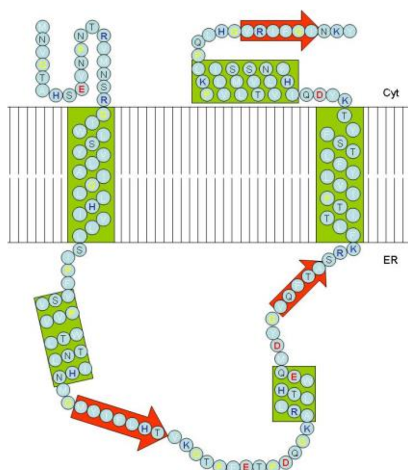


Figure 2. Structure of human ORMDL3. Aminoacid sequence of human ORMDL3. C- and N- terminal tails of the protein are face to citosol. Moreover, this protein has two transmembrane domains with a loop faced to ER.

1.1.2 ORMDL family function

It has been described that ORMDLs participate in several cell functions. The first functional study was performed in yeast, using single- and double- knockout ORM (Hjelmqvist L., et al., 2002). The growth rate was highly affected in the case of double-knockout for ORMs. This phenotype was more pronounced when ER stressors as dithiothreitol (DTT) or tunicamycin, among others, were added to the yeast media. The expression of the human ORMDL3 (hORMDL3) in the double-knockout yeast model, partially rescued this phenotype, suggesting a common functional activity of these family proteins. Therefore, due to location of these family

proteins in ER, the impact of some stimuli in the double-knockout strain, and some other evidences in mammalian cells (Miller M et al., 2012; Cantero-Recasens et al., 2010) this family of proteins have been associated to the ER stress response called Unfolded Protein Response (UPR).

In mammalian cells, ORMDL family has been shown to affect the cellular calcium homeostasis. High ORMDL3 expression levels increase cytosolic calcium content, reducing the calcium content in stores by inhibiting the SECA pump. In addition ORMDLe was shown to impair the buffering capability of mitochondria (Cantero-Recasens G et al.,2010, Carreras-Sureda A et al., 2013).

Finnaly, in 2010, two independent studies in yeast associated both ORM orthologs with alteration in cellular ceramide levels. Thus, double-knockout of ORMs increased sphingolipid levels in the cells. Moreover, it was described that the ORM family proteins were able to interact with both seril palmytoil transferases (SPT1 and 2), the main enzymes that catalyze ceramide synthesis by *de novo* pathway. (Breslow DK et al., 2010, Han S et al., 2010).

In summary, UPR, calcium handling and sphingolipid synthesis are the three processes where ORMDLs have been implicated. An overview of them and the participation of ORMDLs will be described in the following sections.

1.1.2.1 ER stress and UPR

The endoplasmic reticulum has different cellular functions like synthesis, modification, release and trafficking of proteins, calcium storage, metabolism of carbohydrates and lipids and sterols synthesis (Galuzzi L, et al.,2017; Sommer T., et al 1993). A modification in the physiology status of this organelle (normally associated to abnormal protein folding) leads to ER stress response or UPR. This UPR involves a cascade of events to restore ER homeostasis by decreasing the load of proteins in the ER. In mammalian cells this UPR is triggered by three transmembrane proteins: the Inositol-requering protein 1 (IRE1), the double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and the activating transcription factor 6 (ATF6).

The ER stress is recognized by the chaperone BiP, which detach from the inhibitory domains of IRE1, PERK and ATF6 when an accumulation of misfolded proteins take place. This release promotes the activation of the different UPR pathways that implies oligomerization and autophosphorylation of IRE1 and PERK and the translocation of ATF6 to the Golgi apparatus and then activated via proteolytic cleavage.

These three signalling pathways contribute to re-establish the physiological status of the ER, reducing the ER stress and

ensuring cell survival. However, if ER stress is prolonged and cannot be reversed, a cell death program is activated, usually implying autophagy and apoptosis (Bhat TA et al., 2017).

IRE1 pathway

In mammals, IRE1 is formed by two main transmembrane proteins: IRE1α and IRE1β. Upon ER stress, IRE1 is trans-autophosphorylated and activates the C-terminal endoribonuclease domain. It promotes the mRNA excision of X-box binding protein 1 (XBP1), which leads to a frameshift that extends the open reading frame and allows the translation of the spliced XBP1 (XBP1s) transcription factor (Yoshida H et al., 2001). XBP1s induces the expression of different chaperones involved in ER-associated degradation (ERAD), lipid metabolism (Lee AH et al., 2008), proinflammatory cytokines (Martinon F,Nat Immunol 2010) and autophagy mechanisms (Margariti A et al., 2013). Furthermore, IRE1 can contribute to the degradation of mRNAs that are localized to the ER membrane through a process known as regulated IRE1 dependent decay (RIDD) (Kimmig P et al., 2012). IRE1 trans-phosphorylation is also able to recruit TNF receptor-associated factor 2 (TRAF2) to the ER membrane to phosphorylate IκB, therefore activating NF-κB and triggering inflammatory pathways (Hu P et al., 2006) (figure 3).

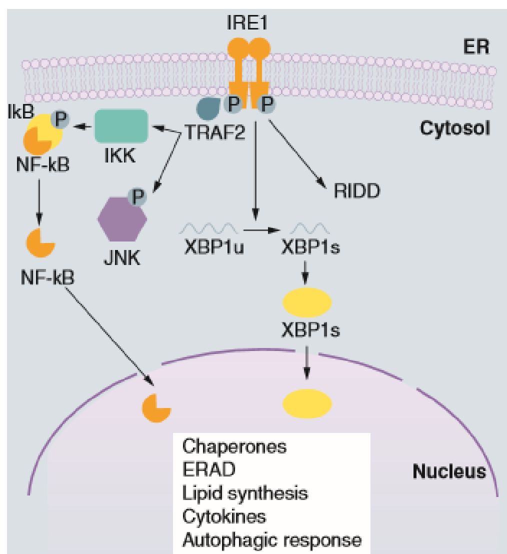


Figure 3. IRE1 pathway in the UPR. Modified image from Galluzzi L *et al* 2017.

PERK pathway

The ER stress induction allows PERK autophosphorilation and the phosphorylation of its substrate, the alpha subunit of eukaryotic translation initiation factor alfa (eIF2α), blocking in general the protein translation. At same time, phosphorylation of eIF2α allows the translation of the ATF4 factor, which induces the expression of the pro-apoptotic gene CHOP. The induction of this pro-apoptotic gene blocks the antiapoptotic activity of B-cell lymphoma 2 (Bcl-2) (McCullough KD et al., 2001;Yoshida H et al., 2007), among other responses in the nucleus. ATF4 factor activity also involves the expression of GADD34 that negatively regulates eIF2α (Novoa I et al., 2003). Moreover, PERK induces the expression of genes

involved in the antioxidant response via phosphorylation of the transcription factor NFE2L2/NRF2 (Cullinan SB et al.,2004) (figure 4).

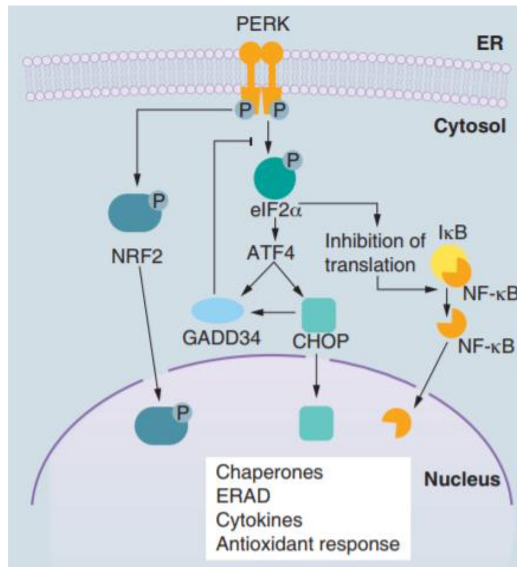


Figure 4. PERK pathway in the UPR. Modified image from Galluzzi L *et al* 2017.

ATF6 pathway

The transmembrane protein ATF6 located in the ER translocates to Golgi apparatus under ER stress conditions. This protein has a N-terminus bZIP transcription factor. After translocation, ATF6 is subjected to proteolysis and the N-terminus fragment is released. The activation of ATF6 N-terminal part induces XBP1 transcription, and optimizes the UPR controlling genes related with protein folding and lipid

synthesis. (Bettigole SE et al., 2015; Maiuolo J et al., 2011; Wu J, Dev. Cell 2007) (figure 5).

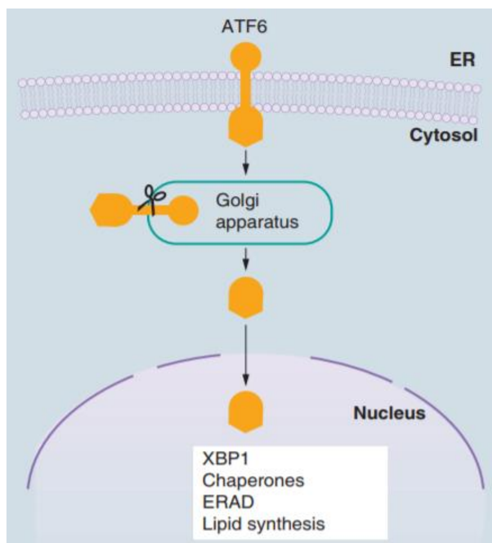


Figure 5. ATF6 pathway in the UPR. Modified image from Galluzzi L *et al* 2017.

Role of ORMDL family in UPR

As previously mentioned, Hjelmqvist and collaborators described the association of ORMDL family with ER stress and UPR response based in the observations with different ER stress inductors like DTT or tunicamycin.

Other studies have supported the idea that ORMDL family is able to modulate ER stress. *McGovern DP et al.* showed that overexpression of ORMDL3 decreased the UPR response monitoring XBP1 splicing and UPR gene transcription in basal

and under tunicamycin and thapsigargin treatments. Opposite results were obtained using, shRNA against ORMDL3. On the other hand, our group demonstrated that overexpression of ORMDL3 in HEK293 cells increase eIF2α phosphorylation. Other UPR pathways than PERK were not affected. (Cantero-Recasens et al., 2009).

ATF6 pathway has also been shown to be altered under ORMDL3 overexpression conditions. In two different studies, overexpression of this protein in lung epithelial cells (A549) and in bone marrow-derived macrophages (BMDM) from transgenic ORMDL3 knock-in mice, it has been demonstrated that nuclear translocation of ATF6 was triggered (Miller M et al., 2012; Miller M et al., 2014).

These different evidences for ORMDL impact in ER stress and UPR response are not totally understood and some are partially contradictory. However, they support the idea that ORMDL family has an important role in maintaining ER homeostasis.

1.1.2.2 Sphingolipid regulation

Sphingolipids are the principal components of cell membranes which regulate the membrane fluidity among other functions. Besides, sphingolipids together with cholesterol contribute to the formation of the detergent-resistant membrane microdomains, that function as

membrane signalling platforms, called lipid rafts (Lingwood D et al., 2010).

Sphingolipid metabolism

These essential lipids are composed by a backbone formed by 18 carbon atoms that contains an amino alcohol N-acylated with diverse fatty acids to form different ceramide species. The ER is the organelle where the sphingolipids are synthetized by *de novo* pathway. The sphingolipid formation starts with a rate-limiting step catalized by the serine palmitoyl transferase (SPT), consisting in the condensation of serine and palmitoyl coenzyme A (CoA) and the production of 3-ketosphinganine. This molecule is then reduced to dihydrosphingosine (DHS), also named as sphinganine, by the 3-ketodihydrosphingosine reductase (KDHR) in a NADPH dependent manner. The subsequent reaction is carried out by the dyhidroceramide synthase (CerS), which acylates sphinganine by adittion of a fatty acyl-CoA, forming dihydroceramides. In mammals, six different CerS are known, each of which uses specific acyl chains, typically with saturated or mono-unsaturated fatty acids with 14 to 26 carbons (Pewzner-Jung Y et al.,2006; Lahiri S et al., 2007).
After that, dyhidroceramide is dehydrogenated by spingosine δ-4 desaturase to ceramide.

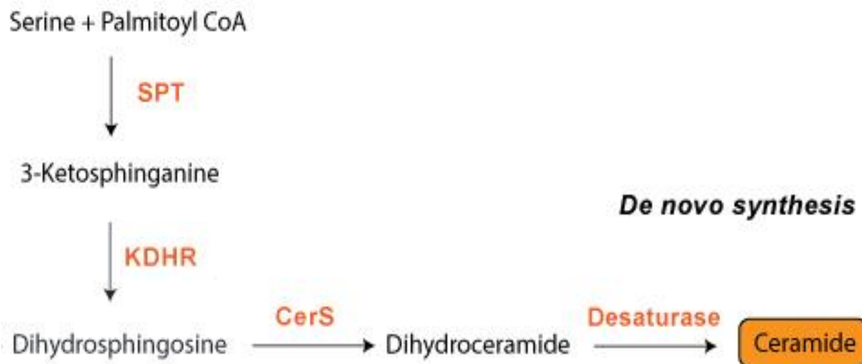


Figure 6. *De novo synthesis* of ceramides in sphingolipid metabolism. *De novo synthesis* starts with the condensation of palmytoil CoA and serine by SPT. This is followed by a series of reactions leading to formation of ceramide.

Ceramides can be transported to Golgi apparatus by two main mechanisms. A non-vesicular transport, which involves the ceramide transfer protein CERT, that comprises donor membrane (ER) and acceptor membrane (Golgi) recognition domains and a hydrophobic pocket for direct ceramide binding. The other transport is vesicular-dependent. This transport despite not being well characterized is the main transport of ceramides to the cis-Golgi (Watson P et al., 2005) Upon reaching Golgi apparatus, ceramides suffer various modifications such as glycosylation resulting in the formation of glycosphingolipids by the glucosylceramide synthase (GCS), phosphorylation resulting in the generation of phosphoceramides by the ceramide kinase (CERK) and addition of phosphoethanolamine (PEA). This PEA addition to ceramide produces sphingomyelin (SM). Moreover, complex sphingolipids can be recycled to ceramide by degradation

enzymes as sphingolmyelinase (SMase), glucosylceraminase (GCase). These recycling pathways from complex sphingolipid together are highly regulated and are important in sphingolipid homeostasis (figure 7).

Opposite to the sphingolipid synthesis is the degradation pathway taking place in lysosomes, on the membranes, and in the nucleus. In this pathway, the ceramidase cleaves the fatty acid from the ceramide producing sphingosine which can be recycled back to ceramide or be further phosphorylated by sphingosine kinase (SK) to sphingosine 1-phosphate.

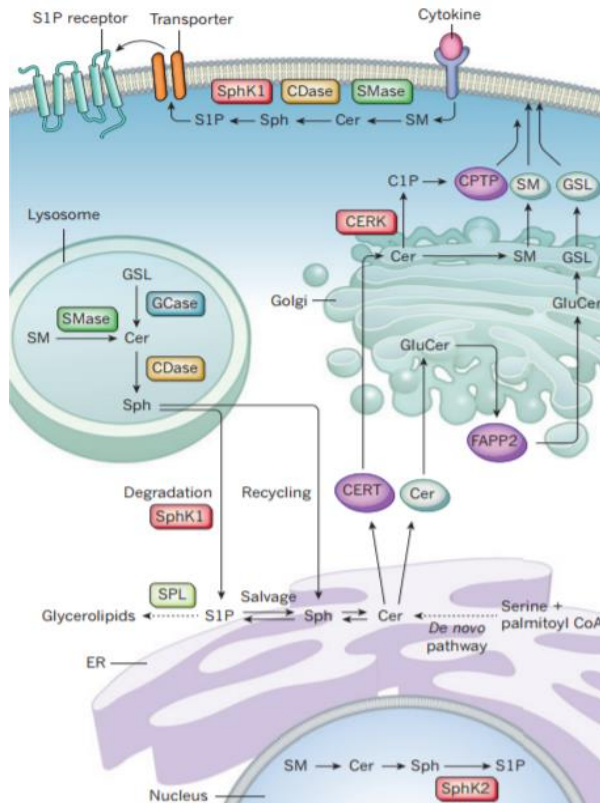


Figure 7. Sphingolipid metabolism in cellular homeostasis. Modified image from Maceyka M *et al* 2014.

Serine Palmytoiltranferase (SPT) complex

The principal reaction that take place to induce ceramide biosynthesis by *de novo* pathway is carried out by the enzyme SPT. In yeast, SPT enzyme works as a heterodimer composed by three subunits, LCB1, LCB2 and TSC3. This last protein, TSC3, is an additional activator of SPT complex (Gable K et al., 2000) but this orthologue in mammals has not been identified. Years after the discovering LCB subunits in

yeast, the human orthologs SPTLC1 (55KDa) and SPTLC2 (65KDa) were described (Nagiec MM et al., 1996; Weiss B et al., 1997/ Hanada K et al., 1997). In mammals, SPT complex is composed by three different subunits; SPTLC1, SPTLC2 and SPTLC3, forming an octamer of four SPTLC1 plus four SPTLC2 and/or SPTLC3. At same time, this octamer is divided in four dimers formed by interaction of SPTLC1 and SPTLC2 or SPTLC1 and SPTLC3. There are different kind of similarity between these subunits. SPTLC1 and SPTLC2 share 20% similarity and are highly conserved among species, while SPTLC2 and SPTLC3 show more than 80% similarity (Hornemann T et al., 2006).

As structure, SPTLC1 is bound to ER membrane. On the other way, SPTLC2 or SPTLC3 are not interacting directly with the ER membrane. SPTLC1 interacts with ER membrane by the N-terminal domain meanwhile the C-terminal forms a cytosol-oriented active site, which is the responsible to interact with the other two subunits, SPTLC2 or SPTLC3 (Hornemann T et al., 2007) (Figure 8).

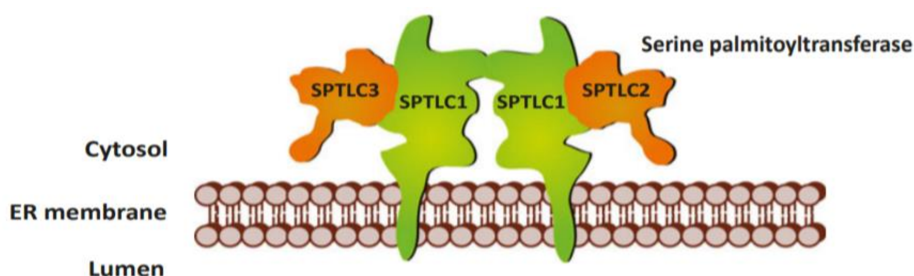


Figure 8. SPT structure. SPT composition liked to ER membrane by the regulatory subunit SPRLC1. SPTLC2 or 3 form the catalitic subunit of the

complex. All of them are oriented to cytosol. Modified image from Paulenda T *et al* 2016.

About SPT activity, it is known to be critical for embryonic development, due to homozygous SPTLC1 and SPTLC2 knock-out mice die in embryogenesis (Hojjati MR et al., 2005). Overexpression of SPTLC1 in transgenic mice and in HEK293 cells do not show increased SPT activity (McCampbell A et al., 2005, Hornemann T et al., 2006) suggesting that SPTLC1 is not directly involved in the catalytic reaction of SPT complex. On the other hand, overexpression or silencing of STLC2 or SPTLC3 subunit alters SPT activity increasing or decreasing it respectively (Hornemann T et al., 2006).
Two small subunits in the human SPT complex are required for maximal enzyme activity: small subunit STPa and small subunit SPTb (ssSPTa, ssSPTb). They activate SPT complex by direct interaction with SPTLC1 after dimerizing with SPTLC2 or SPTLC3. (Han G et al., 2009).

To maintain cellular spingolipid homeostasis, SPT is tightly regulated by negative feedback loop (Breslow et al., 2010). There have been also described several inductors of SPT activity as endotoxins, UVA and tetrahydrocannabinol (Memon RA et al., 1998/Grether-Beck S et al., 2005/Gomez del pulgar T et al., 2002). There are also specific inhibitors. Myriocin is one of the most commonly acting on the catalytic

site of SPT (Miyake Y et al., 1995; Lowther J et al., 2010; Wadsworth JM et al., 2013). However, the exact endogenous mechanism involved in SPT inhibition is not fully understood. In this context, ORMDLs have been described as endogenous inhibitors of SPT complex (Han S et al., 2010; Breslow DK et al., 2010; Siow DL et al., 2012; Kiefer K et al., 2015).

The regulatory mechanism of ORMDLs has been mainly studied in yeast. Thus, it is known that low levels of cellular sphingolipid content promotes ORMs phosphorylation in the N-terminus (Sun Y et al., 2012). Orms are phosphorylated by two different kinases: Ypk1, downstream TORC2 (Roelants FM et al.,2011), and Npr1 downstream TORC1. (Shimobayashi M et al., 2013). The phosphorylation promotes the dissociation of Orms from the SPT complex, releasing its activity to restore the sphingolipid levels. On the other hand, dephosphorylation of Orms is carried out when sphigolipid levels are high. In this case, two different phosphatases act on Orms: Tap42 downstream TORC1 (Liu M et al., 2012) and Cdc45-PP2A activated under the heat stress response (Sun Y et al., 2012).

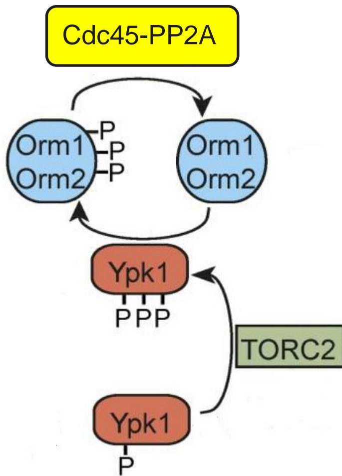


Figure 9. Example of de- and phosphorylation of Orm proteins downstream TORC2. In this case, the kinase Ypk1 phosphorylates Orm1 and 2 and Cdc45-PP2a dephosphorylates them. Modified image from Roelants FM *et al* 2011.

However, an extrapolation of this mechanism to other species is not easy because the N-terminal phosphorylation motif was lost during evolution (Hjelmqvist L et al., 2002; Han S et al., 2010) and still today, a comparable phosphorylation site in mammals has not been discovered. In this respect, our laboratory has demonstrated that the interaction between ORMDL-SPT complex in humans is independent of the sphingolipidic environment. This work suggested the idea of a complex rearrangement between ORMDL-SPTLC structures under changes in sphingolipid contact rather than a binding-release mechanism (Kiefer K et al., 2015).

The degradation of ORMDLs has been also suggested as a mechanism to release the SPT complex. Thus, a recent study showed that degradation of ORMDL proteins was enhanced in RAW247 macrophages exposed to free cholesterol. The high cholesterol content in ER led to initiation of

autophagosome formation and translocation of ORMDL1 to cytoplasmic puncta corresponding to the autophagosome (Wang S et al., 2015).

The general view is that three mammalian ORMDLs have redundant functions (Siow D et al., 2015). Thus, overexpression of individual ORMDL members did not cause a significant decrease in the ceramide content in HEK293 cells while overexpression of all ORMDL members led to complete blockage of de novo ceramide synthesis. However, there is some controversy in this point. A recent publication performed with A549 and RAW264.7 cells showed that ORMDL3 overexpression decreased cellular ceramide levels. In fact, in cells with mid-levels of overexpression of ORMDL3, inhibition of SPT complex activity was observed (Oyeniran C et al., 2015). On the contrary, under a strong overexpression in the same cells, sphingolipids content was elevated (Oyeniran C et al., 2015).

1.1.2.3 Calcium homeostasis

Calcium (Ca2+) is an important cation implicated in intracellular signalling as a second messenger. The cellular calcium signalling network is established by a gradient between extra- and intracellular Ca2+ concentration (from extracellular 100mM to intracellular 100nM) allowing the cell to stimulate Ca2+-sensitive processes depending on speed,

amplitude and spatial-temporal pattern of Ca2+ influx (Jouaville LS et al., 1995; Budd SL et al., 1996). Several external stimuli trigger the Ca2+ ON reactions resulting in an increase of cytosolic Ca2+. This Ca2+ can come from the entry of external Ca2+ or the release of this cation by internal stores as organelles (mitochondria, ER…). The entry of external Ca2+ is performed by the activation of different Ca2+ channels located on the plasma membrane as can be the store-operated channels (SOCs), the volage-operated channels (VOCs) and the receptor-operated channels (ROCs). Besides, the release from internal storages are mainly controlled by two different channel families located in the ER, also called sarcoplasmic reticulum (SR) in muscle cells, the inositol-1,4,5, triphosphate receptors ($InsP_3R$) and the ryanodine receptors (RYR) (Berridge MJ et al., 1993; Clapham DE et al., 1995).

On the other hand side, several pumps and exchangers remove Ca2+ from the cytoplasm to return intracellular resting levels, what is known as Ca2+ OFF reactions (Blaustein MP et al., 1999; Pozzan T et al., 1994). These OFF reactions are associated with the Ca2+-ATPase pumps (PMCA) and Na+/Ca2+ exchangers both of them located at the plasma membranes, while sarco-endoplasmic reticulum Ca2+ ATPase pump (SERCA) returns Ca2+ to internal storages.

Mitochondrias also participate in OFF reactions buffering cytosolic calcium rises by importing calcium throght the VDAC channel at the outer membrane and the mitochondria calcium uniporter (MCU) at the inner membrane. The buffering activity of mitochondria has been shown to be essential to shape several calcium signals and fine tune signalling cascades. (Carreras-Sureda et al., 2012)

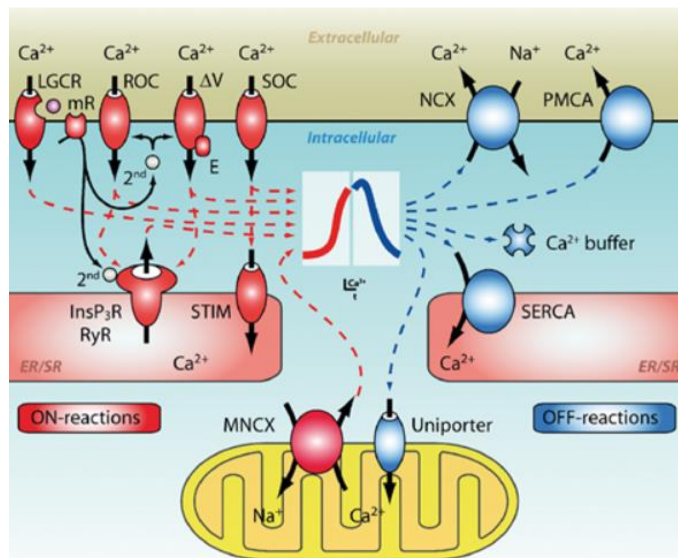


Figure 10. Principal Ca2+ channels, pumps and organelles responsible for intracellular Ca2+ trafficking. Signals for Ca2+ movement are associated to ON or OFF reactions to increase or decrease respectively Ca2+ in the cytoplasm. Modified image from Hörtenhuber M *et al* 2017.

There are several evidences linking ORMDL3 expression levels with altered cellular calcium homeostasis. Our group

have demonstrated in two different models (HEK293 and Jurkat T) that ORMDL3 is able to alter cytosolic calcium levels, ER calcium content and the store-operated calcium entry. Besides, this ER located protein has the capacity to modulate Ca2+ influx in mitochondria. Regarding the domains implicated in this function, the C-terminus of ORMDL3 play a role in the regulation of endoplasmic reticulum Ca2+ content despite the direct interaction of ORMDL3 with SERCA2b was maintained (Cantero-Recasens et al., 2010). The N-terminus was also essential to modify store-operated calcium entry and mitochondrial calcium buffering capability (Carreras-Sureda et al 2013). Confirming the connection between ORMDL3 and calcium homeostasis, hORMDL3 overexpressing mice showed an increase SERCA2b expression. This ATPase has been implicated in airway remodelling in asthma (Miller M et al., 2014). Moreover, knock down of ORMDL3 using siRNA in eosinophils produce a reduction in cytosolic calcium levels (Ha SG et al 2013). However, ORMDL3 has no the same capacity to modulate Ca2+ in all cell types. Control and ORMDL3 knock-down cells after activation through the high-affinity IgE receptor (FceRI) have similar calcium responses in mast cells (Bugajev V et al., 2016).

1.1.3 Pathology associated to *ORMDL3*

Genetic association studies provide us an approach to study the relationship between gene function and the risk of developing a disease. Thus, Genome-wide association

studies (GWAS) allow finding genetic risk factors comparing the genetic sequence of healthy control populations and disease state populations. (Manolio TA et al., 2010). In the case of *ORMDL3* gene, different GWAS studies showed that its locus is associated with numerous pathologies such as rheumatoid arthritis (Kurreeman FA et al., 2012), type 1 diabetes (Saleh NM et al., 2011; Plagnol V et al., 2011), primary biliary cirrhosis (Mells GF et al., 2011), ulcerative colitis (Anderson CA et al., 2011), Crohn's disease (Barrett JC et al., 2008/Franke A et al., 2010), allergic rhinitis (Tomita K et al., 2013), and ankylosing spondylitis (Laukens D et al., 2010).

Asthma is the pathology in which the association with *ORMDL3* has been studied more in detail. Moffatt and colleagues were the first reporting that multiple SNPs on the chromosome 17q21 linked to *ORMDL3* increase expression were associated with a higher risk to develop childhood asthma. In this context, it has been published that ORMDL3 is able to modulate T-lymphocyte activation and that depending on the individual allelic hereditary, this activation can have an important impact in proinflammatory pathologies as asthma (Carreras-Sureda A et al., 2016). Other studies claimed that the expression of ORMDL3 associated to the SNPs in the chromosome 17q21 affect mostly to primary T cells (Schmiedel BJ et al., 2016). Besides, in this study the authors confirmed that asthma-risk variants rs4065275 and

rs12936231 are able to switch CTCF-binding sites in the 17q21 locus, affecting the organization in this locus to favour an enhanced transcription of *ORMDL3*.

Considereing the focus of this thesis on cancer, it is relevant to point out that the risk allele rs7216389 in *ORMDL3* locus, one with high impact in the genetic risk for asthma, presents a significant association with glioma. In addition, four other SNPs mapping to the region of *ORMDL3* (rs2290400, rs8067378, rs11557467 and rs9303277) despite not being statically significant, point at the same direction. (Dobbins SE et al., 2011). This association study implies that higher expression of *ORMDL3* gene would increase the susceptibility to develop glioblastoma.

For the others two ORMDL genes there is almost no data providing evidences for genetic association with pathologies. In the case of *ORMDL1*, there is one study in which this gene is linked with an increased in bilirubin levels (Kang TW et al., 2010).

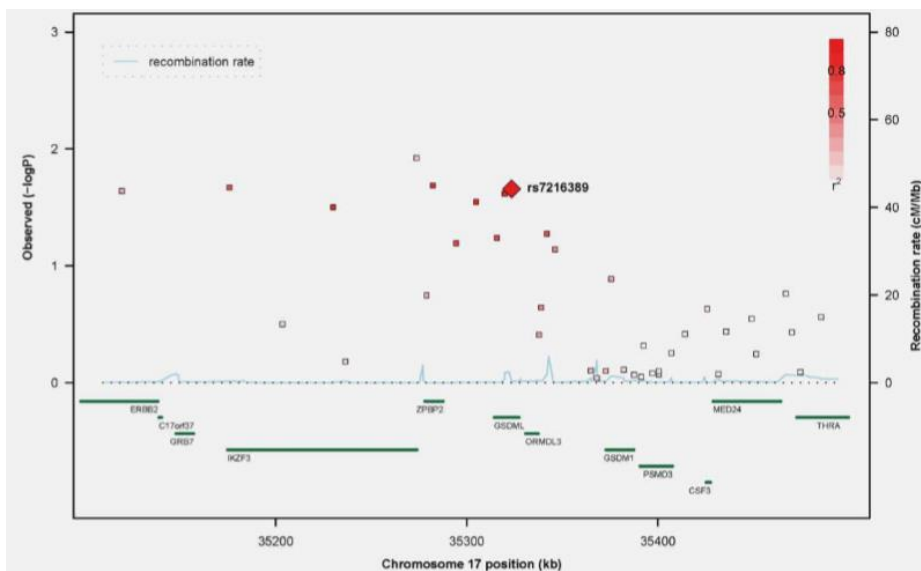


Figure 11. Plot of the 17q21 association with glioma. Test *p* values (represented as -$\log_{10}$ in left y axis) are shown for SNPs analysed in GWAS for glioma study. In the plot are represented all SNPs found in the asthma-risk locus, rs7216389 as a red diamond. Intensity of red colour increase with the increase of the linkage disequilibrium. Recombination rates in HapMap CEU across the region are shown in blue (right y axis). Gen mapping of the region is represented in x axis. Modified image from Dobbins SE *et al* 2011.

1.2. Mitochondria-associated membranes

The intracellular mobility of macromolecules and organelles is hugely limited for the high viscosity of the cytosol by the extreme crowding of intracellular components (Kalwarczyk T et al., 2011/ Ellis RJ et al., 2001). This reduced mobility also influences the strength of interactions between the different intracellular components. Besides, the strong interaction between components as the affinity of protein structures, show an additional factor influencing this strength. The direct interactions between mitochondria and the ER within the cell have been extensively studied. ER and mitochondria interaction was first described by Copeland and Dalton using electron microscopy in cells of the pseudobranch gland of a teleost (Copeland DE et al., 1959). These junctions formed between ER and mitochondria have been named as mitochondria-associated membranes (MAMs). Later, by different types of approaches, this interaction was confirmed by different research groups (Pickett CB et al., 1980; Montisano DF et al., 1982). Functionally, this interaction was initially associated with the direct exchange of ions and metabolites between organelles, mainly Ca2+ ions (Patergnani S et al., 2011; Rizzuto R et al., 1998) but also lipids and lipid-derivated molecules such as ceramides (Grimm S et al., 2012).

1.2.1 MAMs structure and components

The intracellular homeostasis is highly influenced by the ER-mitochondria network. It has been demostrated that around 5–20 % of the mitochondrial surface structure is closely opposed to ER membranes (Rizzuto R et al., 1998). Besides, both, smooth and rough ER forms, interact with mitochondria suggesting that selective recruitment of exclusive domains of ER can be involved in important tethering complexes and as result, that different types of MAMs exists (Csordas G et al., 2006; Wang PT et al., 2015). This connection between these two organelles is supported by a big group of proteins that conform the MAMs.

MAMs have a distinct proteome, specific tethers on the cytosolic face and regulatory proteins in the ER lumen. An isolation of MAM is composed by ER membrane and the outer mitochondrial membrane (OMM) that has been in close contanct at the time of the fractionation (Klecker T, et al., 2014; Marchi S et al., 2014; Naon D et al., 2014). In the last decades, different studies confirm that the molecular composition of the MAMs fraction may contain numerous proteins, from 75 (Raturi A et al., 2013) to more than 1000 by Percoll isolation (Poston CN et al., 2013) what claim that MAMs are an enriched-protein structure. This enrichment of MAM proteins is regulated by lipid modification as palmitoylation, and cytosolic proteins. Besides other proteins of MAM structure are associated with other organelles as

Golgi apparatus, cytoskeleton, lysosomes, ribosomes or plasma membrane (Lebiedzinska M et al., 2009). As a summary, we conclude a brief list of the main MAMs components:

MAMs Proteins	Function
SERCA	ER Ca2+ uptake
GSK 3β	regulator of the VAPB–PTPIP51 interaction
TDP43	Reduce ER-mitochondria associations.
SOD1	Antioxidant enzyme
Fused in sarcoma (FUS)	Accumulation is associated to ALS/FTD
Mitofusins-1/2 (MFN1-MFN2)	ER-mitochondria tether complex
PARKIN	Targeting proteins for degradation by proteasome
FACL4	Ligation of fatty acids to Coenzyme A

IP3R-Grp75-VDAC1	ER-mitochondria tether complex
DJ-1	Redox-sensitive chaperone for oxidative stress. Inhibits aggregations of α synuclein
CNX	Chaperone ensured for protein folding
α Synuclein	Molecular chaperone in the formation of SNARE complexes.
VAPB-PTPIP51	ER-mitochondria tether complex
PACS-2	Regulation of Bap-31
Fis-Bap31	ER-mitochondria tether complex
Beclin1-PINK	Enhance ER-mitochondria interaction, promoting the formation of autophagosomes.

Table 1. Principal proteins located in MAMs and corresponding functions.

1.2.2 MAMs function

MAMs play an important function to maintain cellular homeostasis. These microdomains have emerged as signaling hubs, playing key functions in lipid biosynthesis, calcium signaling, bioenergetics and participating in essential cellular processes like Unfolded Protein Response (UPR), autophagy, and apoptosis. Here we describe in more detail some the functional aspects relevant for this thesis.

1.2.2.1 Calcium regulation

Initial studies in calcium signalling (Somlyo AP et al., 1984) claimed the importance of ER and mitochondria for calcium handling. On one hand the ER is one of the major Ca2+ storage in the cell. On the other hand, depite mitochondria in basal status does not stores high amount calcium (order of nanomolar),,when cytosolic calcium concentration rises, mitochondria is able to increase the intramitochondrial calcium levels until micromolar values (Giacomello M et al., 2010). Nowadays we know that Ca2+ flux between ER and mitochondria is essential for cellular viability. Several molecular players participate in this process.

Ca2+ in the ER determines the correct functions of chaperones in this organelle, like calreticulin (CRT) or calnexin (CNX), involved in the generation of secretory proteins (Michalak M et al., 2002). Both chaperones can

interact with IP_3R and the calcium transport ATPase SERCA2b to regulate ER-mitochondria Ca2+ flux (John LM, et al., 1998; Joseph SK et al., 1999). Besides, the ER protein sigma-1 receptor (Sig-1R) forms a Ca2+sensitive chaperone complex with BiP/GRP78 and prolongs Ca2+ signalling from ER to mitochondria by the stabilization of IP_3R subunit 3 located in MAMs (Hayashi T et al., 2007). In addition,, IP_3R is able to interact with VDAC1 in the OMM through GRP75, a chaperone that is located in MAMs, to enable the Ca2+ mitochondrial uptake from ER. (Szabadkai G et al., 2006).

Another key point in the ER-mitochondria Ca2+ transport is the distance between these two organelles. Thus, a decrease in the ER-mitochondria contact distance, induces an overload of Ca2+ in the mitochondria triggering the opening of the mitochondria permeability pore giving rise to apoptosis (Krols M et al., 2016)

1.2.2.2 Autophagy and apoptosis regulation

Autophagy

Autophagy was firstly described in rat liver cells by an increase of lysosomes formation after glucagon addition (Ashford T et al., 1962). This process is a highly regulated mechanism by which different cellular components (including damaged organelles or incluse pathogens) are targeted to the

lysosomes for degradation. Today we know that exist at least 31 autophagy related genes (Atg) which are involved in the regulation of the different phases of this cellular process: induction, cargo detection and selection, formation of vesicles, elongation, autophagosome-vacuole fusion, and breakdown of the cargo followed by release of the degradation products back into the cytosol (Klionsky DJ et al., 2007).

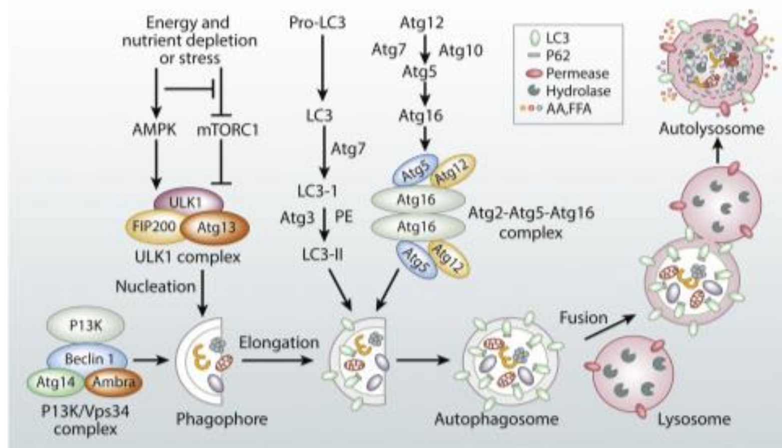


Figure 13. Different steps involved in autophagy. Input of the different components of autophagy in the formation and elongation of the phagophore to form the autophagosome and the posterior fusion with lysosome. Modified image from Kaushal GP *et al* 2016.

MAMs are also involved in the formation and development of autophagic machinery. First, proteins of ER were discovering to localize in the autophagosome membrane. These proteins juxtaposed with the initial membranes of the autophagosome (Ylä-Anttila P et al., 2009; Kornmann B et al., 2009). Besides,

the OMM gives part of mitochondrial membranes for the autophagosome biogenesis. In this context, ER-mitochondria interaction is considered to have an important role in the autophagy induction mechanisms. Thus, there are several evidences showing that autophagosomes are formed at the ER-mitochondrial contact sites under starvation conditions (Hamasaki M et al., 2013). Nevertheless, until now it is not fully undestood the function of MAMs in the molecular mechanisms that leads to autophagy. Remarkably, one of the main regulatory complex of the autophagy is the mechanistic target of rapamycin (mTOR) (Sabatini DM et al., 1995; Thoreen CC et al., 2004). This complex is formed by two subunits: the mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2. It has been published that mTORC2 is located in MAMs activating AKT that acts maintaining MAMs integrity and mitochondria morphology. Besides, this mTORC2-AKT activation modulates IP_3R3 phosphorylation and controls Ca2+ release in MAMs (Betz C et al., 2013).

Finally, some studies revealed that starvation or stress conditions are able to promote autophagy increasing expression of two genes, PINK1 and parkin (Cook KL et al., 2014), located in mitochondrial compartments. Moreover, mitochondria labelled with parkin are not only swallowed by the autophagosomes but are used also to form new autophagosomes.

Apoptosis

Apoptosis is a process of programmed cell death that happens in multicellular organisms. (Elmore S et al., 2007) This mechanism can be initiated by two different pathways: the intrinsic pathway (also known as mitochondrial pathway), activated by intracellular signals processed when cells are stressed,, and the extrinsic pathway, which is activated by extracellular ligands binding to cell-surface death receptors (Alberts B , 2014).

Focusing on the intrinsic pathway, mitochondria is one of the main organelles affected. Stress signals induce mitochondrial membrane permeabilization (MMP) and cytochrome c release.. In the cytosol cytochrome c binds the apoptotic protease activating factor-1 (Apaf-1) and ATP, and as a result, this complex targets pro-caspase-9 forming the apoptosome. This apoptosome cleaves the procaspase-9 to its active form caspase-9 and causes the activation of the effector caspase-3 (Green DR et al., 2004).

The signalling previous to the MMP, occurs in the interface between the ER and the mitochondrial outer membrane. There are several pro-apoptotic and anti-apoptotic proteins described, being the BCL-2 family, which include pro-apoptotic and anti-apoptotic members, one of the most studied. Among its pro-apoptotic members, it is known that

BAX, BAK and BID play an important role in the MMP process.

The relevance of MAMs platforms in apoptosis signalling has been highlighted in several studies. Thus, the voltage dependent anion channels (VDAC1), an important MAM component located in the OMM (Shimizu S et al., 2001), allows, on one hand, the shuttle of ATP from mitochondria matrix to cytosol and, on the other hand, transfers Ca2+ signals to mitochondria. In physiological conditions, the anti-apoptotic family BCL-2 interacts with VDAC1. VDAC1 also interacts with IP_3R promoting ER-mitochondria contacts and calcium transfer between organelles. However, an overload of calcium in the mitochondria promotes VDAC1 oligomerization and causes apoptotic cell death by cytochrome c release (Keinan N et al., 2013; Szabadkai G et al., 2013). VDAC1 is not the only connection between MAMs and apoptosis. Other proteins expressed in MAMs like DRP1 or PACS-2 have been shown to have important roles in apoptosis too (Estaquier J et al., 2007;Simmen T et al., 2005).

1.2.3 MAMs tethering

Some of the listed proteins have an important function regulating the ER-mitochondria tethering. The right control of this process will influence the MAMs dynamics and cellular

homeotasis. Here we describe some examples to understand the role and importance of tether proteins:

Mitofusin 2

This GTPase is one of the most studied tethers. Scorrano group demonstrated that the removal of this protein causes defects in ER morphology (De Brito OM et al., 2008). MFN2 locates in the ER and interacts with other mitofusins located in mitochondria as could be MFN1 or 2, forming trans hetero- or homodimers tethers what is relevant to carry out a proper Ca2+ transfer between these two organelles (Merkwirth C et al., Cell 2008). These two groups also demonstrated by electron microscopy that MFN2 ablation increase ER and mitochondria distance, confirming the tether function of this protein.

IP3R3-Grp75-VDAC1

The inositol-3-phosphate receptor 3 (IP3R3) involved in Ca2+ release from ER interact with the voltage-dependent anion channel 1 (VDAC1) through the molecular chaperone glucose-regulated protein 75 (grp75) to promote a correct Ca2+ flux between ER and mitochondria (De Stefani D., et al 2012). This complex is not a classical tether but it is essential for the proper mitochondrial-ER contact.. Besides, it has been shown that the Sigma-1R (Sig-1R) interacts with VDAC and IP3R stabilizing MAMs and prolongs Ca2+ transfer (Hayashi T et al., 2007).

VAPB-PTPIP51

The Vesicle-associated membrane protein-associated protein B (VAPB) is an ER protein involved in the UPR and cellular calcium homeostasis (Kanekure K et al., 2006). This protein interacts with the tyrosine phosphatase-interacting protein-51 (PTPIP51) located in OMM (De Vos KJ et al., 2012). Modifying the expression these two proteins or downregulating them alters ER-mitochondria distance and Ca2+ flux between organelles (Stoica R et al., 2014). Another tethering complex was recently associated also to VAPB, the VAPB-RMDN3 complex. The loss of this tethering complex was associated to disruption in Ca2+ signalling and an increase in cell autophagy (Gomez-Suaga P et al., 2017).

Fis1-Bap31

The B-cell receptor-associated protein 31 (Bap31) is an integral membrane chaperone protein located in ER controlling new synthetizing membrane proteins. Chandra D et al., demonstrated in 2004 that is the product of the cleavage of Bap31 who play an important role in the ER-mitochondria Ca2+ trafficking. Later, it was demonstrated that mitochondrial fission protein 1 (Fis1) interacts with Bap31 forming an ER-mitochondria association, critical to recruit and activate procaspase 8 and the posterior apoptosis signal from ER to mitochondria (Iwasawa R et al., 2011).

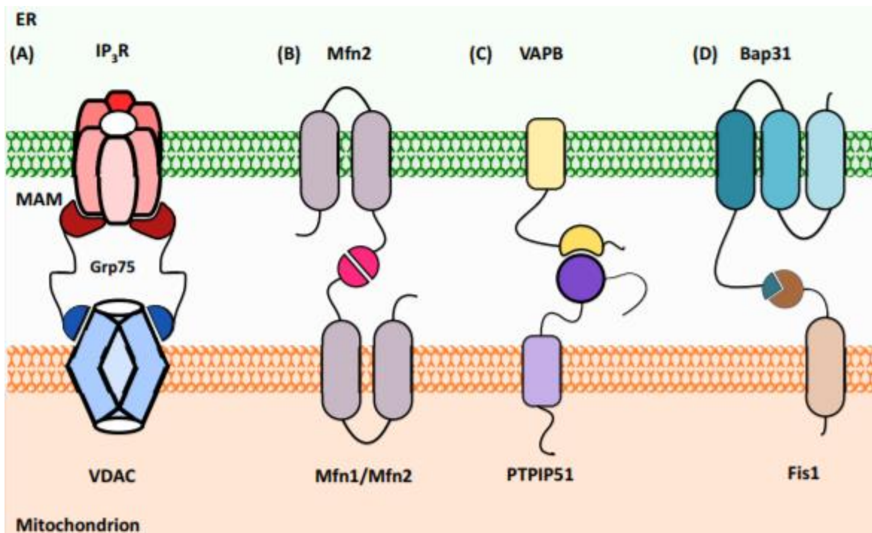


Figure 12. ER-mitochondria tethering complexes. A. IP_3R and VDAC interact through Grp75. B, ER-MFN-2 interacts with mitochondrial MFN-1 or -2. C, VAPB binds to PTPIP51 in mitochondria. D, Bap31 binds to Fis1. Modified image from Paillusson S *et al* 2016.

1.2.4 MAMs in cancer

1.2.4.1 Calcium signalling

Some studies claim that some cellular functions regulated in this ER-mitochondria cross talk might be important in oncogenesis. Some evidences point at the inositol triphosphate receptor (IP_3R). IP_3R is the most important transporter implicated in Ca2+ transport for the maintenance of Ca2+ homeostasis between ER and mitochondria. It is known that the activity of IP_3Rs is modulated by post-transcriptional modification as can be phosphorylation, in

order to modify the amount of Ca2+ efflux from intracellular compartments affecting the cellular response to apoptosis (Mikoshiba K et al., 2007; Vanderheyden V et al., 2009). In this scenario, the proto-oncogene serine/threonine kinase Akt can phosphorylate all the isoforms for IP_3R in a conserved sequence in the C-terminal tail of this receptor (Khan MT et al., 2006; Szado T et al., 2008). In cancer cells in which Akt is upregulated, the phosphorylation of IP_3R increase and, as consequence, Ca2+ efflux from ER to mitochondria is debilitate, acting as a protection in front of apoptotic stimuli. Moreover, the isoform that is enriched in the ER-mitochondria interface is IP_3R3, mainly phosphorylated by Akt, suggesting that the “antiapoptotic” function of Akt requires compartmentalization at MAMs (Marchi S et al., 2008; Marchi S et al., 2012). The phosphorylation of IP_3R3 can be modulated by different tumor supressors as PTEN, p53 and the promyelocytic leukemia protein (PML) (Dahia P et al., 2000; Song MS et al., 2012; Missiroli S et al., 2016; Giorgi C et al., 2010). Interestingly, part of PML is located in MAMs where the role of this protein is relevant to maintain a normal Ca2+ flux between ER and mitochondria.

1.2.4.2 Anticancer therapies targeting MAMs

Due to the high impact of Ca2+ signalling in ER-mitochondria connection, some therapeutic strategies fights to bring

calcium homeostasis to normality in cancer cells to remodel their sensibility for chemotherapeutic drugs.

Thus, some metal compounds can modify Ca2+ signalling and they are used as drugs to treat different types of tumors. For example, platin complexes are used to treat some cancers as sarcomas, lymphomas and germ cell tumors (Desoize B et al., 2002).

To potentiate the effect of these drugs, inhibitors of SERCA pump can be useful to stimulate artificial ER Ca2+-release. A similar strategy uses ER stress inducers like tunycamicin (Hou H et al., 2013) and cannabinoids (Carracedo A et al., 2006). Resveratrol has been also described as possible anti-cancer drug. Resveratrol acts as suppressor of SERCA activity at MAMs, given rise to an increase in mitochondrial Ca2+ uptake and induction of apoptosis in cancer cells (Madreiter-Sokolowski CT et al., 2016; Luyten T et al., 2017).

There are also approaches that target IP_3R specificaly in BH4-domain of this receptor, that is the binding-side of Bcl-2. The disruption of this interaction causes an increase in the Ca2+ release from ER and consequent apoptosis (Rong YP et al., 2008). This mechanism has been proven to induce cancer cell death in lymphocytic leukemia cells (Zhong F et al., 2011) and diffuse large B-cell lymphoma cells (Akl H et al., 2013).

Finally related to mitochondrial Ca2+ uptake, the microRNA miR-25 is a cancer-related drug targeting the mitochondrial inner membrane calcium importer, MCU. It has been described that the overexpression of this miRNA in HeLa cells induces a downregulation of the levels of MCU and mitochondrial Ca2+ uptake, being a possible mechanism for cell death resistance in cancer cells. Targeting this miRNA with anti-miR-25 oligonucleotides can be a potential agent against cancer resistance (Hayes J et al., 2014).

1.3. Cannabinoids

Extracts from the hemp plant *C. Sativa* have been used for recreational and medical purposes for many centuries (Gaoni Y et al., 1964/ Pertwee RG et al., 2008). From the 70 cannabinoids that form part of this plant, THC is the one in which more attention has been paid for its abundance in the plant and its relevance in the biomedical field.

Thus,it has been demonstrated that cannabinoids are able to produce palliative effects in neuropathic pain (Andreae MH et al., 2015), rheumatic diseases (Fitzcharles MA et al., 2016) and also they have been considered as potentials treatments for stroke (England TJ et al., 2015), hypoxic ischemia (Fernández-López D et al., 2013; Castillo A et al., 2010; Pazos MR et al., 2013) and neurodegenerative disorders as Alzheimer (Tolon RM et al., 2009; Aso E et al., 2013), Huntington (Blázquez C et al., 2011; Palazuelos J et al., 2009; Valdeolivas S et al., 2015) Parkinson (Gomez-Galvez Y et al., 2015; Fernandez-Ruiz J et al., 2009) and amyotrophic lateral sclerosis (Shoemaker JL et al., 2007; Witting AJ et al., 2004; Yiangou Y et al., 2006). Moreover, it has been shown that THC can inhibit cancer cell progression (Velasco G et al., 2012).

Besides THC, the endocannabinoids have a wide range of functions in the organims. Anandamide and the 2-AG

(Devane WA, Science 1992) have specific cell-surface cannabinoid receptors (Pertwee RG et al., 2010). Two main receptors have been described, CB_1 and CB_2 (Matsuda LA et al., 1990). They are G protein-coupled receptors (GPCRs). Moreover, other receptors such as the orphan receptor GPR55 and TRPV1 have been also shown to act as endocannabinoid receptors (Fernández-Ruiz J et al., 2007). CB_1 is the main receptor in the nervous system but is also expressed in non-neuronal tissues. CB_2 receptors are expressed primarily in cells of the immune and hematopoietic systems, but also recently they have been described in brain, in nonparenchymal cells of cirrhotic liver, in endocrine pancreas and in bone (Gong JP et al., 2006; Juan-Pico P et al., 2006; Ofek O et al., 2006).

Downstream cannabinoids binding to the receptors, adenyl cyclase is inhibited. The CB_1 receptor also modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of G-protein-activated inwardly rectifying K^+ channels (Howlett AC et al., 2002). Besides these cannabinoid receptors also modulate several pathways that are more directly involved in the control of cell proliferation and survival, including the extracellular signal-regulated kinase (ERK) (Bouaboula M et al., 1995), c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (Tramèr MR et al., 2001), phosphatidylinositol 3-kinase (PI3K)/Akt (Gomez del Pulgar T et al., 2000), focal

adhesion kinase (Derkinderen P et al., 2001) and sphingomyelin cycle (Sánchez C et al., 2001).

1.3.1 THC as antitumoral drug

In the last decades, many studies performed by different groups have shown that cannabinoids have a potent anticancer activity in *in vitro* and *in vivo* models (Munson AE et al., 1975; Galve-Roperh I et al., 2000; Sanchez C et al., Cancer Res 2001; Carracedo A et al., 2006; Salazar M et al 2009; Hernández-Tiedra et al., 2016). The antineoplastic activity of THC is based on the ability to promote cancer cell death and inhibit tumor angiogenesis. The first anticancerogenic observation was described in a study using THC to inhibit lung adenocarcinoma growth rate *in vivo* in mice (Munson AE et al., 1975). These antitumoral effects were later supported by other studies. THC also shows a high impact in the growth of lung carcinoma, glioma, skin carcinoma, lymphoma, thyroid epithelioma, melanoma and pancreatic carcinoma (Munson AE et al., 1975; Galve-Roperh I et al., 2000; McKallip RJ et al., 2002; Carracedo A et al., 2006; Blazquez C FASEB J 2006). Moreover, THC has been reported to exert antiproliferative action on different tumor cells in culture (Guzman M et al., 2003).

There are several studies using THC as anticancer drug in glioma, the model used in this thesis. The first work using this

strategy described an induction of cell death by apoptotic mechanisms in glioma treated cells (Sanchez C et al., Cancer Res 2001; Blazquez C et al., 2004; Galve-Roperh I et al., 2000; Gomez del Pulgar T et al., 2002) . Moreover, *in vivo* experiments it has been shown that intracranial injection of glioma cells and administration of THC or WIN-55,212-2 (synthetic cannabinoid) after tumour appearance, resulted in a reduction in the tumour size, increasing the survival rate in treated rats (Sanchez C et al., Cancer Res 2001).

1.3.2 Antitumoral pathway of THC

Nowadays the molecular mechanisms by which THC induces cancer cell death in different individual tumors is partially known. The anticancer activity of this molecule mainly relies in the induction of autophagy-mediated apoptotic cell death (Salazar M et al., 2009). One of the initial steps after THC binding to cannabinoid receptors is the stimulation of ceramide synthesis by *de novo* pathway. Specifically, the previous metabolite of ceramides, dihydroceramide, increases differentaly in abundance after THC-activity induction (Hernández-Tiedra et al., 2016). This increase in the ratio dihydroceramide: ceramide results in an induction of ER-stress.

The increase of ER-stress by THC leads to the up-regulation of the transcriptional co-activator protein 1 (Nurp1, also named p8) that has been associated in the control of tumourigenesis and tumour progression (Devane WA et al., Science 1992). As results, downstream targets of p8 also are up-regulated, ATF-4, the pro-apoptotic gene CHOP, and the effector of p8, the pseudokinase tribbles homolog 3 (Trib3) (Blazquez et al., 2004; Carracedo et al., 2006; Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002; Velasco G et al., 2012). The stimulation of this pathway leads to a promotion of autophagy/mediated cell death by the inhibition of the AKT/mTORC1 axis by TRIB3 (Salazar et al., 2009; Salazar et al., 2013).

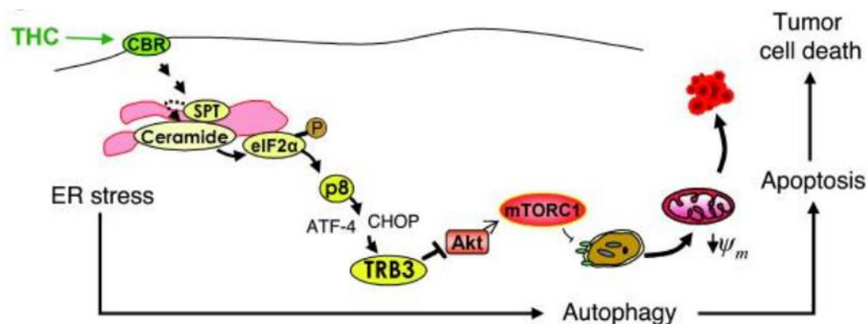


Figure 14. Schematic THC induction of tumor cell death. Signalling pathway by which THC is able to induce tumor cell death by CBRs interaction. After receptor interaction, this cascade promotes ER stress by a previous increase of ceramides what carry out a cytotoxic autophagy

and finally apoptosis and cell death. Modified image from Salazar M *et al* 2009.

Although autophagy is considered to have a cytoprotective role, it has been also shown that this mechanism could lead to cell death (Eisenberg-Lerner et al., 2009; Galluzzi et al., 2015; Mizushima et al., 2008). Different evidences have confirmed that autophagy is upstream of apoptosis in the mechanism of cannabinoid/induced cell death. One clear example is that the pharmacologic or genetic inhibition of autophagy prevents cannabinoid antitumour action in various animal models of cancer (Armstrong et al., 2015; Salazar et al., 2009; Vara et al., 2011). Moreover, blockade of apoptosis prevents cancer cell death but not autophagy. Those results indicate that autophagy is upstream of apoptosis what confirms that autophagy is important to induce cell death by THC administration.

2. MATERIAL AND METHODS

2.1 ORMDL3 constructs

pcDNA, ORMDL3 and ΔN-ORMDL3 plasmids used for cell viability assays were generated by PCR and cloned into pEGFP-N2 vectors. In case of immunofluorescence, ORMDL3-GFP was generated by PCR and cloned into pCDNA3. Delections for ORMDL3 were cloned into pEGFP-N2.

2.2 Cell culture

2.2.1 U87 cells and transfection

The human glioma cell line U87MG was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), penicillin/ streptomycin (100 units/ml), 1% of non-essential amino acids and sodium-piruvate. The cells were maintained in a 5% CO_2 environment at 37ºC.

When indicated, U87MG were transiently transfected with X-Treme gene HP DNA (Roche). DNA amounts in this cell line go between 0.5 and 1 µg of total DNA in the case of 24 well plate and 1.5 µg in the case of 6 well plate or 35mm coverslips. Ratio of X-Treme gene HP reagent and DNA is 2 µl per 1µg of DNA.

2.2.2 Generation of Tet-Off/On cell lines

Clontech Retro-X Tet-On Advanced Inducible Expression System was used to perform diferent control vectors (WT) and hORMDL3 overexpressing (Oe) clones in U87MG cells. In first instance, GP-293 cells were transfected with a pRetroX-Tight-Pur plasmid and an envelope plasmid (in our case VSV-G envelope plasmid). By this way GP-293 cells are able to produce virus with the pRetroX-Tight-Pur system. U87MG were then infected for 48h with the pRetroX-Tight-Pur virus. Selection of positive clones for the integration of pRetroX-Tight-Pur was done with G418. In order to select the best clones, once selected they were transfected with pRetroX-Tight-Pur-Luciferase. Using doxycycline, we could activate the Tight-Pur system and we measured the luciferase activity. The clone with higher luciferase activity was selected to transfect in this case with the pRetroX-Tight-Pur-control and ORMDL3 to obtain WT and Oe clones respectively. Several clones were selected and stored. The Tet-On condition was obtained after 24h of 500ng/ml of doxycycline treatment unless other indications.

2.3 Tetrahydrocannabinol treatment

Δ^9-THC (THC Pharm GmbH, Frankfurt, Germany) is received at 10mg/ml in ethanol. Nitrogen was used to evaporate ethanol and then DMSO as organic solvent was added. Final concentration of stocks was at 50 mM. A second dilution to 10

mM was performed to get working aliquots. We tested the antitumoral efficacy of THC of every new working aliquot in glioblastoma cells by MTT assays using different dilutions. For single concentration experiments, the dose used was the one that caused a decrease of 50% in cell viability.

Before treatments with THC, cells were seeded at a 60-70% density and cultured with medium containing 0.5% FBS, 18-24h before the treatment.

2.4 Expression analysis

2.4.1 Quantitative Real Time PCR Analysis

Cells were seeded in 6 well plates and treated at different time points with THC or vehicle. Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA using the SuperScript-RT system (Invitrogen). Retrotranscription conditions were:

1- A mix with 1μg and water was mixed with random primers and dNTPs (50mM total) for 5 minutes at 65ºC.
2- After that the samples were moved to ice and mixed with Buffer 5x for retrotranscription , DTT at 0.1M and superscript enzyme and continue PCR (12 minutes at 25ºC, 50 minutes at 42ºC, 15 minutes at 70ºC and 4ºC

until the samples were removed from the PCR Machine.

Quantitative RT-PCR was performed on an ABI Prism 7900HT (Applied Biosystems) with SYBR Green (SYBR Green Power PCR Master Mix, Applied Biosystems).

Gene-specific human primers used were:

PCR conditions in all cases were: 95ºC for 5 min, 94ºC for 30 s, 57ºC (p8, CHOP, and BiP) or 52ºC (TRB3) for 30 s, and 72ºC for 1 min, followed by a final extension step at 72ºC for 5 min. The number of cycles was adjusted to 35.

Gene of interest	Fw primer (5’-3’)	Rv primer (5’-3’)
ORMDL1 **ORMDL2** **ORMDL3**	Commercially obtained from QuantiTect Primer Assay (Quiagen)	Commercially obtained from QuantiTect Primer Assay (Quiagen)
SPTLC1	GCGCGCTACTTGGAGAAAGA	TGTTCCACCGTGACCACAAC
SPTLC2	AGCCGCCAAAGTCCTTGAG	CTTGTCCAGGTTTCCAATTTCC
SPTLC3	AGTTGGAAAGGGATGCCTCA	ATGCTGTAGACACCCTCCAC
ssSPTa	GCCTGGAAGCAGATGTCCT	CTGGGGCATGAAGACGTAC
ssSPTb	ATTTCGTGAAGGAGCAAGGC	AACATAGATCGCTCCCAGGG
p8	CTATAGCCTGGCCCATTCCT	TCTCTCTTGGTGCGACCTTT
TRB3	GTCTTCGCTGACCGTGAGA	CAGTCAGCACGCAGGAGTC
CHOP	ATGGCAGCTGAGTCATTGCCT	AGAAGCAGGGTCAAGAGTGGTGAA
BiP	CGGGCAAAGATGTCAGGAAAG	TTCTGGACGGGCTTCATAGTAGAC
β-actin	ACGAGGCCCAGAGCAAGAG	GGTGTGGTGCCAGATTTTCTC

Western Blot

U87MG cells were seeded in 100mm plates. For protein extraction, cells
were washed with cold PBS 1x, scraped in 70 µl of cold lysis buffer (150 mM NaCl, 5mM EDTA, 1% Triton X-100, 10mM Tris-HCl, 1x Complete protease inhibitor) for 20 min with agitation at 4ºC, and then centrifuged at 13,000 rpm at 4ºC for 20 min. Pellet was discarded. The protein concentration in the supernatant was determined using the BCA Assay (Pierce).

Equal amount of protein (around 30µg) was loaded on a 4-12% gradient polyacrylamide gel. Electrophoresis was carried out at 100mV and proteins were transfered to PVDF membrane using iBlot gel transfer system. Immunodetection was carried out using primary rabbit antibodies against ORMDL (1:1000, ABN417 millipore), p-eIF2α (1:500, ab32157 abcam), Bcl-2 (1:1000, 2870S cell signaling), Bax (1:1000, 1672772S cell signalling), LC3 (1:1000, L8918 sigma); and primary mouse antibodies against eIF2α total (1:500, ab5369 abcam) and β-actin (1:3000, A5441 sigma). Secondary antibodies were horseradish peroxide-conjugated anti-rabbit and anti-mouse (1:3000; GE Healthcare). The immunoreactive signal was detected by SuperSignal West Chemiluminescent substrate (Pierce) and visualized using the Molecular Imager Chemidoc XRS system (Bio-rad).

2.5 Cell fate analysis

2.5.1 Cell viability assays

In these experiments 3 x 10^5 U87 cells were seeded in 12-well plates. Cell viability was determined by MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, a yellow tetrazole) (Sigma-Aldrich, M2128). 1:10 of final volume was added in the culture during the last 2h of the experiment. Then, removal of medium was done and 200 µl of acid isopropanol (HCl 0,04N) was added to each well and pipetted. This 200 µl were then measured by absorbance at 570 nm, which is proportional to the number of viable cells in the culture, using a plate reader spectrophotometer. Data was expressed relative to the control condition only treated with vehicle.

2.5.2 Apoptosis assay

Double FITC Annexin V-Propidium Iodide (PI) staining (556420, BD Biosciences) was used in different assays to test apoptosis percentage in U87MG cells. In the case of transfections with plasmids that contain GFP, only simple staining with PI or PE Annexin V (559763, BD Biosciences) was done. As starting material 6 x 10^4 U87 MG cells were seeded in 6-well plates. The day after, transfection with plasmid or addition of doxycycline in case of Tet-On clones was performed for 24 hours. After that, treatments were done at the indicated time points and cells were detached with trypsin. Cells were centrifugated at 1100 rpm and washed

with 1x PBS. A posterior centrifugation at 1100 rpm was done before resuspending them in 200 µl of Annexin V binding buffer mixed with 5 µl of Annexin V and 2 µl of PI The fluorescence detection was acquired by flow cytometry using FACScalibur. Data analysis was carried out using Flowing software (created by Perttu Terho, Turku centre of biotechnology, Finland)

2.6 Ceramide quantification

Tet-Off/On activation of U87MG wild-type (WT) and over-expression (Oe) were treated for 0.5, 1 and 2h with 7µM THC. After the treatments cells were washed twice with 1x PBS and centrifugated at 1200 rpm for 5 min at 4ºC and the pellet was frozen in liquid nitrogen for ceramide quantification. Lipid extraction and processing was performed as reported previously (Munoz-Olaya JM, ChemMed-Chem 2008/Canals D Bioorg Med Chem 2009). Lipid analysis was carried out by ultraperformance liquid chromatography coupled to time-of-flight (TOF) mass spectrometry in positive electrospray ionization mode.

2.7 Fluorescence Microscopy

2.7.1 Immunostaining

For these experiments 1,5 x 10^4 cells were seeded on glass coverslips 24h before transfection or doxycycline addition. Coverslips were washed twice with PBS 1x before fixation

with 4% PFA for 10 minutes. After that, they were washed one time with PBS 1x and permeabilized with 0.1% Triton for 10min. A blocking step was done using 1% BSA ,0.2% FBS in PBS solution for 30min. Primary and secondary antibody staining was done using the same blocking solution. Primary antibody incubation was done of 1h 30min at tested antibody concentrations. Secondary incubation was done using fluorescence Alexa antibodies at 1:2000 dilution. Coverslips were mounted using Mowiol after three washes in PBS 1x.

In some specific experiments, plasmids containing fusion proteins tagged with fluorescence proteins were transfected. In these cases, no immunostaining was performed.
Images were acquired in a SP5 Leica confocal microscope. Image J software was used for image quantification and processing restricted to bright and contrast correction.

2.7.2 FRET analysis of ER-mitochondria proximity

In order to evaluate the distance between both organelles we have used a FRET based indicator of ER–mitochondria proximity (FEMP). This sensor contains a dimerization domain that allows maximal juxtaposition and FRET by brief rapamycin treatment. It codes for the fussion proteins AKAP1 (34-63)-FKBP-YFP and CFP-HA-FRB-Helix-Sac1 (provided by G. Hajnoczky, Thomas Jefferson University, Philadelphia) interlaced with a self-cleaving Tav2A peptide. Following translation, the peptide undergoes autocleavage and releases

YFP and CFP, which are targeted to the outer mitochondrial membrane (OMM) and ER by Akap1 and Sac1 targeting sequences, respectively. Rapamycin incubation forces binding of CFP-FRB-Sac1 with the OMM-FKBP-YFP where the two organelles are juxtaposed.

Fluorescence resonance energy transfer (FRET) signal in the different conditions was analyzed using a PerkinElmer operetta plate reader on 386 well plates. Briefly, 1 x 10^3 U87MG cells were seeded on each well. The day after 100ng of FRET probe DNA was transfected per well with genejet reactive (1:3; DNA:genejet ratio). After 24h, a first measurement was done to obtain the basal FRET in our cell lines. Then, 100nM rapamycin was added in each well for 15 minutes before fixation with 1%PFA in PBS for 10 minutes. Finally, PFA was removed and addition of PBS is done before measuring again the same wells to obtain maximum FRET.
Data is expressed showing basal and maximum FRET efficiencies of the different conditions and an additional index expressing the relative proximity between organelles refereed to the maximum proximity calculated as follows: (FRETmax-FRETbasal) / FRET max. Using this index values near 0 would represent maximum proximity and values close to 1 would indicate minimum proximity.

2.8 Measurement of intracellular Ca2+ concentrations

Cytosolic Ca2+ signal was determined in Tet-On U87 MG cells loaded with 2.5 μM fura-2AM for 30 minutes. The increment of cytosolic Ca2+ is represented by the ratio of emmited fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation (fura-2 ratio 340/380).

Mitochondrial Ca2+ measured in these cells was obtained using the mtPericamR probe transfected 24h before the experiment. Ratiometric pericam-mt imaging was done by excitation at 410 and 488 nm and emission at 520 nm in a Leica TCS SP5 confocal microscope with a 40x oil objective and analized using ImageJ software. Experiments were carried out at room temperature and the cells were bathed in a solution (ISO) containing: 140 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES; 300 mosmol/l, pH 7.4 with Tris). Ca2+ free solutions were obtained by replacing $CaCl_2$ with equal amounts of $MgCl_2$ plus 0.5 mM EGTA.

2.9 Electron microscopy analysis

For these experiments cells were seeded on glass coverslips 24h before fixation with PFA 1%. For 15 minutes. After that, samples were stored at 4ºC in PBS until transfer to electron microscopy facility. ImageJ was used to analyse the images. To calculate ERMICC the following formula was used: MERCs length / (mitochondria perimeter * MERCs width)
where MERCs is mitochondria-ER contacts.

3. HYPOTHESIS

ORMDL family has been involved in a wide spectrum of functions including sphingolipid synthesis inhibition, proteostasis and calcium homeostasis. On the pathological side, *ORMDL3* gene has been associated with several diseases. In this scenario, a GWAS study has shown that this gene is relevant in risk to develope glioma, specificalySNPs that imply a higher *ORMDL3* expression.
The other aspect essential to highlight is related with the antitumoral action of THC. It has been described that THC is able to induce glioblastoma using a pathway that requires ceramide generation. Due to these facts, our initial hypothesis was that the overexpression of ORMDL3 in glioblastoma could increase resistance to antitumoral drugs with a mechanism of action based on cellular ceramide increases such as THC.

We have also focused our attention in the location of ORMDL3 protein. ER-Mitochondrial associated membranes have emerged as important signalling hubs that impact in several cellular processes including cell fate. Interestingly, the ORMDL family functions matches signalling pathways that converge in these microdomains. In this doctoral thesis we have hypothesized that ORMDL3 might be a functional component of the MAMs providing a wider picture of ORMDL3 function and pathophysiology.

4. OBJECTIVES

Taking into account the previous hypothesis, the objectives of this thesis have been organized in two main blocks divided as well in specific objectives:

1. To study the role of ORMDL3 in the antitumoral signalling pathway of cannabinoids in a glioblastoma model.

    1.1. Location and expression of ORMDL3 under THC treatment.

    1.2. Impact of ORMDL3 over-expression in THC antitumoral effect.

2. To study the impact of ORMDL3 expression on ER-Mitochondria Associated Membranes (MAMs) structure and function.

    2.1. To evaluate the impact of ORMDL3 overexpression in apoptosis-mediated by ER stress.

    2.2. To study ER and mitochondria Ca2+ signalling in glioblastoma model of ORMDL3 overexpression.

    2.3. To analyze ER-mitochondria proximity depending on ORMDL3 expression levels.

    2.4. To determine domains of ORMDL3 involved in MAMs function.

5. RESULTS

5.1. Study of the role of ORMDL3 in the antitumoral signalling pathway of cannabinoids.

It has been described that ORMDL family has an impact in cellular ceramide levels possibly by acting as inhibitors of the SPT complex (Siow et al 2012/KK et al 2014). On the other hand, the antitumoral pathway of THC implies an increase of the ratio dihydroceramide/ceramide that promotes of ER stress and finally causes cellular apoptosis (Hernandez-tiedra et al 2016/Salazar et al 2009). Due to this, we decided to study a possible role of these proteins in the antitumoral cascade of THC.

5.1.1 Location and expression of ORMDL3 under THC treatment.

5.1.1.1 SPT-ORMDL complex cellular localization

As a first approach to investigate the effect of THC in ORMDL-SPT complex physiology, we determined the location and expression of ORMDL proteins and SPT subunits by immunofluorescence in U87MG cells under cannabinoid treatment. We transiently transfected the cells with hORMDL3, myc-SPTLC1 and GFP-SPTLC2 constructs. Both SPTLC subunits showed a dramatic cellular reorganization after 6h THC treatment. In this respect, co-localization with calnexin (CNX), a ER resident chaperone, was maintained after treatment (Fig. 1).

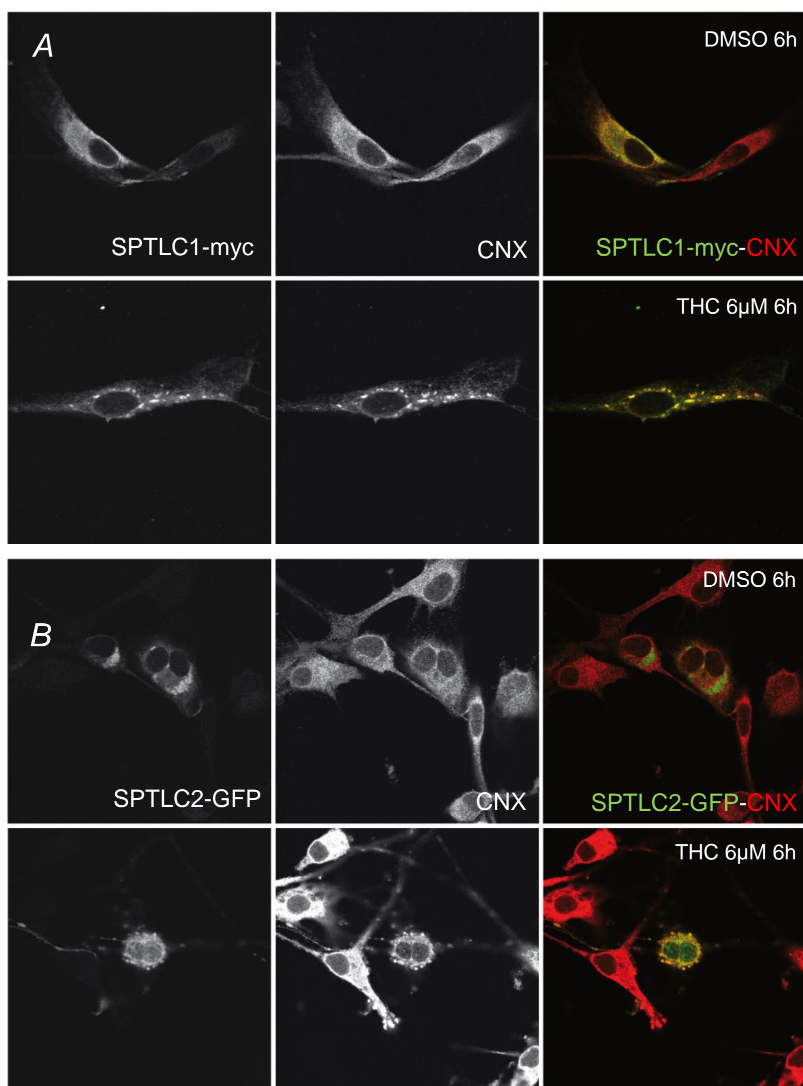


Fig1. Confocal analysis of both SPTLC subunits under THC treatment. U87MG cells transfected with SPTLC1-myc (A) or SPTLC2-GFP (B) were treated with DMSO and THC 6μM for 6h and stained with anticalnexin (CNX) antibody. SPTLC fusion proteins are shown in green and clanexin in red.

ORMDL3 in basal condition has a typical ER pattern, colocalizing with SPT subunits (Fig. 2A).

Following the previous behavior observed for SPTLC subunits, after THC treatment, ORMDL3 ER pattern dramatically changed forming puncta pattern associated to stress (Fig. 2B) (Varadarajan S et al., 2012).

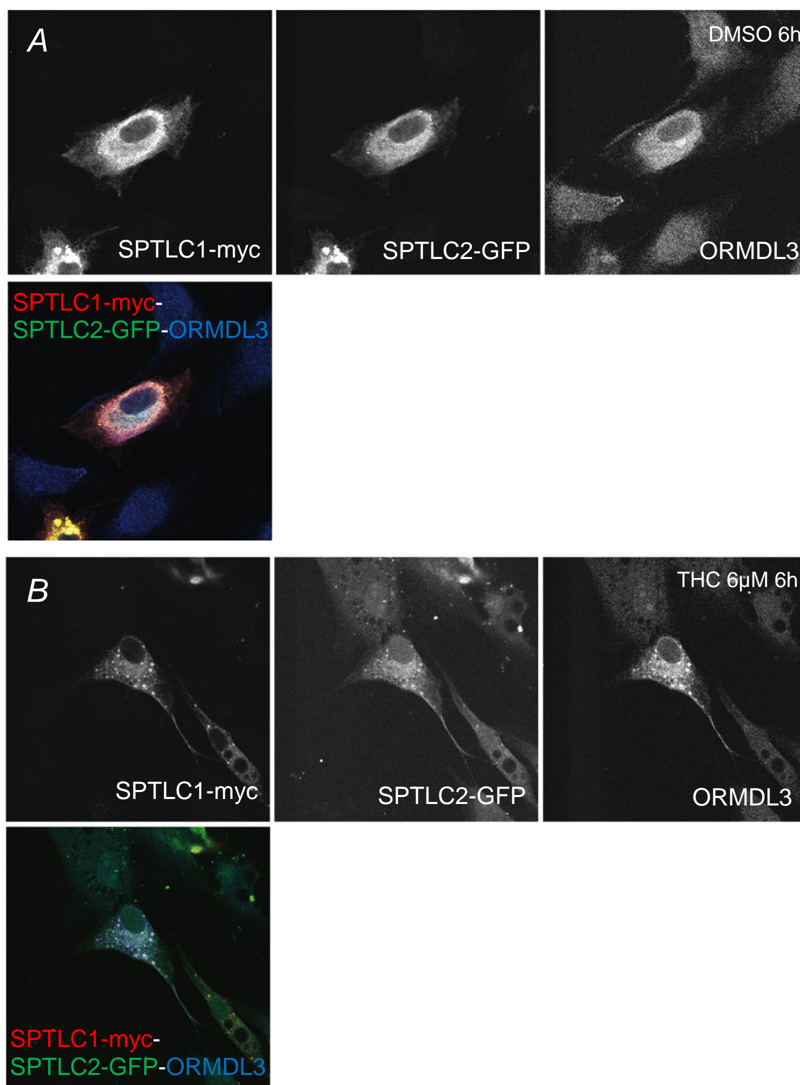


Fig2. Confocal analysis of SPTLC-ORMDL3 complex under THC treatment. U87MG cells transfected with SPTLC1-myc (red), SPTLC2-

GFP (green) and ORMDL3 (blue) were treated with DMSO (A) and THC 6μM (B) for 6h.

5.1.1.2 ORMDLs expression under THC treatment

It has been reported that THC antitumoral pathway implies an increase in de novo ceramide synthesis. We have investigated the possible regulation of OMDL family as endogenous inhibitors of the SPT enzyme. First, we characterize the expression of ORMDLs and the components of the SPTLC complex by real time PCR in order to study a possible transcriptional regulation after THC addition. We did not observe mayor changes in the RNA expression of the ORMDL members or the SPTLC main subunits. Besides, the treatment with THC slightly increased ssSPTb regulatory subunit (Fig 3A).

We have also studied endogenous protein expression of ORMDLs under THC treatment using an antibody that recognizes the three members (Fig. 3B). We observed a marked reduction in ORMDL levels in U87MG cells after 6h THC treatment. Given the different cellular targets of THC, we demonstrated that the effect observed was produced through cannabinoid receptors because the reduction in ORMDL expression was avoided when using in combination THC and CBRs antagonists (Fig. 3B). These results pointed at a post-translational mechanism to decrease ORMDL expression because RNA levels were not modified by THC incubation.

We tested this hypothesis by using pharmacological inhibitors as MG132 to block proteasome activity. Figure 3C shows that THC was unable to reduce ORMDL levels when proteasome was inactive. Taking together all these results, we concluded that one likely mechanism behind the increase in the cellular ceramide caused by THC is through the degradation of ORMDL proteins and the consequent release of ceramide synthesis by *de novo* pathway.

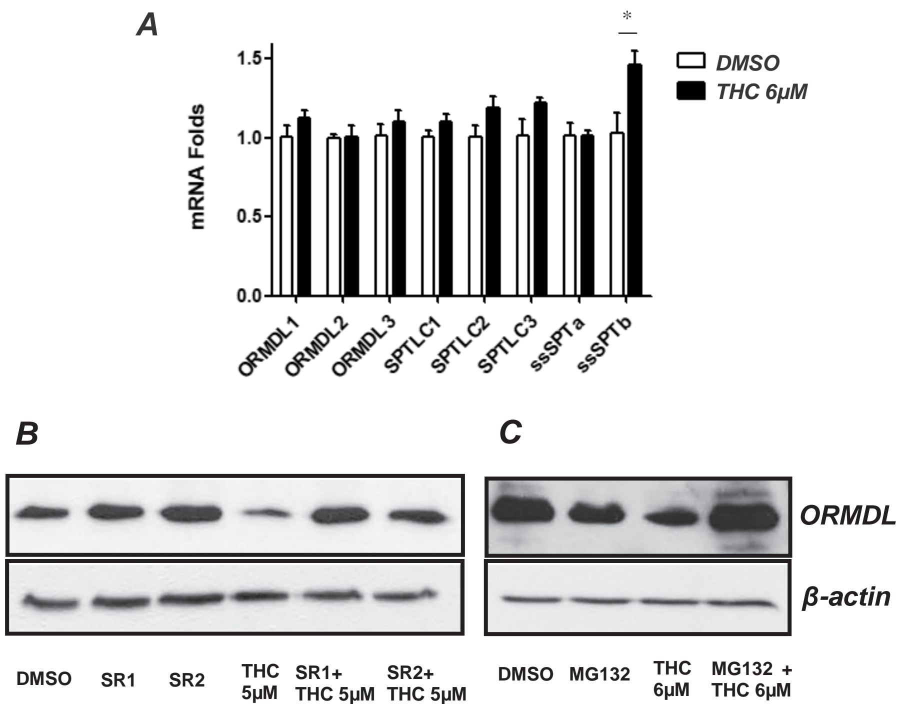


Fig 3. THC reduces ORMDL protein levels by proteasome degradation. A, mRNA levels of the different ORMDL proteins, SPT subunits and SPT small subunits. (C, *n* = 4 for DMSO; *n* = 5 for THC; analysis between DMSO and THC treatments, * p<0.05). *Error bars* represent SEM.B, C, Representative western blot of ORMDL levels in U87MG under 5μM THC treatment for 6h. B, In some experimental

conditions the CB1 antagonist SR1 and SR2 were added together with the THC treatment. C, Proteasome inhibitor MG132 was added with and without THC to test possible modifications of ORMDL proteins by proteasome.

5.1.2 Impact of ORMDL3 over-expression in THC treatment as antitumoral drug.

Genome-Wide Association study linked a high expression of ORMDL3 with higher risk of glioma. On the other hand, increased expression of ORMDLs might create form a brake in the THC antitumoral signalling pathway. We decided to design cellular models with highORMDL3 expression simmilar to the SNP linked to risk of glioma condition to test viability under antitumoral action.

5.1.2.1 Cell viability and apoptosis assays

Our first approach was to transfect transiently hORMDL3 in a glioma model. U87MG cells were transfected with pcDNA-GFP, ORMDL3-GFP and ΔN-ORMDL3-GFP. This last construct lacks the first sixteen aminoacids in the N-tail of ORMDL3, what produces a lack of function in the protein. Apoptosis analysis after THC treatment was evaluated by flow cytometry using propidium iodide (PI) of the green cells. Our results showed that the increase of PI incorporation under THC treatment was reverted in ORMDL3 over-expressing cells. When ΔN-ORMDL3 was over-expressed only partial of PI incorporation was observed (Fig 4).

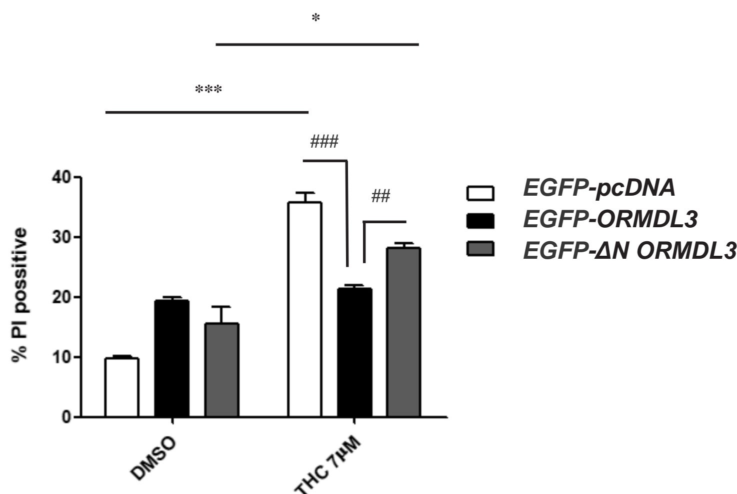


Fig 4. ORMDL3 induces resistance to cancer cell death under THC treatment. EGFP-ORMDL3, EGFP-ΔN-ORMDL3 and EGFP were trantly transfected in U87 cells treated wth DMSO and THC 7μM for 24h after transfection. After that, PI staining was done to determinate cell death. Data is normalized at 24h DMSO treatment for all three constructs. (*n* = 3; analysis between DMSO and THC treatments, * p<0.05, *** p<0.001; analysis between THC treatments, ## p<0.01, ### p<0.001). *Error bars* represent SEM.

After these observations, we decided to work with an inducible stable cell line model to continue our experiments. A Tet-Off/On ORMDL3 overexpressing model in U87 cells was generated. Figure 5 shows the overexpression levels by western blot (Fig. 5A). Immunostaining of Oe clon before and after dococyclin treatment showed that the ORMDL3 overexpression remains in the endoplasmic reticulum (Fig. 5B).

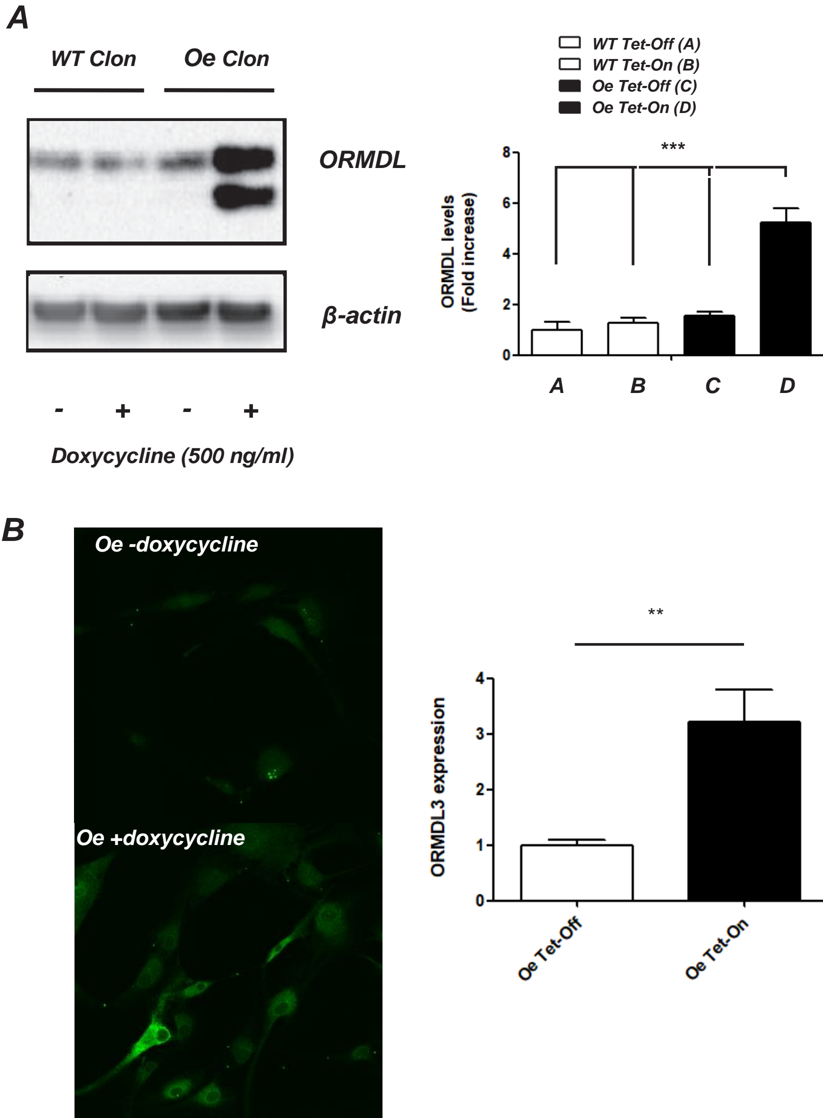


Fig 4. Expression of ORMDL protein in a Tet-On/Off system. A, western blot of ORMDL levels in WT and Oe Tet-Off/On cell lines after 24h 500 ng/ml doxocycline treatment. Data is normalized at WT ORMDL levels without doxycycline. (*n* = 3; analysis between DMSO and THC treatments, *** p<0.001; *Error bars* represent SEM.) B, ORMDL levels by immunostaining detection in Oe Tet-Off/On conditions. Quantification by folds of Oe Tet-Off/On cell line (*n* = 5; ** p<0.01; *Error bars* represent SEM.)

We then analysed the effect of THC in our Tet-Off/On system. For this purpose, we monitored cell viability doing an MTT assay at different THC concentrations (Fig 6A). Our data showed that at high THC concentrations Tet-On conditions was able to reduce cell death (Fig. 6A). Moreover, we decided to validate these results without the possible effect of doxycycline in the model (Fig. 6B). Therefore, we tested the same treatment comparing mock Tet-On cell line (WT) and the ORMDL3 Tet-On line (Oe) both with the same concentration of doxycycline. Our MTT experiments showed that Oe line had a marked cell death resistance compared with WT line.

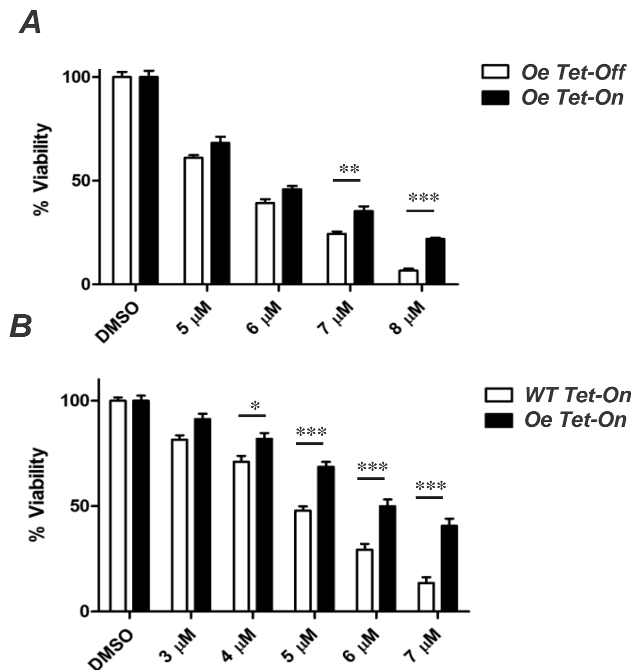


Fig 6. Overexpression of ORMDL3 induces cancer cell death resistance under THC treatment. A, MTT experiment in Tet-Off/On

induction in ORMDL3 overexpressing clon (Oe). THC was used as stimulus in different concentrations. Data is normalized at 24h DMSO in both conditions. (*n* = 6; analysis between DMSO and THC treatments, ** p<0.01, *** p<0.001; *Error bars* represent SEM.) B, MTT experiment in Tet-On for WT and Oe clones with different THC concentrations. Data is normalized at 24h of both clones. (*n* = 10; analysis between DMSO and THC treatments, * p<0.05, *** p<0.001; *Error bars* represent SEM.)

5.1.2.2 Ceramide production measurements

It has been described previously that one of the early steps in THC antitumoral pathway is the detection of a ceramide imbalance due to a specific increase of dihydroceramide content in the cells (Hernández-Tiedra S et al., 2016). In our Tet-On models we have characterize ceramide production after THC treatment by mass spectrometry. Our results confirmed that in WT Tet-On cells, the dihydroceramides increases after THC treatment. However, no change was observed in ORMDL3 overexpressing cells (Oe) (Fig 6A). In a similar manner, total ceramide content increase was only observed in the WT clone probably caused by the increase produced in the previous metabolite (Fig 6B).

Our results showed that increase levels of ORMDL3 blocked the activation of the de novo ceramide synthesis pathway caused by THC treatment.

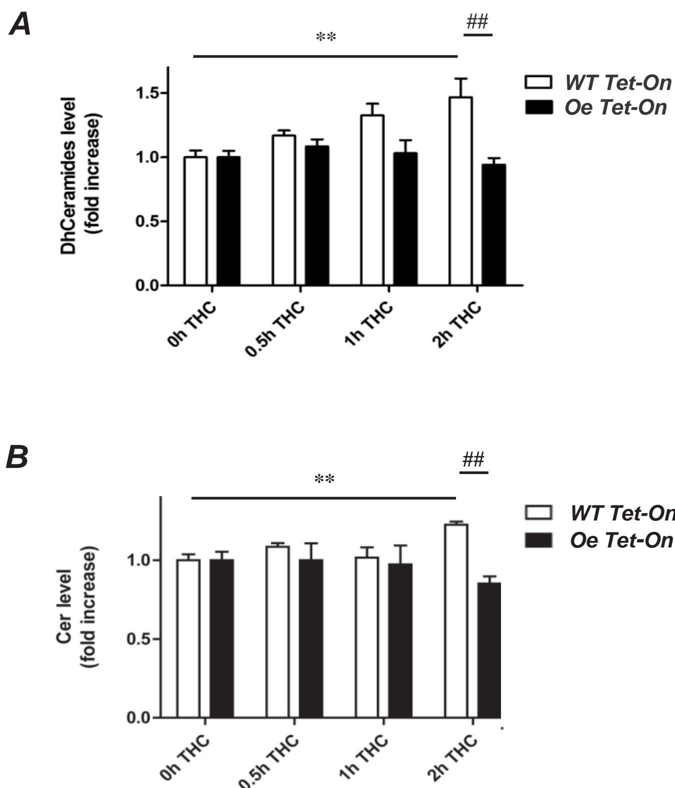


Fig7. Ceramide synthesis by THC treatment. DihydroCeramides (A) and total Ceramides (B) quantification were done at 0, 0.5, 1 and 2h after 6μM THC treatment in both, U87MG WT and Oe Tet-On cell lines. Data is normalized at 0h THC treatment of WT and Oe Tet-On clones respectively. (*n* = 3; analysis between DMSO and THC treatments, ** p<0.01; analysis between THC treatments, ## p<0.01). *Error bars* represent SEM.

5.1.2.3 ER stress induction evaluation

In order to further study the antitumoral pathway of cannabinoids we focused on the generation of ER stress caused by THC. It has been demonstrated that an increase of

dihydroceramides in the cells treated with THC leads to ER stress and activation of the PERK pathway of the Unfolded Protein Response (UPR). We evaluated the phosphorilation of PERK substrate, the eIF2α, to monitor its kinase activity in our cell models treated with THC. Our results revealed an increase p-eIF2α/total eIF2α ratio in WT cells after THC treatment (Fig. 8). On the contrary this effect was not observed in the Oe Tet-On line. Remarkably, the overexpression of ORMDL3 produced a p-eIF2α increased in basal condition that was attenuated in the THC treated condition. These data support the idea that an increment of ORMDL3 levels blocks the induction of ER stress by THC.

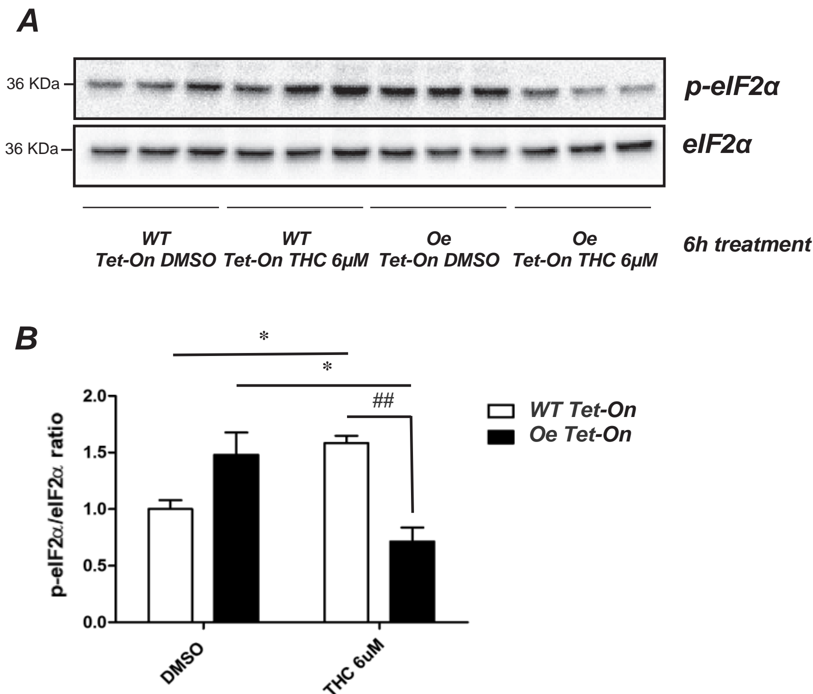


Fig 8. ORMDL3 reduce ER stress under THC treatment. A, Representative Western Blot of the ratio p-eIF2α/eIF2α in U87MG WT

and Oe Tet-On clones under DMSO and 6μM THC treatment for 6h. B, Quantification of the ratio p-eIF2α/eIF2α after the different treatments. Data is normalized at DMSO 6h treatment of WT Tet-On clon. (*n* = 3; analysis between DMSO and THC treatments, * p<0.05; analysis between THC treatments, ## p<0.01). *Error bars* represent SEM.

We have also measured mRNA levels of genes downstream the PERK pathway at different time points of THC treatment (Fig 9). Our results demonstrated that p8, TRIB3, CHOP and BiP mRNA levels are increased in WT treated cells at 3h and 8h. In the same direction than previous results, the overexpression of ORMDL3 prevented the upregulation of all these stress markers.

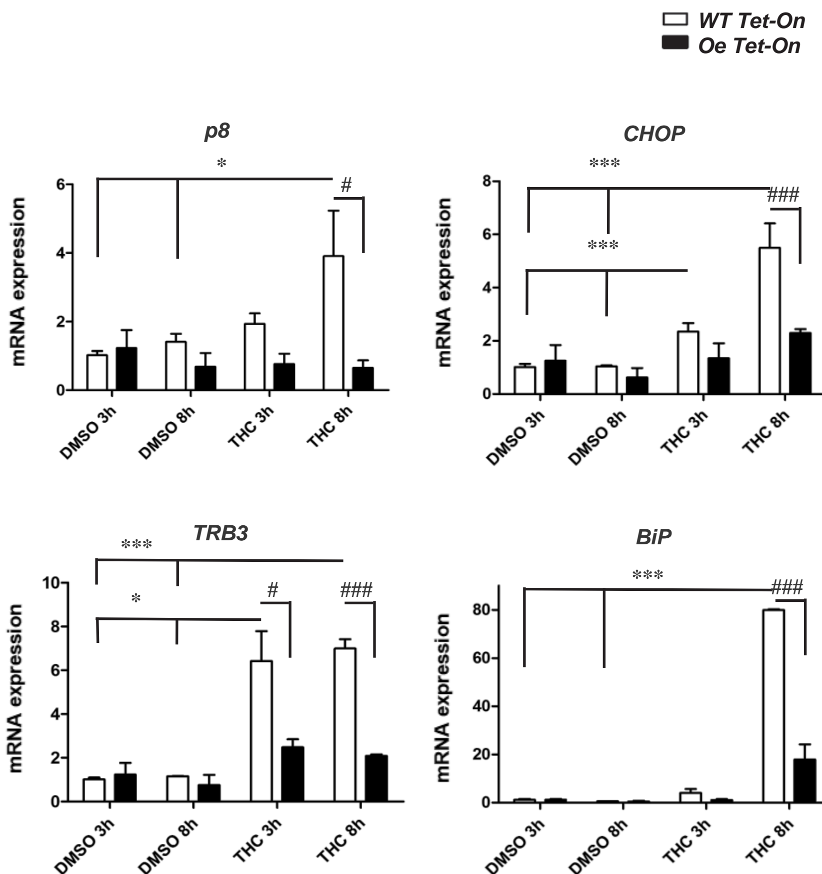


Fig 9. Overexpression of ORMDL3 induce a resistance in the increase of ER stress genes under THC treatment. mRNA levels of different ER stress genes are represented for WT and Oe Tet-On clones at 3 and 8h after DMSO and THC 6μM addition. Data is normalized at DMSO 6h treatment of β-actin of WT Tet-On clon. (*n* = 6; analysis between DMSO and THC treatments, * $p<0.05$, *** $p<0.001$; analysis between THC treatments, # $p<0.05$, ### $p<0.001$). *Error bars* represent SEM.

5.1.2.4 Autophagy initiation analysis

Following the THC antitumoral pathway, we decided to analyse the initiation of a deleterious autophagic flux described previously in this cascade (Salazar M et al., 2009; Hernández-Tiedra S et al., 2016). Thus, we have monitored LC3 recruitment to autophagosomal membranes. We first transfected LC3-GFP construct in our tet-On clones and observed that at basal levels Oe tet-On clone presented higher number of puncta per cell (Fig 10A). We then wanted to corroborate this by western blot as can be observed in figure 10 B-C, ORMDL3 overexpression increased total LC3 levels. However, under THC treatment LC3II levels in Oe model were maintained meanwhile in WT cells increased. These results suggested that ORMDL3 blocked the autophagic flux induction produced by THC.

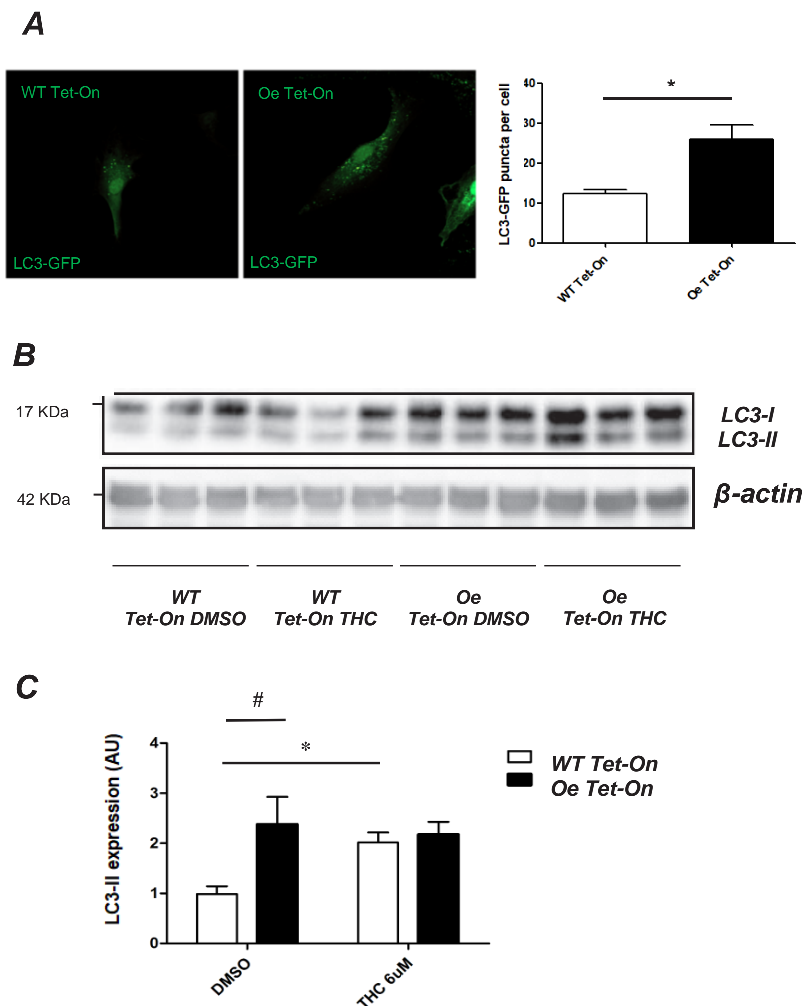


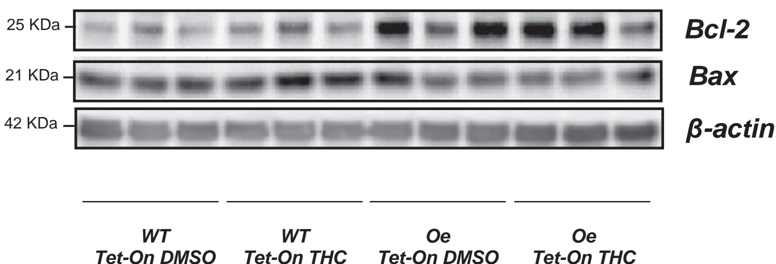
Fig 10. ORMDL3 impairs autophagy flux generated by THC. A, Confocal images for LC3-GFP in WT and Oe Tet-On clones 24h after transfection. Puncta per cell corresponded to LC3-GFP were quantified to correlate autophagy. (n = 3, 28 individual cells, analysis between both cell lines, * $p<0.05$). *Error bars* represent SEM. B, Representative Western Blot of LC3 in U87MG WT and Oe Tet-On clones under DMSO and 6µM THC treatment for 6h. C, Quantification of LC3 after DMSO and THC treatments. Data is corrected by β-actin and normalized at 6h DMSO treatment of WT Tet-On clone. (n = 3; analysis between DMSO and THC

treatments, * p<0.05; analysis between THC treatments, # p<0.05). *Error bars* represent SEM.

5.1.2.5 Apoptosis induction quantification

Our first approach to characterize apoptosis induction was to quantify expression of anti- and pro- apoptotic proteins, like Bcl-2 and Bax respectively, in our cells in Tet-On conditions. We detected increased levels of Bcl-2 expression in ORMDL3 overexpressing cells (Oe) both in basal and under THC addition compared with WT cells (Figure 11). in addition, only WT cells showed an increase in Bax expression when treated with THC. (Fig 11A and 11B).

In order to confirm these results, we performed an annexin V apoptosis experiment by flow cytometry treating our clones with THC (Figure 11C). Supporting the werten blot results, we obtained an increase in apoptosis in WT cells under THC treatment whereas the ORMDL3 overexpressing line (Oe) had no significant increase in annexin staining (Fig 11C).



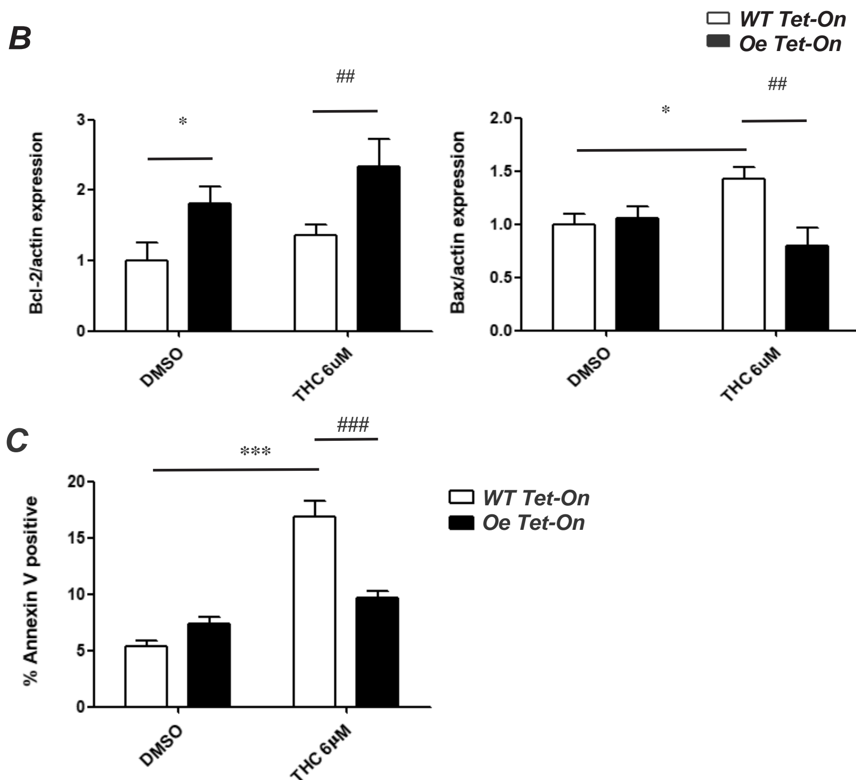


Fig 11. ORMDL3 blocks THC apoptosis induction. A, Representative western blot Bcl-2, Bax and β-actin as loading control in U87MG WT and Oe Tet-On clones under DMSO and 6μM THC treatment for 6h. B, Quantification analysis of Bcl-2 and Bax expression. Data is normalized at DMSO 6h treatment of WT Tet-On clone. (*n* = 3; analysis between DMSO and THC treatments, * p<0.05; analysis between THC treatments, ## p<0.01). *Error bars* represent SEM. C, Percentage of Annexin V positive cell for WT and Oe Tet-On cell clones under DMSO and 6μM THC treatment for 24h. (*n* = 3; analysis between DMSO and THC treatments, *** p<0.001; analysis between THC treatments, ### p<0.001)

5.2. Study of the role of ORMDL3 in MAMs

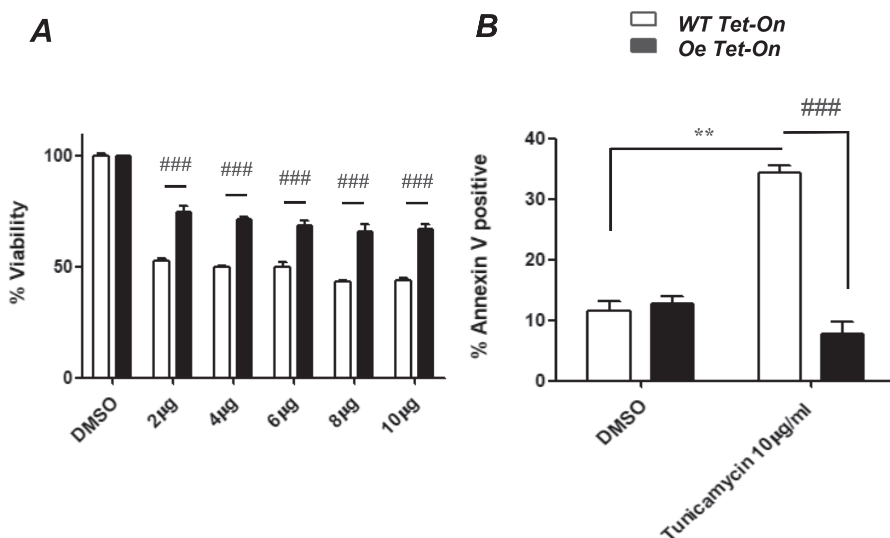
ER-mitochondria associated membranes (MAMs) are cellular signalling hubs involved in many different cellular processes that determine cell fate. In the last years the molecular composition of these platforms has been characterized and different types of proteins have been shown to play an important role in the communication between ER and mitochondria participating in the structure, dynamics and function of MAMs.

ORMDL3, an ER-resident transmembrane protein has been shown by previous studies in our laboratory to localize in the proximity of ER-mitochondria contacts. Moreover, changes in ORMDL3 expression alter both ER and mitochondria calcium dynamics (Carreras-Sureda A, HMG 2013). In this context, we have further studied the consequences of altering ORMDL3 expression in MAMs structure and function using our U87 glioma cell models.

5.2.1 Impact of ORMDL3 over-expression in apoptosis-mediated by ER stress.

Many components of the apoptosis intrinsic pathway have been described to localize in MAMs. The disruption of these structures alters this signalling cascade and has been suggested to be an important pro-survival mechanism in

tumoral cells. Besides sustained ER stress leads to program cells death by some branches of UPR that have been also described at MAMs. Taking together all this, we have addressed the possibility that ORMDL3 expression might determine resistance to apoptotic stimulus by altering the ER-mitochondria pro-apoptotic signalling pathway. With that purpose, we have used different stimuli causing ER stress to test cell death induction in our U87 ORMDL3 overexpression cell model. Viability and apoptosis induction by H_2O_2 and tunicamycin were tested using MTT and Annexin V staining assays. Figure 12 A and B shows an increase in the viability of Oe clone at different doses of H_2O_2 and tunicamycin compared with WT cells. Moreover, our data using annexin stainingconfirmed thatthe apoptosis induction obtained with 10ug tunicamycin and 0.5mM H_2O_2 in WT cells wasblocked in ORMDL3 over-expressing cells supporting the idea that this protein induces resistance to cell death by deleterious stimuli affecting ER homeostasis.



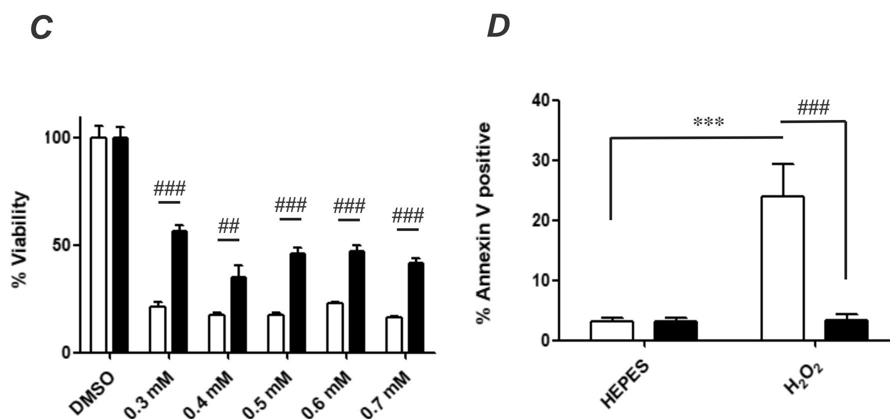


Fig 12. ORMDL3 promotes resistance to apoptosis induced by ER stressors. Viability percentage by MTT assay (A,C) and and apoptosis induction by Annexin V staining (B,D) in U87MG WT and Oe Tet-On clones treated with tunicamycin (A-B) and H_2O_2 (C-D) for 24h and 5h respectively. Data is normalized to DMSO treatments. (*n* = 4 for MTT assays. n = 3 for Annexin V in tunicamycin, and n = 5 for Annexin V in H_2O_2; analysis between DMSO or H_2O and Tunicamycin or H_2O_2 treatments, ** p<0.01; analysis between different concentration of Tunicamycin or H_2O_2 treatments, ## p<0.01; ### p<0.005). *Error bars* represent SEM.

We then wanted to characterize whether, this antiapoptotic effect caused by ORMDL3 overexpression was a general effect or specific for ER stress. Therefore, we studied the extrinsic apoptosis pathway in our cell models. We treated our different clones with TNF-α. This factor binds to TNFR-1 or 2 receptors and enables the adaptor protein TRADD to bind to FADD, recruiting this way caspase-8 and leading to programmed cell death. The results we obtained showed no

difference in viability when we compared WT and Oe clones (Fig 13).

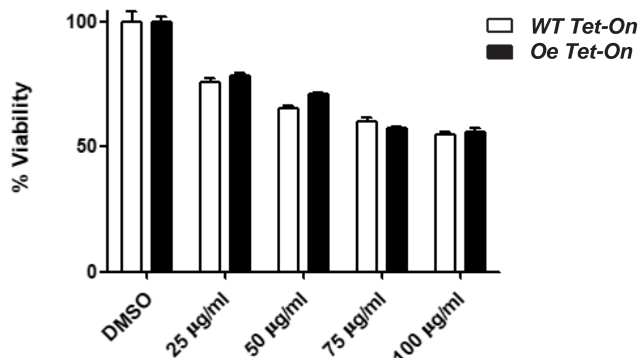


Fig 13. ORMDL3 does not affect apoptotic extrinsic pathway. Viability percentage and apoptosis induction by MTT assay at different concentrations of tunicamycin in U87MG WT and Oe Tet-On clones for 24h. Data is normalized at 24h DMSO in WT and Oe Tet-On clones. (*n* =4) *Error bars* represent SEM.

5.2.2 Ca2+ signalling in ER-mitochondria network.

Ca2+ signalling is the communication mechanism between ER and mitochondria. Disruption of ER calcium homeostasis leads to mitochondria calcium overload inducing apoptosis. Several components in MAMs participate in this process tuning calcium fluxes and affecting mitochondria dynamics. We have characterized calcium homeostasis in our cells to further explore the possible impact of ORMDL3 on MAMs function (Fig 14). Cytosolic calcium measurements using Fura 2AM showed

that after 24h of ORMDL3 overexpression basal cytosolic calcium levels were increased compared to WT cells confirmed what has been published previously about ORMDL3 function (Carreras-Sureda A et al., 2013) (Fig 14A). We then measured ER calcium content in our U87 cell clones by adding ionomycin to the cells in the absence of extracellular calcium to produce ER calcium depletion. Our results showed no differences in ER calcium levels between WT and ORMDL3 overexpressing cells (Fig 13B). Finally, we performed mitochondrial calcium measurements after 24 hours of overexpression using a ratiometric calcium sensor transiently transfected to our cells. Our measurements showed a lower signal in cells overexpressing ORMDL3 compared to control cells (Fig. 14C). Moreover, we monitored mitochondrial calcium buffering capacity by depleting ER calcium content and triggering the stored operating calcium entry followed by an addition of external calcium. Our data showed that mitochondria of ORMDL3 overexpressing cells reached lower levels of calcium concentration than WT cells. These results revealed an alteration in the mitochondrial machinery for calcium influx in cells that express high levels of ORMDL3 as it has been previously described (Carreras-Sureda A et al., 2013) (Fig 14C).

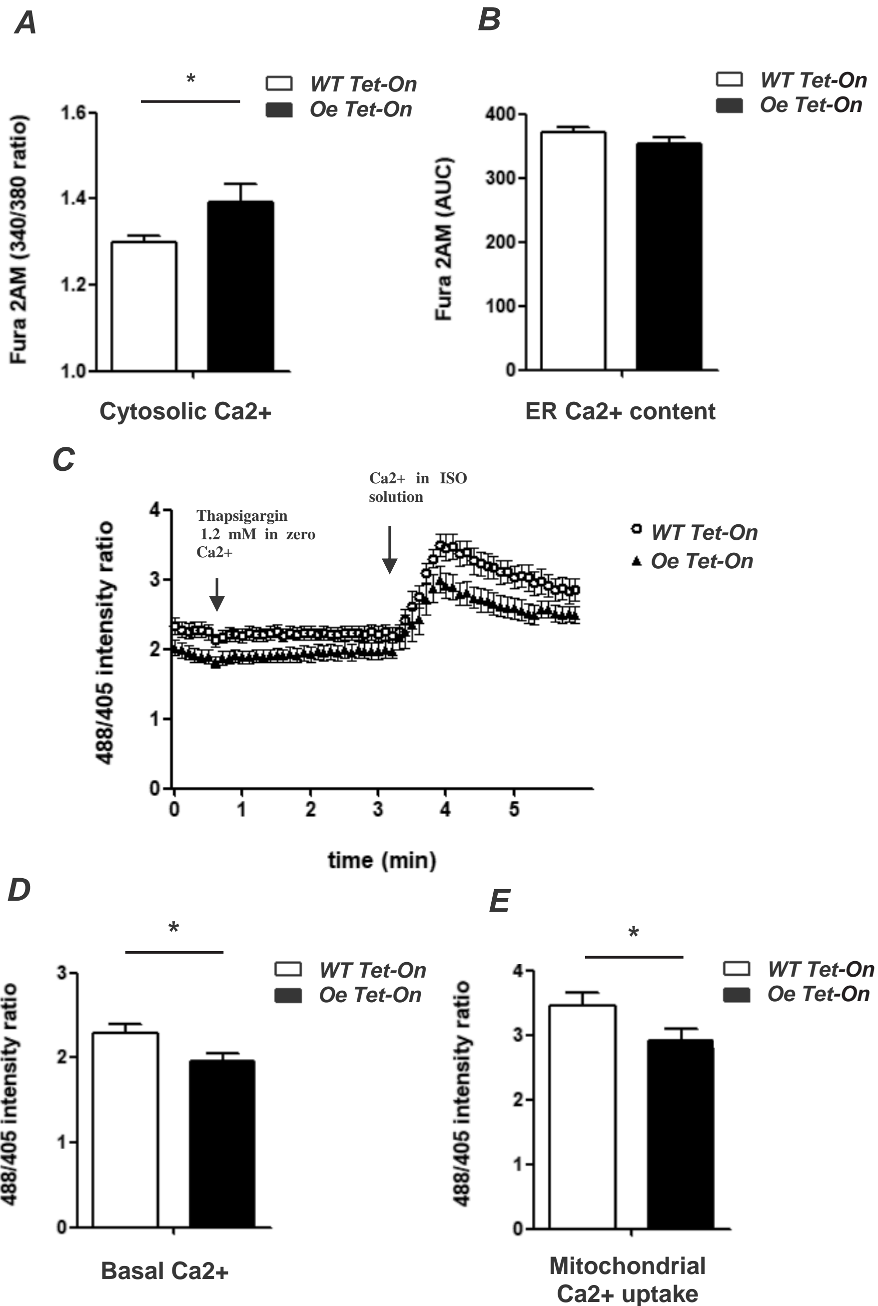


Fig 14. Overexpression of ORMDL3 alters cytosolic Ca2+ content and prevent mitochondrial Ca2+ uptake. Ca2+ analysis in A, cytosol, B, ER, and C, mitochondria in WT and Oe Tet-On clones 24h after

doxycycline addition. Fura ratio was used to Ca2+ measurement in cytosol (A and B). Pericam probe was transfected 24h before starting the experiment to measure mitochondrial Ca2+ (C). Fura 2 raw data is represented in A. Area under the curve after ionomycin addition is represented in B. (n = 100 in WT and 97 in Oe clones, * p<0.05). *Error bars* represent SEM. C, Mitochondria calcium recording in extracellular 0 calcium solution before treatment with 1uM thapsigargine. At minute 3 extracellular solution with 1.2mM calcium was added. Initial mitochondria calcium levels (D) and maximum peak after calcium addition (E) was represented. (n = 13 in WT and 16 in Oe clones, * p<0.05). *Error bars* represent SEM.

In summary, overexpression of ORMDL3 alters cytosolic and mitochondria basal calcium levels. Moreover, the lower Ca2+ mitochondrial uptake observed could explain in part the resistance to pro-apoptotic stimuli.

5.2.3 ER-mitochondria distance in ORMDL3 down- and over-expression.

Taking in consideration all the previous results, we decided to explore the possibility that ORMDL3 would alter the integrity of MAMs. Therefore, we carried out experiments to analyse the distance between ER and mitochondria in our ORMDL3 Tet-Onn cell lines. The distance was estimated using a FRET based sensor developed in Luca Scorrano lab in which the CFP and the YFP target the ER and the mitochondria respectively. Moreover, we normalized our FRET efficiencies to the maximum interaction obtained by adding rapamycin

and promoting dimerization of the FRET probe. Figure 15 shows that the FRET probe targeted properly in our U87 cell line. We observed CFP localizing at ER, YFP localizing at mitochondria and stablishing the FRET channel we could observed the ER-mitochondria interaction spots. This FRET channel revealed the MAMs. Remarkably when we stained ORMDL3 in our Tet-On cells we could observe also the presence of this protein colocalizing with the FRET channel revealing that a fraction of ORMDL3 was present in MAMs. We then decided to compare FRET efficiency between our cells models. Our results showed that ORMDL3 expressing cells had a lower FRET efficiency that WT cells at basal condition (Fig. 16A). When adding rapamycin both, WT and ORMDL3 cells, increased FRET efficiencies compared to basal. We then normalized the difference to the maximum FRET and obtained that ORMD3 overexpressing cells presented a higher relative distance between organelles that WT cells (Fig. 16B). We also studied the relative distance using a Tet Off-Tet-On approach. We found out that doxycycline altered the distance promoting organelle proximity in WT and ORMDL3 cells. Therefore, we only observed an increased distance between organelles in our ORMD3 Tet-On cell line with the lower doxycycline dose. Nevertheless, the ORMDL3 line kept a higher organelles distance compare to WT cells at the different concentrations studied.

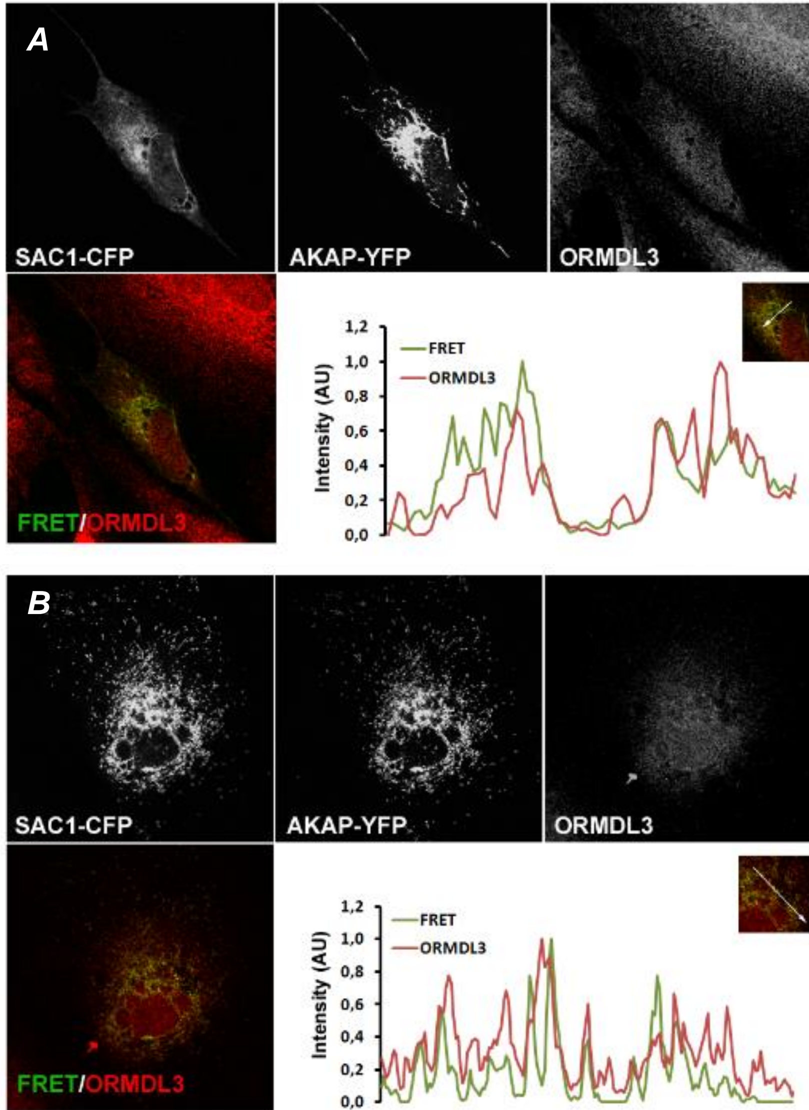


Fig 15. Immunofluorescence images for FRET ER-mitochondria probe and ORMDL3. Analysis of FRET probe localization and ORMDL3 immunostaining before (A) and after (B) rapamycin. FRET probe was transfected in U87 Oe ORMDL3 Tet-On cells for 24h. Immunostaining protocol was performed after 24h transfection of FRET probe. ORMDL3 antibody was used to stain this protein in this cell line. Colocalization of FRET (SAC1-CFP (ER)/AKAP-YFP-(mito) and ORMDL3 is shown in right bottom.

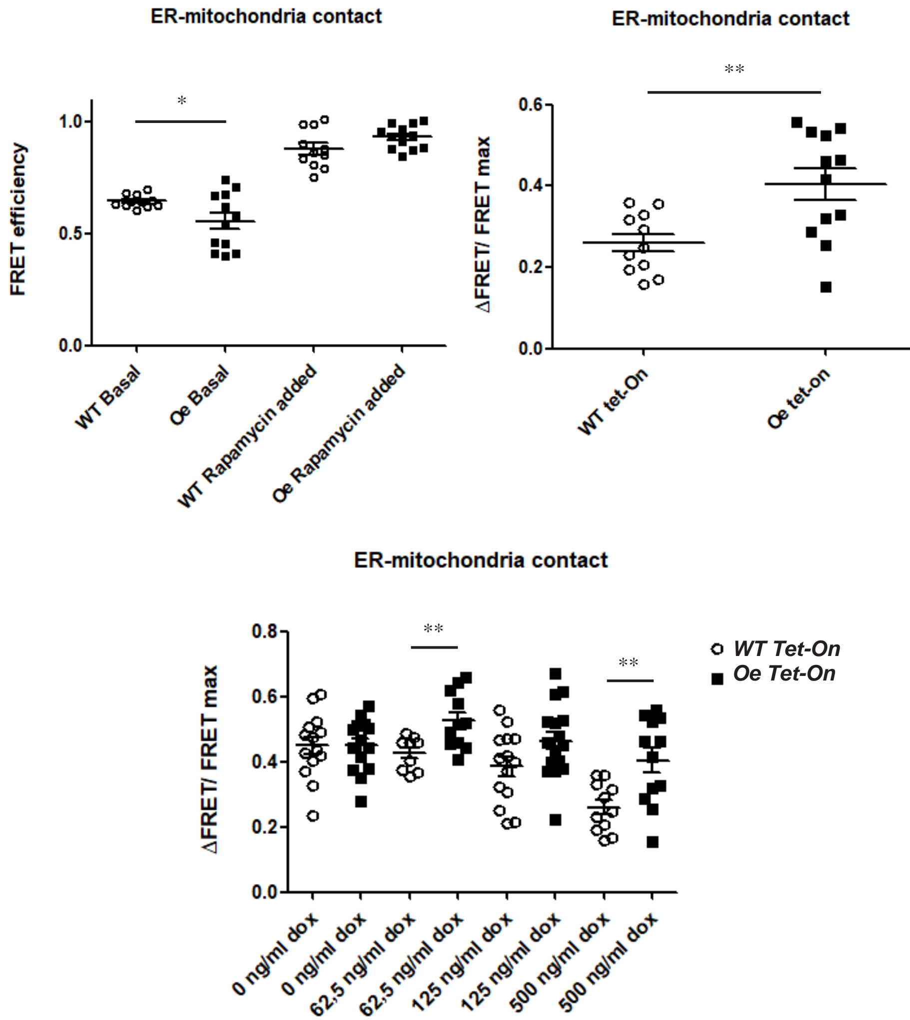


Fig 16. Overexpression of ORMDL3 alters ER-mitochondria contacts. FRET experiments after 24h of transfection probe in U87MG WT and Oe Tet-On clones for A, Basal and maximum FRET signal, B, FRET Coefficient proportional to ER-mitochondria distance and C, FRET coefficient using different concentrations of doxycycline to activate Tet-On system. Raw data of all experiments is represented for WT and Oe Tet-On clones. * $p<0.05$, ** $p<0.01$; analysis between both cell lines. *Error bars* represent SEM.

In addition to these results, ER-mitochondria distance was measured in MEFs KO for ORMDL3 (model in which Scorrano lab are carrying ORMDL3 experiments) by electron microscopy. In this case, data analysis measuring the ER-mitochondria contact coefficient (ERMICC) showed a contact in KO ORMDL3 MEFs compared with WT (Figure 17).

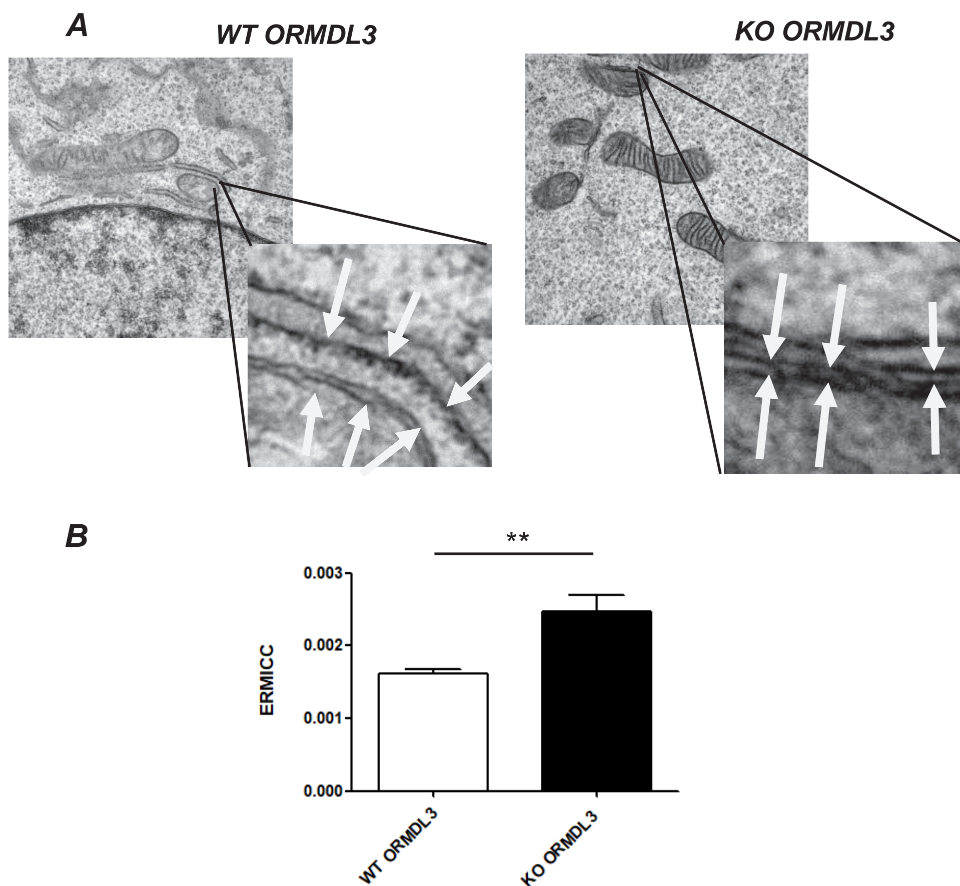


Fig 17. ORMDL3 act as spacer between ER and mitochondria. A, Electron microscopy images of MEFs WT and KO of ORMDL3. White arrows mark contact points between ER and mitochondria. B, Quantification of ERMICC in WT and KO ORMDL3. (n = 5; analysis between WT and Oe clones, ** p<0.01). *Error bars* represent SEM.

5.2.4 ORMDL3 domains involved in the function of this protein in MAMs.

Having in mind the changes observed in the interaction experiments for the different ORMDL3 models, we decided to generate different constructs with deletions of ORMDL3 to detect the important motifs responsible for these changes in MAMs. We studied the targeting of these constructs doing colocalization experiments with the ER marker protein disulphide isomerase (PDI) and mitotracker to stain mitochondria in the U87 cell model (Fig 18).

Four principal deletions were tested for these experiments. First, last 32 amino acids of the C-terminal from the 153 total amino acids of the protein were removed (Fig 18A). Colocalization of this construct with PDI and mitotracker shows an ER typical pattern expected as ORMDL3 protein.

Next, 90 amino acids were removed also from the C-terminal of the protein, resulting in the same pattern as the previous deletion (Fig 18B).

However, when we studied the localization deleting the N-termunal of the protein the localization pattern changed. Thus, the Delta1-88 ORMDL3 mutant tresulted in an increased colocalization with mitotracker marker and decreased with the endoplasmic reticulum one (Fig 18C). Moreover, we confirmed this result with another deletion in

which we removed 29 amino acids more, with a total of 117 amino deletion (Fig 18D). For this delta 1-117 ORMDL3 mutant deletion we had a complete mimitochondrial pattern.

Observing these results, we concluded that there must be a C-terminus mitochondrial interacting domain that allowed ORMDL3 constructs to target to mitochondria and probably that was an important domain for the MAMs associated ORMDL3 functions observed. In WT protein the structure of the ORMDL3 N-terminus part must be essential to retain the protein in the ER.

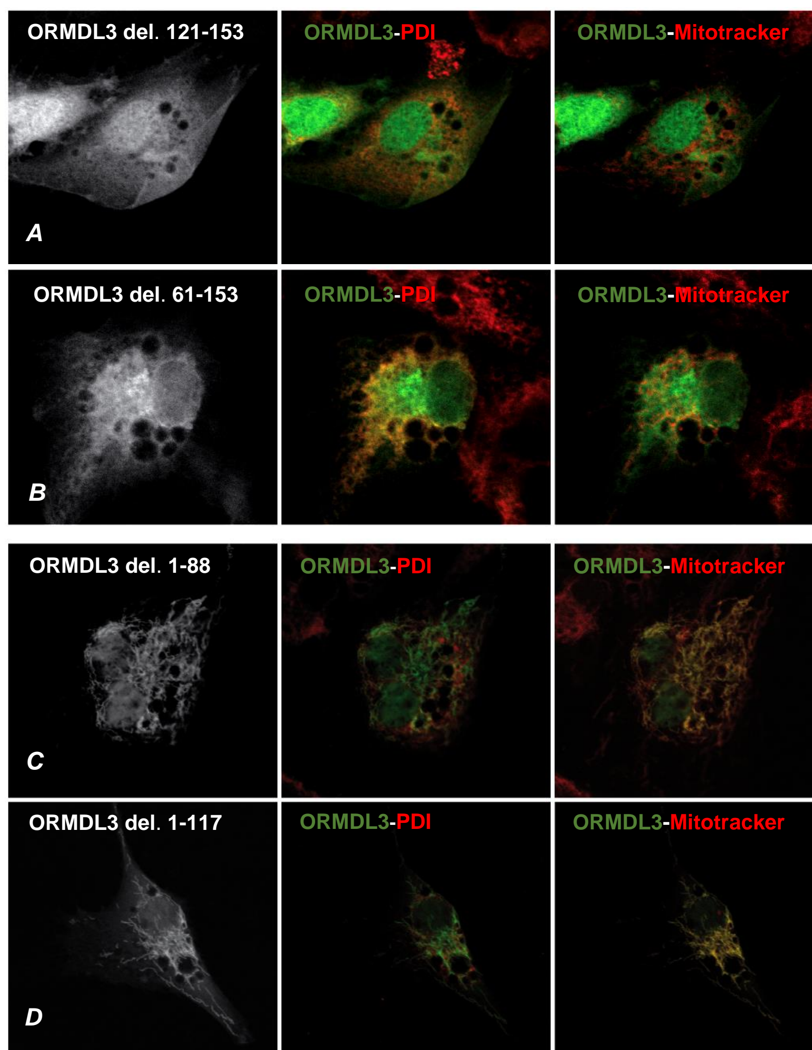


Fig 18. ORMDL3 mutants locate differentially in ER or mitochondria organelles. Different ORMDL3 deletions from the 153 amino acids of the protein were transfected in U87 MG cells for 24h. A, deletion from 121 to 153; B, deletion from 61 to 153; C, deletion from 1 to 88 and D, deletion from 1 to 117. Immunostaining protocol were performed after the 24h transfection of the different deletions anchored to GFP. The chemic compound mitotracker has fluorescence emission at 555 nm and PDI were label with Alexa 555.

With this scenario in mind, we run preliminary apoptosis experiments with the different constructs for ORMDL3 using U87 cell line (Fig 19). In this case, as ER-targeting ORMDL3 construct, deletion from 121 to 153 was selected. As mitochondria-targeting ORMDL3 construct, deletion from 1 to 117 was selected. Our annexin V staining analysis using H2O2 as a pro-apoptotic stimulus showed that overexpression of Delta 1-117 ORMDL3 mutant induced a pronounced cell death resistance compared with pcDNA transfected cells and the mutations of ORMDL3 that also locate in ER.

All these results suggested that over-expression of ORMDL3 can alter MAMs homeostasis by directly interacting mitochondria from the ER and producing cell death resistance.

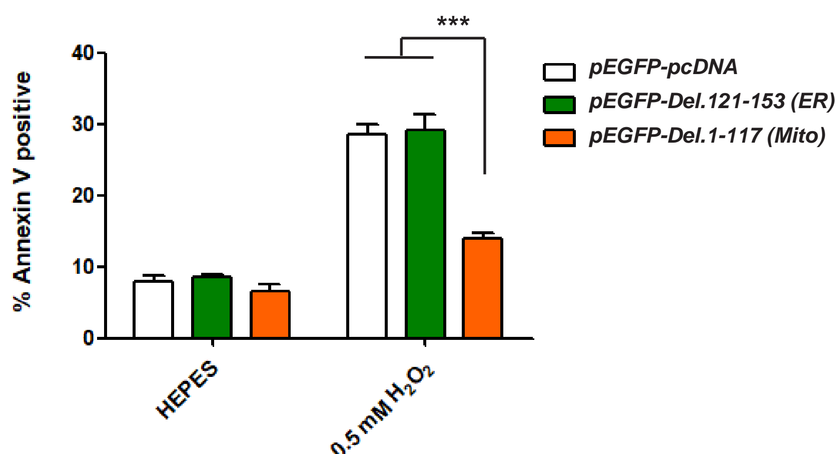


Fig 19. Delta1-117 ORMDL3 deletion promotes apoptosis resistance. Annexin V staining was performed to test apoptosis induction by H_2O_2 in U87 cells transiently overexpressing of different ORMDL3 constructs f:

del.121-153 ORMDL3, del 1-117 ORMDL3 and empty vector. *Error bars* represent SEM.

6. DISCUSSION

Since the last decade, GWAS studies have emerged as essential tools to elucidate genetic causes for complex diseases. High frequency variation in single nucleotides (SNPs) when different human control and disease genome are compared, can be identified as possible causative of specific diseases. In this context the starting point of this thesis was the study showing that an allele variant of the SNP rs7216389 present in the 17q21 chromosome close to the coding region of *ORMDL3* was genetically associated to higher risk of glioma (Dobbins SE et al., 2011). Moreover, this link implied an increase of ORMDL3 expression levels (Moffat MF et al., 2007; Schmiedel BJ et al., 2016). We decided to focus our interest in understanding why ORMDL3 expression levels might play a relevant role in cancer development and progression.

The genetic association of ORMDL3 in different GWAS studies implicated not only cancer pathology risk, also asthma and inflammatory diseases as rheumatic arthritis, inflammatory bowel disease and diabetes type I were reported (Moffat MF et al., 2007; Stahl EA et al., 2010; Kurreeman FA et al., 2012; Franke A et al., 2010; Barrett JC et al., 2008). Other studies link also inflammatory profiles and cancer. For example, in breast cancer ciclooxigenase-2 (COX-2) has an important impact in the malignancy of this tumor (Wang X et al., Oncotarget 2017). Besides, COX-2 also is involved in the infiltration of macrophages in the metastasis of lung cancer (Che D et al., Mol immunol 2017).

The ORMDL family members have been implicated in calcium homeostasis, ER stress regulation and *de novo* sphingolipid biosynthesis control. In the present thesis we have focused on this last function because several antitumoral agents like resveratrol or tamoxifen, exert their pro-apoptotic action by altering cellular sphingolipid content (Scarlatti F et al., 2003; Kartal M et al., 2011; Corriden R et al., 2016). In this context, in the last years THC has been studied as a potential supplement to target glioblastoma, a very aggressive cancer with limited options for treatment. It has been shown that in U87 MG cells this drug is able to increase ceramide content in the cell to induce ER stress and cell death (Salazar M et al., 2009). More specifically, THC disturbs *de novo* ceramide synthesis pathway, where ORMDLs are endogenous inhibitors, producing a dihydroceramide-ceramide imbalance (Hernández-Tiedra et al., 2016). We decided therefore that this model could be interesting in order to test whether ORMDL3 acted as a break in the THC antitumoral action increasing cell death resistance. Taking this pathway as a core structure in the present thesis we have done new contributions in the field of ORMDL3 by studying its role in the ORMDL-SPT complex function, ER stress induction, apoptosis resistance, calcium homeostasis and MAMs structure.

Location and physiology of ORMDL3 and SPT complex

As described in 2010 by two different groups (Breslow DK et al., 2010; Han S et al., 2010) the two ORMDL orthologs in yeast, Orm1 and Orm2, are required to inhibit the serine palmitoyltranferase (SPT), the first rate-limiting enzyme implicated in *de novo* synthesis of ceramides. Moreover, posterior studies revealed that interaction of Orm proteins on SPT, can be regulated by phosphorylation mechanisms depending on the cellular sphingolipid content (Roelants FM et al., 2011; Gururaj C et al., 2013). These studies confirmed that Orm proteins act as negative regulators of SPT complex by a phosphorylation feedback loop in yeast.

In this scenario our localization results in U87 cells agreed with existing data (Siow DL et al., 2012) in which ORMDL3 and SPTLC1 are able to colocalize in ER organelle. Moreover, we also show for the first time that SPTLC1 and ORMDL3 have similar staining pattern that SPTLC2, the other subunit of SPT complex. Moreover, under THC treatment we have demonstrated that ORMDL3 and SPT complex maintain their expression in ER when this organelle suffers stress (Figure 1). Similar puncta pattern has been already described in the ER when cells are treated with different compounds from diverse chemical classes (Varadarajan S et al., 2012).

ORMDL regulation in the THC antitumoral pathway

Our expression studies revealed that ORMDLs are degraded upon THC treatment (Figure 2A). This downregulation of ORMDL proteins could explain in part the increase of ceramide content published by Salazar et al., 2009 that leads to apoptosis and and cancer cell death. We have confirmed by blocking with an antagonist that this effect was cannabinoid receptor dependent similarly to the increase in ceramides described in Hernández-Tiedra et al., 2016.

Finally, we have discarded the transcriptional regulation as a possible mechanism to downregulate ORMDL proteins (Figure 2B). On the contrary we have shown that there is a post-translational regulation that ends with the degradation by the proteasome. However, the molecular determinants and enzymes involved in this shut down pathway are still unknown. In general, these results support the data published in Salazar M et al., 2009 and Hernández-Tiedra et al., 2016 in which THC increase dihydroceramide and ceramide content in U87MG cells, now creating a new mechanistical scenario in which THC might increase these sphingolipids by downregulation of ORMDL proteins, inhibitors of SPT enzyme.

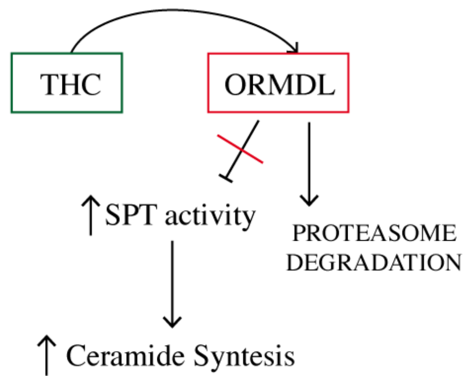


Fig 1. Downregulation mechanism of ORMDL proteins induced by THC. THC induces degradation of ORMDL proteins by proteasome realising SPT to increment ceramide content in the cell by *de novo* pathway.

Functional role of ORMDL3 in glioma risk

It has been shown by different laboratories that SNPs in the chromosome region 17q12-q21 form a *cis* regulatory haplotype that changes the expression levels of ORMDL3 (Carreras-Sureda A et al., 2016; Schmiedel BJ et al., 2016). The genetic association of the SNP rs7216389 that increases to ORMDL3 expression to glioma risk has opened a new scenario connecting the function of this of this protein with cancer. In this context we have developed ORMDL3 overexpression models in glioma cells (Figure 4) mimicking the effect of the rs7216389 SNP risk allele in order to explore the acquisition of resistance to antitumoral and pro-apoptotic treatments.

Combining the results obtained with viability studies and apoptosis analysis of U87 MG cells we can conclude that ORMDL3 over-expression induce resistance to cell death (Figure 6 and 11). Until now, there were reports showing that the absence of yeast homologues of ORMDLs were more sensitive to toxic agents including ER stressors (Hjelmqvist L et al., 2002; Han S et al., 2010).

Moreover, McGovern and collaborators showed that overexpression of ORMDL3 was able to block UPR when using tunicamycin (McGovern DP et al., 2010) and thapsigargin. However, this work provides for the first-time

evidences of how a single ORMDL member can promote cell death resistance.

Our experiments following the antitumoral pathway of THC has allowed us to better understand the mechanism behind cell death resistance observed in ORMDL3 overexpressing cells.

Impact on ceramide production

The role of ORMDL protein family as inhibitors of SPT, the first enzyme that catalyses ceramide synthesis by *de novo* pathway, has been reported by different studies mainly working using yeast KO models (Breslow DK et al., Nature 2010; Han S et al., PNAS 2010). Remarkably, in mammalian systems the literature is still controversial. Single overexpression of the different ORMDL members failed to cause alteration in cellular ceramide content in previous studies with cellular models (Kiefer K et al., 2015; Gupta SD et al., 2015) or with transgenic animals (Zhakupova et al., 2016) being necessary a triple overexpression to modify SPT activity (Kiefer K et al., 2015). However, analysis of SPT activity under ORMDL3 overexpression showed a decrease activity and a reduce levels in cellular ceramide content in HeLa cells (Siow DL et al., 2015). Similarly, a different study in epithelial cells and macrophages showed that high expression of ORMDL3 results in a decrease ceramide production (Oyeniran C et al., 2015). In the same direction the

plasma analyses of the ORMDL3 transgenic mouse model of Miller and colleagues revealed reduced levels of ceramides (Miller M et al., 2017). In this complex scenario, we have demonstrated that under induction of ceramide synthesis by THC, our overexpression Tet-On model failed to increase nor ceramide synthesis neither dihydroceramide, previous metabolite (Figure 6). Thus, in our U87 model ORMDL3 overexpression has an impact by itself on SPT activity. The different results obtained in the literature might be explained depending of the basal expression levels of the different members of the ORMDL family in each model. In this respect, Oyeniran and collaborators (Oyeniran C et al., 2015) showed that the impact of ORMDL3 on ceramide production was very different depending on the degree of overexpression.

Impact on ER stress induction

Previous studies have described the alteration of stress-regulated proteins under THC treatment (Carracedo A et al., 2006). Interestingly the degree of p8 protein induction upon THC treatment has been shown to determine the sensitivity to THC antitumoral action because p8 mediates the apoptotic effect of THC via upregulation of the endoplasmic reticulum stress-related genes ATF-4, CHOP, and TRB3. We have validated this pathway in our WT cells analysing p8, TRB3, CHOP and BiP. On the contrary, overexpression of ORMDL3

turned in a lower increase of the expression compared to WT of these four genes at 3 and 8 hours under THC treatment (Figure 8).

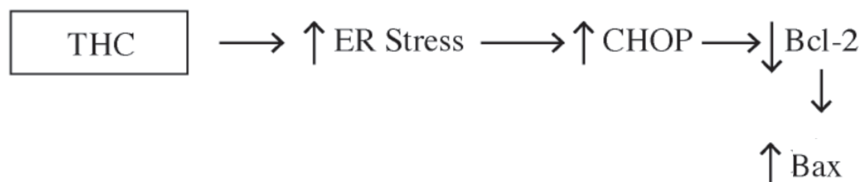
The trigger of the increase expression of genes related with the ER stress relays in the PERK branch of the unfolded protein response (UPR). Thus, it has been previously shown that THC produces phosphorylation of eIF2α (Salazar et al., 2009). Interestingly, our ORMDL3 overexpressing model presented higher levels of phospho-eIF2α as has been previously described in HEK293 cells (Cantero-Recasens et al., 2010). However, upon THC treatment phospho-eIF2α increased only in the WT clone whereas under ORMDL3 overexpression this phosphorylation decreased (Figure 7). This data showing a the UPR tuning caused by ORMDL3 is in agreement with McGovern and collaborators previous work (McGovern DP et al., 2010) and explains the lower stress cascade observed in our ORMDL3 overexpressing cell system.

Impact on apoptosis

Cell death induction is caused by an activation of pro-apoptotic or inhibition of anti-apoptotic proteins. One important protein determining cell fate is the anti-apoptotic Bcl-2. It is known that this protein localizes at the ER membrane in close proximity with mitochondria and prevents the intrinsic pathway of apoptosis (Krajewski et al., 1993;

Chipuk JE et al., 2010) by inhibiting pro-apoptotic proteins like Bax and Bak. Moreover, Bcl-2 is inhibited by the pro-apoptotic gene CHOP, downstream ATF6 and PERK/eIF2/ATF4 (Malhotra JD et al., 2011). We observed higher levels of Bcl-2 in our ORMDL3 overexpressing cells than in WT cells. This would explain why under THC stimulus, whereas WT cells increases proapoptotic protein Bax, the Tet on ORMDL3 clone maintained Bax levels (Figure 11). Increase in Bax levels are associate to a pro-permeabilization of OMM and release of mitochondrial cytocrome C, activating caspase pathway and apoptosis induction (Welch C et al., 2009).

In summary, ORMDL3 impacts at different levels on the antitumoral action of THC. However, the effect that ORMDL3 has at basal levesl on the PERK pathway and the expression of Bcl-2 protein could be a more general explanation of the apoptosis resistance observed in our cells. In this sense this antiapoptotic effect might be the underlying mechanism behind the relationship between higher risk of glioma and higher levels of ORMDL3.



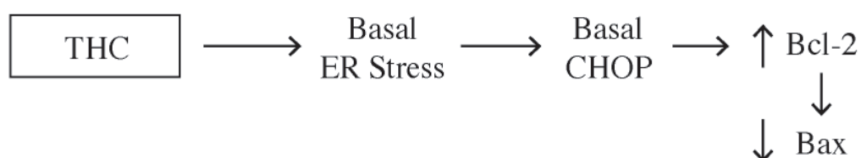


Fig 2. Modulation of ER stress, anti- and pro-apoptotic proteins under THC treatment in different ORMDL level conditions. Overexpression of ORMDL3 is able to reduce ER stress under THC treatment, not increasing the proapoptotic gene CHOP. It maintains high Bcl-2 levels what blocks Bax and stops the posterior apoptosis induction.

Role of ORMDL3 in MAMs physiology

The endoplasmic reticulum and mitochondria have an important role in apoptosis-cell death induction. Moreover, the signalling pathways of these organelles are interconnected by physical junctions that tune the cellular homeostasis. These junctions are the signalling hubs called ER mitochondria-associated membranes (MAMs). Given the results obtained pointing at ORMDL3 as a modulator of the ER-mitochondrial driven apoptotic pathway we wanted to explore possible implication of this protein in MAMs physiology.

The ER-motochondria communication code is based on Ca2+ signalling. Moreover, disturbances in Ca2+ fluxes, causing a mitochondrial calcium upload, are considered the trigger of several apoptotic stimuli. In this context and taking into account the increased expression of Bcl-2 observed in our Tet on overexpressing model, it is important to mention that Bcl-2 inhibits IP3R, the efflux pathway of calcium in MAMs, decreasing Ca2+ release from this organelle and downregulating mitochondrial Ca2+ uptake (Rong YP et al., 2008). Therefore, we wanted to study the calcium homeostasis in our cells models. We found, as published before (Cantero-Recasens G et al., 2010; Carreras-Sureda A et al., HMG 2013), a cytosolic calcium imbalance in ORMDL3 overexpressing cells (Figure 13A). On the contrary, we could not relate that increase with a lower content in ER calcium levels. It was previously shown in our laboratory that

ORMDL3 inhibited SERCA activity, the pump responsible of calcium uptake in the ER (Cantero-Recasens G et al., 2010). However, in our U87 cell model the calcium dynamics seemed to be affected in a different way (Figre 13B). One possibility that would explain this discrepancy would be the inhibition of IP3R produced by high Bcl-2 levels acting as a counterpart of the SERCA blockade mediated by ORMDL3.

Besides, mitochondrial basal Ca2+ levels and uptake were tested in both clones. We observed reduced calcium content and lower calcium buffering capability in ORMDL3 overexpressing cells (Figure 13C). These results confirmed a calcium imbalance caused by ORMDL3 expression affecting mainly mitochondria and cytosol as previously described. This also suggests that part of the apoptosis resistance observed in our cells could be explained by an impaired calcium uptake system. Further analyses must be done to better understand the contribution of the different players involved in MAMs calcium fluxes like IP3R or MCU activity.

ER-mitochondria distance plays a considerable function in maintaining Ca2+ signalling homeostasis. MAMs distance has been described to be dependent of the presence and/or activity of proteins like MFN1 and 2, VAPB, PTPIP51 among others. We have demonstrated with our experiments that ER-mitochondria distance is modified depending on the ORMDL3 expression levels. Thus, basal signal was increased after ORMDL3 overexpression (Figure 15). Moreover, we

demonstrate that our protocol for FRET analysis works properly when the signal increase between FRET probe and ORMDL3 after rapamycin addition (Figure 14).

These results about ER-mitochondria distance would provide a clue about the mechanism behind the altered Ca2+ flux and diminish apoptosis induction by ER stress stimuli observed in ORMDL3 overexpressing cells. Thus, previous reports have shown that MFN-2 actuates as tether between ER and mitochondria altering Ca2+ flux between both organelles (Naon D et al., 2016). Downregulation of MFN-2 decrease ER-mitochondria contact coefficient and also decrease mitochondrial Ca2+ uptake. Comparing this with our results, we demonstrate that ORMDL3 has a role reducing the tethering what would imply that ORMDL3 acted as spacer between ER and mitochondria organelles. Our results in MEFs KO ORMDL3 model pointed at that direction because we observed that ERMICC decrease in case of ORMDL3 KO (Figure 17).

Interestingly, PERK has been shown to act as a tether in MAMs allowing the cross-talk between organelles and favoring the propagation of ROS signals (Verfaillie T at al. 2012). In our cells we have seen monitored phospho-eIF2α and we have seen that PERK activity was altered. At basal conditions there was an increase of phospho-eIF2α and under THC stimulus the phophorilation levels decreased. We also showed that the H2O2 apoptotic pathway was

dramatically altered in our ORMDL3 ovrexpressing cells. These data points again a proximity problem and point at PERK as a candidate tether affected by ORMDL3 expression.

Alteration in ER-mitochondria distance can affect other important pathways in the cell. Thus, loss of tethering complex VAPB-PTPIP51 stimulates autophagy (Gomez-Suaga P et al., 2017) by an alteration in Ca2+ flux between both organelles. Another tethering complex is the one formed by VAPB-RMDN3. Loss of this interaction between ER and mitochondria results with low Ca2+ levels in mitochondria and autophagy stimulation (Gomez-Suaga P et al., 2017). In our model, we have demonstrated that when we overexpressed ORMDL3, causing an increase in ER-mitochondria distance, we observed lower calcium levels in mitochondria and autophagy stimulation too. In this context, resistance to cancer cell death in our model of overexpression of ORMDL3 can also be partially caused by a prosurvival autophagy stimulation, providing energy to the tumor. This tumorigenic role for autophagy has been described in some cancer cells. (Guo JY et al., 2016; White E et al., 2015).

Another aspect that centers the antiapoptotic role of ORMDL3 in the context of MAMs physiology is the stimuli specific protective effect. Thus, we have observed that increase levels of ORMDL3 reduce apoptosis mediated by stimuli that cause ER stress and converge in MAMs like THC, H2O2 or tunicamycin (Figure 12). On the contrary when TNFα was

used to promote apoptosis causing a direct caspase activation (Rath PC et al., 1999) we did not observed protection in our cell model indicating that this proapoptotic stimulus was upstream the ORMDL3 overexpression effect (Figure 13).

Once proved that ORMDL3 interfered the induction of cell death under ER-mitochondria stress pathway, we tried to elucidate the motif of the protein involved in this. Different constructs were performed but the ones on which at least 117 amino acids were removed from the N-terminal of the protein, modified completely the patter of the protein, targeting completely to mitochondria (Fig 18 C and D). On the other hand, deletions produced in the C-terminal of the protein did not change the ER pattern. Finally, we performed Annexin V staining in cells treated with H2O2 with these deletions. We observed that the deletion that localizes the protein in mitochondria increased the resistance to cell death compared with the one that localized in ER (Figure 19). That would suggest that the C-terminus of ORMDL3 in implicated in the apoptosis resistance and probably interacts with elements at the outer membrane of the mitochondria.

In summary, our results demonstrate that ORMDL3 expression levels must be considered as determinants of cancer cell physiology. The underlying mechanism behind the increased glioma risk observed with high expression levels of

ORMDL3 produced by the SNP allele variant could be dependent on the prosurvival and antiapoptotic effect that produces this protein. Moreover, ORMDL3 expression levels should be considered also when adjusting the dose of any antitumoral drug involving ER stress of that implies the activation of the intrinsic apoptosis pathway. Besides this thesis provides important evidences of the role of ORMDL3 in MAMs, the signalling hub between ER and mitochondria that affects many cellular processes that determine cell fate like calcium homeostasis, autophagy and apoptosis.

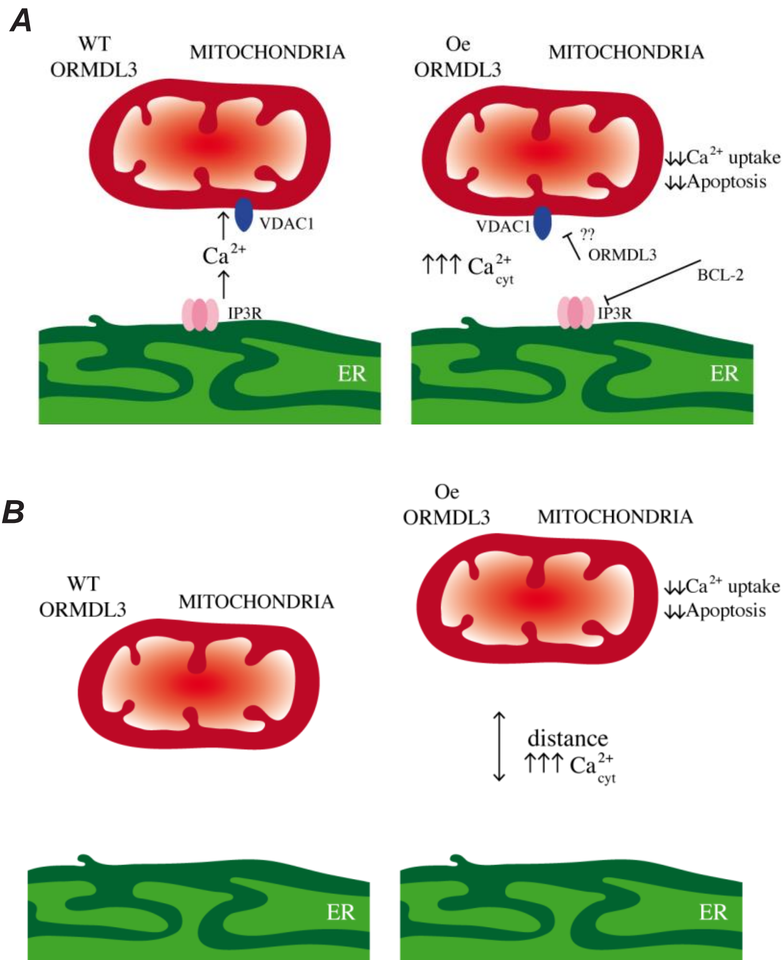


Fig 3. Complementary mechanisms by which ORMDL3 have an influence in Ca2+ trafficking and apoptosis induction in WT and Oe ORMDL3 Tet-On cell lines. A, Over-expression of ORMDL3 increase Bcl-2 levels what decrease ER Ca2+ release by IP_3R, incrementing cytosolic Ca2+. Moreover, the possible interaction between ORMDL3 and VDAC1 also can modulate mitochondrial Ca2+ uptake, decreasing mitochondrial Ca2+ and downregulating apoptosis. B, Increase in ER-mitochondria by ORMDL3 when is over-expressed can be another alternative condition to alter Ca2+ trafficking between these two organelles, decreasing apoptosis under the over-expression as consequence

7. CONCLUSIONS

1. THC antitumoral pathway implies a downregulation of ORMDL proteins by proteasomal degradation, releasing this way the activity of the serine palmitoyltransferase.
2. Overexpression of ORMDL3 blocks dihydroceramide and ceramide synthesis under THC treatment in the U87 glioblastoma cell line.

3. Over-expression of ORMDL3 reduces ER stress and deleterious autophagy triggered by THC treatment in the U87 glioblastoma cell line.

4. Overexpression of ORMDL3 produces a resistance in cell death induction under stimuli that involve the intrinsic apoptotic pathway like THC, tunicamycin and H_2O_2 in U87 cells.

5 ORMDL3 expression levels impact negatively on the degree of interaction between the endoplasmic reticulum and mitochondria.

6 ORMDL3 overexpression alters MAMs function modifying pathways that involve calcium fluxes, autophagy and apoptosis.

7 The C-terminus of ORMDL3 contains an interacting domain with mitochondria responsible for the apoptosis resistance observed overexpressing the full length ORMDL3 in U87 cells.

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