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# ROLE OF THE ORMDLS IN KERATINOCYTES

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# ROLE OF THE ORMDLS IN KERATINOCYTES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

At
VIRGINIA COMMONWEALTH UNIVERSITY

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**List of Abbreviations**

| | |
|---|---|
| AD | Atopic dermatitis |
| ALI | Air- Liquid Interface |
| CERT | Ceramide transfer protein |
| DAG | Diacyglycerol |
| ECM | Extracellular matrix |
| ER | Endoplasmic reticulum. |
| GlcCer | Glucosylceramide |
| HDR | Homology- directed repair |
| LSD | Lysosomal storage disorders |
| MHC | Histocompatibility complex |
| NHEJ | Nonhomologous end joining |
| PAM | Protospacer adjacent motif |
| PLP | Pyridoxal 5′-phosphate |
| S1P | Sphingosine-1-phosphate |
| SC | Stratum corneum |
| SM | Sphingomylen |
| SPT | Serine Palmitoyl-transferase |
| ULCFA | Ultra long chaine fatty acid |
| ARCI | Autosomal recessive congenital Ichthiosis |

**Abstract:**

The epidermis, the outermost layer of the skin, serves as a vital barrier protecting the body from environmental stressors, pathogens, and dehydration. Keratinocytes, the predominant cell type in the epidermis, play a central role in maintaining skin integrity and barrier function. In normal human skin, keratinocytes move from the basal layer to the stratum corneum, the top layer of the epidermis. In their travel, keratinocytes undergo a process of terminal differentiation, loss their ability to proliferate, secrete sphingolipids into the extracellular space, and gradually loss their nucleus and organelles. Sphingolipids, particularly ceramides, are key components of the intercellular lipid lamellae and contribute to the formation of the skin barrier permeability. The biosynthesis of sphingolipids occurs within keratinocytes through intricate enzymatic pathways. Serine palmitoyltransferase SPT is the first and rate-limiting enzyme in the *de novo* synthesis pathway of sphingolipids. SPT is regulated by ORMDL to maintain the balance of sphingolipids within cells. Perturbations in sphingolipid metabolism have been linked to skin disorders characterized by impaired barrier function, such as atopic dermatitis and psoriasis. In the skin, knocking out ORMDL could lead to increased SPT activity and altered levels of sphingolipids, including ceramides. Changes in ceramide composition may influence skin barrier function and improve its permeability. Understanding the role of ORMDL knockout in the skin could have implications for the development of therapeutic strategies. Modulation of sphingolipid metabolism or manipulation of ORMDL may be explored for managing skin conditions.

# Chapter 1 Introduction

## 1.1 Introduction of the sphingolipid biosynthesis:

Eukaryotic cell membranes are composed by a complement of lipids and proteins indispensable for their function. These lipids are divided into three main classes: sterols, glycerolipids and sphingolipids (Breslow et al., 2013). Sphingolipids are a group of lipids essential to the composition of the plasma membrane of many cell types. Sphingolipids hold both hydrophobic and hydrophilic properties. They contribute to a number of different cellular function including cell proliferation, death, migration, inflammation, and central nervous system development (Quinville et al.,2021).

Sphingolipids are composed of backbones called “sphingoid bases” synthesized from serine and a long-chain fatty acyl-CoA. Sphingolipid metabolism is highly regulated and complex, with many stimuli and agents affecting one or more enzymes. Importantly, all key enzymes of sphingolipid metabolism have now been identified at the molecular level, revealing the vast complexity of these metabolic pathways and their precise subcellular compartmentalization (Hannun & Obeid, 2017).

The biosynthesis of sphingolipids involves a series of enzymatic reactions that takes place primarily in the endoplasmic reticulum (ER) and Golgi apparatus of eukaryotic cells. Sphingolipid synthesis starts in the endoplasmic reticulum, where the condensation of C16 fatty acid palmitoyl-CoA and the amino acid L-serine is catalyzed by serine palmitoyltransferase (SPT). The product, 3-ketodihydrosphingosine is reduced to dihydrosphingosine by 3-ketodihydrosphingosine reductase. Then ceramide synthase converts dihydrosphingosine to dihydroceramide. Dihydroceramide desaturase introduces a double bond into dihydroceramide

resulting to the formation of the central molecule in this pathway, ceramide ( Wigger et al, 2019). Ceramide is then transferred to the Golgi via vesicular transport or the ceramide transfer protein (CERT). When transported to the trans-Golgi apparatus, and plasma membrane from the Endoplasmic reticulum, ceramides can be converted into sphingomyelins by sphingomyelin synthases. These sphingomyelins gradually increase in concentration to compose most of the sphingolipids in the plasma membrane (Lee et al., 2023).

On the other hand, Sphingolipid catabolism is essential for maintaining cellular homeostasis and preventing the accumulation of sphingolipids. The primary pathway for sphingolipid catabolism involves the breakdown of sphingomyelin by the action of sphingomyelinase. This enzyme cleaves the head group from sphingomyelin, producing ceramide. Ceramide can be derived from simple precursors such as serine and palmitoyl-CoA in the *de novo* pathway or can be derived by sphingomyelins and sphingosine in the catabolic pathway. This ceramide is further broken down by ceramidase enzymes yielding sphingosine. The latter can be phosphorylated to form sphingosine-1-phosphate (S1P), an important signaling molecule (Fig.1). Sphingosine and its phosphorylated form, S1P, have important roles in cellular signaling pathways. S1P, in particular, can bind to specific cell surface receptors (S1P receptors) and modulate various cellular processes, including cell growth, differentiation, migration, and immune responses.

It has long been known that cells adjust sphingolipid production in response to metabolic needs. However, dysregulation of sphingolipid homeostasis may have damaging effect. Today, sphingolipids are known to be involved in neurodegeneration, inflammatory processes, cancer metastasis, lysosomal storage disorders, and skin diseases (Quinville et al, 2021).

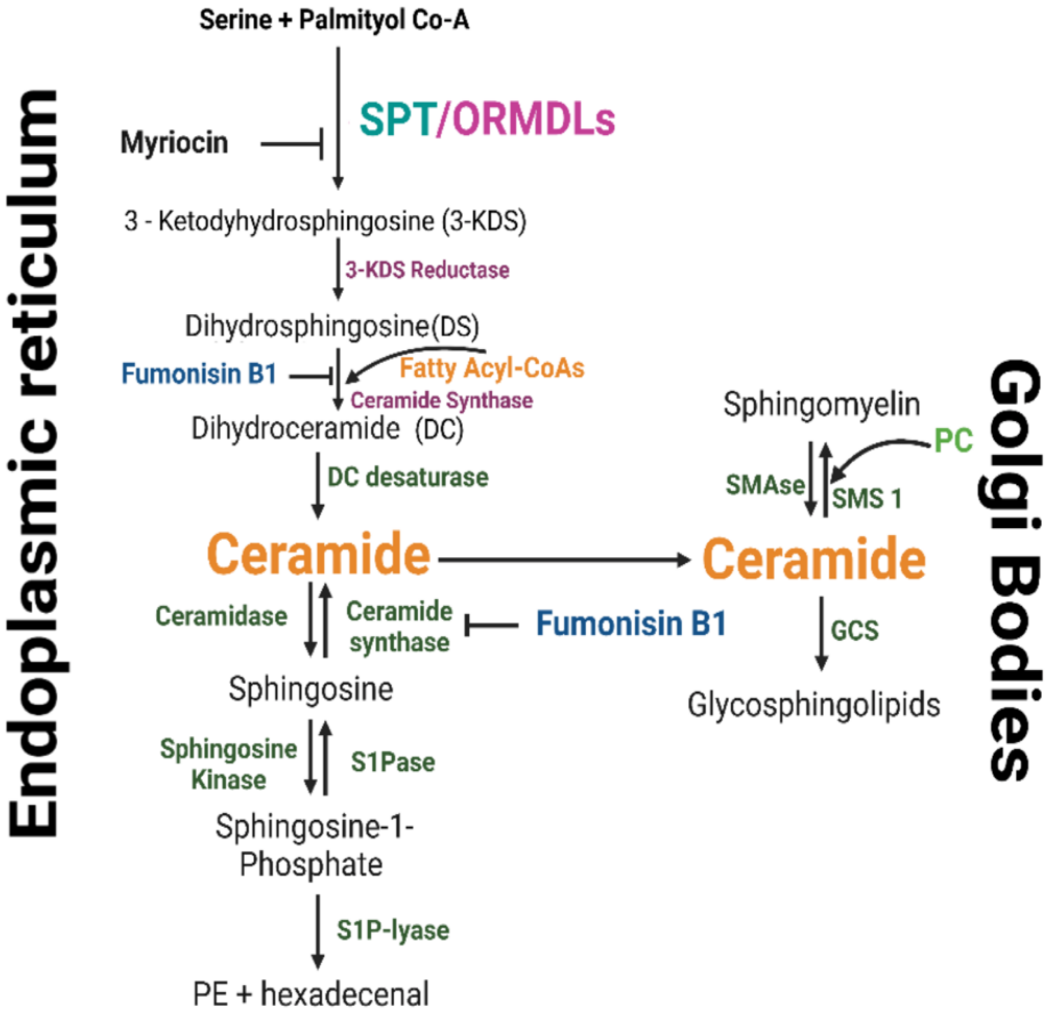



**Fig 1. Sphingolipid synthesis and degradation in cellular region pathway.** Sphingolipids can be synthesized by different enzymes starting from the ER and transporting to the Golgi. Sphingolipids can also be degraded from sphingomyelin and ceramide to small sphingolipids acting as a signaling molecule (Wattenberg.,2021) .

### 1.2 Serine palmitoyltransferase SPT is controlled by ORMDL.

#### 1.2.1 SPT is the rate-limiting step in the synthesis of sphingolipids.

Serine Palmitoyltransferase is a key enzyme in sphingolipid production. SPT belongs to a family of pyridoxal 5′-phosphate (PLP)-dependent α-oxoamine synthases (POAS) (Hanada, 2003). The primary function of SPT is to catalyze the initial and rate-limiting step in the *de novo* synthesis of sphingolipids. This step involves the condensation of two precursor molecules, L-serine and palmitoyl-CoA to form 3-ketosphinganine. SPT is homeostatically controlled in both yeast and eukaryotes. In yeast, the enzyme is a multisubunit membrane composed of two major subunit LCB1 and LCB2, and a small regulatory subunit that influences the substrate specificity of the enzyme, whereas the human enzyme is composed of three large subunits and two small subunits. The large subunits are called SPTLC1 and SPTLC2 or SPTLC3. The small subunits are called ssSPTa and ssSPTb. SPTLC1 is the supporting subunit, it keeps the protein attached to the membrane and makes it functional. SPTLC2 is the catalytic subunit, it contains the binding site where the substrates serine and palmitoyl-CoA bind. SPTLC2 may be substituted for SPTLC3. The two small subunits ssSPTa and ssSPTb increase the catalytic efficiency of the enzyme and provide some fatty acyl-CoA preference to the complex. SPT resides in the endoplasmic reticulum. The regulation of the SPT is critical for maintaining cellular sphingolipid levels and overall sphingolipid homeostasis. The activity of Serine Palmitoyltransferase is controlled by its regulatory subunit called the ORMDLs. (Siow & Wattenberg, 2012). ORMDL proteins are linked to the modulation of sphingolipid levels in cells through their influence on the enzymatic activity of SPT, resulting in a reduction in its functionality.

### 1.2.2 ORMDL/Orm proteins are part of the endoplasmic reticulum (ER) membrane.

ORMDL/Orm proteins are a conserved new family of endoplasmic reticulum membrane proteins. Discovered in 2002, ORMDL/Orm is found in yeast, mammals and vertebrates. The yeast genes Orm1 and Orm2 encode proteins sharing approximately 70% identity with each other(Hjelmqvist et al., 2002). Whereas, in human, the three isoform proteins: ORMDL1,ORMDL2,ORMDL3 are highly homologous and share more than 80% identity with each other (Hjelmqvist et al., 2002). ORMDL3 has gained significant attention since it has been strongly associated with elevated risk of asthma. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma (Moffatt et al., 2007). Knocking down or inhibiting ORMDL genes, particularly ORMDL3, can lead to specific changes in ceramide metabolism within cells. Breslow et al found that ORMDL knock-down causes an increase in ceramide level in the cell (Brown & Spiegel, 2023). Whereas Orm inhibition occurs through the phosphorylation. The phosphorylation of Orm reduces the ability of the protein to inhibit SPT activity and increases sphingolipids biosynthesis (Breslow et al., 2010).

### 1.2.3 The ORMDL-SPT complex controls sphingolipid biosynthesis.

ORMDL is the regulatory subunit of ORMDL-SPT complex. This regulation is essential for maintaining the balance of sphingolipids within cells. ORMDL associates with SPT to form the ORMDL-SPT complex. Upon this binding, ORMDL proteins exert their inhibitory effect on SPT activity. This inhibition is thought to occur through conformational changes in SPT or by preventing SPT from properly binding to its substrates, such as L-serine and palmitoyl-CoA. Davis et al observed that these conformational changes result in reducing SPT activity (Davis et al., 2019). The structural nature of the change in SPT was unknown until recently. An interesting study revealed that SPT-ORMDL complex is inhibited by the central molecule in sphingolipid

metabolism, ceramide. Ceramide can induce and lock the N-terminus of ORMDL3 into an inhibitory conformation (Xie et al., 2023). There are differences between the isoforms, The inhibition of SPT-ORMDL1 and SPT-ORMDL2 by ceramide does not exceed 10% and 30% respectively. However, SPT-ORMDL3 inhibition reached 60% (Xie et al., 2023). This percentage shows that ORMDL3 is more responsive to ceramide than ORMDL1 and ORMDL2. The SPT-ORMDL complex appears able to sense ceramide level in the cell. At low levels of ceramide, the complex becomes active since the N-terminus ORMLD remains flexible for the substrate to enter and get catalyzed by SPT to produce more ceramide. However, at high levels of ceramide, ceramide binds with the complex, lock the N- terminus of ORMDL and turning the complex into the inactive state (Xie et al., 2023). This study focused on the short-chain C6 ceramide to examine SPT-ORMDL response. As mentioned above, the research finding revealed that ORMDL3 is more sensitive to C6 ceramide. However, it will be interesting to examine the response of the three isoforms with different ceramide long-chain or other structural differences(Xie et al., 2023)

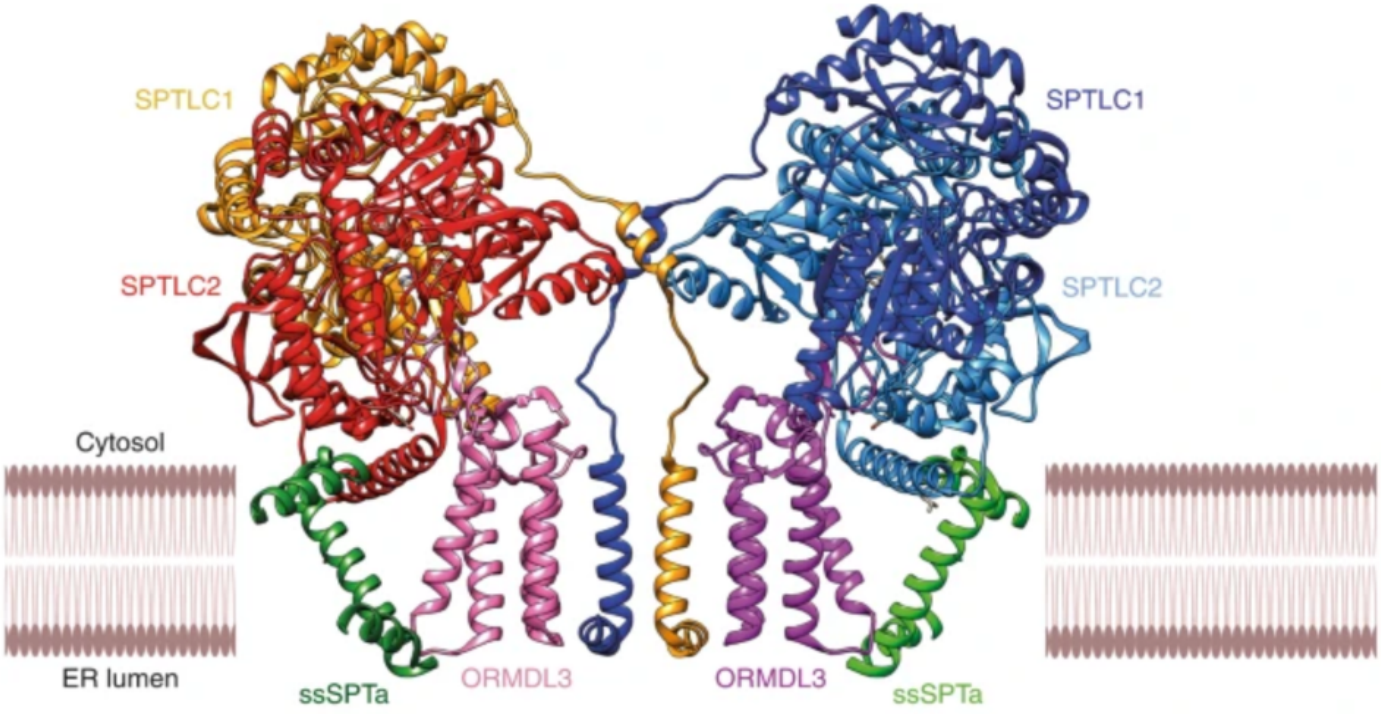



**Fig2 : structure of SPT- ORMDL complex.** SPT is a homodimer composed of two large subunits. SPTLC1, SPTLC2 and two small subunits called ssSPTa and ssSPTb. SPTLC2 may be substituted by SPTLC3. ORMDL is the regulatory subunit embedded in the ER membrane and forms a complex with SPT.

### 1.3 The role of ceramides in skin function:

#### 1.3.1 The epidermis is the rich lipid layer of the skin:

The epidermis is indeed the outermost layer of the skin. It contains a rich lipid layer that is essential for the skin's function and integrity. This lipid-rich layer is primarily found in the stratum corneum, which is the uppermost layer of the epidermis. The lipid layer in the stratum corneum acts as a protective shield, preventing excessive water loss from the body, and serving

as a barrier to external environmental factors such as microbes and pathogens. Keratinocytes are the primary type of cells found in the epidermis. They constitute 90% of epidermal cells. Keratinocyte differentiation begins in the stratum basal. As keratinocytes move upward from the basal layer, they enter the spinous layer. In this layer, the cells begin to flatten and lose their ability to divide. They become connected to neighboring keratinocytes by structures called desmosomes. These desmosomes contribute to the strength and integrity of the epidermis. When keratinocytes reach stratum granulosum, they fill with keratin granules. The final stage of keratinocyte differentiation is the stratum corneum. In that layer, keratinocytes are fully differentiated, have become flattened and filled with keratin. These cells are referred to as corneocytes.

Corneocytes are surrounded by a lipid extracellular matrix(ECM) organized into lamellar membranes (Morganti et al., 2019). The primary lipid classes in the extracellular matrix ECM are ceramides, cholesterols, and free fatty acids. However, ceramides are the most abundant lipid class in the ECM. The organization of these lipids is crucial for the skin barrier. Disruptions in the composition or structure of the lipid matrix can lead to skin conditions such as dryness, increased sensitivity, and impaired barrier function. (Knox & O'Boyle, 2021).

**1.3.2 Ceramide biosynthesis in keratinocytes.**

Skin ceramides are synthesized in the endoplasmic reticulum. Ceramides that are a minor in most tissues, are considered the dominant lipid in the cornified layer. They represent 50% of the lipids by weight (Vavrova et al., 2017). Chemically, ceramides are composed of a long fatty acid FA linked by an amide bond to spingoid base. Skin ceramides are heterogeneous. The different FA long chain, hydroxylation, as well as various degree of saturation, all together yield

to hundreds of distinct chemical structure (Rabionet et al., 2014). Among the huge variety of sphingoid bases, stratum corneum  contains mainly sphingosine (d18:1), dyhydrosphingosine (d18:0), phytosphingosine ( t18:0), and a skin specific 6-hydroxy-sphingosine (t18:1) (Robson et al., 1994). In addition to non-hydroxylation and alpha-hydroxylation, where the chain long in FA range between 16 and 26 carbon atoms, epidermal ceramides are unique to contain a hydroxyl group in ω-position. The ultra-long chain ULC in ω-hydroxylation is between 28 and 38 carbon atoms. The synthesis of long-chain fatty acid is performed by a family of enzymes (ELOVL1-7) called " Elongase of very long chain fatty acid" (Wang et al., 2022). The esterification of the ω-hydroxyl group generates a major class of skin ceramide: ω-FA esterified ULC- ceramide, where linoleic acid is the predominant species of this class of ceramide (Coderch et al., 2003).

The initial steps of ceramide synthesis occur in the lower layers of the epidermis, primarily in the stratum basal and stratum spinosum. As keratinocytes undergo differentiation and migrate upwards through the epidermal layers, they continue to synthesize ceramides. Ceramide synthesis is a multi-step process that involves the acylation of sphingoid bases with fatty acids. Different ceramide synthase CerS (CerS1-6) enzymes are responsible for incorporating various fatty acids into the ceramide molecules. CerS3 was considered as the most important enzyme since it is involved in the synthesis of all major ceramides in the epidermis.

Unlike the above ceramides synthesizing in the ER, glucosylceramide (GlcCer), an important stratum corneum ceramide having a crucial role in maintaining skin hydration and barrier function, is synthesized in the membrane of the Golgi. GlcCer involves the addition of a glucose (sugar) residue to ceramide. This reaction is catalyzed by an enzyme called

glucosylceramide synthase. Along with GlcCer, sphingomyelin (SM) is another lipid synthesizing in the Golgi. GlcCer and SM get transported into lamellar bodies (LB) and are exocytosed into extracellular space (Rabionet et al., 2014). Thus, GlcCer and SM, are converted back to ceramide by the action of beta-glucocerebrosidase and acid-sphingomyelinase, making ceramide the major component of the intercellular lipid lamellae in the stratum corneum. This operation results in protecting keratinocytes from cytotoxic ceramide effects (Rabionet et al., 2014).

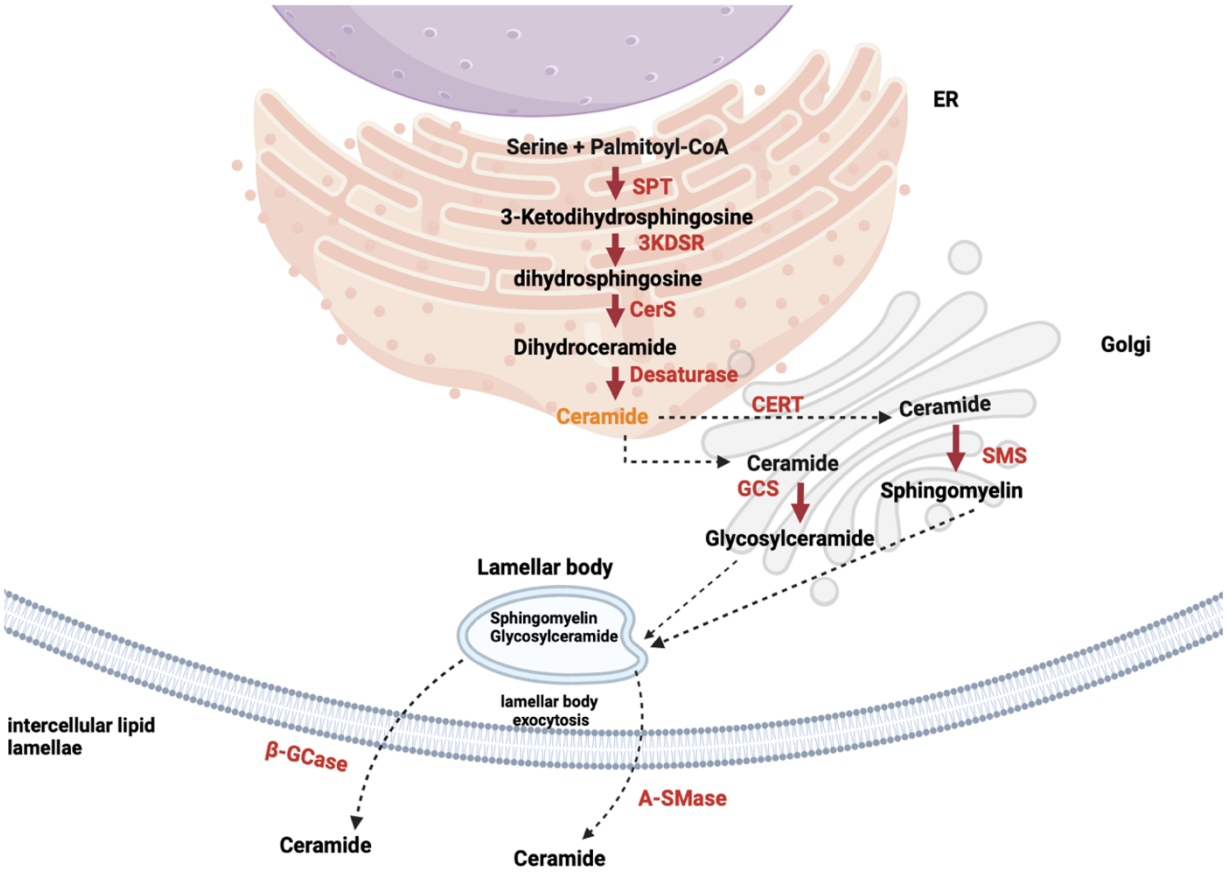



**Fig 3: Sphingolipids secretion by lamellar bodies**. GlcCer and SM are synthesized on the cytosolic side of the Golgi apparatus by glucoceramide synthase and sphingomyelinase. GlcCer and SM are then transported out of the cell by lamellar bodies, converted back to ceramide to form the intercellular lipid lamellae of the cornified layer.

### 1.3.3 Role of ceramides in barrier function of healthy and disease skin:

Ceramides are a fundamental component of the stratum corneum, the outermost layer of the epidermis. They are a key component of the skin's lipid matrix, where they are organized into lipid lamellae. This organized structure forms an effective hydrophobic barrier that helps in preventing dehydration, protecting against irritants, and maintaining barrier homeostasis (Elias et al., 2014). The stratum corneum consists of corneocytes entirely surrounded by lamellar lipid regions. This is called “bricks and mortar” where lipids are the ‘mortar’ of the stratum corneum holding the corneocyte cells ‘bricks’ in place (Knox & O’Boyle, 2021). Bouwstra et al found that a 13nm lamellar lipid exists in murine and human SC(Bouwstra & Ponec, 2006). This phase was considered important for maitaining the skin barrier. Furthermore, Coderch reported that intercellular lipids comprise of 15 % of the cornified layer weight (Coderch et al., 2003).They concluded that modification of intercellular lipid organisation and composition may impair the skin barrier properties. This is true since disruptions in ceramide levels or composition can lead to various skin conditions characterized by dryness, sensitivity, inflammation, and compromised barrier function.

Diseased skin is often characterized by an altered lipid composition and organization including atopic dermatitis AD, psoriasis, acne vulgaris, recessive X-linked ichthyosis, aged dry skin (Bouwstra & Ponec, 2006). Although there are other components in the skin to consider like water and protein, a decrease in total lipid concentration and a reduction in the barrier function are the main cause of skin disorder. In atopic dermatitis where more studies are done than any other skin disease, total lipids in the skin mesured at three anatomical locations were significantly lower in patients with AD than control (Sator et al., 2003). A number of studies found that the decrease in ceramides disturbs the highly ordered lipid lamellae, upsetting the skin

barrier function (Knox & O'Boyle, 2021). Furthermore, the lipid: protein ratio was also decreased in skin of AD patients (Knox & O'Boyle, 2021). In psorisis disease, Motta et.al found that the distribution of ceramides in psoriatic skin are lower than in healthy epidermis (Borodzicz et al., 2016.). Another study reported that higher levels of sphingosine and sphinganine were found in psoriatic lesions compared to non-lesional full-thickness epidermis from the same patient (Bochenska & Gabig-Ciminska, 2020.). Acne vulgaris is a common skin condition that start in puberty. Acne has always been associated with lipids. It is characterised by overproduction of sebum by sebaceous glands giving the formation of comedones and pus-filed spots (Clayton et al., 2019). Sebum composition is clearly different in adolescent patient with acne comapred to healthy skin. Diacyglycerol DAG were very abondant in juvenil with acne. Squaline and non-esterified fatty acid NEFAs were showing elevated levels in sebum skin affected by acne (Knox & O'Boyle, 2021).

Another skin disease is called Hereditary ichthyoses. It is a genetic skin condition that causes widespread and persistant scaling of the skin. Ichthyoses is also associated with altered lipids in the skin. Many genes are  involved in this disorder such as fatty acid transport protein 4 (FATP4), short-chain dehydrogenase/reductase family 9Cmember 7( SDR9C7) and others (Takeichi et al., 2019). Moreover, mutation of the triacylglyceride lipase activator coded by ABHD5 causes Autosomal recessive congenital ichthyoses (ARCI) (Uchida & Park, 2021).

Ceramides are an essential molecule modulating an epidermal permeability barrier in the stratum corneum. Heterogenous species of ceramide are required for generating a defence system in the outer most layer of the skin,whish serves as " the first line of defence and the last line for preserving life" (Uchida & Park, 2021). In addition to ceramides role in keratinocytes

proliferation and differentiation, they are involved in other cell fuctions like cell cycle arrest, cellular signaling, and appoptosis. Recent research reveals that ceramides are playing a detrimental role in the pathogenesis of several diseases including cardiovascular disease, type II diabetes and obesity(Shalaby et al., 2022). Therefore, it has become necessary to study in depth this small molecule that keeps fascinating the biochemistry community.

### 1.4 Crispr Cas-9: history and mechanism.

#### 1.4.1 Short history of Crispr- Cas9

In 1987, Yoshizumi Ishino and his team of researchers from the Osaka University in Japan first reported the presence of Clustred Regularly Interspaced Short Palindromic Repeats, abbreviated as CRISPR, in the Escherichia coli genome(Gostimskaya, 2022). These represent short, repeated sequences of DNA nucleotides found within the genome of prokaryotes. These sequences are the same when read from 5' to 3' on one strand of DNA, and from 3'to 5' in the complementary strand, and are therefore described as palindromic repeats. Later on, the functionality and importance of CRISPR was first realized in prokaryotes. Scientists found that the CRISPR system is a key part of the bacteria adaptive immunity, which protects these bacteria from attack by viral DNA. During a viral infection, bacteria take a small piece of the foreign viral DNA, and integrate it into the CRISPR locus to generate CRISPR array. These consist of duplicate sequences, which are the palindromic repeats flanked by variable sequences called spacers. These spacers were an exact match to DNA found in viruses, especialy viruses that infect bacteria. In this way, bacteria retain a memory of a past infection and be able to defend against it in the future.

### 1.4.2 Crisp- Cas 9 mechanism.

The CRISPR array will undergo transcription to form CRISPR RNA (crRNA), also called pre-crRNA. Then the protein Cas9 gets involved. Cas refers to CRISPR-associated nuclease protein, which is a nuclease capable of cleaving DNA at specific sites. Along with Cas9, there are also molecules of tracRNA. tracRNA have sections complementary to the palendromic repeats. For each spacer and palindromic repeat, we end up with a complex composed of the segment of pre-crRNA, a tracrRNA, and a Cas9 protein. Then another enzyme called ribonuclease three(RNaseIII) comes and cleaves the strand between these complexes leaving us with individual crRNA complexes. When the crRNA complex encounters a section of viral DNA having a sequence complementary to crRNA,the complexe will unwind and hybridyze with DNA. When the complex recognizes a short sequence unique to the viral genome called a protospacer adjacent motif (PAM), it will cut both strands of the DNA, just a few base pairs upstream from the PAM. This process will neutralize the virus. Its genome can no longer be transcribed properly to create more viral particles. This stops the infection. Scientists were inspired by this bacterial immune defence. They adapted its mechanism and used it as a genome-editing tool in various organisms, including humans. Scientists realize that in bacteria, the crRNA and tracrRNA are separate molecular entities. They assumed that these two molecules could be combined into a single molecule to generate a molecule that can be synthesized in the lab called single guide RNA or sgRNA(Asmamaw & Zawdie, 2021). If the sgRNA complexes with a Cas9 protein, this two-component system will be able to cleave DNA just as the three-component system does in bacteria. So when the complex forms, it will scan DNA until it finds the appropriate sequence along with PAM. binding will occur, and DNA will be cleaved at precisely the desired location in the two strands.

After the incision is made, the natural DNA repair can occur via two routes. Either by homology-directed repair, abbreviated as HDR, or by non-homologous end joining, abbreviated as NHEJ. The NHEJ pathway repairs double-strand breaks in DNA by directly ligating without the need for a homologous template, a DNA strand with similar sequence that can act as a template. The NHEJ mechanism can also introduce insertion or deletion of specific sequences at the joining ends. Thus, NHEJ produces DNA strands with non-uniformity in size.

While the NHEJ is more common in a eukaryotic domain, the homology-directed repair HDR process requires a large amount of donor (exogenous) DNA templates containing a sequence of interest(Asmamaw & Zawdie, 2021). Gene editing using the CRISPR-Cas9 system is an extremely efficient tool for generating mutations within the genomic DNA of cell lines. We used this approch to generate knockout of our protein of interest ORMDL in keratinocytes. The knockout of the regulatory subunit of SPT will reveal the role of this protein in sphingolipids biosynthesis in skin cells and therefore in the barrier permeability of the epidermis.

### 1.5 Thesis question and hypothesis

ORMDL proteins are known to play a crucial role in the regulation of sphingolipid metabolism. ORMDLs are involved in the homeostasis of sphingolipids by regulating the activity of serine palmitoyltransferase (SPT), a key enzyme in sphingolipid. The precise functions of ORMDLs and the regulation of sphingolipid biosynthesis may vary in different cell types and tissues. The purpose of this thesis is primarily to investigate the role of ORMDLs in keratinocytes, being the predominant cell type in the epidermis and involved in the formation of the skin barrier. To address the above question, we have generated ORMDLs knock-out in keratinocytes: ORMDL single knock-out, ORMDL double knock-out, and ORMDL triple knock-out. Then, we have examined the activity of SPT in these ORMDLs knockout cell lines. We hypothesize that ORMDLs knockout will increase the impermeability of the barrier and improve skin health by synthesizing more sphingolipids, including ceramides, essential for the formation of intercellular lipid lamellae of the stratum corneum of the epidermis.

## Chapter 2 Experimental Methods

### 1. Cell culture conditions:

Cell culture conditions for keratinocytes:

- To passage/ split:

Keratinocytes media was removed from a 10 cm dish. Cells were washed with 10 ml of 1X-PBS. PBS was aspirated, then 4 ml of 0.25% trypsin was added to cells. Incubated in the $CO_2$ incubator for 10 mins with gentle agitation every 5 mins. Cells were taken out from the incubator. 5 ml of DMEM was added to quench trypsin. Cells were moved to a 15 ml Falcon tube, then spun at 500 rpm for 5 mins. The supernatant was removed. Cells were resuspended in 10 ml of keratinocytes complete media, split in two 10 cm dishes containing 5 ml of keratinocytes complete media. 5 ml of cells were added to each dish. The media was changed every two days.

- To freeze:

After trypsinizing and spinning cells, the supernatant was removed. Cells were resuspended in 1 ml of serum-Free cell Freezing medium (BAMBANKER #302-14681(CS-02-001)) aliquoted into labelled cryovial, then stored at -80°C and transferred to liquid nitrogen after two days.

- To revive from frozen:

Cryovial was thawed at room temperature. As soon as fully thawed, dropwise was added to 5 ml of pre-warmed Keratinocytes complete media contained in a 15 ml Falcon tube. The tube was centrifuged at 500 rpm for 5 minutes. The supernatant was removed. Cells were resuspended in 10 ml of Keratinocytes complete media, then plated onto a 10 cm dish. The media was changed the next day.

Cell culture conditions for HEK 293T:

- To passage/split

DMEM media was removed from a 10cm dish. Cells were washed with 10 ml of 1X-PBS. PBS was aspirated, then 1ml of 0.025% trypsin was added to cells. Incubated in the $CO_2$ incubator for 1 mins. Then, cells were taken out from the incubator. Trypsin was aspirated. HEK 293T Cells were resuspended in 10 ml of DMEM complete media and were split in two 10 cm dishes containing 5ml of complete media. 5 ml of cells were added to each dish. Dishes were incubated in the $CO_2$ incubator, and the media was changed after two days.

- Freeze of HEK 293T.

HEK 293T cells were trypsinized and spun. The supernatant was removed. Cells were resuspended in1ml of DMSO freeze media, aliquoted into labelled cryovial, stored at -80°C and transferred to liquid nitrogen after two days.

- Revive HEK293T from frozen.

200ul of DMEM complete media was taken from a Falcon tube containing 5ml of complete media, then added to the cryovial and resuspended with the pipette. Then, 200ul of cells was taken from the cryovial and added to the Falcon. This step was repeated 5 to 6 times. Then, the remaining cryovial cells were added to the Falcon tube, spun at 12000 rpm for 5 min. The supernatant was removed. Cells were resuspended in 10 ml of DMEM complete media, plated onto a 10cm dish. The media was changed the next day.

**2- DNA extraction, elution, purification, and sequencing.**

**DNA extraction :**

DNA was extracted from a 6 well plate of keratinocytes. First, media was removed. Cells were washed with 2 ml of PBS. Then, 100ul of 0.25% trypsin was added, and the plate was incubated for 10 minutes at room temperature. 900ul of non-sterile DMEM complete media was added to collected cells. Then cells were moved to 1.5 ml Eppendorf tubes and spun at 5000 rpm for 5 minutes. Media was discarded and 1ml of PBS was added to cells. Then tubes were spun again at 500 rpm for 5 minutes. PBS was removed and 25ul of quick extract DNA (Biosearch Technologies # SS000035-D1) was added to pellets. Tubes contents were mixed by vortexing for 15 seconds, incubated at 65°C for 10 minutes, then vortexed for 15 seconds, and incubated at 98°C for 5 minutes. 25ul of water Milli-Q was added to each tube, vortexed for 15 seconds, then spun at 2000 rpm for 5 minutes at room temperature. DNA was stored at -80°C.

**Agarose gel preparation**:

A 100 ml 1X-TAE was added to a 500ml Erlenmeyer flask. 1g agarose (Bioexpress #C455235) was added to the flask. The flask was microwaved in 15 second intervals and swirled until the solution was completely cleared. When the solution was cooled (bearable on skin).10ul Ethidium bromide (Sigma # 5302-642366)was added and swirled till clear.

The ends of gel container were taped, the comb was placed, and the solution was added to the container. The gel was left to solidify. After gel had set. Tape was removed and the gel container was placed into large apparatus where 1X-TAE was completely covering gel. 20ul of 1kb plus ladder (Invitrogen # 01294818)was added to first lane of gel. 5ul of purple (Invitrogen #10816015) loading dye was added to each sample. Mixed with pipet. All the reaction mix in

PCR tubes were added to respective wells. Gel was running at 120V until samples were 2/3 across gel.

**DNA purification:**

After elution, DNA bands were cut and placed in Eppendorf tubes. Capture buffer (Cytiva # 17511371) was added to each tube. Then, tubes were placed into thermomixer at 60°C for 30 mins. 500ul of DNA containing tubes were added to DNA elution tubes, then, centrifuged for 13000 rpm for 1 min. This step was repeated twice. DNAs were washed with 500ul of buffer wash type 1(Cytiva # 17511371) to remove any impurity. DNA elution tubes were centrifuged for 1 min at 5000 rpm. This step was repeated twice. DNA columns were placed in new labeled Eppendorf tubes. 20ul of elution buffer type 6(Cytiva # 17511371) was pipetted in the middle of the columns, Incubated at room temperature for 5 mins. Then Centrifuged at 13000 rpm for 2 mins. DNAs were eluted in the Eppendorf tubes then taken for quantification.

**DNA sequencing:**

DNAs were first amplified and eluted before sending for sequencing. PCR reactions were prepared in PCR tubes as follows: 15.25ul of nuclease free water, was added to 5ul 5X fusion reaction buffer B0518S (BioLabs #10081385), 0.5ul 10mM Deoxynucleotides (dNTP) solution N0447S (BioLabs #0941705), 1ul forward primer (IDT #462016438), 1ul reverse primer(IDT #462016439), 100 ng DNA, 0.75ul 100% DMSO B0515A (BioLabs #10081387), and 0.5ul fusion DNA polymerase M0530S (BioLabs #10058480).

The PCR tubes were running in the PCR machine using the following program:

| | |
|---|---|
| 98°C | 3 mins |
| 98°C | 10 sec |
| 60°C | 10 sec |

| | |
|---|---|
| 72°C | 20 sec |
| 72°C | 5 min |
| GOTO 2 | 35x. |
| 4°C | ∞ |

DNAs were eluted in agarose gel (see protocol above). Bands were cut, purified (see protocol above). DNAs were quantified using Nanodrop software, then DNA sequencing tubes were prepared and sent for sequencing.

**3- Protein extraction:**

keratinocytes were cultured in a 6-well plate. On the bench, the media was removed from the plate and cells were washed with 1ml of 1X-PBS. PBS was aspirated and 150ul of 0.25% trypsin was added. The plate was incubated at room temperature for 10 mins. 1 ml of DMEM complete media was added to quench trypsin, then cells were collected in 1.5 ml Eppendorf tubes. Cells were centrifuged at 5000 rpm for 5 mins at 4°C. The media was removed, and cells were washed with 1 ml of 1X-PBS. Then, keratinocytes were centrifuged at 5000 rpm.4°C for 5 min to remove PBS. Cell pellets were kept on ice. The pellets were resuspended in 150ul of lysis buffer and incubated for 5 mins on ice. The resuspended cells were passed through 26g needles for 30 times. Then centrifuged at 700 rpm for 5 mins at 4°C. protein lysate was collected from the top to avoid collecting DNA. Proteins were stored at -20°C, and Bradford was performed prior running western blot.

- Lysis buffer preparation:

For 1 ml of lysis buffer, the following components were mixed:

- 938ul resuspension buffer
- 40ul of 25X protease inhibitor
- 20ul triton 10X
- 2ul of 1M DTT

- Protease inhibitor preparation:

1 tablet of protease inhibitor (Thermo #A32965) was diluted in 2 ml Mill-Q water. Vortexed until the whole tablet was dissolved.

**4- RNA extraction, cDNA preparation, and qPCR conditions.**

**RNA isolation:**

Keratinocytes were lifted by 0.25% trypsin, quenched with DMEM, pelleted and washed with 1X-PBS. 1ml of Trizol (Ambion #250408) solution was added to cell pellets and mixed until homogeneous. The Trizol/cell mix was sat for 2 minutes then moved to Eppendorf where they sat for 8 minutes at room temperature. Meanwhile, the 2 ml phase lock gel (5 PRIME #2302830) was centrifuged at 10000 rpm, 4°C for 2 minutes. 200ul of chloroform/isoamyl alcohol (Sigma #102487059) (49:1, v/v) was added to the sample, vortexed for 15-30 seconds, then incubated for 3 minutes at room temperature. Phases were separated by centrifuging at 13000 rpm for 10 minutes at 4°C. The upper (clear aqueous) phase was transferred to a sterile 1.5 ml microfuge tube. Then 500ul isopropanol was added to the tube and vortexed lightly. Then, 150ul of Na-acetate (Fisher # 6131-90- 4) and 3ul of Glyco-bleu were added to the tube and refrigerated at -20°C for 15minutes. RNA pellet was obtained by spin at full speed (13000 rpm) for 20 minutes. The supernatant was discarded, and the RNA pellet was washed with 500ul of ice-cold 70% ethanol. The pellet was centrifuged for 3 minutes to resettle the pellet then ethanol was carefully removed. This process was repeated for 3 full washes with ethanol. After the last ethanol wash was removed, a dry spin of 2 minutes was done to remove excess ethanol from the sides of the tube. The RNA pellet was placed in the hood to dry for 10 minutes. DEPEC nuclease free water was pre-warmed at 55°C in the thermomixer heater. The pellet was resuspended in 25ul of DEPEC water. RNA was stored at -20°C.

**cDNA preparation:**

RNA was diluted to obtain a volume of 10ul. cDNA reaction was prepared as follows:

| Component | Volume |
|---|---|
| 10X PCR buffer (Applied Biosystem#2644800) | 2ul |
| 10X dNTP (Applied Biosystem#2633820) | 0.8ul |
| 10X RT random primers(Applied Biosystem#2651246) | 2ul |
| Multiscrib RT ase (Applied Biosystem#2648047 | 1ul |
| RNase inhibitor (Applied Biosystem#2635324) | 1ul |
| DEPEC $H_2O$ | 3.2ul |
| Total volume | 10ul |

**Table1: components and volume for cDNA preparation**

cDNA components were mixed with RNA, then PCR machine run using the following program:

| | |
|---|---|
| T= 25°C | 10 mins |
| T= 37°C | 2 hours |
| T= 85°C | 5 mins |
| 4°C | ∞ |

**Real- time PCR:**

Primers used are: ORMDL1. ORMDL2, ORMDL3, and HPRT.

QPCR program:

| | |
|---|---|
| T= 95°C | 3mins |
| T= 95°C | 10 sec |
| T= 55°C | 30 sec |
| GOTO 2 | 55X |
| End | |

**5-Lenti-CRISPR Guide Puro Construct.**

**Synthesize of oligos:** oligos were synthesized in the following form:

1st 5'- CACCGNNNNNNNNNNNNNNNNNNN-3'
2nd 5'- AAAC (reverse complement of 20 Ns) C -3'

Upon arrival, each oligo was resuspended in nuclease free water to make 100uM (final concentration).

**Digestion and dephosphorylation:** CrisprV2 was purchased from Add gene (14.873 bp, #52961). CrispV2 were digested and phosphorylated by taking 1ug of the plasmid DNA, adding 1ul of the restriction enzyme BsmBI (NEB #R058), 0.2ul DTT (100mM), 1ug CIP (NEB #M0290), 2ul NEB buffer 3, in addition to nuclease free water to make up to 20ul. The reaction was incubated at 37°C for 1hour.

**Gel purification and elution of (CrisprV2 plasmid):** The digested product was run on 1% agarose gel. Digested product was shown about 2Kb band. Only high molecular weight band was cut and the 2Kb band was ignored. Bands were Purified (see above DNA purification) and stored at -20°C.

**Phosphorylation and annealing of gRNA oligos:** the phosphorylation reaction was prepared by the addition of 1ul of 1st oligo (100uM), 1ul of 2nd oligo (100uM), 0.5ul T4PNK (NEB #M0201), 1ul of T4 buffer (NEB #B0202). 6.5ul of Nuclease free water was added to make 10ul total volume.

PCR was running for annealing oligos in Nuclease free water.

37°C 30 min
95°C 5min and then was ramped down to 25°C at 5°C/min.

After annealing, oligos were diluted in nuclease free water (1:200)

**Ligation of digested Crispr V2 Lenti plasmid and annealed oligos:** ligation reaction consists of inserting the diluted oligos into the plasmid. 50ng of the digested V2 plasmid was added to 1ul of diluted oligos, 5ul ligation buffer (NEB # B2200), 1ul quick ligase, (NEB# M2200) and nuclease free water to make up to 10ul total volume. The ligation reaction was incubated overnight at 8°C.

**Transformation:** competent cells were thawed on ice. The ligation reaction was chilled in a 1.5 ml microcentrifuge tube. 25ul of competent cells (Invitrogen one shot Stbl # 2612941A) were added to DNA (ligation reaction). Cells and DNA were mixed gently by pipetting up and down or flicking the tube 4-5 times. The mixture was placed on ice for 30 minutes. Without mix, the tube was placed in a thermomixer heater for a heat shock at 42°C for 45 seconds, then placed on ice for 5min. 950ul of room temperature media was added to the tube. Then, cells and DNA tube was placed in the thermomixer heater at 37°C for 60 minutes. Shaked vigorously (250 rpm). The tube was centrifuged for 2 minutes at 13000 rpm. The supernatant was removed keeping 50-100ul in the tube to resuspend cells. Agar petri dish with ampicillin antibiotic was warmed at room temperature. Cells and ligation mixture were spread onto the plate. The plate was incubated overnight at 37°C.

**6- Plasmid Maxi prep protocol:**

**Primary culture**: 4 ml of LB broth media containing 4ul of Carb antibiotic and bacterial clone was inoculated and incubated at 37°C, overnight at 175rpm.

**Secondary culture**: in a 400 ml flask, the primary culture was added to 400 ml of LB broth media (Fisher #221648) containing 400ul of Carb antibiotic(Gemini #400-105P). The flask was incubated overnight at 37°C, and 175rpm.

The following day, the secondary culture was moved to a 250 ml Beckman capped bottle, and spun at 6000 g for 20 mins at 4°C. This step was repeated twice to spin all the media. The supernatant was discarded, and bacteria pellet was resuspended in 10 ml of buffer 1 from the QIAGEN Maxi kit (QIAGEN #172041507) . Then, 10 ml of buffer 2 was added and mixed thoroughly by vigorous mixing. The bottle was incubated at room temperature for 5 mins. 10 ml of pre-chilled buffer 3 was added and the mixture was incubated on ice for 10 mins. The Beckman capped bottle was centrifuged at 15000g for 30 mins at 4°C.

Meanwhile, QIAGEN tip was equilibrated with 10 ml QBT buffer and column was allowed to empty by gravity flow. The supernatant was applied to the equilibrated tip and column was allowed to empty by gravity flow. Then QIAGEN was washed with 30 ml buffer QC two times and column was allowed to empty by gravity flow. DNA was eluted in a 50 ml Falcon using 15 ml buffer QF (pre-warm) by gravity

flow. DNA was precipitated by adding 10.5 ml room temperature isopropanol to the eluted DNA. Mixed well. (at this point DNA can be stored). The 50 ml Falcon was then centrifuged at 15000 g for 30 mins at 4°C. The supernatant was discarded carefully. Pellet was resuspended in 500ul of 70% ethanol and moved to 1.5 ml Eppendorf, centrifuged at 13000 rpm for 30 mins at room temperature. DNA pellet was washed twice with 70% ethanol and centrifuged at 13000

rpm for 5 mins at room temperature. Pellet was placed in the hood to dry for 10 mins. DNA was redissolved in 200ul of Mill-Q and was stored at -20°C.

### 7- Western blots conditions

### SDS- PAGE gel preparation:

Three gel concentrations were prepared in separate Falcons. 15%, 12%, and 10%.

| | 15% | 12% | 10% |
|---|---|---|---|
| **Mill-Q** | 5.8 ml | 7.8 ml | 10 ml |
| **1.9M Tris (KD medical # RGF-3340)** | 4 ml | 4 ml | 4 ml |
| **Bis/Acrylamide (Fisher #218816)** | 10 ml | 8 ml | 6 ml |
| **10% SDS** | 200ul | 200ul | 200ul |
| **APS (TCI America # 7727-54-0)** | 150ul | 150ul | 150ul |
| **TEMED (Thermo # XB343505)** | 15ul | 15ul | 15ul |

**Table 2: Gel concentrations used in Western blots electrophoresis, 15%, 12%, and 10%.**

Two glass plates were clamped into the casting frames and placed on the casting supports. 4 ml of the 15% was poured into the glass plate assemblies. Then, 2 ml of 12% gel concentration was carefully added into the glass plate. The 10% gel concentration was added right on the top on the 12% solution. 1ml of butanol was added over the gel to make the top of the gel be horizontal. The gel was left 30 mins for solidification. After 30 mins, butanol was removed from the casting plate by inverting. Then, the stacking gel was prepared:

| | Stacking gel |
|---|---|
| **Mill-Q** | 5.8 ml |
| **0.5M Tris** | 2.5 ml |
| **Bis/Acrylamide** | 1.67 ml |
| **10% SDS** | 100ul |
| **APS** | 100ul |
| **TEMED** | 10ul |

**Table 3: stacking gel concentration used in western blots electrophoresis.**

The solution was stirred gently but carefully. The stacking gel was Pipetted until an overflow. The gel comb was carefully inserted and adjusted above the casting plate. The plate was incubated for 45 mins for stacking gel to polymerase. After complete gelation, the gel can be used for western blot or stored at 4°C.

**Conditions for running western blot:**

The gel was secured into the electrode assembly and placed inside the tank. The position of the comb side of the gel was toward the assembly. The comb was removed. The inner chamber was filled with running buffer, then the outer buffer chamber was filled with buffer until covering the gel. 2.5ul of ladder was loaded in the first well. 5ul of samples were carefully loaded in the wells. Leads were connected and the run began at 50V for 30 min, then at 110V for 1h20mins.

PVDF membrane was soaked in methanol for 5mins, then soaked in the transfer buffer for another 5 mins. The blotting sandwich was prepared by placing the gel holder with the white side down soaked in transfer buffer. A pre-wet sponge was placed onto the white plastic, followed by a pre-wet sheet of blotting paper. The pre-soaked membrane was placed on the sheet, then the gel was carefully placed onto the membrane to avoid trapping excessive air bubbles. A second sheet of blotting paper was placed on the top of the gel, then the final sponge on the top. The gel holder was closed and inserted into the inner module so that the black side of the holder faced the black side of the module into the transfer chamber. A frozen cooling unit was added, and the chamber was filled with transfer buffer. The lead was attached and connected to power supply. Red to red and black to black. The power supply was placed in a packed of ice and was set at 250 mA for 2h. After 2h of transfer. The membrane was cut right at 70KDa to get the loading control membrane section, and at 35KDa to separate SPTLC and ORMDL membrane sections. The

loading control and SPTLC membranes were blocked in a 10 ml of 5% milk. ORMDL membrane was blocked in a 10 ml of 5% Free Fat BSA. The blots were incubated at room temperature for 2h.

After 2h, the blocking solutions were removed. Then 10 ml of primary anti-bodies was applied to the membrane, then took to the cold room for overnight incubation. The next day, primary anti bodies were removed to be used later. Membrane sections were washed three times with TBS-T for 10 mins, then the secondary antibody was applied to the membrane. The membrane was again incubated overnight in the cold room. The following day, blots were washed with TBS-T three times for 10 mins, then washed with water for 15 mins. At this point, the blots were ready to be developed.

**Primary antibodies dilutions:**

- Vinculin (Protein tech #66305-1-Ig)110 KDa (anti-mouse): dilution 1:10000 in 5% biorad milk in TBST
- Calnexin(Enzo # ADISPA-860-F) 98KDa (anti-rabbit): dilution 1:3000 in 5% biorad milk in TBST
- SPTLC1, SPTLC2, SPTLC3 (Protein tech #66305-1-Ig), (cloud -clone- corp# A20230831945) 52KDa (anti-rabbit): dilution 1:3000 in 5% biorad milk in TBST
- ORMDL(Sigma # 4028594) 17KDa (anti-rabbit): dilution 1:3000 in 3% fat- free BSA

**secondary antibodies dilutions:**

Anti-mouse ( for vinculin): dilution 1:10000 in 5% biorad milk in TBST

Anti-rabbit ( for calnexin, SPTLC1, SPTLC2): dilution 1:10000 in 5% biorad milk in TBST

Anti-rabbit ( for SPTLC3): dilution 1:3000 in 5% biorad milk in TBST

Anti-rabbit ( for ORMDL) : dilution 1:10000 in 3% fat-free BSA

**8- Virus particles construct and keratinocytes infection.**

**Virus preparation :**

HEK 293T cells were seeded in p100 tissue culture dish to be 90% confluent on the transfection day ($3x10^6$ cells in 7 ml P/S free media). On day 2, transfection mix was prepared by adding 90ul PEI to 1.5ml of pre-warmed Opti-mem. Mixed well by flicking tube. 9ug of target plasmid and 3ug of each of the packaging plasmids (gag/pol, Rev, Env plasmid, gag encodes structural proteins, pol encodes enzymes required for reverse transcription and integration into the host cell genome, and env encodes the viral envelope glycoprotein) were added to 1.5ml pre-warmed Opti-mem. Mixed well by flicking tube. After 5mins, DNA containing tube was added to the transfection mix. Incubated 20mins at room temperature. Then drop wise of the mixture was added to the p100 dish. The dish was incubated in $CO_2$ incubator for 24h. On day 3, the transfection mix was removed, and 10ml of P/S free media was added. On day 4, the media was collected and replaced with 10ml P/S free media. On day 5, the media was collected and added to previously collected media. The lentivirus media was filtered through 0.45uM filter. The lentivirus media can be stored at -80°C until needed.

**Keratinocytes viral infection:**

Keratinocytes were seeded for infection in a 6-well plates at $1x10^5$ cells per well. The next day, Cells were 50-70% confluent. The old growth media was removed from keratinocytes. 1ml of lentivirus media was added. Cells were incubated at $CO_2$ incubator overnight. The next day, the lentivirus media was removed and replaced by keratinocytes fresh media and incubated overnight. The day after, the media was removed, cells were washed with 2ml of 1X-PBS. Then puromycin (2 ug/ml of media) antibiotic selection was added to cells. The selection media was

changed every 48h. cells were treated with puromycin Sigma # P4512-1MLX10)for three weeks. After three weeks, cells were moved to 25T flasks to grow.

**9- SPT assay:**

**Preparation of additive treatment media ( for 24 well plate, 500 ul was used for each well):**

For C8 (Avanti #860508): in 15 ml falcon, 5ul C8 from stock was added to 250ul BSA/PBS and mixed well. 10ml pre-warm opti-mem was added to this.

For Myriocin(caymanchem #63150): in 15 ml falcon, 5ul of myriocin was added to 5ml of pre-warm opti-mem.

**Preparation of labelling media ( for 24 well plate, 250ul was used for each well):**

35ul $^{3}$H- serine was added to 7ml MEM .

For C8: in 15 ml falcon, 1.3ul C8 from stock was added to 75ul BSA/PBS and mixed well. 2.5ml pre-warm labelling media containing $^{3}$H- serine was added to this.

For Myriocin: in 1.5 ml Eppendorf, 1uM myriocin (1ul myriocin from stock) was added to 1ml labelling media.

For no additive wells: labelling media was used as it is.

Keratinocytes was seeded on a 24 well plates with a density of $1x10^{5}$ cells. Media was removed from wells. Additive media was added to respective wells and complete media to non-treated wells. The plate was incubated for 1 hour at 37°C in CO2 incubator. The radioactive waste was aspirated in radioactive waste container. Cells were washed with 1XPBS. 200ul of 1xPBS was added to each well. 400ul alkaline methanol(0.7g KOH/100 methanol) was added to each well. Then 200ul chloroform was added and cells were scraped off and transferred to 2ml screw tubes. (at this point, samples can be stored at -20°C).

**Lipid extraction:**

Samples were centrifuged at 13000 rpm for 1min at room temperature . 300ul of freshly prepared alkaline water, 500ul chloroform, and 100ul 2N NH4OH were added to each tube. Tubes were vortexed vigorously for 1 min and spun down at 13000 rpm for 2min. The upper phase was aspirated with vacuum equipped with radioactive waste container. The organic phase was washed by adding 750ul alkaline water. Tubes were vortexed vigorously for 30 second and spun down at 13000 rpm for 2min. The upper phase was aspirated. The wash step was repeated, and the upper phase was aspirated. 1ml tip was pre-wet in chloroform and 450ul of organic phase was taken to scintillation vial. Scintillation vials were dry under liquid nitrogen using low heat setting for 5 min. 3-4ml scintillation fluid was added to each vial, capped and vortexed for few seconds. Each vial has been read for 1min on scintillation counter using flag 13'.

# Chapter 3 Results

## 3.1 Experimental identification of keratinocytes:

The observation of keratinocytes in cell culture showed that keratinocytes are growing more slowly compared to other cell lines such as Hela cells. Keratinocytes have a doubling time of approximately 60 hours (Liu et al., 1979). The slow rate is a characteristic of these cells. The beauty of keratinocytes lies not only in their aesthetic appearance under the microscope but also in their crucial role in skin biology. Due to the role of ORMDLs in regulating sphingolipids in the cells, we first started by comparing ORMDLs in keratinocytes and Hela cells. We used western blots by probing for ORMDL protein, SPTLC1, SPTLC2, and SPTLC3 which are subunits of serine palmitoyltransferase (SPT), the enzyme targeted by ORMDL . We used vinculin as the loading control to ensure that equal amounts of protein were loaded in each lane of Western blot. The expression of ORMDLs in keratinocytes appears to be similar to that in HeLa cells ( Fig.3.1). This suggests that, under the experimental conditions we investigated, the levels of ORMDLs are comparable between these two cell types.

For keratinocytes protein extraction, we used Urea sample buffer USB and the regular lysis buffer. Urea is a strong chaotropic agent that disrupts protein-protein interactions and helps solubilize proteins. It is often used in denaturing conditions for protein extraction and solubilization. We compared the two different lysis buffer to assess which one yields better signals in our Western blot analysis. The regular lysis buffer has proven to be effective in providing good signals for our Western blot experiments ( Fig 3.2). The choice of an effective lysis buffer was crucial for obtaining high-quality results in applications like Western blotting.

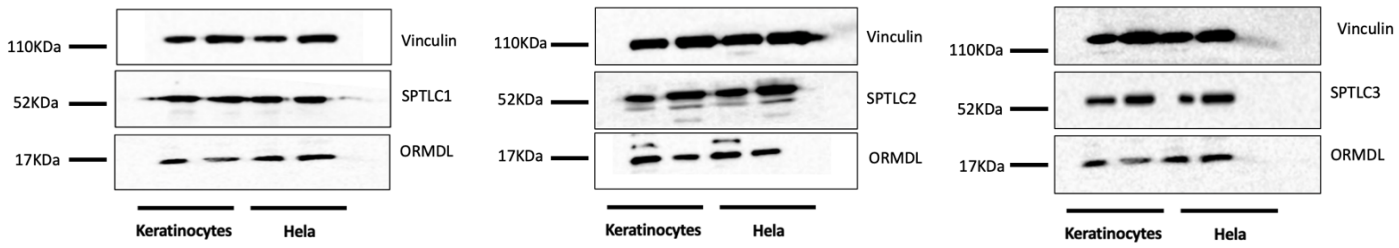



**Figure 3.1: Western blots of keratinocytes and Hela wild type**. We probed for ORMDL, SPTLC1, SPTLC2, SPTLC3. Vinculin is the loading control. Western blots showed similar expression at the ORMDL level. Primary anti-bodies used are anti- mouse for vinculin, SPTLC2 and SPTLC3. Anti-rabbit for SPTLC1 and ORMDL.

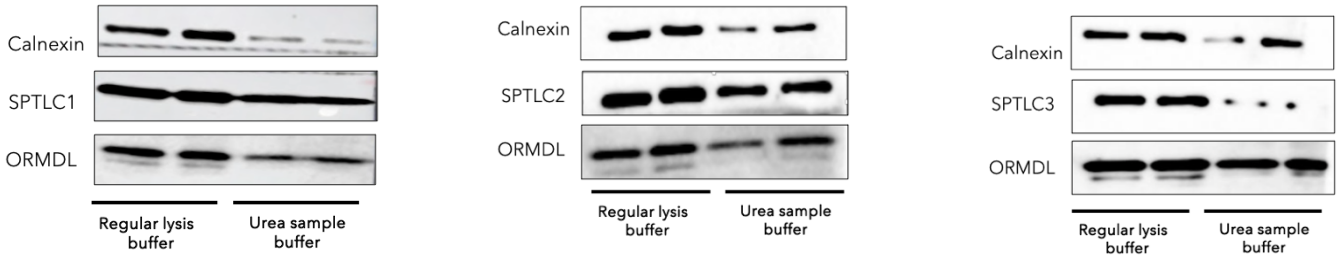



**Figure 3.2: Western blots of protein extraction buffer analysis.** Urea sample buffer showed a weak signal compared to the regular lysis buffer in keratinocytes protein extraction.

### 3.2 Construction of Lenti-CRISPR Guide Puromycin plasmid:

We purchased Crispr V2 Lenti plasmid from Addgene (14,873 bp. #52961) (Fig 3.3), and we wanted to insert the gRNA into that plasmid. All the gRNAs were designed by a previous Post Doc form our lab (Fig 3.4). To knockout ORMDL1, ORMDL2, and ORMDL3, we used three gRNAs for each ORMDL.

We digested and dephosphorylated three Crispr V2 plasmid, and then eluted in 1% agarose gel (Fig 3.5). Meanwhile, we phosphorylated the nine gRNAs using T4 polynucleotide Kinase, and annealed them in the PCR machine. The ligation of digested Crispr V2 Lenti plasmids and annealed oligos took place overnight at 8°C in the PCR machine. We used 200ng of digested plasmid in ligation reaction. The next day, the ligation reactions was transformed into bacterial competent cells. Cells were spread into Ampicillin antibiotic petri dishes, and incubated overnight at 37°C. The clones we had were valuable because of the challenges we faced during the cloning process. The number of these clones ranged between ten and twenty in each dish. Gentle handling during the transformation step was crucial for maintaining cell viability and increasing the chances of successful transformation. We used Carbenicillin antibiotics petri dishes to compare with Ampicillin dishes. We observed the same number of clones in Carbenicillin antibiotic plates. We therefore continued to do the cloning in Carbenicillin plates since Carbenicillin is known to be more stable than Ampicillin.

# CrisprV2 Lenti plasmid (Addgene #52961)

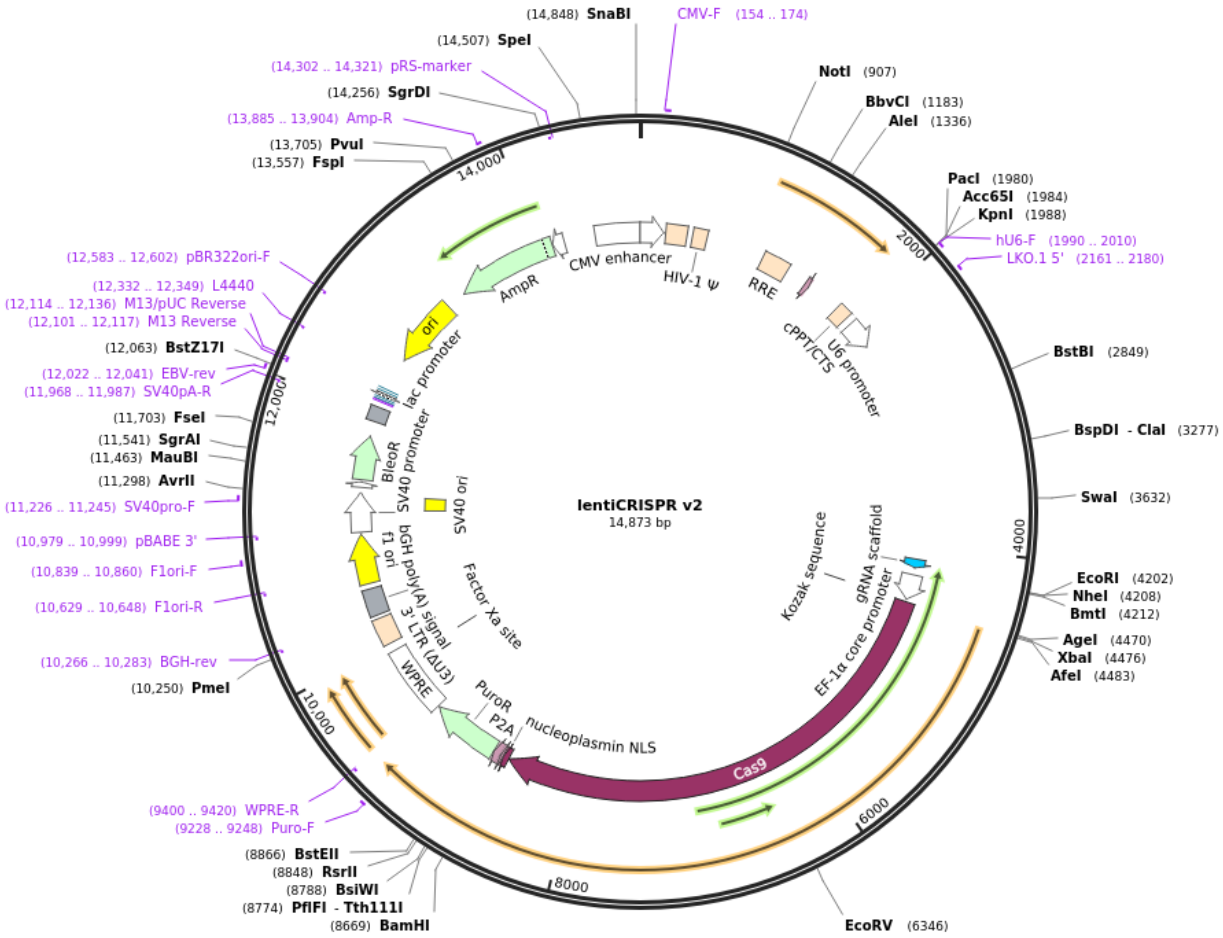



**Fig 3.3: Crispr V2 Lenti plasmid n the CRISPR-Cas9 system for lentiviral delivery (Addgene. 14,873 bp).** The plasmid contains Ampicillin resistance for bacteria cloning, Puromycin resistance to select infected cells, Cas9 nuclease, and gRNA location.

## gRNAs for ORMDL 1

| | |
|---|---|
| hORMDL1-g1-FOR | CACCGGGCCTGAGGGCGTGTATCCG |
| hORMDL1-g1-REV | AAACCGGATACACGCCCTCAGGCCC |
| hORMDL1-g2-FOR | CACCGGTATCCGCGGCCGTAGCAGC |
| hORMDL1- g2-REV | AAACGCTGCTACGGCCGCGGATACC |
| hORMDL1-g3-FOR | CACCGGGCCGCGGATACACGCCCTC |
| hORMDL1-g3-REV | AAACGAGGGCGTGTATCCGCGGCCC |

## gRNAs for ORMDL 2

| | |
|---|---|
| hORMDL2-g1-FOR | CACCGTCAGATCCCCGTCCGGCTAT |
| hORMDL2-g1-REV | AAACATAGCCGGACGGGGATCTGAC |
| hORMDL2-g2-FOR | CACCGCTCAGATCCCCGTCCGGCTA |
| hORMDL2-g2-REV | AAACTAGCCGGACGGGGATCTGAGC |
| hORMDL2-g3-FOR | CACCGACCCGAGTGATGAATAGCCG |
| hORMDL2-g3- REV | AAACCGGCTATTCATCACTCGGGTC |

## gRNAs for ORMDL 3

| | |
|---|---|
| hORMDL3-g1-FOR | CACCGTGTTCATCACCCGCGTGTTG |
| hORMDL3-g1-REV | AAACCAACACGCGGGTGATGAACAC |
| hORMDL3-g2-FOR | CACCGGCTGTTCATCACCCGCGTGT |
| hORMDL3-g2-REV | AAACACACGCGGGTGATGAACAGCC |
| hORMDL3-g3-FOR | CACCGCTGTTCATCACCCGCGTGTT |
| hORMDL3-g3-REV | AAACAACACGCGGGTGATGAACAGC |

**Fig 3.4: gRNAs designed for knocking out ORMDLs in keratinocytes.** gRNAs have 25 bp each. Each ORMDL has three gRNAs. Forward and reverse. We had 9gRNAs in total.

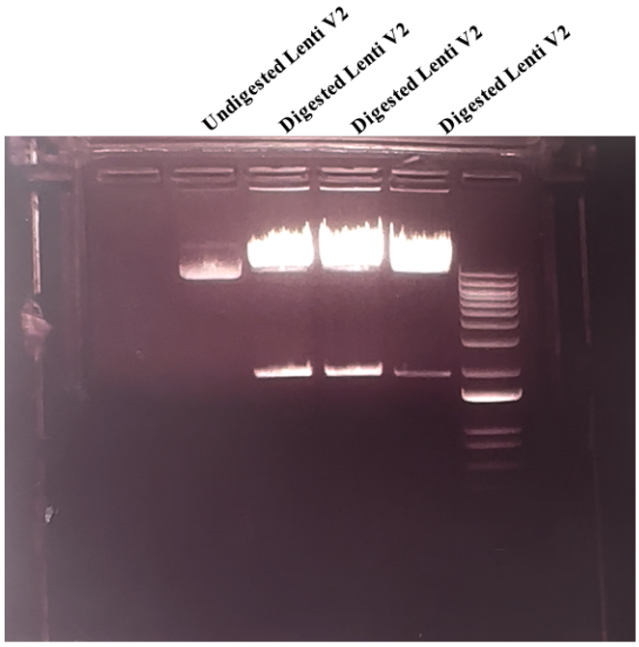
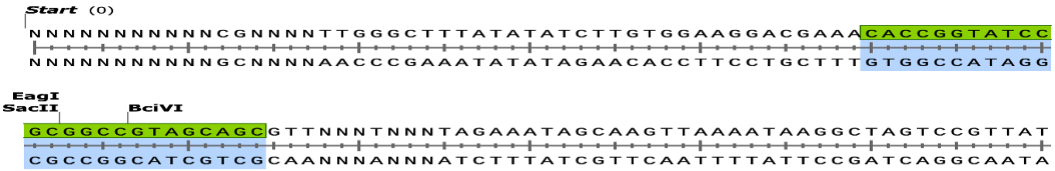



**Fig 3.5: Elution of three digested Lenti V2 plasmids on 1% agarose gel.** Digested products show ~ 2kb bands. The high molecular weight bands were cut. 2Kb bands were ignored. Undigested Lenti V2 was eluted for comparison.

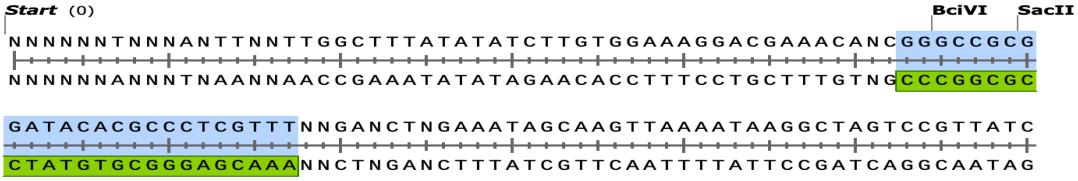
From the clones obtained, we created master plates by isolating individual clones from the cloning plates to ensure the purity and identity of each clone for further analysis. Then we picked clones from the master plates and performed mini-preps. After getting positive clones by DNA sequencing, we proceeded to maxi-preps in order to isolate larger quantities of plasmid DNA from bacterial clones. We send our plasmids again for sequencing to confirm the incorporation of gRNAs in the Lenti V2 plasmids. The DNA sequencing analysis revealed that The genetic constructs we intended to generate were successfully present in our plasmids We verified the gRNA incorporation by using Snap gene software (Fig 3.6).

## ORMDL1 gRNA1

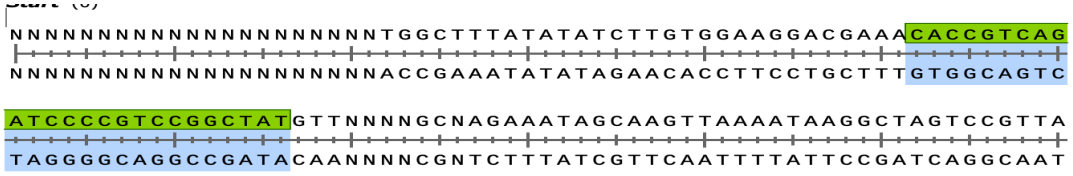

## ORMDL1 gRNA2



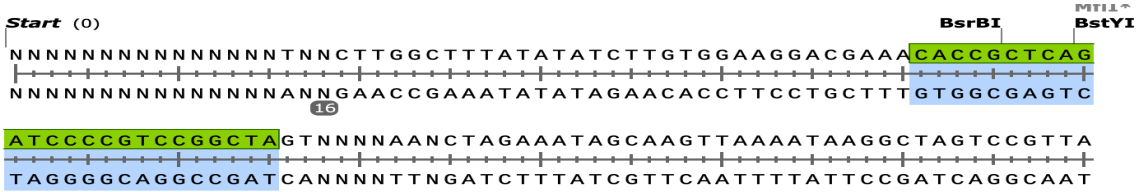

## ORMDL1 gRNA3




## ORMDL2 gRNA1

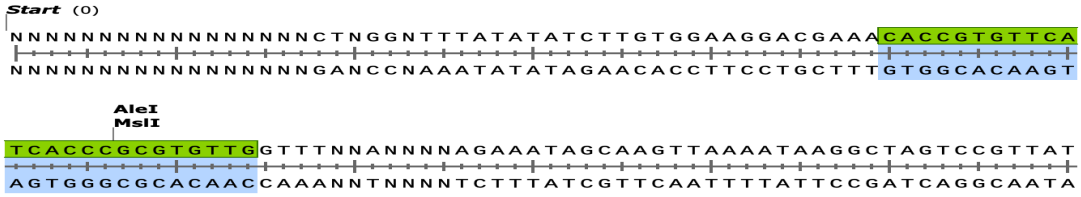


## ORMDL2 gRNA2

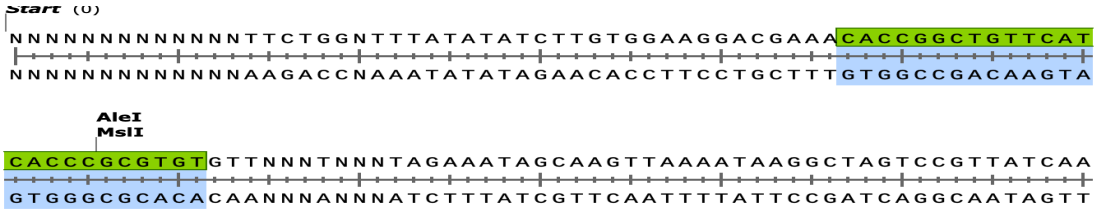


**ORMDL2 gRNA3**


**ORMDL3 gRNA1**




**ORMDL3 gRNA2**




**ORMDL3 gRNA3**

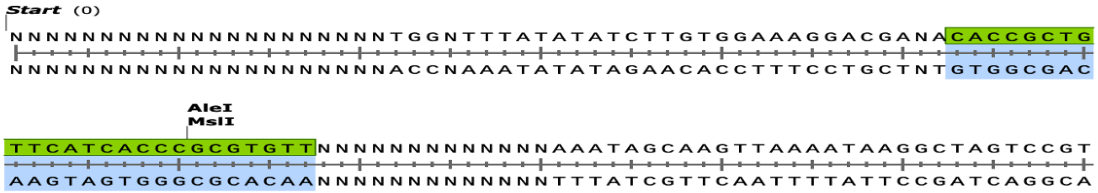



**Fig 3.6: Verification of the nine gRNAs insertion into the Lenti-V2 plasmids using SnapGene Viewer software for plasmid mapping**. The nine gRNAs were successfully incorporated into the plasmids. The plasmids will be used to prepare virus particles.

### 3.3 Lentiviral transduction and keratinocytes infection:

Lentiviral transduction was performed as described previously on chapter 2. We obtained 20 ml of viral supernatant that we aliquoted in 2ml Eppendorf tubes. Before infecting cells, we filtered the viral media and added 8ug polybrene per ml. Keratinocytes were cultured in 6 well plates with a density of 1x105 cells per well. We infected cells with viruses and allowed them to recover in fresh media. Then we started treatment with puromycin antibiotic. We used 2ug/ml concentration. During puromycin treatment, we encountered issues of contamination and sometimes cell death. We tried different antibiotic concentrations to determine the lowest effective dose that allows for positive selection without causing excessive cell death. We filtered puromycin to ensure that the puromycin solution is sterile before adding it to the cells. Contaminated puromycin solution can introduce microbial contaminants into the cell culture. We repeated the infection five to six times for infection to work. We think that the contamination likely occurred because the cells were stressed and were not in optimal conditions, or perhaps the keratinocytes were more sensitive to the cytotoxic effects of puromycin.

After about two weeks of treatment, we had 20 to 30% of cells left in the plates. We continued with the selection media till we observed cell clustered in the plates. Then, we moved the cells to 25T flasks and gave them enough time to grow. We observed that the new cell lines grow faster than wild type. The 25T flasks were about 80% confluent at the end of the week which is not very common in keratinocytes. The faster growth of the new cell lines compared to the wild type suggests that the absence of ORMDLs may be influencing cellular processes related to proliferation and growth. We think that changes in sphingolipid levels resulting from

ORMDL knockout may influence the progression of the cell cycle, leading to an increased rate of cell division.

We prepared two viruses that we called virus 1 and virus 2. The purpose of affecting cells with these two viruses is to test the efficiency of these viruses in the modification of the target gene. We run western blots for double and triple knockout. We observed a reduction of ORMDLs in both double and triple knockout cells infected with virus 1 and virus 2 cells ( Fig 3.7). The reduction in ORMDL levels indicates that the viruses (virus 1 and virus 2) are efficiently delivering the genetic material responsible for the knockout. This is a positive indication of the efficacy of the viral vectors in manipulating the cellular gene expression.

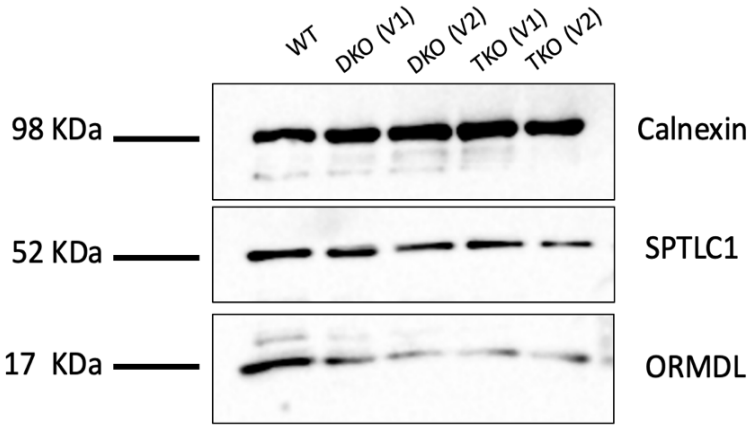



**Figure 3.7: Western blots for double and triple knockout cells infected with virus 1 and virus 2.** ORMDL expression was reduced in both viruses- infected cells. Wild type was loaded for comparison.

### 3.4 DNA cell lines sequencing:

#### 3.4.1 ORMDL1 gRNAs were found in the non-coding region:

ORMDL1 is situated on chromosome 2. ORMDL2 is located on chromosome 12, and ORMDL3 on chromosome 17. For each ORMDL we designed three gRNAs. These gRNAs were designed by a previous post. Doctorate from our lab. By miscommunication, instead of having the three gRNAs of ORMDL1 in the coding region, they were located in the 5'untranslated on the gene. Since the gRNAs of ORMDL1 are in the non-coding region of the gene, we focused in most of our experiments on ORMDL2 and ORMDL3.

#### 3.4.2 DNA sequencing of pools and clones:

We then proceeded by preparing DNAs for sequencing. We extracted DNAs from pools, amplify DNAs in PCR machine (see program on chapter 2), and run PCR products on agarose gel electrophoresis( Fig 3.9). The bands were then purified to remove impurities and sent for sequencing to Eurofins genomics, then we analyzed sequences using Crisp ID program. The outcomes were interesting. Sequence changes were observed in some gRNA cell lines like ORMDL1/3 KO but not in others ( Fig 3.10a). The variations in results could be attributed to several factors such as the efficiency of CRISPR-Cas9 editing which can vary between different gRNAs and target sites. Some gRNAs may lead to more efficient editing, resulting in detectable changes in the cell lines, while others may not induce significant alterations. We translated ORMDL1/3 double KO and we observed alteration in the sequences of the Amino acids.

We continued our analysis by isolating clones from the pools. We transferred 100 cells in a p100 dish. We selected this number of cells based on the potential cell death. We allowed clones

to seed for two weeks and then we moved them into 6-well plates to have only one clone per well. Clones were cultured for two weeks and then transferred to T25 flasks for an additional week. When we observed confluency in the flasks, we proceeded by extracting DNAs to start the sequencing process ( Fig3.10.(c, d)). While examining the region targeted by the guide RNA after sequencing, no mutations were observed. However, alterations were identified upstream of our guide RNAs. It's important to acknowledge that these observations may be influenced by sequencing reads and could potentially be artifacts. We think that we need to increase the number of sequenced clones to enhance the possibility of identifying positive clones.

a

ORMDL1

GGGCGTGTATCCGCGGCCGTAGCAGCCGGGCTGGTCCTGCTGCGAGCCGGCGGCCCGGAGTGGGGCGGCGGGTAAGT
GGGCACGAACGCGGTGGACGCTGTACAGTCGCCTTCGTCGGTAGGGAACTGCTGGCGGCCTAGGGGTCGTCCTGAGA
GGGAAAAGCGGGCTCCGGCTGCATCTGCGGCGCCCTGCGTGGCCGTAAGAGCCAAGTGGCCTCAGAGGCTCGCCCCC
TACGCAAATCCTCGTTGTCTTGCCAGGGTCCTGACGTGACGTGCGGCCGCGAGAAGGAAGGGGCGGGTTGGGGGAGG
CGCTGAGGTGGTAGCAGCGGCGGCTTTTGCTGAGTTTTCTCTGGCCCAGAGCGCTCGGGCTGTCGGTCTAGCAGGGG

b

ORMDL2

GTTACTTCCTGGAGACTTCAGGTGTGGTAGCCGGCGCCGCGCCCCATAGCCGGACGGGGATCTGAGCTGGC
AGGTAGGAGCTGCAAAGACTGTAAGGGTTGCTGAGGAAAAATGGTGGGAGGCGAAAGGGGCGCCGAGGGA
GTGGTGACCTAAATACTGAGAGGGGAGCGCTGAAGGGAGCCCTGAAAAAACCGATGAACATGAGGAGGAA
GCTGGGAGGGGCATGGGGCGGGCTTAAGGAACTGGCGGTGGGGCTGGGGCGGGCAAGAGCCGCTTCCTAA
CCAGACATCATCAGCTTTAGAGCTACCACAATTTTAAAGATCCGAGTTCAGATAATCACTGCTGCCCCTG

c

ORMDL3

ACTTCTCTGCCCTGGGGCTGCGTGCCTCTTGATGACCTGTCCTCCGATGCCCTGCCAGGGCCCTGTCCCAATGGGAT
GATGCATGGGGCAGCCTGTTGTTTATCCCAGCCCAGCAGCAGGAAATGAGTAGAGCTAAGTCCACATGGACTTTCTC
CACTTTCCATGCTTCAGAATCAAAAGAGTGGAACTGCCTGGAGTTCTCAGGGAAACAAGGGCCTGCCTCCCAGCGGG
ATCCGAGGGTGAGAGGGGGCAGGGAAAGCAGTGTGACCTGTCTGACCTGTGCTGCTTGTTTGCAGAACAGCAGAGAG
GGGCAGCAGGATGAATGTGGGCACAGCGCACAGCGAGGTGAACCCCAACACGCGGGTGATGAACAGCCGTGGCATCT
GGCTCTCCTACGTGCTGGCCATCGGTCTCCTCCACATCGTGCTGCTGAGCATCCCGTTTGTGAGTGTCCCTGTCGTC
TGGACCCTCACCAACCTCATTCACAACATGGTGAGAGCCTGTCTGCTGCCTGCCCCTGCCCCTGCCCCTGCCCTGTG

**Figure 3.8: gRNAs (25bp) regions in ORMDLs gene.** a) The three ORMDL1 gRNAs were located in the very beginning of the gene, the 5' untranslated region. b) ORMDL2 gRNAs were located in two different regions. Two primers were designed for ORMDL2. c) all the ORMDL3 gRNAs overlap in the same coding region.

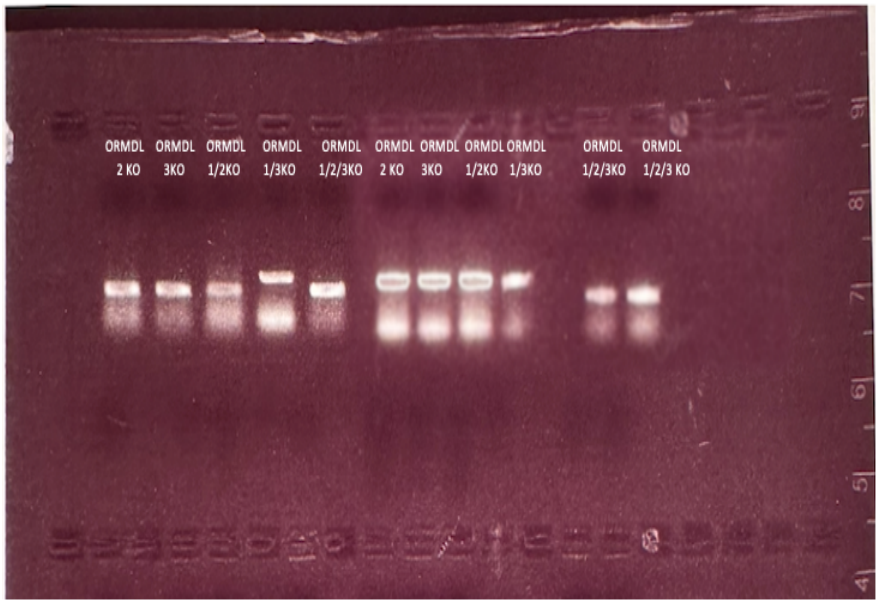
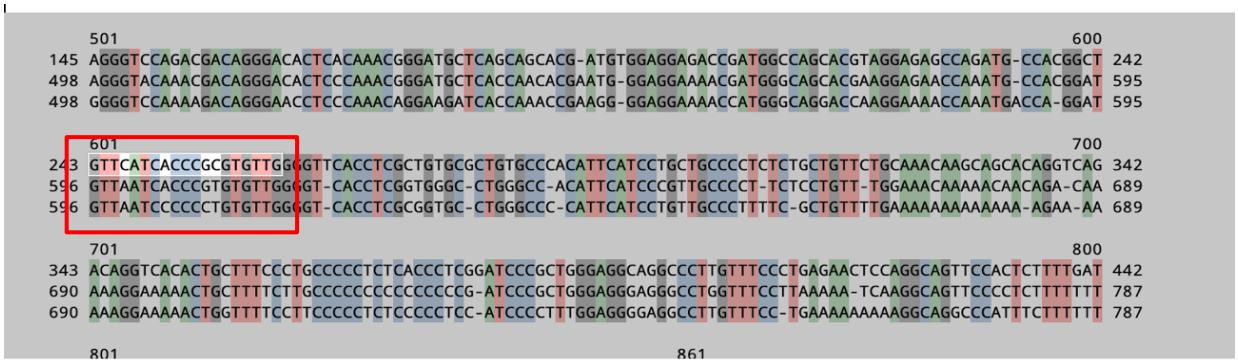



**Figure 3.9: PCR products running on agarose gel electrophoresis.** DNA bands represent ORMDL 2 and ORMDL3 single KO. ORMDL1/2 and ORMDL1/3 double KO. ORMDL1/2/3/ triple KO. ORMDL2/3 double KO was still puromycin treatment. DNA bands were cut, purified and send for sequencing.

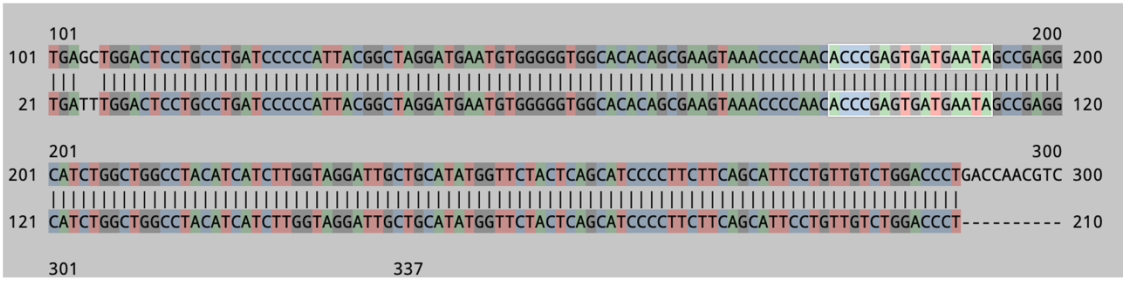
**a Pools : ORMDL1/3 KO ( ORMDL3 is the primer)**




**b**

```
MNVGTAHSEVNPNTRVMNSRGIWLSYVLAIGLLHIVLLSIPFVSVPVVWTL
MNV  AH   +PNTRV+N RGIW S+VL I  L  VL+SIPF SVPVV TL
MNVAQAHRG-DPNTRVINIRGIWFSFVLPIVFLPFVLVSIPFGSVPVVCTL
```

**c Clones : ORMDL 2KO**




**d. Clones : ORMDL1/2/3 KO**

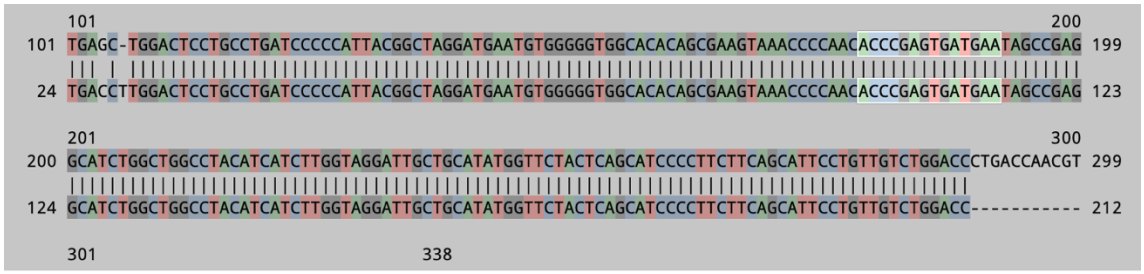



**Figure 3.10: ORMDL knock out sequencing of the pools.** Top line of sequencing is the reference sequence. Bottom lines are the sample sequencing.
a) ORMDL double knock-out 1/3 gRNAs from the pool. Couple of nucleotides were changed in the gRNA. ORMDL3 is the primer.
b) Translation of the sequenced ORMDL double knock out 1/3. Alteration in the amino acid sequencing..
c) ORMDL single knock-out 2 gRNA clone. No changes.
d) No alteration observed in the gRNA of ORMDL triple knock-out 1/2/3 clone.

### 3.5 ORMDL knockout cell lines analysis:

#### 3.5.1 Real time-PCR analysis:

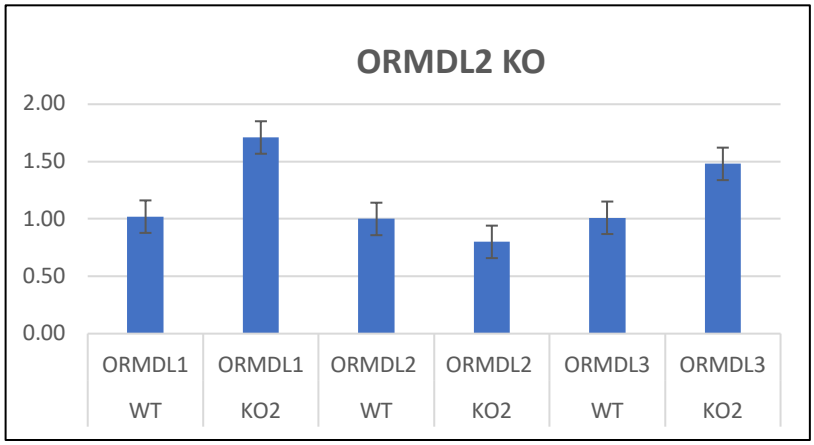
We wanted to run real time-PCR for our cell lines to assess the impact of ORMDL gene knockout on mRNA expression levels. First, we extracted RNAs from our cell lines. Then we prepared cDNA reaction and run PCR (see cDNA reaction and PCR program in chapter 2). We run 15ng cDNA reaction in Q-PCR. The primers we used are ORMDL1, ORMDL2, ORMDL3 for single knockout cell lines. In addition to these primers, we used SPTLC1, SPTLC2, SPTLC3, ssSPTa, and ssSPTb primers for double and triple KO.

Real time analysis showed that there was a decrease of ORMDL2 gene in ORMDL2 KO. This reduction of ORMDL2 was compensated by the increase of ORMDL1 and ORMDL3 (Fig 3.11). We believe that there is a possibility of functional redundancy among the ORMDL family members. If one family member is knocked out, others may compensate, leading to minimal changes at the mRNA level for the targeted gene (Baker et al., 2021). For ORMDL3 KO, we did not observe significant changes at the level of mRNA for that cell line.

We then continued with double, and triple knock out. In figure (3.12.a), there is a significant upregulation of ORMDL2 mRNA levels in the cells that just have one isoform of ORMDLs, but no changes were observed in the potential TKO cells. The possible explanation of this observation is that cells might activate compensatory mechanism when two isoforms are knocked out. The upregulation of ORMDL2 in the ORMDL1/3 knockout cell line could represent a compensatory response to maintain overall ORMDL expression. In the figure (fig 3.12.b) we observed an upregulation of ORMDL3 in the double knockout ORMDL1/3KO. We think that the cell senses the lack of ORMDL3 and produced more of ORMDL3 in that cell line.

We did not see any changes in the ORMDL1/2 KO, but we did see changes in the potential TKO. We know that at least ORMDL1 was not knocked out since their gRNAs are in the non-coding region. So, here also the cell compensates the absence of the other isoforms and that's why we see changes in the TKO.

Since ORMDL is the regulatory subunit of SPT, we wanted to examine the effect of ORMDL knockout on SPT at the mRNA level. In figure (3.12.c), and as compared to wild type, there were no significant changes in ORMDL1/3 KO and ORMDL1/2 KO. However, there were changes in the triple knockout. We believe that in TKO cells, where the three isoforms are potentially eliminated, there was compensatory effect from the remaining isoform. This explanation may apply to figure (3.12.e) where SPTLC3 is upregulated in the TKO as well. These changes can be examined by using western blots to see if there are any changes at protein level. As for SPTLC2, there is no changes in the three cell lines. in ssSPT figures, we can see upregulation of ssSPTa and ssSPTb in both isoforms ORMDL2 and ORMDL3. Unfortunately, there is no antibody available to determine if there are any changes at protein level.



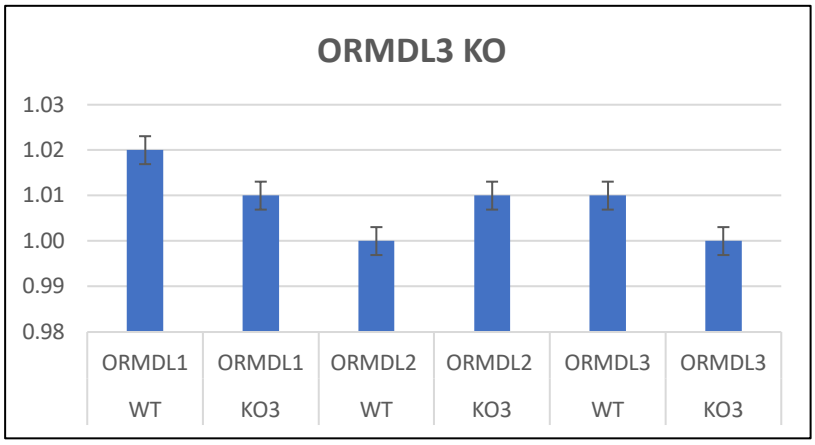




**Figure 3.11: q-PCR analysis for single knockout cell lines.** The decrease of ORMDL2 in ORMDL2 KO was compensated by an increase of ORMDL1 and ORMDL3 isoforms. No changes were observed in ORMDL3 at mRNA level in the single knockout cell lines.

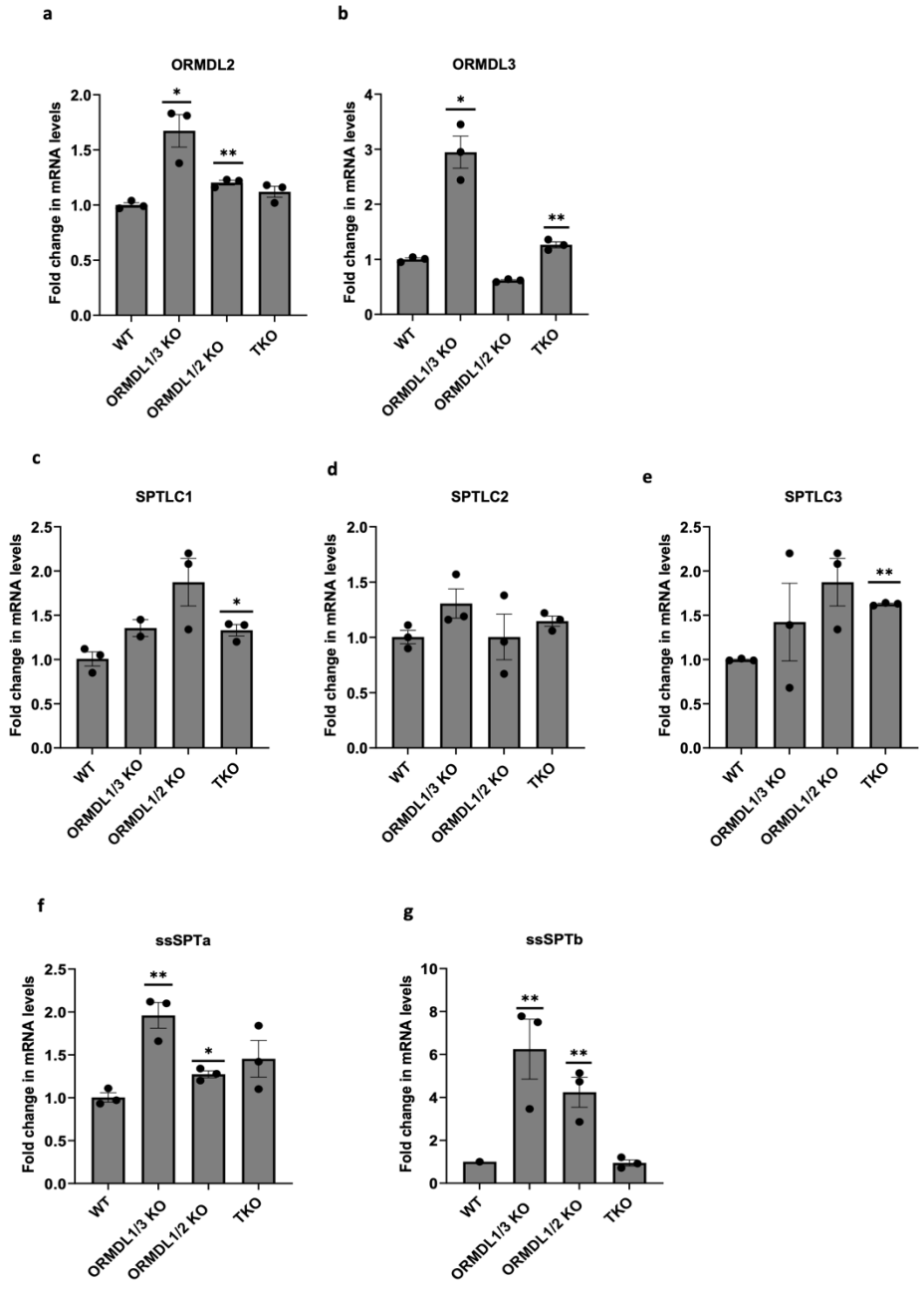



**Figure 3.12: real time analysis done on double knockout ORMDL (1/3), ORMDL ( 1/2 ) and triple knockout (1/2/3).**

a) Compensatory response of ORMDL2 in both double knockout to maintain overall ORMDL expression.
b) Compensatory mechanism of ORMDL3 in the double KO ORMDL1/3 KO and the potential TKO.
c) and d) potential ORMDL TKO affects SPTLC1 and SPTLC3 at mRNA level.
d) No effect of ORMDL KO on SPTLC2 in the real-time.
f) and g) changes of ssSPTa and ssSPTb at mRNA in both isoforms.
Statistical analysis was done by measuring T-test (significance means p=0.05)

### 3.5.2 SPT activity analysis for ORMDL knockout cell lines:

SPT assay is a suitable approach to determine SPT activity in our ORMDLs knockout cell lines. Assessing SPT activity in cells with ORMDL knockout can provide valuable insights into the impact of ORMDLs on sphingolipid biosynthesis. We performed SPT for ORMDL 2KO, ORMDL 3KO, ORMDL 1/2KO, ORMDL 1/3KO,ORMDL TKO in addition to keratinocytes wild type. Cells were treated with C8 ceramide, myriocin, and no treatment. Figure (3.13) shows that there is a consistent inhibitory action by C8 ceramide across each knockout. The absence of specific ORMDL isoforms does not seem to alter the efficacy of C8 ceramide in inhibiting SPT. Each individual ORMDL isoform is able to regulate SPT and have a ceramide response which is similar to background myriocin (Siow & Wattenberg, 2012). The low expression of serine in knockout cells may be due to the selection. Probably the treatment of cells with puromycin altered the effect of the enzyme and decreased its activity.

### 3.5.3 Western blot analysis for ORMDL knockout cell lines:

We conducted Western blot analyses on ORMDL knockout cell lines with the aim of assessing the expression levels of SPTLC1 and ORMDL. However, the obtained blots did not reveal any visible alterations in the protein levels of SPTLC1(fig 3.14). Additionally, drawing definitive conclusions about the reduction of ORMDL was challenging due to the experimental setup involving pooled samples, which comprised a mixture of both wild-type and knockout cells. To gain more precise insights into the protein-level changes, it is essential to replicate the experiment using individual clones instead of pooled samples.

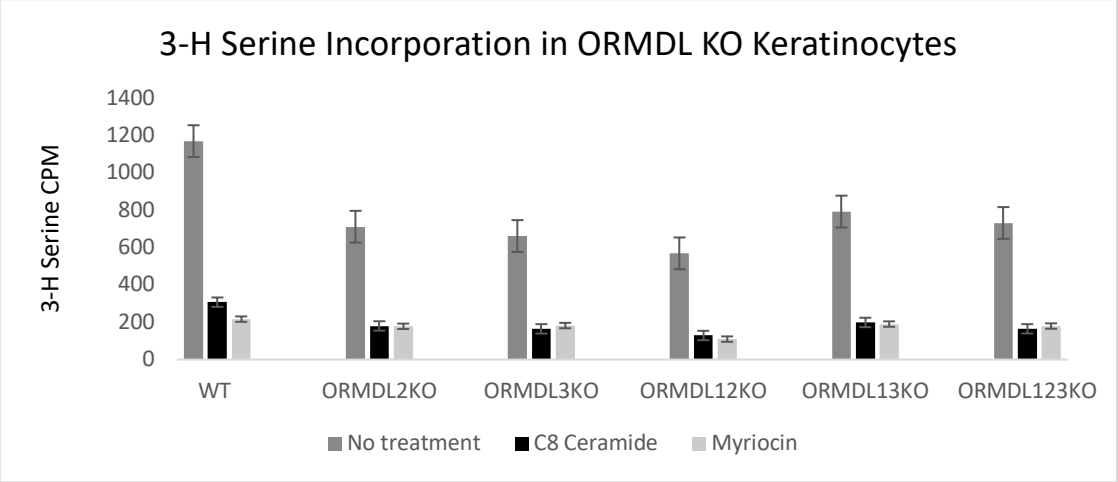



**Figure 3.13: SPT assay for ORMDL single, double, and triple knockout.** There is a similar inhibitory action by C8 ceramide across each knockout. We also observe the inhibition of myriocin in the samples and a decrease in serine incorporation in ORMDLs knockout.

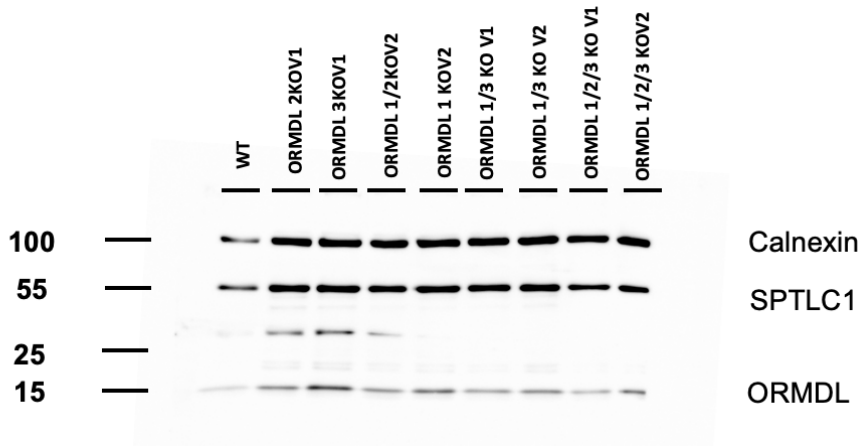



**Figure 3.14: Western blots for keratinocytes cell lines.** Blots did not reveal any discernible alterations in the protein levels of SPTLC1. Conclusive statements regarding the reduction of ORMDL could not be made, as the experiment was conducted on pooled samples.

## Chapter 4 Discussion

In reflecting on the study, we encountered certain challenges that we plan to address in future investigations. Firstly, the guides targeting ORMDL1 were positioned in the 5' untranslated region and did not produce the desired effect on mRNA levels, resulting in an unsuccessful knockdown. Additionally, the sequencing of both pools and clones did not provide conclusive evidence of the intended knockdown. To overcome these challenges, we intend to design new guide RNAs for ORMDL1, potentially targeting different regions, and consider sequencing a larger number of clones to enhance the robustness of the results in future experiments.

### 4-1 The ORMLDs knockout stimulate sphingolipid biosynthesis in the skin and improve epidermal barrier function:

ORMDL proteins are involved in the regulation of sphingolipid biosynthesis. They are the regulatory subunits of SPT, the initial rate limiting enzyme in the sphingolipid biosynthesis. In normal cells, ORMDLs knockout lead to an increase in sphingolipid levels. However, in keratinocytes, ORMDLs knockout are expected to have an effect on differentiation and regulatyion of cell growth. Our expectation is likely based on the known roles of sphingolipids and the regulatory functions of ORMDLs in sphingolipid biosynthesis. Sphingolipids, including ceramides, are crucial signaling molecules involved in various cellular processes, including differentiation and cell growth regulation. We know that all of the epidermal layers contain sphingolipids consisting of ceramides. The basal and spinous layers, which are involved in cell proliferation and differentiation, contain fewer sphingolipids compared the granular and the cornified layer. The synthesis and processing of sphingolipids primarily occur in the stratum granulosum, contributing to the lipid composition of the stratum corneum which is essential for maintaining skin barrier function. ORMDLs knockout increase SPT activity and leads to more

ceramide biosynthesis. Ceramides play a vital role in forming the lipid matrix within the stratum corneum. An increase in ceramide levels we believe would help to reinforce this lipid barrier, improving its integrity and functionality. This contributes to a more robust barrier against water loss and external stressors. In certain cells, the excessive accumulation of ceramides can lead to cellular stress. The relationship between ceramides and cellular toxicity is complex and can be influenced by various factors, including the specific cell type and the overall cellular environment. However, In the skin, the cell has a specific mechanism to temporary store excess sphingolipids. The high concentration of ceramide results in producing more sphingomyelins and glycosphingolipids in the Golgi. Both sphingolipids are stored in the lamellar bodies. The lamellar bodies that appears in the granulosum layer, secrets sphingolipids such as glucosylceramide and sphingomyelin in the intercellular lipid lamelae in the cornified layer. These sphingolipids are processed into their sphingolipid products, ceramides by glucocerebrosidase and sphingomyelinase. The integrity of the skin barrier is strongly dependent on the lipid components of the SC, particularely Ceramides(Ge et al., 2023). There are at least 15 subclasses of ceramides in human SC, including ceramide EOS, known as acylceramide, an esterified-hydroxy acid sphingosine consisting of a sphingosine base bearing a very long omega-hydroxy fatty acid with a chain length distribution between 26C and 36C atoms (Ramos et al., 2018). Ceramide EOS creates hydrophibicity by their omega-hydroxy long chain which promotes the interaction of sphingolipids with each other. The hydrophobic environment prevents the transepidermal water loss TEWL from the cell and improve epidermal barrier function. We expect that the expression of ceramide due to ORMDLs knockout would help to improve barrier permeability since ceramides are a crutial components of the intercellular lipide lamelae in the stratum corneum. The arrangement of these ceramides in lamellar structures

provides a cohesive and highly ordered matrix, creating an effective barrier, strenght layers. and healthy skin.

**4.2 The knockout of ORMDLs can change the morphology of the skin:**

We anticipate that the knockout of ORMDLs may impact skin morphology, potentially influencing the structure and function of the skin. Alterations in ORMDLs expression may improve the formation and the maintenance of the skin barrier, which manifests as changes in skin texture, hydration levels, and sensitivity to environmental factors. Previous studies revealed that providing cells with ceramides, increases the expression of Filaggrin, the late keratinocytes differentiation marker(Hirabayashi1 et al., 2017). Filaggrin is an essential component of keratohyalin granules (Ipponjima et al., 2020). Filaggrin monomers condense keratin filaments which could provide the force required for cell flattening. Filaggrin deficiency extended the flatenning time in the granular layer due to the abnormal keratin condensation granules (Ipponjima et al., 2020). The elongated time frame of the morphology change would delay the cornification process, since cells are not competelly flattened (Ipponjima et al., 2020). ORMDL Knockout expectation is to rescue abberant differentiation by supplememtation of ceramides. We anticipate that ceamides flow will increase the frequency of transformation and regulate terminal keratinocytes differentiation through modulating filaggrin expression.

**4.3 ORMDLs knockout can resolve skin diseases and inhance the skin immune system:**

Psoriasis is immune-mediated skin disorder associated with a reduction in ceramides containing long-chain fatty acids in psoriatic lesions, which may be dependent on the reduction in the gene expression of ELOVL (long-chain fatty acid elongase) and ceramide synthase

observed in the in vitro culture of keratinocytes (Borodzicz et al., 2016). The lack of ORMDLs protein increases the activity of ceramide generating enzymes such as serine palmitoyl transferase in the *de novo* biosynthesis pathway, which increase ceramides level in the epidermis. Given the complexity of psoriasis and the multiple factors contributing to its development, the potential involvement of ORMDL knockouts in therapy would likely be part of a broader investigation into the role of sphingolipids, immune function, and skin barrier integrity in the condition.

Autosomal recessive congenital ichthiosis ARCI is a skin disease consisting of the malformation of the corneocytes lipid envelope and impaired biosynthesis of acylceramide. Considering the role of ORMDL knockout in sphingolipid synthesis, the latter could contribute to the development of therapies aimed at adjusting lipids in affected individuals, thereby potentially improving skin health.

The skin is an active immune organ. Skin acts as the first line of defence protecting the body from infection. A unique study revealed that CD1d expression in epidermis is regulated by ceramide synthesis, which is known to increase during kerstinocytes differentition. (Fishelevich et al., 2006). CD1d molecules are a group of glycoproteins that play a unique role in the immune system. Unlike the more well-known major histocompatibility complex (MHC) molecules, which present peptides to T cells, CD1d molecules bind to and present lipid antigens to T cells. The study showed the important role of ceramide-dependent signaling pathways in controlling CD1d expression. One of the experiment revealed that “Fumonisin B1 blocked the increase in gene expression and total cellular CD1d Ag expression, which was reversible by the addition of exogenous ceramides”( Fishelevich et al., 2006). We expect that ORMDL knockout cell lines

can enhance the immune system response by generating enough ceramide capable to increase CD1d antigen expression. It's essential to consider that while ORMDLs knockout may influence sphingolipid levels, the downstream effects on immune responses are likely multifaceted and may involve various signaling pathways and cellular processes. Further studies are needed to elucidate the detailed mechanisms and potential therapeutic implications of manipulating ORMDL proteins in the context of skin immune system modulation.

**4.4 Limitations and future directions:**

In order to deeply study the role of ORMDLs in the skin, we intend to generate genomic mutation in mice by knocking-out ORMDL genes. Mice are a valuable model for studying genetics and genomics, providing insights into human biology and potential therapeutic approaches for various genetic conditions and diseases. Mice skin share a significant portion of their genetic makeup with human skin (Monaco et al., 2015). Unfortunatelly, human skin diseases does not occur in mice. Therefore, it is imperative to carry out genome editing in mice and manipulate the targeted gene. Gene inactivation in mice can be obtained by Crispr-Cas 9 technique, like the one we used for keratinocytes, or by other techniques such as transgenic overexpression of Cre-recombinase, or RNA interferase.

By analyzing mouse skin mutations, we can assess skin barrier permeability, for example by staining using a toluidine blue exclusion test or measuring transepidermal water loss. Phenotypic differences can be observed in the integrity and elasticity of the skin as well as in the size of the mutant mouse, due to the loss of water through the defective barrier. Skin cross section could reveal lipid structure in the epidermis, particularely in the stradum corneum.

Furthermore, analysis of keratinocyte differentiation markers could demonstrate the role of ORMDL in early and late keratinocyte differentiation.

To mimic the process of keratinocyte differentiation as it occurs in the skin, we intend to culture keratinocytes in vitro under conditions that promote their differentiation and stratification, similar to what happens naturally in the epidermis. To do so, there is a method called Air-Liquid Interface (ALI) Cultures. ALI allows keratinocytes to grow on a porous membrane and be exposed to air from one side while being fed nutrients from the other (Yee et al., 2010). This method encourages stratification such as the organization of its various layers, and differentiation, leading to the formation of multiple layers of cells, somewhat similar to the layers in the epidermis (Yee et al., 2010).The ALI culture technique for keratinocytes provides a more relevant and physiologically accurate environment for studying their behavior, differentiation, and response to various stimuli, making it a valuable tool for skin biology research and therapeutic development.

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