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Final Thesis

The effect of SMPD4 absence on mitosis and neuronal progenitor cell number

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The effect of *SMPD4* absence on mitosis and neuronal progenitor cell number

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Preface

This project is part of the research group “Genetics of Brain Malformations” of Grazia Mancini in the department of Clinical Genetics of the Erasmus MC in Rotterdam. The focus of this research group is to study genetic mutations involved in brain malformations and to characterize the function of these genes and the disease mechanism involved in their genetic mutations. This way, the research group wants to improve diagnosis and medical care for its patients. This project is focused on the further characterization of the *SMPD4* gene.

I am very much thankful to Daphne Smits, Jordy Dekker, Rachel Schot and Grazia Mancini for letting me be part of this research group and for all the guidance they gave me during my internship. I would like to thank them for providing me with an opportunity to gain a lot of knowledge and for teaching me a lot of new laboratory techniques.

Abstract

SMPD4 is a neutral sphingomyelinase that plays an important role in sphingolipid metabolism. Sphingolipids are a family of lipid molecules abundantly present in the brain where they play an important role in the brain development and function due to their essential cellular role as structural components of cell membranes (Ledeen 2008). SMPD4 catalyzes the hydrolysis of membrane sphingomyelin, the most abundant eukaryotic sphingolipid, to form ceramide. The generated ceramide works subsequently as a second messenger in several neurodevelopmental processes such as neuronal cell proliferation and neuronal survival (Krut O 2006).

The Mancini research group has reported that bi-allelic loss-of-function variants in the *SMPD4* gene lead to a severe neurodevelopmental disorder including microcephaly, arthrogryposis, and a profound developmental delay (Magini P 2019). The first objective of this project is to study the effect of *SMPD4* knockdown on the homeostasis of the nuclear envelope (NE) during mitosis, most specifically during the nuclear envelope breakdown (NEBD). This project also aims to study the effect of SMPD4 loss-of-function on neuronal cell proliferation and apoptosis, as both these processes are involved in the pathogenesis of microcephaly. Lastly, the sub-cellular localization of patient-specific missense SMPD4 mutant proteins was studied and compared to wild-type SMPD4.

Firstly, a NEBD assay was performed on *SMPD4* knockdown Hela cells to observe the effect of the SMPD4 loss-of-function on the duration of the NEBD process through time-lapse confocal microscopy. Unfortunately, this project is still in progress and no conclusions can be drawn on the effect of the *SMPD4* knockdown on the NEBD. Secondly, to study the effect of SMPD4 loss-of-function on neuronal cell proliferation, an EdU proliferation assay was performed on *SMPD4* knockdown NSCs. From this assay, it can be concluded that *SMPD4* knockdown leads to decreased proliferation in NSCs, which would explain the patients' phenotype, as reduced proliferation of neuronal cells leads to congenital microcephaly. Next, a TUNEL apoptosis assay was performed to detect apoptotic DNA fragmentation in *SMPD4* knockdown NSCs. This assay shows an increase in apoptosis in *SMPD4* knockdown NSCs when compared to control NSCs, indicating that *SMPD4* knockdown cells are more susceptible to apoptotic cell death. This is another neurodevelopmental process involved in the pathogenesis of microcephaly. Finally, to study the effect of patient-specific SMPD4 missense mutants on the protein's sub-cellular localization, Hek cells transfected with the mutant SMPD4 were stained with an SMPD4, and an ER (Calnexin) or nuclear pores (mAb-414) antibody and imaged through confocal microscopy to determine the colocalization of SMPD4 with the ER and nuclear pores. This experiment showed that the SMPD4 Pro446Leu mutant results in decreased SMPD4 colocalization with the ER when compared to wild-type SMPD4, this is hypothetically caused by protein misfolding.

Together, these findings support that SMPD4 loss-of-function leads to decreased proliferation and increased apoptosis in neuronal cells, these are both processes known to be involved in the development of congenital microcephaly. Furthermore, the missense Pro446Leu mutation was observed to have an effect on the SMPD4 protein subcellular localization.

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1. Introduction

Malformations of cortical development (MCD) are a group of rare human brain anomalies that can be divided into three different categories: malformations caused by abnormal neuronal and glial progenitor cell proliferation or apoptosis, malformations caused by abnormal neuronal migration, and malformations caused by abnormal cortical organization. These abnormalities can cause a wide range of developmental defects of the cortex that are a common cause of intellectual disability, cerebral palsy and seizures. During this project, the focus will be on malformations caused by abnormal neuronal cell proliferation or apoptosis. This abnormality leads to microcephaly, a rare brain developmental disorder, that is characterized by too-small head circumference (Desikan 2017).

The Mancini research group at the Erasmus MC Clinical Genetics department has reported children, from 12 unrelated families, presenting with a severe neurodevelopmental disorder including microcephaly, arthrogryposis, hypomyelination and a profound developmental delay. Genomic analysis revealed that 23 of these affected individuals harbor bi-allelic loss-of-function variants in the *SMPD4* gene (Magini P 2019). Several neurodevelopmental processes that are involved in the pathogenesis of microcephaly, such as neuronal progenitor cell proliferation and neuronal survival, are controlled by sphingolipid metabolism (Colombaioni L 2004).

Sphingolipids are a family of lipid molecules, and they are particularly abundant in the brain. They are important for proper brain development and functions due to their essential cellular role as structural components of cell membranes (Ledeen 2008). This project's gene of interest, *SMPD4*, is a putative transmembrane neutral sphingomyelinase that plays an important role in sphingolipid metabolism. SMPD4 catalysis the hydrolysis of membrane sphingomyelin, the most abundant eukaryotic sphingolipid, to form ceramide and phosphorylcholine. (Krut O 2006). It was hypothesized by the Mancini research group that SMPD4 has an important role in the homeostasis of membrane sphingolipids, thereby influencing membrane integrity, and endoplasmic reticulum (ER) function and organization (Magini P 2019). Furthermore, previous research performed by the Mancini group has shown several defects in the nuclear envelope (NE) structure and reassembly process in *SMPD4* knockdown cells during mitosis (unpublished data).

The Mancini research group is focusing on characterizing and determining the function of the *SMPD4* gene with the goal to gain more knowledge about biological mechanisms underlying normal and abnormal brain development and improve diagnosis and medical care for their patients. This project aims to study the effect of SMPD4 loss-of-function on the homeostasis of the NE during mitosis, as this process is important for normal cell proliferation. This will be done by performing a *SMPD4* knockdown by means of short interfering RNA (siRNA) transfection on Hela cells. The effect of the *SMPD4* knockdown on the nuclear envelope breakdown (NEBD) will be observed through time-lapse confocal microscopy after performing a NEBD assay. This project also aims to study the effect of the *SMPD4* knockdown on neuronal cell proliferation and apoptosis, as both processes are involved in the pathogenesis of microcephaly. So far, most *in vitro* discoveries done by the Mancini research group were done on (patient) fibroblasts, but to better understand the effect of the SMPD4 loss-of-function on neuronal cells, human neural stem cells (hNSCs) will be used for this project. To observe the effect of the SMPD4 loss-of-function on cell proliferation, an EdU assay will be performed on *SMPD4* knockdown hNSCs. A TUNEL apoptosis assay will be performed on *SMPD4* knockdown hNCSs to observe the effect on cell apoptosis. Lastly, this project aims to study the localization of missense mutant SMPD4 and if it differs from wild-type SMPD4, as missense mutations are known to have an effect on protein subcellular localization and expression. This will be done by performing a site-directed mutagenesis procedure to create a patient-specific missense mutation in a *SMPD4* plasmid, which will subsequently be transfected into Human Embryonic Kidney cells (HEK293T). The localization will be observed by means of immunofluorescence staining followed by confocal microscopy.

2. Theoretical Background

2.1 Malformations of the cerebral cortex

Cerebral cortical development is a complex process, where neuronal cell proliferation, cell migration and cortical organization play an essential role. Disruptions of the normal development of the human cerebral cortex can lead to malformations of the cortical development (MCDs). These malformations can occur between the 8th and 22nd week of gestation and they compromise 2-5% of the general population (Romero DM 2018). Patients show a wide range of developmental disorders of the cortex that are common causes of intellectual disability, cerebral palsy, and epilepsy. These malformations can be categorized into three different groups, each one happening during a different stage of gestation. A schematic overview of these malformations can be seen in figure 1 (Desikan 2017).

Group I. Malformations caused by abnormal cell proliferation or apoptosis

This group of malformations is characterized by neurogenesis disorders. Reduced proliferation of neuronal and glial cells or accelerated apoptosis lead to congenital microcephaly (a small brain). Increased proliferation of neuronal and glial cells or reduced apoptosis lead to macrocephaly (large brain). And lastly, abnormal neuronal and glial cell proliferation leads to dysplastic malformations. These malformations can occur from week 8 until week 16 of gestation (Pang 2008).

Group II. Malformations caused by abnormal neuronal migration

Several stages are involved for successful neuronal migration and disruptions at any of these stages will result in malformations. Incomplete neuronal migration leads to heterotopia (neuronal cell accumulation in abnormal locations). Motility disruption during migration to the cortical plate leads to lissencephaly (smooth brain). And abnormal neuronal migration arrest causes overmigration of neurons leading to cobblestone lissencephaly. These processes occur between week 12 and 20 of gestation (Desikan 2017).

Group III. Malformations caused by abnormal cortical organization

The process of cortical organization happens after the 24th week of gestation. Abnormalities in this process are known to cause polymicrogyria. Polymicrogyria is characterized by abnormal lamination and the large formation of small cortical folds (V. Fernández 2016).

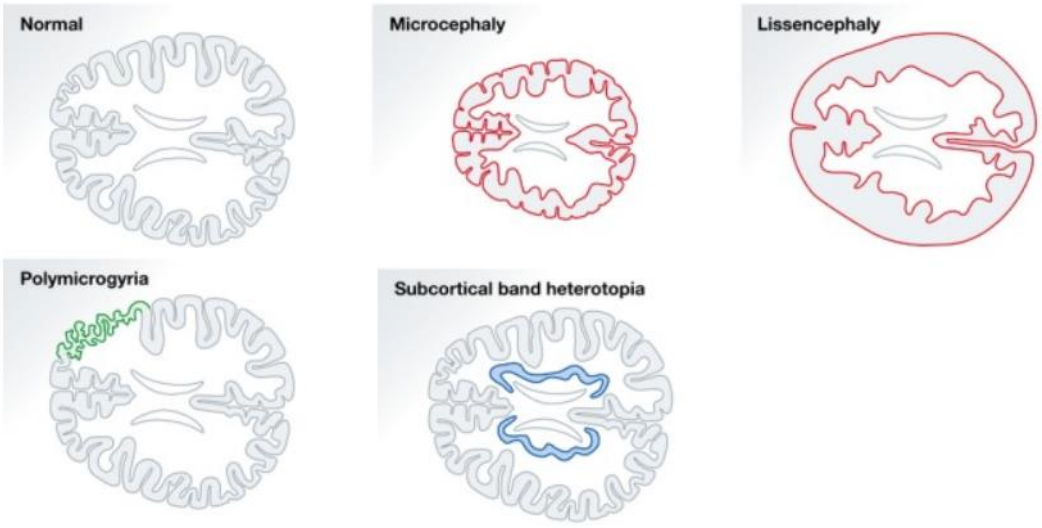

Figure 1. Schematic overview of a normally developed brain compared to the different MCDs (V. Fernández 2016).

2.2 Microcephaly

Primary microcephaly, also known as congenital microcephaly due to its presence in gestation or at birth, is characterized by a defect in which an infant's head circumference is >2 standard deviations below the mean, compared to infants of the same age, sex and ethnic background (DeSilva 2017). Microcephaly has also been associated with reduced myelination, also known as hypomyelination (Nakayama 2015). Microcephaly leads to variable severities of intellectual disabilities. Other health problems linked to microcephaly are epilepsy, cerebral palsy, developmental delay, and ophthalmologic disorders (Ashwal 2009). Most of the genetic causes of microcephaly are associated with cell cycle defects, such as centrosomal abnormalities. Centrosomal proteins have as function to control the mitotic spindle, an important process for normal cell proliferation during mitosis. During this project, the Mancini research group focuses on characterizing and finding the function of the *SMPD4* gene and how its malfunction can influence normal neural stem cell proliferation and apoptosis, causing microcephaly (Magini P 2019).

2.3 Sphingolipids

Sphingolipids constitute a class of bioactive lipid mediators and they are abundant in cell membranes of the central nervous system (CNS), where they play an essential role in the brain development and functioning of the nervous system (Olsen 2017). The most abundant eukaryotic sphingolipid is sphingomyelin, and its metabolism regulation is essential for cell functioning, as it controls the regulation of neurodevelopmental processes like neuronal migration (Buccoliero 2002). Sphingomyelin metabolism is mediated by sphingomyelinases, which hydrolyze sphingolipids to phosphorylcholine and ceramide. Ceramide is a lipid signalling molecule and has been suggested to play an essential role in apoptosis, cell cycle arrest, inflammation and autophagy (Krut O 2006). Sphingomyelinases are a family of enzymes that consist of one acidic sphingomyelinase (*SMPD1*) and four neutral sphingomyelinases (nSMases): nSMase1 (*SMPD2*), nSMase2 (*SMPD3*), nSMase3 (*SMPD4*) and mitochondria-associated MA-nSMase (*SMPD5*) (Airola 2013). Sphingomyelinases are acidic or neutral depending on the pH in which their enzymatic activity is optimal (Jenkins 2009).

2.4 *SMPD4* gene

The *SMPD4* gene encodes nSMase3, one of the neutral sphingomyelinase enzymes. SMPD4 is 866 aa long protein that contains a protein-protein interaction domain and a C-terminal transmembrane domain near the enzyme active site. Research on the subcellular location of SMPD4 has shown that the protein is localized at the ER and the Golgi-apparatus (Krut O 2006). In other studies, a proteomics analysis detected SMPD4 (by then referred to as NET13) as a component of the outer nuclear envelope (Dreger M. 2001). The Mancini research group has also been able to detect the presence of SMPD4 in the ER and NE, as seen in figure 2, and confirmed binding of SMPD4 to proteins from the ER, the NE and to several components of the nuclear pore complex (Magini P 2019).

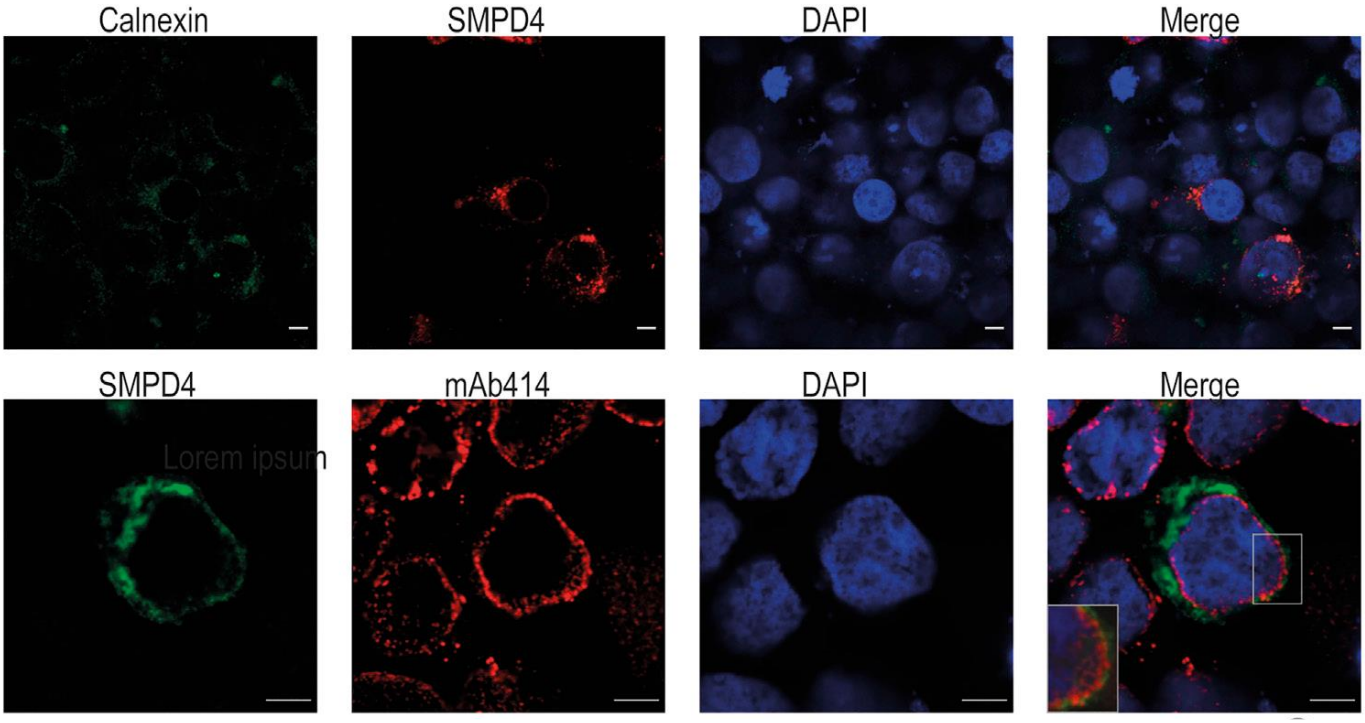

Figure 2. Immunofluorescence staining of overexpressed Myc-labelled SMPD4 in HEK293T cells. The upper panels show the staining of the ER with calnexin (in green), SMPD4 (in red), and the cell nucleus with DAPI (in blue), along with a merged image (far right). The lower panel shows colocalization of SMPD4 (in green) and the nuclear pore marker (mAb414) (Magini P 2019).

2.5 Nuclear envelope

The NE consists of two membranes, the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), which separate the nucleus from cytoplasmic materials. The ONM is continuous with the rough ER, while the INM contains many specific integral membrane proteins associated with chromatin and nuclear lamina, both key proteins important for nuclear architecture (Boni 2005). At multiple sites in the NE, these two membranes are fused via membrane-lined channels called nuclear pore complexes (NPCs). During mitosis, major structural cell changes occur, such as the nuclear envelope breakdown (NEBD) (Figure 3). This dismantling of the NE is essential for species that perform an open-mitosis and starts with the crucial disassembly process of NPCs, which leads to increased NE permeability and the mixing of cytoplasmic and nuclear components (Laurell E 2011). Further, the nuclear lamina is depolymerized, and the nuclear membrane components are detached and removed from chromatin, leading to the distribution of these components into the ER (Marino J 2014).

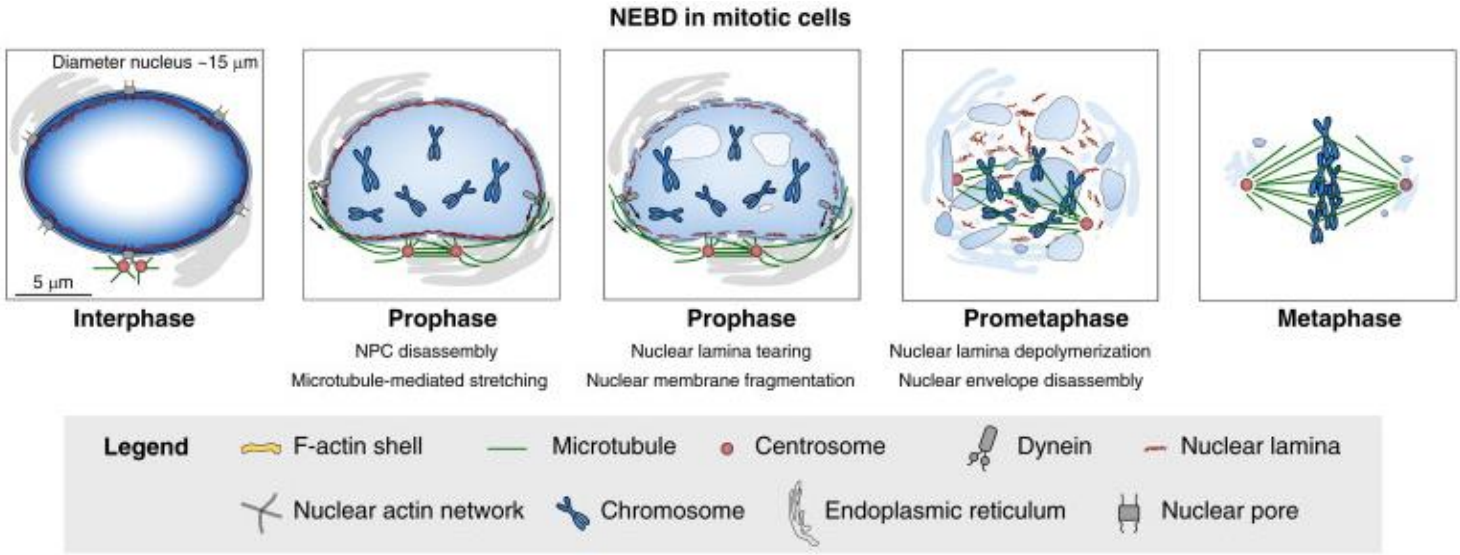

Figure 3. Different stages of the NEBD process in mitotic cells. Legend below shows the different structures involved in the NEBD and mitosis. (Mogessie B 2014)

2.6 Nuclear pore complex

Nucleocytoplasmic transport of macromolecules, such as nuclear proteins, signaling molecules and transcription factors is facilitated by NPCs. NPCs are large protein structures that fuse the INM and ONM to create a channel. NPCs consist of roundabout 30 different nuclear pore proteins, so-called nucleoporins (Nups). These Nups are contained at the nuclear ring, the inner pore ring and the cytoplasmic ring, the cytoplasmic filaments, and the nuclear basket (Figure 4) (Beck 2017). As mentioned before, the NEBD during mitosis starts with the crucial NPC dismantling step (Laurell E 2011). Furthermore, NPCs have a key role in DNA replication, as their integrity is necessary for a proper DNA-damage response and genome stability (Whalen 2020). DNA replication is a crucial cell division cycle step regulated by multiple signaling pathways that control several processes, such as cell proliferation (Stoeber K 2001). During DNA replication, several replication fork barriers can be encountered which can lead to fork stalling. However, the replication fork mostly maintains the ability to continue DNA replication once such a barrier is overcome. However, when the stalled replication fork cannot be restarted it can turn into a collapsed replication fork, which no longer has the ability to perform DNA replication, and this leads to replication stress. Studies have shown that NPCs have a crucial role in the maintenance of genome stability by protecting against replication stress, as stalled or collapsed replication forks relocate to NPCs during such an event (Whalen 2020).

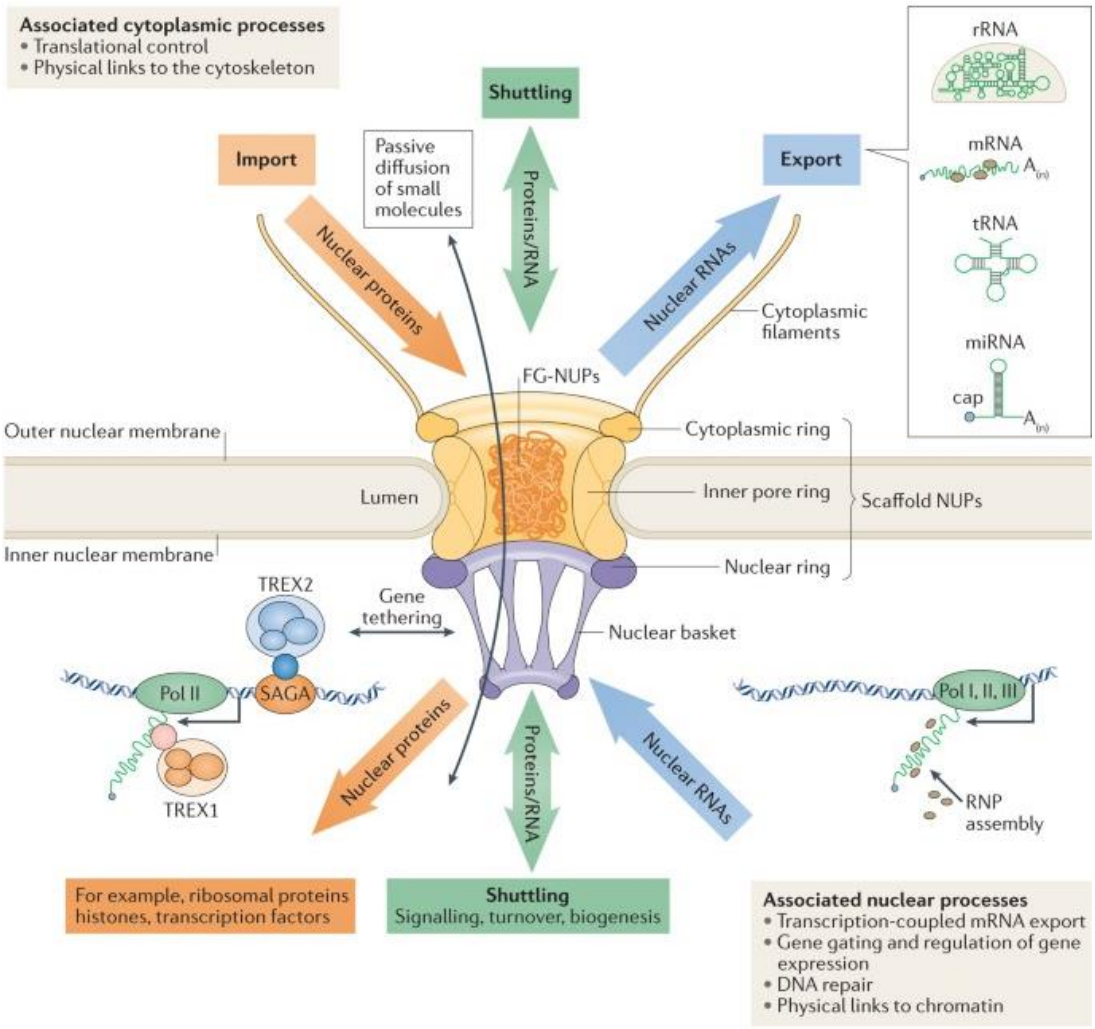

Figure 4. Schematic overview of the NPC substructures, as well as the nucleocytoplasmic transport process via NPCs (Beck 2017).

2.7 Cell Apoptosis

Programmed cell death, also known as apoptosis, is a vital mechanism that occurs during normal cell development and aging. Apoptosis plays an essential role in several processes, such as embryonic development, normal cell turnover, immune system development and functioning, and tumorigenesis protection (S. 2007). The apoptosis mechanism of unnecessary, aged, or damaged cell removal involves a cascade of multiple events, as seen in figure 5. Initially, the cell shrinks due to decreased cell volume and the chromatin along the nuclear membrane condensates. Afterwards, the cell nucleus collapses and cellular fragments are generated followed by DNA fragmentation. These generated cellular fragments may appear on the cell surface as blebs or vesicles. The last morphological change is the phagocytosis of apoptotic bodies which blocks inflammatory responses (T.A. 1997). Neuronal cell apoptosis during embryogenesis is a crucial process for proper nervous system development and function. During normal brain development, approximately half of the neurons are eliminated by apoptosis after extensive neural progenitor cell proliferation. This happens to remove excessive cells during neurogenesis and to eliminate differentiated migrating neurons that did not establish proper synaptic connectivity (Blomgren K 2007).

As mentioned before, SMPD4 mediates sphingomyelin metabolism to produce the lipid signaling ceramide, which is suspected to play a crucial role in apoptosis (Krut O 2006). Studies show that stress activates sphingomyelinases which leads to the accumulation of ceramide. Subsequently, ceramide works as a second messenger to promote apoptosis. A dysregulation in sphingomyelinase activity can lead to increased apoptosis or autophagy in cells (Mullen TD 2012).

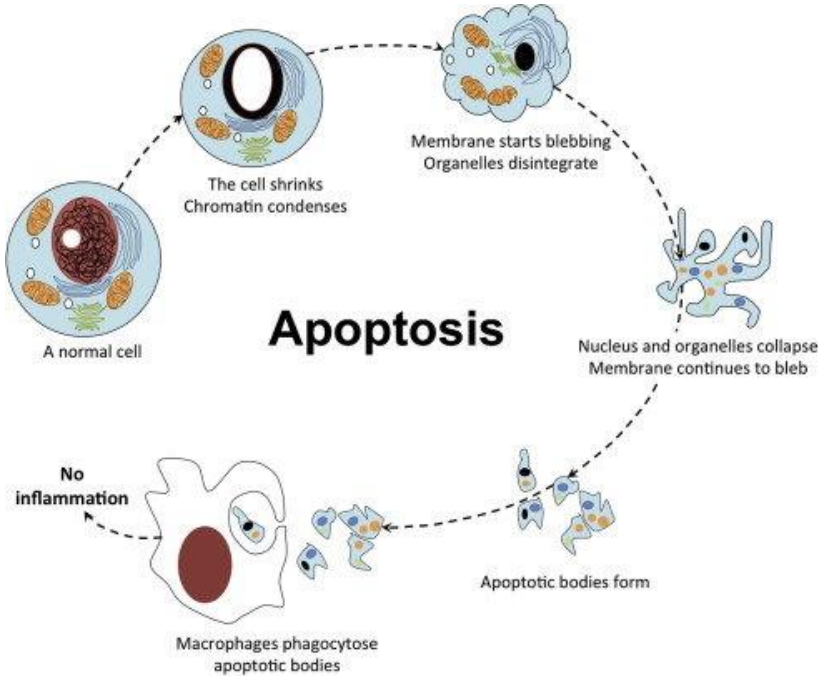

Figure 5. The different stages of cell apoptosis and morphological changes (Abou-Ghali 2015)

2.8 Previous research Mancini group

The Mancini research group is focused on the characterization and identification of disease-causing genes involved in malformations of the brain. Their main focus momentarily is the characterization of the *SMPD4* gene and to determine its function and localization. They have found 32 individuals from 12 different families that present microcephaly, arthrogryposis (contractures of the joints), severe neurodevelopment delay, and early death. Genomic analysis revealed that 23 of these affected individuals harbor bi-allelic loss-of-function variants in the *SMPD4* gene. The affected individuals have different *SMPD4* mutations: nonsense, missense, frameshift, splice and deletions. Three patients identified with missense mutations present a milder phenotype, while patients with nonsense, splice or deletion mutations show a more severe phenotype. This suggests that missense mutations lead to a dysfunctional SMPD4 protein that still presents some of the normal functioning, while the other variants lead to a complete loss of the SMPD4 protein. Furthermore, during a flow cytometry study for cell cycle investigation, fibroblasts were treated to knockdown *SMPD4* and this resulted in a lower percentage of fibroblasts in the G2/M phase (Figure 6A). While a higher percentage of cells in the SubG1 phase was observed in fibroblasts from individuals with *SMPD4* variants (Figure 6B). The lower percentage of cells in G/M phase suggests that the absence of SMPD4 causes a cell cycle delay. In addition, the increase of cells in the SubG1 phase suggests the activation of cell apoptosis. A FAM-FLICA assay confirmed this, as an increased number of apoptotic cells were observed in patient fibroblasts (Magini P 2019). From a cytoSMART cell cycle analysis, it was also

concluded that the mitosis duration is increased in patient fibroblasts compared to control fibroblasts, indicating a possible cell cycle delay (Figure 6C). The increase of apoptotic cells could be explained by the cell cycle delay since cell cycle delays can cause cells to undergo apoptosis (Pilaz LJ 2016).

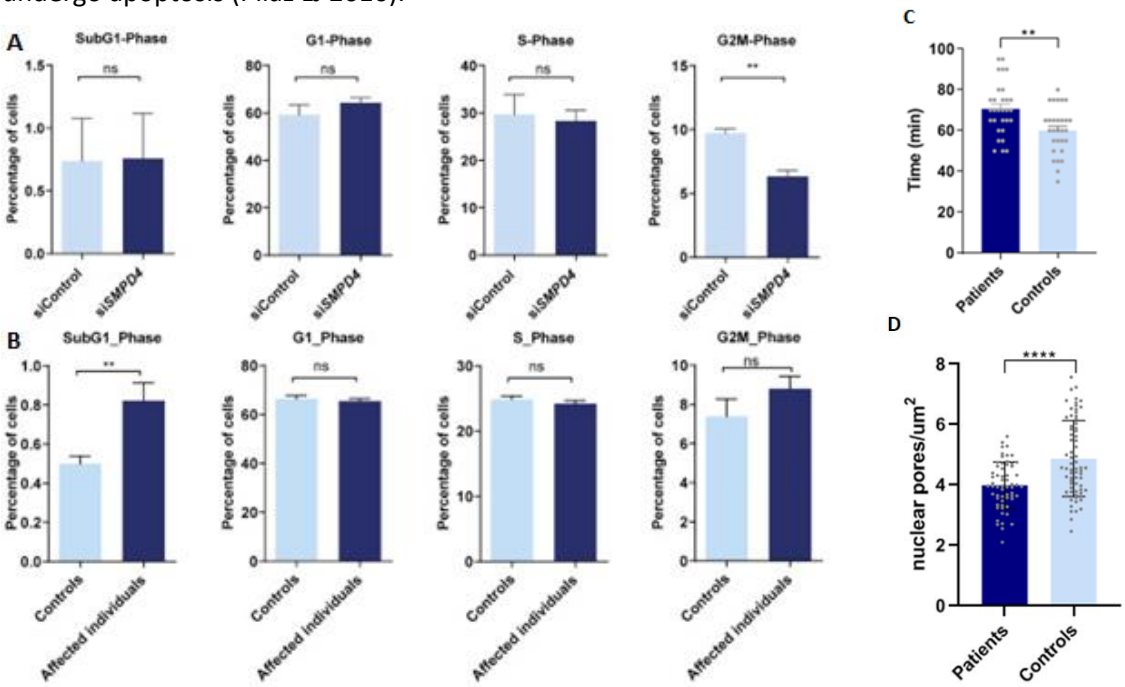

Figure 6. ***(A)*** *Flow cytometry analysis of control fibroblasts after SMPD4 knockdown by siRNA;* ***(B)*** *Flow cytometry of control fibroblasts and three individuals with SMPD4 variants;* ***(C)*** *Statistical analysis of mitosis duration between patient cell lines and control cell lines (**p<0.01).****(D)*** *Statistical analysis of the counting of the number of nuclear pore complexes per micron² for three patient cell lines and three control cell lines (****P=<0.001) (Magini P 2019)*

Previous research focused on the effect of the *SMPD4* knockdown on the NE, showed several defects in the NE during mitosis. Firstly, increased nuclear envelope invaginations were observed during mitosis while using a Lamina-associated polypeptide 2 (LAP2) marker for the NE (Figure 7). These invaginations can be a result of NE reassembly during mitosis when the fusion of NE sheets does not happen correctly or when there is a delayed NE breakdown (Drozdz 2017). Secondly, a delay in NE reassembly and NE irregularities were observed during mitosis in *SMPD4* knockdown cells. Previous research using electron microscopy on fibroblasts from individuals with *SMPD4* variants showed cellular abnormalities, dilated rough ER cisternae, and a decrease of structurally normal rough ER (Magini P 2019). These discoveries could explain the NE reassembly defects, as the ER is the membrane source for NE reassembly (Anderson DJ 2008). It was also discovered that SMPD4 colocalizes with centrosomes during mitosis (Figure 8). Centrosomes are believed to dictate the site of NEBD, as they push on the NE to form prophase invaginations during the G2/M transition. During this stage, centrosomes organize the microtubules, which were also found to contribute to the NEBD by pulling on the NE (Bolhy S 2011). Moreover, the Mancini research group has also found out that patients' fibroblast cells have a lower number of NPCs (Figure 6D).

These recent discoveries lead to this project's aim to focus on the effect of *SMPD4* knockdown on the NE (dis)assembly.

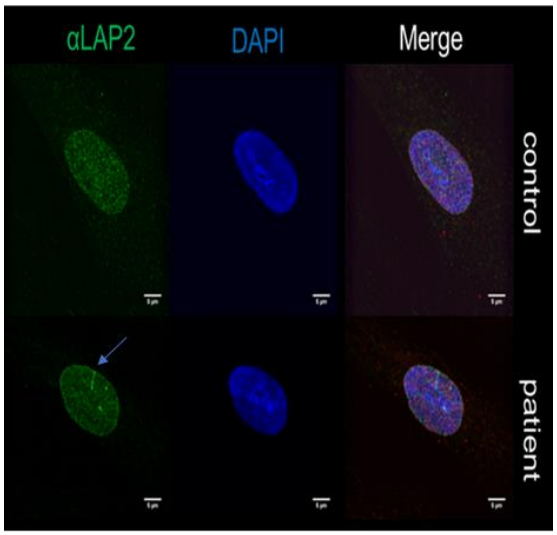

Figure 7. NE immunofluorescence staining in control fibroblasts and fibroblasts of individuals with SMPD4 variants. Panel shows staining of the NE with αLAP2 (in green) and the cell nucleus with DAPI (in blue), and a merged image (far right). NE invaginations were observed in cells of individuals with SMPD4 variants (marked with arrow).

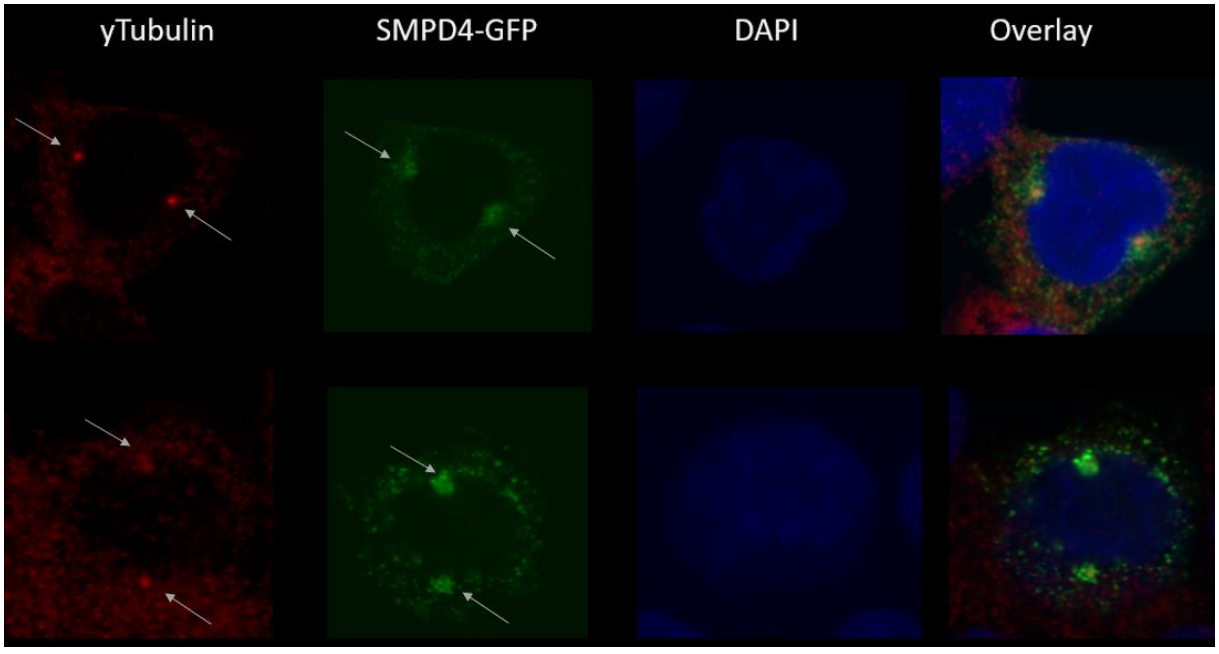

Figure 8. Immunofluorescence staining shows SMPD4 colocalization with centrosomes in NSCs during mitosis. Staining of centrosomes is shown with γTubulin (in red), SMPD4 is shown with SMPD4-GFP (in green), the cell nucleus with DAPI (in blue), and a merged image is shown on the far right. Centrosome and SMPD4 colocalization is marked with arrows.

3. Methodology

The protocols for these methods can be found in Appendix II.

3.1 Cell culture

3.1.1 General info

Before thawing or splitting NSCs, desired flask or well were coated with 0.5% Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco, USA) in KnockOut™ DMEM/F12 cell medium (Gibco, USA). When using NSCs for imaging, coverslips coated with Poly-L-lysine (PLL) (Sigma, UK) and subsequently coated with 0.5% Geltrex were used. The cell medium used for NSCs was KnockOut™ DMEM/F12 supplemented with 2mM L-Glutamine, 20ng/ml EGF (Peprotech, UK), 20ng/ml bFGF (Peprotech, UK), and 2% StemPro® Neural Supplement (Gibco, USA). For Hela and Hek293T cells, +/+ DMEM (1x Dulbecco's Modified Eagle Medium (Gibco, UK) with 10% Fetal Calf Serum (FCS; Thermo Fisher Scientific) and 1% Pen-Strep (PS; Cambrex BioWhittaker, Belgium) was used. During transfection procedures, -/- DMEM (1x Dulbecco's Modified Eagle Medium without FCS and PS) was used for Hela and Hek293T cells, as FCS and PS negatively interact with the transfection reagent.

3.1.2 Thawing cells

The frozen cell vial was thawed quickly by gentle agitation in a 37°C water bath. Cells were slowly incorporated in 10ml pre-warmed cell medium. The cell suspension was centrifuged for 5min at 1000 rpm. Supernatant was aspirated and cell pellet was dissolved in 1ml pre-warmed cell medium. NSCs were transferred to a T25 flask containing 4ml medium and 1% PS was added. Hela and Hek293T cells were transferred to a T75 flask containing 12ml pre-warmed cell medium. Cells were incubated at 37°C and 5% CO_2 with caps loosened.

3.1.3 Splitting cells

All cell culture medium was removed from cells and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS; Lonza, USA). Cells were detached by adding 0.25% Trypsin/EDTA to Hela and Hek293T cells or Accutase (Sigma, UK) to NSCs with 5min incubation at 37°C. After cell detachment, 10ml pre-warmed +/+ DMEM was added to Hela and Hek293T cells or 10ml PBS (Sigma) was added to NSCs. Cell suspension was centrifuged for 5min at 1000 rpm. Supernatant was aspirated and cell pellet was dissolved in cell medium. The cells were then added to desired flask or well and 1% PS (Gibco) was added to NSCs. Cells were incubated at 37°C and 5% CO_2 with caps loosened until further use. Hela cells were split every 72hours at a 1:8 ratio, Hek293T were split at a 1:5 ratio and NSCs were split every 72hours at a 1:2 ratio.

3.1.4 Freezing cells

Cells grown in a T75 flask were washed with PBS (Sigma, UK). Cells were detached by adding 0.25% Trypsin/EDTA to Hela and Hek293T cells or Accutase (Sigma, UK) to NSCs with 5min incubation at 37°C. After cell detachment, 10ml pre-warmed +/+ DMEM was added to Hela cells or 10ml PBS (Sigma) was added to NSCs. Cell suspension was centrifuged for 5min at 1000 rpm. Supernatant was aspirated and cell pellet was dissolved in 1ml 10% DMSO in cell medium. 0.5ml of cell suspension in 10% DMSO was transferred to a cryovial. Cryovials were placed in an isopropanol chamber and stored at –80°C overnight. Afterwards, frozen cells were transferred to liquid nitrogen.

3.2 *SMPD4* knockdown

During this project, a knockdown was performed on the *SMPD4* gene to study its function. For this procedure, cells were plated in a concentration of $3.0x10^5$ cells/well in 2ml supplemented cell medium in a 6-well plate (surface 9,6 cm^2). Cells were grown on sterilized 24mm coverslips when used for imaging. From this point on, medium without FCS and PS (-/- DMEM for Hela cells; KnockOut™ DMEM/F12 with supplements for NSCs) was used during this procedure. One day after plating the cells, the cells were washed slowly with 2ml pre-warmed medium. Afterwards, 1.8ml pre-warmed medium was added to cells. A *SMPD4* siRNA mix was prepared by adding 2.5 µl si*SMPD4* (5 nmol; Dharmacon, USA) to 100µl cell medium. During this experiment, a pool of 4 individual siRNAs targeting *SMPD4* was used to improve the gene silencing effectiveness and to reduce off-target effects (Hannus 2014). A control siRNA mix was prepared by adding 2.5 µl siGENOME Non-Targeting Control siRNA #3 (5 nmol; Dharmacon, USA) to 100µl cell medium. Control siRNAs were used as a negative control, which are designed to target no known genes in humans. The transfection reagent mix was prepared by adding 5 µl DharmaFect 3 (Dharmacon, USA) to 200µl medium. These mixes were incubated for 5 min at RT before adding 100µl of DharmaFect mix to siRNA mixes. Afterwards, reaction mixes were incubated for 20min at RT. After incubation, 200µl of reaction mix was added to well for a total volume of 2ml. Plates were incubated at 37°C and 5% CO2 until further use. After 3hrs of incubation, 10% FCS (200 µl) and 1% PS (20 µl) were added to Hela cells. For NSCs, this procedure was repeated after 24hrs to increase transfection efficiency.

3.3 Quantitative PCR (qPCR)

3.3.1 RNA extraction and purification

The RNeasy mini kit (Qiagen; Hilden, Germany) was used to extract and purify RNA from siRNA transfected cells, which will be used for qPCR to quantify *SMPD4* expression and determine if the *SMPD4* knockdown was successful. Medium was removed from cells grown in 6-well plate (surface 9,6 cm^2). 350 µl RLT buffer was added, and cells were scraped. Cells were transferred to a RNase-free microcentrifuge tube and 1 volume of 70% ethanol was added. Cell suspension was mixed by pipetting up and down and by vortexing. Afterwards, the protocol provided by the RNeasy mini kit (Qiagen; Hilden, Germany) was used without modifications. The concentration and purity of the purified RNA were determined with a Nanodrop™ Spectrophotometer, Model DS-11 FX (DeNovix, USA).

3.3.2 cDNA synthesis

cDNA was synthesized by adding the following: 4 µl 5x iScript buffer, 1 µl iScript reverse transcriptase, 1 µg purified extracted RNA, and Milli-Q for a total reaction volume of 20 µl. The samples were placed in a thermocycler machine, C1000 Touch (Bio-Rad, USA) and the following program was used: 25°C for 5 min, 42°C for 30 min, and 95°C for 1 min. The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used for this procedure.

3.3.3 qPCR

For the qPCR procedure, a primer mix was made by adding the following: 1 µl forward (fw) primer (10µM), 1 µl reverse (rv) primer (10µM) and 98 µl Milli-Q. Two housekeeping gene primers were used for this experiment while using Hela cells: *CLK2* (fw primer 5'-TCGTTAGCACCTTAGGAGAGG -3', rv primer 5'-TGATCTTCAGGGCAACTCG-3'), and *RNF111* (fw primer 5'- GCAGAATGCAGCAGAAGTTG -3', rv primer 5'- CCATTCTTGCAGAAGTGGTTG -3'). When working with NSCs, the following housekeeping genes were used: *HPRT1* (fw exon 3 primer 5'- TTGCTGACCTGCTGGATTAC -3', rv exon 4 primer 5'-TGTTGACTGGTCATTACAATAGCTC -3'), and *TBP* (fw exon 2 primer 5'-

CTTACGCTCAGGGCTTGG-3', rv exon 3 primer 5'- ATAGGCTGTGGGGTCAGTCC -3'). Housekeeping genes were used to normalize mRNA levels between the control and the *SMPD4* knockdown samples. Housekeeping genes are used as their expression shows low variance under experimental and control conditions (Turabelidze A 2010). The qPCR *SMPD4* primers used for this experiment were fw primer 5'- GACTCCCAGCCCCGGTGT-3', and rv primer 5'- CCACTCGGAACACCATGAG -3'. The cDNA mix was made with 79.86 µl iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, USA), 6.39 µl cDNA, and 41.25 µl Milli-Q. The negative control consisted of 12.68 µl iTaq Universal SYBR Green Supermix and 21.42 µl Milli-Q. The cDNA and primer mixes were combined using 9.9 µl of the primer mix and 39.6 µl of the cDNA mix. Afterwards, 15 µl of each sample was added in triplicate in a qPCR plate. The following qPCR program was used: 1x 95°C for 3min; 39x 95°C for 5 sec, 60°C for 30 sec; 1x 65°C for 5 sec; and 1x 95°C for 5 sec. CFX software was used for the SYBR green qPCR measurements.

3.4 NEBD assay

3.4.1 LBR-GFP transfection

During this experiment, a NEBD assay was performed on *SMPD4* knockdown Hela cells and a control group, to observe the effect of the *SMPD4* knockdown on the NE breakdown during mitosis. To analyse this process, an LBR-GFP construct was transfected into *SMPD4* knockdown Hela cells to observe the NE during imaging. The LBR-GFP construct is based on the Lamin B receptor (LBR), a characterized INM protein, and when fused with the green fluorescent protein (GFP) it shows fluorescence distributed throughout the NE (Boni 2005). Prior to the transfection, Hela cells were plated on a 6-well plate ($3.0x10^5$ cells/well) on a 24mm coverslip and a *SMPD4* knockdown was performed. 24 hours after the knockdown, the Hela cells were washed slowly with 2ml complete medium and 1.8ml fresh complete medium was added. The transfection reaction mix was prepared by adding the following in 2 different tubes: a) 200µl medium to 1.66 µl LBR-GFP construct (1.2µg/ml); b) 200µl medium to 4 µl Lipofectamine 3000 Reagent (Invitrogen, Lithuania). These mixes were incubated for 5min at RT before adding 200µl of the Lipofectamine mix to the LBR-GFP mix. Afterwards, the reaction mix was incubated for 20min at RT. After incubation, 200µl of reaction mix was added to well for a total volume of 2ml. Plates were incubated at 37°C and 5% CO2 until further use. After 3hrs of incubation, 10% FCS (200 µl) and 1% PS (20 µl) were added to Hela cells.

3.4.2 NEBD assay (Mühlhäusser P 2007)

24 hours after LBR-GFP transfection, Hela cells were semi-permeabilized in ice-cold permeabilization buffer (20 mM Hepes, pH 7.4, 110 mM KoAc (Honeywell, The Netherlands), 5 mM MgOAc (Sigma, USA), 0.5 mM EGTA (Sigma, USA) and 250 mM sucrose (Sigma, Switzerland) containing 40µg/ml digitonin (Promega, USA) for 10min at RT. A low concentration digitonin was used to perforate the plasma membrane while keeping the NE intact. Afterwards, cells were washed with permeabilization buffer without digitonin for 2, 5 and 10 minutes on ice. In-between wash steps, NEBD reaction mix was prepared by adding the following (1 well): 20µl CSF mitotic extract, 2.75µl permeabilization buffer (without digitonin), 1µl 155kDa TRITC-dextran (25mg/ml; Sigma, Sweden), and 1.25 µl 20x Energy mix (200mM creatine phosphate (Sigma, USA), 10mM GTP, and 10mM ATP in 50mM HEPES pH 7.0; add 1mg/ml creatine kinase and 250mM sucrose)). After washing steps, cells were used for disassembly reaction within 10 min. Coverslips were mounted on a microscope chamber together with 20µl NEBD reaction mix to induce mitosis. Fluorescent 155kDa-TRITC-dextran was used to act as a permeability marker for the NE, since they can only enter the nucleus if NEBD is induced, and the NPCs become dismantled. Laser-scanning microscopy was performed of *in vitro* disassembly reaction, using a confocal microscope (Leica TCS SP5), to follow the fate of the NE over time and to observe the gradual loss of the NE permeability barrier by monitoring the gradual diffusion of fluorescent

dextran into the nuclei. Quantification of both processes in both the control group and the *SMPD4* knockdown group was performed and compared with Fiji software.

3.5 EdU proliferation assay

To study the effect of the SMPD4 loss-of-function on neuronal cell proliferation, the Click-iT EdU assay (Thermo Fisher Scientific, USA) was performed on *SMPD4* knockdown NSCs. After plating NSCs on a 12-well plate ($1.5x10^5$ cells/well) on 18mm coverslips and performing a siRNA transfection, the cells were allowed to rest overnight prior to the Click-iT EdU assay. 1ml of 10µM EdU solution was added to the cells and the cells were incubated for 24hrs at 37°C and 5% CO_2. During incubation, EdU (5-ethynyl-2'-deoxyuridine) is incorporated into newly synthesized DNA. After incubation, the cells were fixed in 4% PFA in PBS for 15min at RT. The cells were washed twice with 1ml 3% Bovine serum albumin (BSA) in PBS. Cells were permeabilized by adding 1ml of 0.1% Triton in PBS and incubating for 20min at RT. During incubation, the Click-iT reaction cocktail mix was prepared by adding the following (for 2 coverslips): 860µl 1x Click-iT reaction buffer, 40µl Copper protectant, 3.25µl Alexa Fluor 647 picolyl azide, and 100µl 1x EdU reaction buffer additive. The reaction cocktail mix was used within 15min of preparation. The cells were washed with 3% BSA in PBS after permeabilization. Afterwards, 0.5ml Click-iT reaction cocktail mix was added to each well. The cells were protected from light and incubated for 30 minutes at RT with gentle rocking. EdU contains an alkyne, which will couple with the fluorescent azide dye by means of a copper-mediated "click" reaction (Krishan A 2010). After 30 min incubation, the cells were washed once with 3% BSA in PBS. Finally, coverslips were mounted on microscopy slides using 20 µl ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, USA) to stain nuclei. Cells were imaged by means of confocal microscopy (Leica TCS SP5) and the Fiji software was used to quantify the number of cells that proliferated during the assay (EdU+) and the number of DAPI+ cells. GraphPad Prism Software will be used to perform an unpaired t-test to determine if there is a significant decrease in proliferating cells in the *SMPD4* knockdown group while comparing to the control group.

3.6 TUNEL click-It apoptosis assay

To determine if there is an increase in apoptosis in *SMPD4* knockdown NSCs, a TUNEL click-iT apoptosis assay (Thermo Fisher Scientific, USA) was performed to detect apoptotic DNA fragmentation. After plating NSCs on a 12-well plate ($1.5x10^5$ cells/well) on 18mm coverslip and performing a siRNA transfection, the cells were treated with 0.1mM hydrogen peroxide to induce apoptosis and incubated for 20hrs at 37°C and 5% CO_2 prior to the TUNEL click-iT apoptosis assay. Cell medium was removed, and cells were washed once with PBS. Afterwards, cells were fixed by adding 1ml 4% PFA in PBS for 15min at RT. The cells were washed with 3% BSA in PBS and subsequently permeabilized in 1ml 0.1% Triton X-100 in PBS for 20min at RT. After permeabilizations, the cells were washed twice with 3% BSA in PBS. 100 µl TdT reaction buffer was added to the coverslips and incubated for 10min at RT. In the meanwhile, the TdT reaction buffers were prepared by adding the following (for 2 coverslips (18mm)): 94 µl TdT reaction buffer, 2 µl EdUTP, and 4 µl TdT enzyme. 50 µl of the TdT reaction cocktail was added to each coverslip and incubated for 1hr at 37°C in a humidified chamber. During this step, the TdT enzyme incorporates EdUTP nucleotides into fragmented DNA. After incubation, cells were washed twice with 3% BSA in PBS for 2min. The Click-iT reaction cocktail was prepared by adding the following (for 2 coverslips (18mm)): 97.5 µl Click-iT reaction buffer (containing Alexa Fluor 647 azide), and 2.5 µl Click-iT reaction buffer additive. 50 µl of the Click-iT reaction cocktail was immediately added to the cells and incubated for 30 min at RT in the dark. The modified dUTPs contain an alkyne, which couples with the fluorescent azide dye by means of a copper-mediated "click" reaction. Afterwards, the cells were washed with 3% BSA in PBS for 5min. Finally, coverslips were mounted on microscopy slides using 20 µl ProLong Gold antifade

reagent with DAPI (Thermo Fisher Scientific, USA) to stain nuclei. Cells were imaged by means of confocal microscopy (Leica TCS SP5) and the Fiji software was used to quantify the number of cells that present apoptotic DNA fragmentation (TUNEL+ foci) and the total number of cells (DAPI+ cells). To determine if there is a significant increase in apoptotic cells in the *SMPD4* knockdown group, GraphPad Prism Software was used to perform an unpaired t-test.

3.7 Exogenous expression of *SMPD4* mutants

3.7.1 Site-directed mutagenesis (SDM)

To create the patient-specific mutations in wild-type (WT) *SMPD4* plasmid, the Quick-Lightning Site-Directed Mutagenesis (SDM) Kit (Agilent Technologies, USA) was used. The synthetic primers used for this procedure that contain the desired mutation can be seen in table 1. The reaction mix was prepared by adding the following: 5 µl 10x reaction buffer, 0.5 µl Wild-type human *SMPD4* Myc-DKK-tagged ORF plasmid (100ng; Origene, USA), 1.25 µl fw primer (125ng; IDT, Belgium)), 1.25 µl rv primer (125ng; IDT, Belgium)), 1 µl dNTP mix, 1.5 µl QuickSolution reagent, 1 µl QuickChange Lightning Enzyme, and 38.5 µl ddH_2O (for a total volume of 50 µl). The following thermal cycling program was used: 1x 95°C for 2min; 30x 95°C for 20sec, 60°C for 10sec, 68°C for 3min; and 1x 68°C for 5 sec. Afterwards, 2 µl Dpn1 restriction enzyme was added to PCR mix and incubated at 37°C for 5min to digest the WT *SMPD4* plasmid.

Table 1. Synthetic primers used to create patient-specific missense mutation.

Mutation	**Site-directed mutagenesis primer (5'-3')**	
Leu231Pro	FW	GGT GGA CAG GTA CCC GTC ATG GTT CCT GC
	RV	GCA GGA ACC ATG ACG GGT ACC TGT CCA CC
Pro446Leu	FW	GCG TAC CGC CAC AGC TGC AGG TAG C
	RV	GCT ACC TGC AGC TGT GGC GGT ACG C
Pro192Leu	FW	GAA TAT GTA ATA CTC GAA CAG ATT CAG GGC CAA GTT CAG
	RV	CTG AAC TTG GCC CTG AAT CTG TTC GAG TAT TAC ATA TTC

3.7.2 Transformation

70 µl XL10-Gold *Escherichia coli* bacteria ultracompetent cells (Agilent Technologies, USA) were thawed on ice and subsequently incubated with 5 µl SDM PCR product for 30min on ice. Transformation was performed by means of heat shock at 42°C for 45 sec subsequently with 2min incubation on ice. 500 µl LB broth (EZ Mix, Lennox) was added to bacterial cells and incubated under agitation (200rpm) at 37°C for 1h. 100 µl of transformation mix was added to LB-Ampicillin (0.1% Ampicillin) agar plates and incubated overnight at 37°C.

3.7.3 DNA isolation

Mutated *SMPD4* plasmid was isolated with the NucleoBond Xtra Maxi kit for transfection-grade plasmid DNA (Macherey-Nagel; Düren, Germany). Firstly, transformed single bacterial colonies were picked from agar plate and inoculated in 3ml LB medium with Ampicillin and incubated overnight at 37°C with shaking (300rpm). Transformed bacterial cells were diluted 1/500 and grown to 50ml overnight at 37°C with shaking (300rpm). The next morning, bacterial culture was harvested by centrifuging at 4000 x g for 15min at 4°C. Pellet was resuspended in 10ml Resuspension Buffer by vortexing. Afterwards, 20ml Lysis Buffer was added and gently mixed by inverting and incubated for 5min at RT. 15ml Neutralization Buffer was added to lysate and mixed by inverting 15 times and incubated on ice for 15 min. Cell suspension was centrifuged at 4,000xg for 15min at 4°C. The supernatant containing plasmid DNA was collected and loaded on an equilibrated NucleoBond Xtra Column Filter. The filter was washed twice with 15ml Wash Buffer. Next, the plasmid DNA was eluted with 15ml water and precipitated by adding 10.5ml isopropanol and mixed. The precipitated DNA

was centrifuged at 4,000xg for 30 min at 4°C. The supernatant was removed, and the pellet was washed with 5ml 70% ethanol and centrifuged at 4,000xg for 10 min at 4°C and the supernatant was removed. Finally, the pellet was air-dried for 10 min and redissolved in 0.5ml water. The concentration and purity of the purified DNA were determined with a Nanodrop™ Spectrophotometer.

3.7.4 Sanger sequencing

PCR reaction

To determine if the SDM procedure was successful, isolated plasmid DNA was sequenced by means of the Sanger Sequencing method to check if the desired mutation is present. Firstly, the isolated DNA plasmid was amplified through a PCR reaction. During this experiment, a PCR was performed using multiple primers (Appendix III table 1) to amplify the entire *SMPD4* plasmid to confirm the SDM procedure was successful and if undesired mutations were created. A PCR reaction mixture was created by adding the following substances: 13.75µl Milli-Q water; 2µl 10x FastStart Buffer (Roche; Mannheim, Germany); 1.6µl 10mM dNTPs (Roche; Mannheim, Germany); 1µl fw primer (10µM) (IDT; Leuven, Belgium); 1µl rv primer (10µM) (IDT; Leuven, Belgium); and 0.1µl FastStart Taq Polymerase (Roche; Mannheim, Germany). 20µl of the PCR master mix was used for each sample and 0.5µl of the isolated DNA sample was added. The samples were placed in a thermocycler machine, C1000 Touch (Bio-Rad, USA). An initial denaturation step was performed at 95°C for 5min. Next, 25 amplifying cycles consisting of a denaturation step at 95°C for 30sec; an annealing step at 58°C for 30sec; and an elongation step at 72°C for 2min were performed. Finally, a final elongation step was performed at 72°C for 5min.

Gel Electrophoresis

Before the PCR product can be used for sequencing, it was run through an agarose gel to confirm the PCR reaction was successful. The loading mixture was prepared by adding 3µL of sample to 10µL loading dye (1.5ml 3x OrangeG (BHD Laboratory Supplies) + 5µl Gelred (Biotum). 12 µL sample was loaded in each slot of a 1.5% agarose gel. 1kb+ Marker; 10 µl kb+/1000 µl Milli-Q (Invitrogen) was used as a molecular weight marker, DNA ladder. Electrophoresis was carried out and the gel was afterwards visualized with a UV-molecular imager, Molecular Imager®Gel Doc™ XR System (Bio-Rad, USA).

Exo-Sap

Exo-Sap-it reagent was used to clean up the amplified PCR product, as it hydrolyses excess primers and nucleotides from the previous PCR reaction. (ExoSAP-IT™ PCR Product Cleanup Reagent sd). 1 µl Exo-Sap-it® Affymetrix (Thermo Fisher) was added to 5 µl PCR product together with 5 µl Milli-Q. The samples were placed in a thermocycler machine, C1000 Touch (Bio-Rad, USA) and the following program was used: I. 37°C for 45 min; II. 80°C for 15 min to deactivate the Exo-Sap-it.

BDT reaction

BDT reaction mix was prepared by adding the following substances: 3.5µl 5x Sequence buffer (400mM Tris-HCl pH 9.0, 10mM $MgCl_2$, Applied Biosystems®); 1 µl either fw or rv primer (10µM) used in PCR reaction (IDT; Leuven, Belgium); and 0.5 µl Big Dye Terminator (BDT) v3.1 (Applied Biosystems®). 5 µl BDT mix was added to 5 µl DNA from Exo-Sap. The samples were placed in a thermocycler machine, C1000 Touch (Bio-Rad, USA) and the following program was used: 1x 96°C for 45 sec; 24x 96°C for 10 sec, 58°C for 5 sec, 60°C for 2 min.

Sephadex purification of BDT product

Sephadex is a gel filtration medium that desalts, removes contaminants, and exchanges buffers (Sephadex® G-50 sd). Sephadex™ G-50 Superfine powder (GE Healthcare Bio-Sciences; Sweden) was added to metal plate mold, MACL09645 (Millipore Merck; Darmstadt, Germany). The Sephadex

powder was then transferred from mold to a filtration plate, MAHV N45 (Millipore Merck; Darmstadt, Germany). 300µl demi water was added to the Sephadex powder and the filtration plate was placed on a transparent collector plate, Nunc conical well 442587 (Thermo Fisher Scientific). After waiting 30 min at RT, the plate was centrifuged at 910rcf for 5 min and the flow-through was discarded. Afterwards, 10µl of the BDT sample was added into the middle of the Sephadex gel. The filtration plate was then placed on a purple sample collector plate and centrifuged at 910rcf for 5 min to collect purified BDT product in collector plate. 10 µl Milli-Q water was added to each well for a total volume of 20 µl. The samples were analysed by the diagnostics department and run through an ABI 3730 genetic analyser (Applied Biosystems®).

3.7.5 Transfection *SMPD4* mutants

HEK293T cells were plated on a 6-well plate (surface 9,6 cm^2) at 1.5x10^5 cells/well on a 24mm coverslip in +/+ DMEM one day prior to the transfection procedure. For the cell transfection with the mutant *SMPD4*, 1.6ml -/-DMEM medium was added to cells. For the DNA mix, 1µg isolated mutated *SMPD4* plasmid was added to 200 µl DMEM -/-. Wild-type human *SMPD4* Myc-DKK-tagged ORF plasmid was used as a control. The transfection reagent mix (for 1 DNA mix) was prepared by adding 200µl medium to 3µl Lipofectamine 2000 Reagent. These mixes were incubated for 5min at RT before adding 200µl of the Lipofectamine mix to the DNA mix. Afterwards, the reaction mix was incubated for 20min at RT. After incubation, 400µl of reaction mix was added to well for a total volume of 2ml. Plates were incubated at 37°C and 5% CO2 for at least 24 hours prior to immunofluorescence staining. After 3hrs of incubation, 10% FCS (200 µl) and 1% PS (20 µl) were added to Hek293T cells.

3.7.6 Antibodies

For immunofluorescence staining of HEK293T transfected cells the following primary antibodies were used: polyclonal anti-human SMPD4 (EMC Biosciences, 1:300), mouse monoclonal anti-Myc (Cell signaling technologies, 1:1000), mouse monoclonal anti-414 covans MMS-120P (Biolegend, 1:500), and monoclonal rabbit anti-human Calnexin (Cell signaling, 1:50). The following secondary antibodies were used: Red Cy3 affiniPure Donkey Anti-Mouse IgG (Jackson Laboratories, 1:100) and goat anti-Rabbit (IgG) Alexa fluor 488 (Thermo Fisher Scientific, 1:300).

3.7.7 Immunofluorescence of exogenous SMPD4

HEK293T cells transfected with mutated Myc-SMPD4 were fixed with 4% PFA in PBS for 20min on ice. Afterwards, cells were washed once with PBS, and blocked and permeabilized in blocking buffer (50 mM Tris HCl [pH 7.4], 0.9% NaCl, 0.25% gelatin, 0.5% Triton X-100) for 1hr on ice. Primary antibodies dissolved in blocking buffer were added to cells and incubated overnight at 4°C. The next day, coverslips were washed once, and the secondary antibodies dissolved in blocking buffer were added to cells. Coverslips were incubated for 1hr at RT in the dark. Coverslips were mounted with ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, USA) to stain nuclei. The coverslips were dried at 37°C before analyzing through confocal microscopy (Leica TCS SP5). The Fiji software was used to determine the colocalization of the SMPD4 mutants with the ER and nuclear pores. To determine if there is a significant difference in colocalization between the SMPD4 wild-type and mutants' expression, GraphPad Prism Software was used to perform an unpaired t-test.

3.8 Fiji software

The Fiji software was used to make a z-stack of the images acquired through confocal microscopy and to visualize the influx of TRITC-labelled 155kDa dextran into segmented nuclei through time by using the Otsu threshold method. To determine the number of proliferated cells during the EdU assay, the colour channels (EdU and DAPI) were split, and the threshold was adjusted for each

channel to automatically measure the Edu+ cells and total cell count (DAPI+) by using the particle analyser application. To quantify the percentage of apoptotic NSCs during the TUNEL apoptosis assay, the particle analyser application was used to automatically count the cells in the DAPI colour channel for the total cell count. Afterwards, the multi-point tool was used to mark and count the cells that presented apoptotic DNA fragmentation (TUNEL+ foci). To determine the colocalization of the mutant SMPD4 with the ER and the nuclear pores, the following steps were performed: the area and slice of interest (transfected cell) were selected, the colour channels were split and the colour brightness was adjusted, and finally, the JACoP plugin was used to adjust the threshold and to determine the Pearson's coefficient.

4. Results

4.1 NEBD

The first aim of this project was to further elucidate the function of SMPD4 in the homeostasis of the nuclear envelope during mitosis, as SMPD4, a transmembrane protein, is hypothesized to have an important role in this process thereby influencing membrane integrity. To study this, a NEBD assay was performed on *SMPD4* knockdown Hela cells transfected with LBR-GFP construct for NE imaging. During this assay, mitotic extracts derived from Xenopus eggs were used to induce mitosis and NEBD, and fluorescent 155kDa-TRITC-dextran was used to observe the gradual loss of the NE permeability barrier by monitoring the gradual diffusion of the fluorescent dextran into the nuclei. The efficiency of the *SMPD4* knockdown on Hela cells can be seen in Appendix III, figure 1. This project is momentarily in progress, and this assay still needs to be performed on control knockdown Hela cells. *In vitro* NEBD process on *SMPD4* knockdown Hela cells was observed through time-lapse confocal microscopy and analyzed with the Fiji Software (Figure 9). The first visible indication of nuclear disassembly was the influx of the fluorescent dextran into the nuclei along with the shrinkage of the nuclei. A decrease in LBR-GFP signal was also observed over time. It was observed that dextran entered 50% of the nuclei 32min after adding the mitotic extracts (Figure 10). After 72min of adding the mitotic extracts, 100% of the analysed cells presented fluorescent dextran in the nuclei.

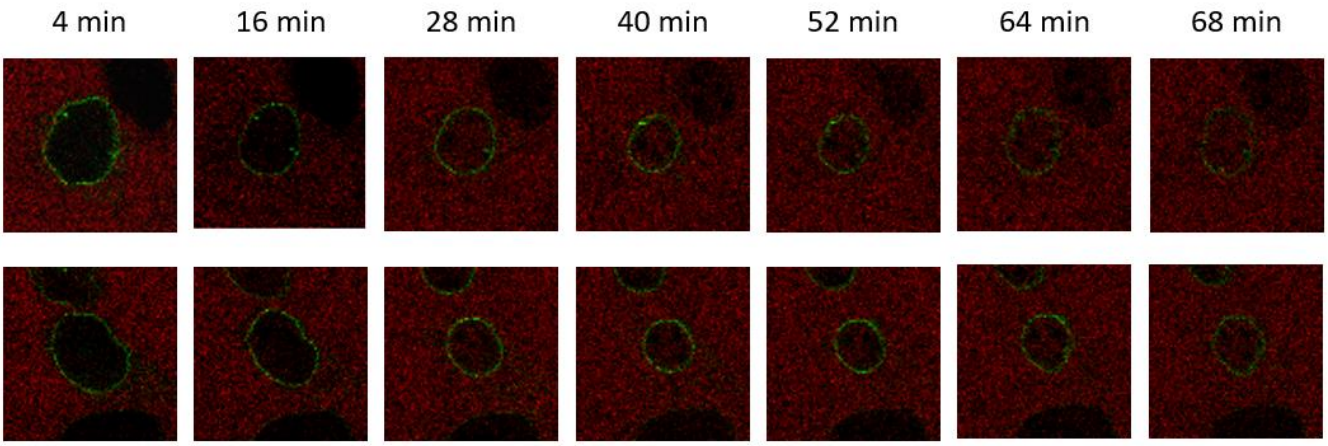

Figure 9. In vitro NEBD in SMPD4 knockdown Hela cells. *Time course of in vitro NEBD of SMPD4 knockdown Hela cells transfected with LBR-GFP (green). Hela cells were permeabilized and incubated with mitotic extract and an energy-generating system to induce NEBD. Mitotic extract was supplemented with fluorescent 155kDa-TRITC-dextran (red) for monitoring of NE permeability. NEBD process was visualized through time-lapse laser-scanning confocal microscopy.*

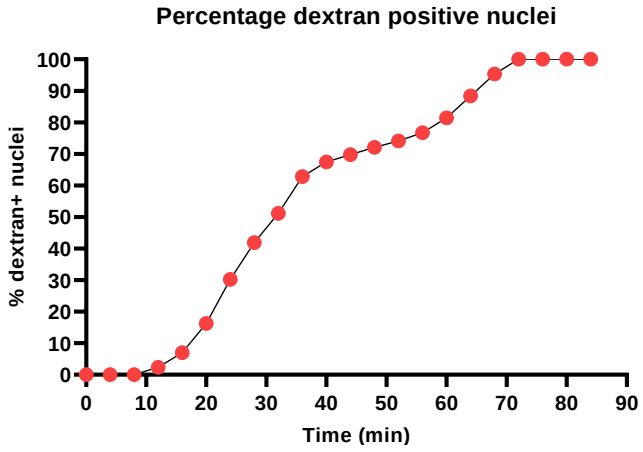

Figure 10. Quantification of dextran-positive nuclei of SMPD4 knockdown Hela cells over the time course of experiment. N=1, n>40 cells

4.2 Proliferation

Decreased neuronal cell proliferation is one of the processes that can lead to congenital microcephaly. To determine the effect of the *SMPD4* knockdown on neuronal cell proliferation a EdU proliferation assay was performed on *SMPD4* knockdown (si*SMPD4*) NSCs to detect DNA replication. After EdU treatment, cell nuclei were stained with DAPI and NSCs were imaged through confocal microscopy. The Fiji Software was used to to analyze and quantify cell proliferation on *SMPD4* knockdown and control NSCs (Figure 11A; and Appendix III figure 3 & 4). The efficiency of the *SMPD4* knockdown on NCSs can be seen in Appendix III, figure 2. *SMPD4* knockdown NSCs show a significant decrease in proliferation when compared to control NSCs, as shown in figure 11B (****p=<0.0001). This experiment was performed in biological duplicate (n=2 experiments), with reproducible results.

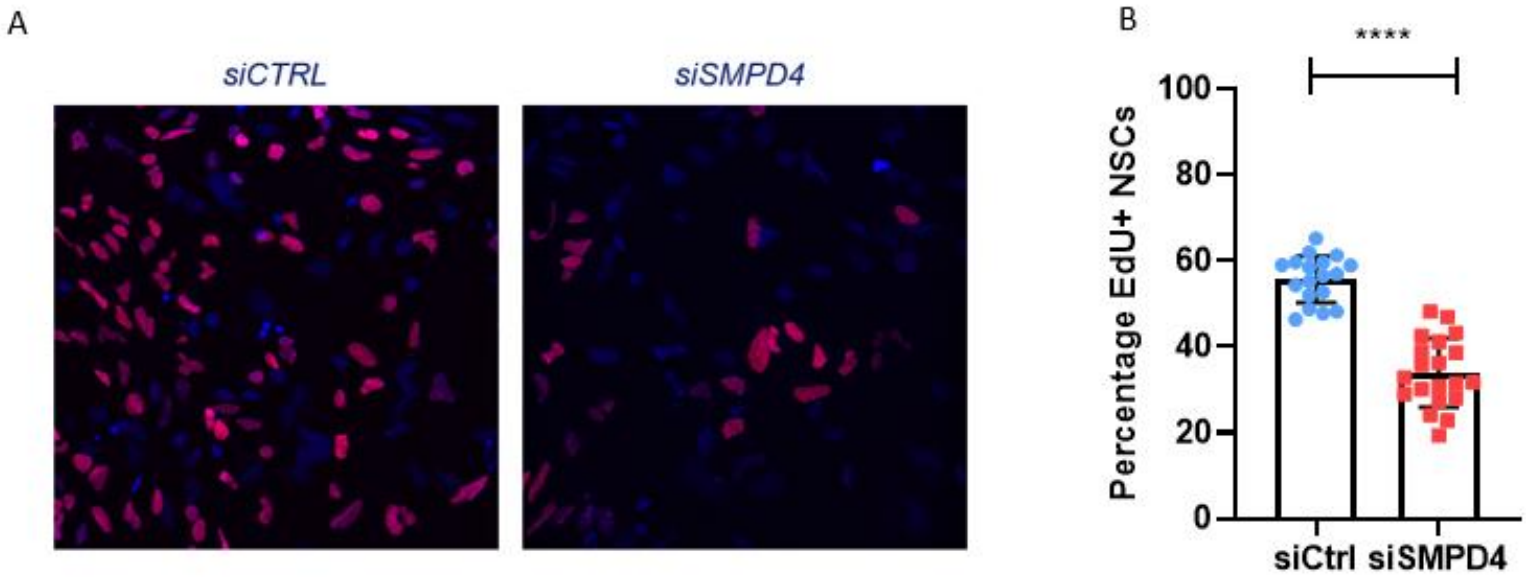

Figure 11. Confocal imaging of control and SMPD4 knockdown proliferating NSCs. *A) After performing a SMPD4 knockdown on NSCs, a EdU proliferation assay was used to detect DNA replication. EdU positive NSCs were visualized with confocal microscopy, shown in red, and the cell nuclei are shown in blue with DAPI. B) Quantification of proliferated NSCs (EdU+) in control and SMPD4 knockdown group. Fiji software was used to make a z-stack of every image and for automated cell count. GraphPad Prism Software was used to perform an unpaired t-test: p= <0,0001 (****).*

4.3 Apoptosis

An increased number of apoptotic cells has been observed in *SMPD4* patients' fibroblasts. Increased apoptosis is another process involved in the pathogenesis of microcephaly. To better understand the effect of the *SMPD4* loss-of-function on neuronal cells, a TUNEL apoptosis assay was performed on *SMPD4* knockdown NSCs 48h after siRNA treatment. The TUNEL method was used to label DNA fragmentation in apoptotic cells, after inducing apoptosis with 0.1mM hydrogen peroxide for 20h in *SMPD4* knockdown NSCs. Afterwards, cell nuclei were stained with DAPI and NSCs were imaged through confocal microscopy. The Fiji Software was used to make Z-stack images and to analyze and quantify *SMPD4* knockdown and control NSCs that present apoptotic DNA fragmentation (Figure 12; and Appendix III figure 5 & 6). *SMPD4* knockdown NSCs show a significant increase in apoptosis when compared to control NSCs, as shown in figure 13 (****p=<0.0001). This experiment was performed in biological duplicate (n=2 experiments), with reproducible results.

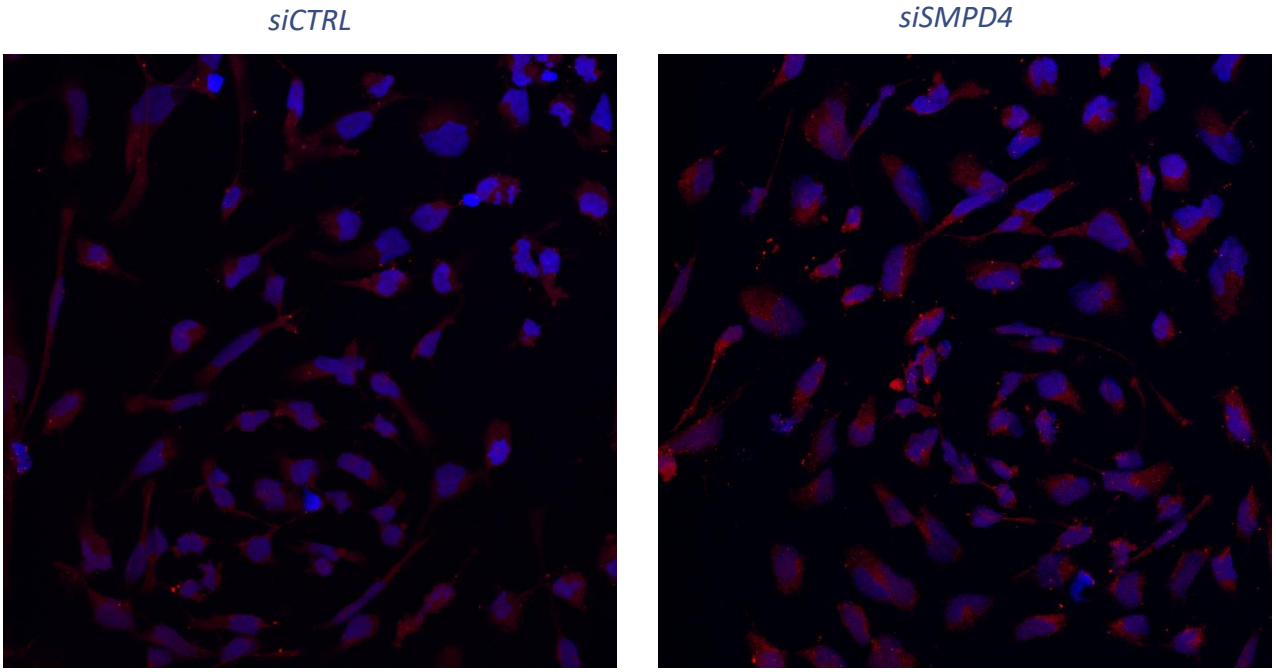

Figure 12. Confocal imaging of control and SMPD4 knockdown apoptotic NSCs. *After SMPD4 knockdown, cells were treated with 0.1mM hydrogen peroxide to induce apoptosis and the TUNEL staining was performed. Apoptotic NSCs presented with apoptotic DNA fragmentation foci (shown in red fluorescence foci) were visualized with confocal microscopy, and the cell nuclei are shown in blue with DAPI.*

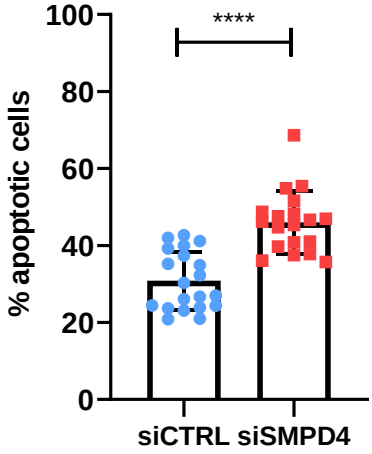

Figure 13. Quantification of apoptotic cells in control and SMPD4 knockdown group.
Fiji software was used to quantify the percentage of cells that presented apoptotic DNA fragmentation (TUNEL+ foci). GraphPad Prism Software was used to perform an unpaired t-test: p= <0,0001 (****).

4.4 Subcellular localization of mutant SMPD4

Previous studies have shown that wild-type SMPD4 is localized to the ER in HEK293T cells. To study the sub-cellular localization of patient-specific missense mutants, HEK293T cells were transfected with either the Myc-tagged wild-type *SMPD4* or the missense mutants. Afterwards, cells were stained with an anti-Myc and a calnexin ER antibody and imaged through confocal microscopy. To determine whether the SMPD4 mutants also colocalize with the ER, the colocalization coefficient of overexpressed wild-type SMPD4 and the patient-specific mutants with the ER was determined with the Fiji Software. Quantitative analysis of colocalization was performed by measuring the Pearson's coefficient of the overlapping area of the Myc-*SMPD4* transfected cell signal with the calnexin ER marker (Figure 14A, and Appendix III figure 7-9). Compared to wild-type SMPD4, the missense Pro446Leu mutation shows significantly less colocalization with the calnexin ER marker (**** p=<0.0001, Figure 14B). It was also observed that the Pro446Leu SMPD4 mutants show a distinct network like structure compared to the wild-type SMPD4 expression. The Leu231Pro SMPD4 mutant shows no significant difference in colocalization with the ER (p=>0.9999, Figure 11B) when compared to wild-type SMPD4 colocalization. This experiment was performed in biological duplicate (n=2 experiment), with reproducible results.

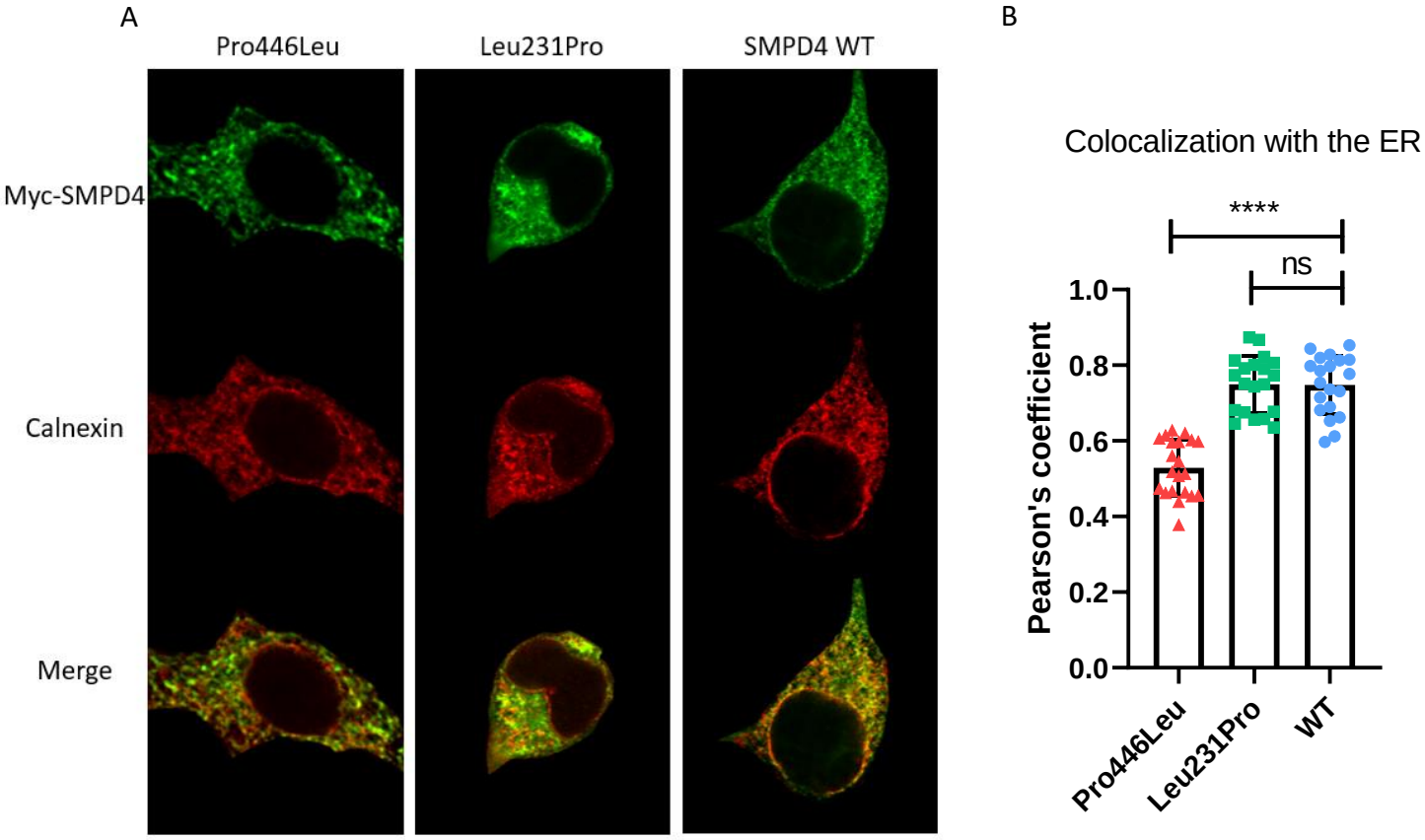

Figure 14. Colocalization of overexpressed Myc-tagged wild-type and mutant SMPD4 with the ER. *A) HEK293T cells were transfected with wild-type Myc-tagged SMPD4 or the patient-specific missense mutants. Cells were fixed and stained with anti-Myc and calnexin antibodies and analysed through confocal microscopy. Colocalization of the ER showed with calnexin (red) and the Myc-tagged SMPD4 (green) was analysed with the Fiji Software. Colocalization can be observed in the lower panel with the merged images (shown in yellow). B) Correlation coefficient analysis of colocalization of Myc-tagged wild-type SMPD4 and its mutants with calnexin. GraphPad Prism Software was used to perform a one-way ANOVA Kruskal-Wallis test: Pro446Leu p=<0.0001 (****); Leu231Pro p=>0.9999 (ns).*

The same procedure was performed to study the colocalization of SMPD4 mutants with the nuclear pores since wild-type SMPD4 has also been localized in the NE. To do this, HEK239T cells transfected with Myc-tagged wild-type and mutant SMPD4 were stained with anti-SMPD4 and anti-414 antibodies to stain SMPD4 and the nuclear pores and imaged through confocal microscopy (Figure 15A, and Appendix III figure 10-12). Compared to wild-type SMPD4, the missense Pro446Leu and Leu231Pro mutations show no significant difference (Pro446Leu p=0,4321, Leu231Pro p=0,5496; Figure 15B) in colocalization with the nuclear pores when compared to wild-type SMPD4 (n=2 experiments).

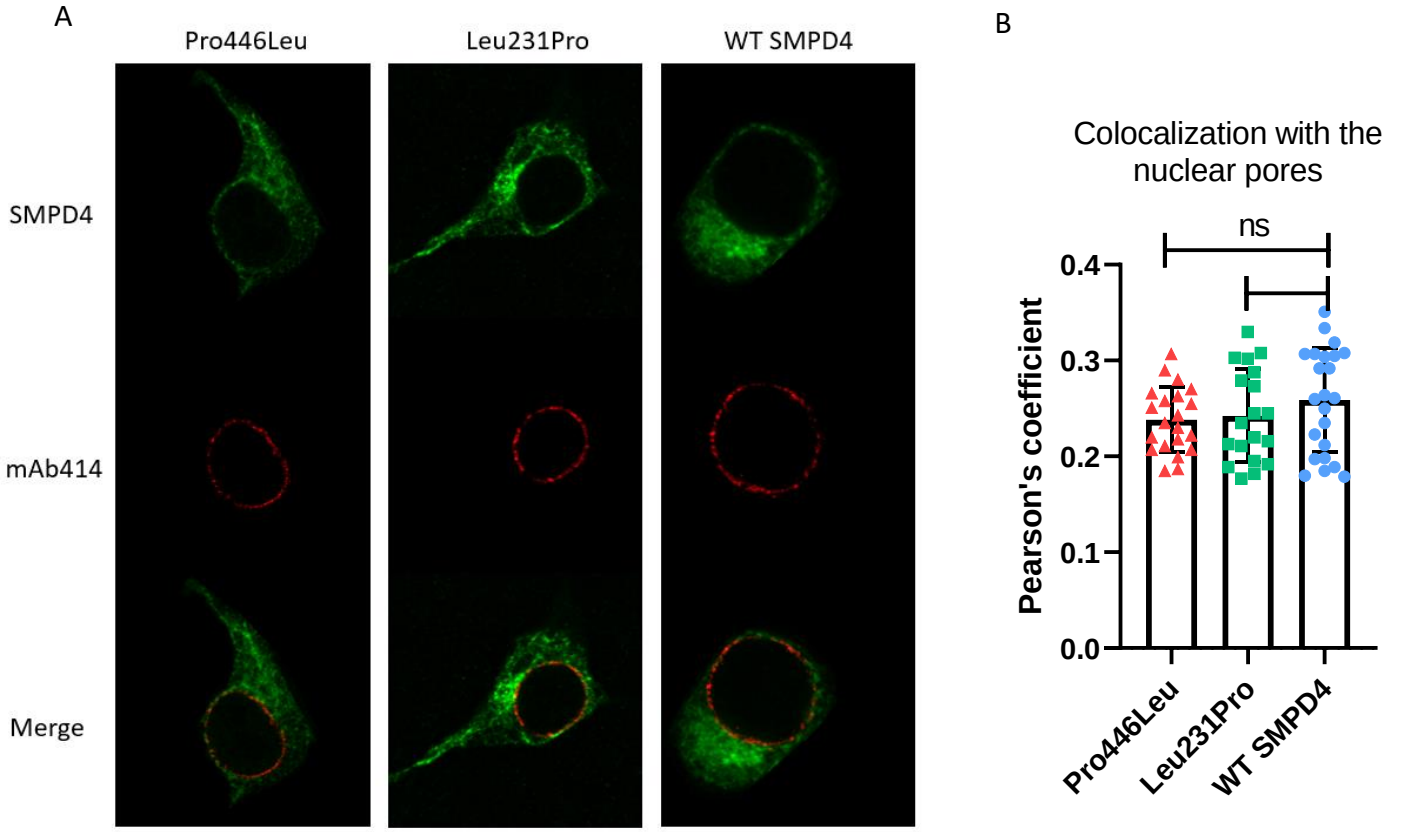

Figure 15. Colocalization of overexpressed exogenous wild-type and mutant SMPD4 with the nuclear pores. *A) HEK293T cells were transfected with wild-type Myc-tagged SMPD4 or the patient-specific missense mutants. Cells were fixed and stained with an anti-SMPD4 and anti-414 antibodies and analysed through confocal microscopy. Colocalization of the nuclear pores showed with mAb414 (red) and SMPD4 (green) was analysed with the Fiji Software. Colocalization can be observed in the lower panel with the merged images. B) Correlation coefficient analysis of colocalization of exogenous wild-type SMPD4 and its mutants with the nuclear pores. GraphPad Prism Software was used to perform a one-way ANOVA Kruskal-Wallis test: Pro446Leu p=0,4321 (ns) and Leu231Pro p= 0,5496 (ns).*

5. Discussion

The Mancini research group has reported that bi-allelic loss-of-function variants in the *SMPD4* gene lead to a severe neurodevelopmental disorder including microcephaly, arthrogryposis, and a profound developmental delay. The focus of this study was to gain more knowledge about the biological mechanisms underlying normal and abnormal brain development and to further characterize and determine the function of the *SMPD4* gene with the goal to improve diagnosis and medical care for these patients. By knowing the processes involved in the pathogenesis of these patients, further research can be done to discover the best treatment for them.

5.1 NEBD

As the homeostasis of the NE during mitosis is an important process for normal cell proliferation, the effect of the SMPD4 loss-of-function on the NEBD was studied by observing this process through time-lapse confocal microscopy in *SMPD4* knockdown Hela cells. Unfortunately, this project is still in progress and this assay still needs to be performed on the control Hela cells, therefore no comparisons can be made to a control group, and no conclusions can be drawn on the effect of the *SMPD4* knockdown on the NEBD process. Furthermore, NEBD results cannot be compared to other literature results as this project used a different experimental setup, as a different NE marker was used which involved an additional transfection procedure and not a stable cell line, and this could have an effect on the results. However, it can be concluded that during this assay the NEBD was successfully induced and observed. The first indication showing nuclear disassembly was the gradual diffusion of fluorescent 155kDa-TRITC-dextran into the nuclei, since the dextran can only enter the nucleus if NEBD is induced, and the NPCs become dismantled. The second indication is the cell shrinkage observed over time. Studies have shown that chromatin condensation during NEBD provides a driving force for nuclear shrinkage (Beaudouin J. 2002). Lastly, all the analysed cells presented fluorescent dextran in the nuclei, after 72min of adding the mitotic extracts, this duration is comparable to other research literature (Linder MI 2017).

As mentioned before, the disassembly of NPCs is a crucial event during NEBD, as this process is required for the permeabilization of the NE barrier and the mixing of cytoplasmic and nuclear components. Little is known about how the NPC disassembly process takes place, but it is known that proper phosphorylation of NPC components is required for efficient NPC disassembly, as delayed NPC disassembly has been observed in phosphorylation defective Nup mutants (Laurell E 2011). It is also known that phosphorylation of NE proteins is a crucial regulatory mechanism that leads to NEBD during mitosis (Beaudouin J. 2002). This is a reason to think that SMPD4, a NE protein, is involved in the NEBD process, as SMPD4 was also found to be enzymatically activated during mitosis by phosphorylation (Kettenbach 2011). SMPD4 catalyzes the hydrolysis of sphingomyelin to produce ceramide. The production of ceramide is known to lead to the permeabilization of membranes and for inducing membrane bending (Goñi FM 2006). This could be an important process for the NEBD to initiate mitosis and the reason why SMPD4 must be enzymatically activated by phosphorylation during mitosis.

Furthermore, previous research performed by the Mancini group, which was focused on characterizing the effect of the *SMPD4* knockdown on the NE, showed several defects in the NE during mitosis. It was observed that *SMPD4* knockdown cells presented an increased number of NE invaginations which correlates to a delayed NEBD or to a NE reassembly defect when the fusion of NE sheets does not happen correctly (Drozdz 2017). It was also found that SMPD4 localizes in the surroundings of centrosomes during mitosis. Centrosomes are believed to dictate the site of NEBD, as they recruit membrane remodeling factors and lipid enzymes, and they push on the NE to form

prophase invaginations during the G2/M transition. During this stage, centrosomes organize the microtubules, which were also found to contribute to the NEBD by pulling on the NE (Bolhy S 2011).

5.2 Cell proliferation

Congenital microcephaly, which is found in all SMPD4 loss-of-function patients, is caused by decreased proliferation in neuronal cells. During this project, the effect of *SMPD4* knockdown on the cell proliferation of NSCs was studied by performing an EdU proliferation assay. This assay is used to detect DNA replication which happens during the Synthesis(S)-phase of the cell cycle. DNA replication is a highly regulated and conserved process to ensure accurate genome duplication and successful cell cycle progression (Takeda 2005). The EdU proliferation assay showed a decrease in proliferation in SMPD4 knockdown NSCs when compared to control NSCs. These results are in line with the observations that loss of SMPD4 in patients leads to microcephaly.

This could be explained by the decreased number of NPCs in *SMPD4* knockdown cells. An intact NE and proper nucleocytoplasmic transport facilitated by NPCs is essential for the DNA replication process. Studies have found that the import of structural and regulatory factors through nucleocytoplasmic transport is necessary for the initiation of DNA replication (Hughes M 1998). Furthermore, it has been observed that SMPD4 patient fibroblasts show a decrease in NPC number not only after mitosis but also at the end of the interphase, and they present a lower nuclear volume (unpublished data). This is known to be caused by decreased nucleocytoplasmic transport (Maeshima 2011) (Mukherjee 2016).

Other studies show that NPCs and Nups play a central role in proper replication fork progression, DNA-damage response and genome stability and integrity in proliferating cells (Whalen 2020). During DNA replication, several replication fork barriers can be encountered which can lead to fork stalling and replication stress. For instance, transcriptional activities and chromatin tethering happening at the NPC regions represent a barrier for proper passage of the replication fork, which leads to increased replication stress. This stress is relieved by the S-phase phosphorylation of Nups checkpoint, which leads to the detachment of tethered chromatins from the NPCs, lessening the replication stress and preventing replication fork collapse (Ibarra 2015).

Furthermore, the core Nup84 complex, the largest nuclear pore subcomplex, is involved in dealing with replication stress and DNA-damage response. During replication stress, the Nup84 complex regulates the recruitment and relocation of collapsed replication forks and persistent DNA lesions to NPCs. Studies have shown that down-regulation of several subunits of this complex leads to hypersensitivity to DNA-damaging agents and accumulation of DNA lesions (Whalen 2020). Interestingly, one of these subunits is Nup133 which has been reported to interact with SMPD4 (Magini P 2019). Downregulation of Nup133 results in the accumulation of spontaneous DNA damage, which suggests an involvement in repair pathways or DNA-damage responses (Whalen 2020).

From these findings it can be hypothesized that the decrease in NPCs in *SMPD4* knockdown cells has a negative effect on the DNA replication process, leading to decreased cell proliferation and subsequently to the patients' phenotype.

5.3 Cell apoptosis

To study the effect of the *SMPD4* knockdown on apoptosis in NSCs, a TUNEL apoptosis assay was performed as increased apoptosis is another process involved in the pathogenesis of microcephaly. This assay was performed to detect DNA fragmentation after inducing apoptosis with 0.1mM hydrogen peroxide in NSCs for 20 hours.

This assay showed that a *SMPD4* knockdown on NSCs leads to increased apoptosis when compared to control NSCs. Ceramide, which is formed when SMPD4 catalyses the hydrolysis of sphingolipids, is suspected to play a role in apoptosis, autophagy and inflammation. Dysregulation of the sphingolipid metabolism can result in a build-up of ceramide, which acts as a proapoptotic lipid signalling molecule, and subsequently lead to increased cell death (Mullen TD 2012). However, the absence of SMPD4 would lead to decreased ceramide levels in the cell, as SMPD4 is necessary for the hydrolysis of sphingolipids. Studies have shown that decreased mitochondrial ceramide levels result in functional and structural alterations in mitochondria leading to neuronal cell death and neurodegeneration. (Schwartz 2018). There are two main apoptosis pathways, the extrinsic death receptors activation pathway and the intrinsic mitochondria pathway. Mitochondrial dysfunction is known to induce apoptosis by activating the intrinsic mitochondrial apoptosis pathway. During the mitochondrial apoptosis pathway, stress signals induce mitochondrial outer membrane permeabilization (MOMP). This permeabilization process allows the release of apoptogenic proteins to the cytosol and the activation of the caspase cascade which leads to cell apoptosis. Several studies have reported that ceramide accumulation induces mitochondrial apoptosis by directly inducing and/or regulating MOMP or by activating proteins that regulate this process (Patwardhan 2016).

It was formerly hypothesized that the lower number of NPCs found in *SMPD4* knockdown cells leads to increased DNA damage. Persistent DNA lesions during DNA replication can trigger DNA damage-induced apoptosis, as DNA damage is one of the stress signals that can induce MOMP and promote caspase activation (Norbury 2004). It is believed that DNA double-stranded breaks arise from recurrent DNA lesions during DNA replication, as this process leads stalled or collapsed replication forks to be attacked by nucleases. The formed DNA double-stranded breaks were found to correlate with intrinsic apoptosis induction (B. 2003).

As previously mentioned, SMPD4 loss-of-function causes prolonged mitosis in patient fibroblasts and *SMPD4* knockdown NSCs, indicating a possible cell cycle delay in M-phase. This could also explain the apoptosis increase in SMPD4 knockdown cells since cell cycle delays can induce apoptosis. Prolonged mitosis of progenitor cells during neurogenesis is known to have an effect on cell fate alterations associated with microcephaly. Prolonged mitosis of radial glial progenitor cells leads to decreased generation of new progenitor cells by directly producing neurons. Furthermore, during prolonged apoptosis, radial glial cells are more likely to produce apoptotic progeny (Pilaz LJ 2016).

5.4 *SMPD4* patient-specific missense mutations localization

The Mancini group has been able to localize wild-type SMPD4 in the outer NE and the ER. Moreover, a proteomics analysis showed that SMPD4 interacts with multiple NPC proteins (Magini P 2019). This project aimed to determine the effect of SMPD4 mutants on protein localization and if it differs from the wild-type SMPD4's subcellular localization.

Missense mutations can influence protein stability, structure, dynamics, subcellular localization and expression. A change in subcellular localization of a protein can have a detrimental effect on a cell, as this localization provides a specific environment for proper protein function, activity in signaling pathways, and interactions with other proteins (Zhang 2012). To study this, two patient-specific missense SMPD4 mutants, Pro446Leu and Leu231Pro, were created through site-directed mutagenesis. A third mutant, Pro192Leu, is still in the process of being created and was not included in the analysis yet. These mutations were introduced into a Myc-tagged wild-type *SMPD4* plasmid, which was subsequently transfected into HEK293T cells. Afterwards, an immunofluorescence staining was performed to detect the mutant SMPD4 and the ER or the nuclear pores. Colocalization analysis determined that the Leu231Pro SMPD4 mutant shows similar colocalization with the ER and the nuclear pores as the SMPD4 wild-type colocalization. Therefore, it can be concluded that the Leu231Pro has no effect on the subcellular localization of the protein. The pathogenesis of this missense mutation could be caused by an alteration on the binding site of the protein effecting protein–protein or protein-membrane interactions (Zhang 2012). The Pro446Leu mutant shows no significant difference in its colocalization with the nuclear pores, yet it did show significantly less colocalization with the ER when compared to wild-type SMPD4.

SMPD4 synthesis happens at the ribosomes, which can be found in the cell cytoplasm or attached to the ER. Afterwards, newly synthesized SMPD4 enters the lumen of the ER where SMPD4 is folded. Protein folding is a crucial process that regulates the proteins biological activity and stability and its final cellular location (Dobson 2003). Missense mutations are also known to cause misfolding of proteins and ER retention since misfolded proteins are usually unable to present exit signals. These processes are known to cause ER stress in cells (Kincaid 2007). This phenomenon could explain why the Pro446Leu SMPD4 mutant shows decreased localization throughout the whole ER. Misfolded protein gets retained and accumulates at the lumen of the ER and does not get released and diffused to the rest of the ER where it normally localizes. The retention of SMPD4 in the ER lumen may lead to loss-of-function of the SMPD4 protein as it never reaches its final subcellular localization, and it may result in toxic accumulation in the ER (Stefl 2013). These events would most likely clarify the pathogenicity of this missense mutation.

6. Conclusion

The first aim of this project was to determine the effect of the *SMPD4* loss-of-function on the homeostasis of the NE during mitosis. This project also aimed to study the effect of the *SMPD4* loss of function on neuronal cell proliferation and apoptosis. Lastly, this project aimed to study the localization of patient-specific mutant SMPD4 and if it differs from wild-type SMPD4.

The NEBD assay is momentarily still in progress and therefore no conclusions can be drawn.

It can be concluded from the EdU proliferation assay that *SMPD4* knockdown leads to decreased proliferation in NSCs, which would explain the patients' phenotype, as reduced proliferation of neuronal cells leads to congenital microcephaly. It is hypothesized that the decrease in NPCs in *SMPD4* knockdown cells has a negative effect on the DNA replication process, leading to decreased cell proliferation. Furthermore, an increase in apoptosis was observed during the TUNEL apoptosis assay in *SMPD4* knockdown NSCs when compared to control NSCs, indicating that *SMPD4* knockdown cells are more susceptible to apoptotic cell death. This is another neurodevelopmental process involved in the pathogenesis of microcephaly. Finally, it can be concluded that the missense Leu231Pro mutation has no effect on the subcellular location of the SMPD4 protein. However, the Pro446Leu mutation leads to mislocalization of the SMPD4 protein, most likely due to protein misfolding and ER retention.

7. Recommendations

Firstly, it would be recommended to further look into the replication stress process to determine if the loss-of-function of SMPD4 does indeed lead to increased replication stress, leading to decreased cell proliferation and subsequently to the patients' phenotype. Replication stress recruits several DNA repair checkpoint proteins, such as the ataxia telangiectasia and Rad3-related protein (ATR). ATR is the central kinase that detects DNA damage and once activated it induces a signaling cascade that promotes cell survival by restarting/rescuing stalled replication forks (Zeman 2014). To determine if the activation of the ATR pathway is increased in SMPD4 loss-of-function cells, a qPCR analysis could be performed to detect ATR expression (Fischer 2016).

Secondly, it would be recommended to determine if SMPD4 loss-of-function leads to increased activation of the intrinsic mitochondrial apoptosis pathway, as this project has reported that *SMPD4* knockdown in NSCs leads to increased apoptosis. This could also be determined by performing a qPCR analysis. The activation of the intrinsic mitochondrial apoptosis pathway causes cytochrome c to be released into the cytosol, where it subsequently binds to Apaf-1 leading to the formation of apoptosomes. Apoptosome formation leads to the activation of the executioner caspases and finally to cell death (Johnson CE 2007). A qPCR analysis of cytochrome c and Apaf1 expression levels in *SMPD4* knockdown cells would indicate if SMPD4 loss-of-function leads to increased activation of the intrinsic mitochondrial apoptosis pathway.

Lastly, it was previously hypothesised that the Pro336Leu missense mutation leads to mislocalization of the SMPD4 protein, most likely due to protein misfolding and ER retention. The activation of the autophagy-lysosome pathway is required for the clearance of accumulated misfolded proteins in the ER. Autophagy is an intracellular catabolic process that plays a crucial role in the removal of misfolded and accumulated proteins by delivering these components to the lysosomes for digestion (Metcalf 2021). A colocalization experiment could be performed with a lysosome-associated membrane protein 2 (LAMP2) antibody as a marker for lysosomes, simultaneously with an SMPD4 marker to confirm the presence of misfolded SMPD4 in lysosomes.

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Appendix I. Abbreviations

Abbreviation	Description
BSA	Bovine Serum Albumin
CNS	Central nervous system
ER	Endoplasmic reticulum
FAM	Carboxyfluorescein
FCS	Fetal Calf Serum
FLICA	Fluorochrome-Labeled Inhibitor of Caspases
Fw	Forward primer
GFP	Green fluorescent protein
HEK	Human Embryonic Kidney
hNSCs	Human neural stem cells
INM	Inner nuclear membrane
LBR	Lamin B receptor
MCD	Malformations of cortical development
MOMP	Mitochondrial outer membrane permeabilization
NE	Nuclear envelope
NEBD	Nuclear envelope breakdown
NPCs	Nuclear pore complexes
ONM	Outer nuclear membrane
PBS	Phosphate-Buffered Saline
PDL	Poly-D-lysine
PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
Rv	Reverse primer
SDM	Site-Directed Mutagenesis
S-phase	Synthesis-phase
siRNA	Short interfering RNA

Appendix II. Protocols

1. Cell culture

Chemicals, solutions and samples

- Complete cell medium Hela cells (+/+ DMEM: 1x Dulbecco's Modified Eagle Medium with 10% Fetal Calf Serum (FCS) and 1% Pen-Strep, Gibco)
- Complete cell medium hNSCs (KnockOut™ DMEM/F12 supplemented with: 2mM L-Glutamine; 20ng/ml EGF (Peprotech); 20ng/ml bFGF (Prepotech); and 2% StemPro® Neural Supplement.
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membare Matrix, 0.5% in cell medium (Gibco)
- Phosphate Buffered Saline (PBS) (Sigma Life Science)
- Cell detachment solution: Hela cells → Trypsin/EDTA; hNSCs → Accutase (Sigma)
- Pen-Strep (Gibco)
- 10% DMSO

1.1 Thawing cells

1. Prior to thawing hNSCs, a desired flask must be coated with Geltrex for 1h at 37°C. Hela cells do not require pre-coating.
2. Take your vial of frozen cells out of the -80°C freezer or nitrogen and thaw them quickly by gentle agitation in a 37°C water bath.
3. When the cells are almost thawed move the vial to the hood.
4. Dissolve the thawed cells in 10ml pre-warmed complete medium.
5. Centrifuge cell suspension for 5min at 1000 rpm.
6. While centrifuging, prepare your desired flask. Fill a (pre-coated) T75 flask with 12ml pre-warmed complete medium.
7. After centrifugation, aspirate the supernatant without disturbing the cell pellet.
8. Dissolve the pellet in 1ml of pre-warmed complete medium and add cell suspension to prepared flask.
9. Check the cells under the microscope.
10. Incubate at 37°C and 5% CO_2 with caps loosened.

1.2 Splitting cells

1. Prior to splitting hNSCs, desired flask or well must be coated with Geltrex for 1h at 37°C.
2. Remove all medium from culture flask.
3. Wash cells with PBS.
4. Add cell detachment solution (Hela: Trypsin/EDTA; hNSCs: Accutase) and incubate for 5 min at 37°C.
5. After cell detachment, add 10 ml complete cell medium to Hela cells or PBS to NSCs.
6. Centrifuge cell suspension for 5min at 1000 rpm.
7. Remove supernatant and resuspend pellet in complete medium.
8. Add cells to flasks or wells at desired concentration.
    a. Split NSCs every 72hrs at 1:2.
    b. Split Hela cells every 72hrs at 1:8.
9. Add 1% Penstrep to NSCs.
10. Incubate at 37°C and 5% CO_2 with caps loosened.

1.3 Freezing cells

1. Prepare 10% DMSO in complete medium. You need 0.5ml 10% DMSO per vial; 1ml per T75 flask.
2. Wash cells with PBS.
3. Add cell detachment solution (Hela: Trypsin/EDTA; hNSCs: Accutase) and incubate for 5 min at 37°C.
4. After cell detachment, add 10 ml complete cell medium to Hela cells or PBS to NSCs.
5. Centrifuge cell suspension for 5min at 1000 rpm.
6. Remove supernatant and resuspend pellet in 10% DMSO. Add 1ml for a t75 flask.
7. Transfer 0.5ml to cryovial.
8. Place cryovial in an isopropanol chamber and stored at –80°C overnight.
9. Transfer frozen cells to liquid nitrogen.

2. Transfection

2.1 *SMPD4* knockdown

Chemicals, solutions and samples

- Complete cell medium for hNSCs (KnockOut™ DMEM/F12 supplemented with: 2mM L-Glutamine; 20ng/ml EGF (Peprotech); 20ng/ml bFGF (Prepotech); and 2% StemPro® Neural Supplement).
- DMEM (1x Dulbecco's Modified Eagle Medium) for Hela cells. Use medium without supplements for transfection mic preparation!
- *SMPD4* siRNA (5 nmol, Dharmacon)
- siGENOME Non-Targeting Control siRNA #3 (5 nmol, Dharmacon)
- DharmaFect 3 transfection reagent (Dharmacon,
- FCS (Gibco)
- Pen-Strep (Gibco)

Procedure

One day prior to the siRNA transfection procedure, split cells to a 6-well plate (3-4x10^5 cells/well) or to a 12-well plate (1.0x10^5 cells/well). Grow cells on sterilized coverslips when using for imaging. When working with NSCs, Poly-D-lysine (PDL) coated coverslips are used and subsequently coated with Geltrex for 1h at 37°C prior to procedure. During this procedure, DMEM medium without extra supplements is used for Hela cells because serum contains albumin which negatively interacts with your lipo-reagent. siRNA transfection procedure for 6-well plate (2 wells; 1 si*SMPD4* & 1 siCTRL):

1. Wash cells slowly with 2ml medium.
2. Add 1.8ml fresh medium to wells.
3. Prepare knockdown reaction mix by adding the following in 3 different tubes:
    a. 100µl medium to 2.5 µl si*SMPD4*
    b. 100µl medium to 2.5 µl siCTRL
    c. 200µl medium to 5 µl DharmaFect.
4. Incubate mixes for 5 min.
5. Add 100µl of DharmaFect mix (c) to tube a and b.
6. Incubate reaction mix for 20min at RT.
7. Add 200µl of the corresponding tube to the corresponding well for a total volume of 2ml.
8. Incubate plate at 37°C and 5% CO2 until further use.
9. This procedure needs to be repeated the next day on NSCs to increase transfection efficiency.
10. (For Hela cells → After 3hrs of incubation, add 10% FCS (200 µl) and 1% Pen-Strep (20 µl)).

2.2 LBR-GFP transfection

Chemicals, solutions and samples

- DMEM (1x Dulbecco's Modified Eagle Medium) for Hela cells. Use medium without supplements for transfection mix preparation!
- LBR-GFP construct (1210 ng/ml)
- Lipofectamine 3000 Reagent, 1 mg/ml (Thermo Fisher Scientific, Lithuania)
- FCS (Gibco)
- Pen-Strep (Gibco)

Procedure

For the NEBD assay, *SMPD4* knockdown Hela cells need to be transfected with a LBR-GFP construct to be able to visualize the NE during breakdown. The following protocol is for 6-well plate (2 wells; 1 si*SMPD4* & 1 siCTRL)

1. Wash Hela cells slowly with 2ml medium.
2. Add 1.8ml fresh medium to wells.
3. Prepare transfection reaction mix by adding the following in 2 different tubes:
    a. 200µl medium to 1.66 µl LBR-GFP construct
    b. 200µl medium to 4 µl Lipofectamine 3000 Reagent
4. Incubate mixes for 5 min.
5. Add 200µl of Lipofectamine mix to LBR-GFP mix.
6. Incubate reaction mix for 20min at RT.
7. Add 200µl of the transfection mix to cells for a total volume of 2ml.
8. Incubate plate at 37°C and 5% CO2 until further use.
9. After 3hrs of incubation, add 10% FCS (200 µl) and 1% Pen-Strep (20 µl) to Hela cells.

3. qPCR

3.1 RNA extraction

To determine if the *SMPD4* knockdown was successful, extract RNA from the transfected cells and perform a qPCR. The RNeasy mini kit (Qiagen; Hilden, Germany) will be used to extract and purify RNA:

1. Remove medium from cells.
2. Add 350 µl RLT buffer to well and scrap cells.
3. Transfer cells to microcentrifuge tube and add 1 volume of 70% ethanol (350 µl). Mix well by pipetting up and down, and vortexing.
4. Transfer up to 700 µl of your sample to a RNeasy Mini spin column in a collection tube and centrifuge for 15sec at >8000g. Discard flow-through.
5. Add 700 µl RW1 buffer to spin column and centrifuge for 15sec at >8000g. Discard flow-through.
6. Add 500 µl RPG buffer to column and centrifuge for 15sec at >8000g. Discard flow-through.
7. Repeat step 6 but centrifuge column for 2min.
8. Place column in a new empty collection tube and centrifuge column for 1min at >8000g.
9. Place column in a new 1.5ml collection tube. Add 20 µl RNase-free water directly to membrane. Centrifuge for 1min at >8000g to elute RNA.
10. Determine the concentration and purity of the purified RNA with a Nanodrop™ Spectrophotometer.

3.2 cDNA synthesis

Make cDNA from the extracted RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA):

1. Add the following: 4 µl 5x iScript buffer, 1 µl iScript reverse transcriptase, 1 µg purified extracted RNA, and Milli-Q for a total reaction volume of 20 µl.
2. Place samples in a thermocycler machine, C1000 Touch (Bio-Rad, USA) and use the following program: 25°C for 5 min, 42°C for 30 min, and 95°C for 1 min.

3.3 qPCR

1. Prepare primer mix by adding 1µl forward (fw) primer, 1 µl reverse (rv) primer and 98 µl Milli-Q. Two housekeeping gene primers are used for this experiment when working with NSCs: *HPRT1* (fw exon 3 primer 5'- TTGCTGACCTGCTGGATTAC -3', rv exon 4 primer 5'- TGTTGACTGGTCATTACAATAGCTC -3'), and *TBP* (fw exon 2 primer 5'- CTTACGCTCAGGGCTTGG-3', rv exon 3 primer 5'- ATAGGCTGTGGGGTCAGTCC -3'). When working with Hela cells, the following qPCR primers are used: *CLK2* (fw primer 5'- TCGTTAGCACCTTAGGAGAGG -3', rv primer 5'-TGATCTTCAGGGCAACTCG-3'), and *RNF111* (fw primer 5'- GCAGAATGCAGCAGAAGTTG -3', rv primer 5'- CCATTCTTGCAGAAGTGGTTG -3'). The qPCR *SMPD4* primers used for this experiment were fw primer 5'- GACTCCCAGCCCCGGTGT-3', rv primer 5'- CCACTCGGAACACCATGAG -3'.
2. Prepare cDNA mix by adding:
    - 79.86 µl iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, USA)
    - 6.39 µl cDNA
    - 41.25 µl Milli-Q.
3. Prepare negative control mix by adding: 12.68 µl iTaq Universal SYBR Green Supermix and 21.42 µl Milli-Q.
4. Combine DNA and primer mixes using 9.9 µl of the primer mix and 39.6 µl of the DNA mix per primer.
5. Afterwards, add 15 µl of each sample in triplo in a qPCR plate.
6. Use the following qPCR program: 1x 95°C for 3min; 39x 95°C for 5 sec, 60°C for 30sec; 1x 65°C for 5 sec; and 1x 95°C for 5 sec.
7. Use CFX software to analyze the SYBR green qPCR measurements.

4. NEBD assay

Chemicals, solutions and samples

- Permeabilization buffer (20 mM Hepes, pH 7.4, 110 mM KoAc, 5 mM MgOAc, 0.5 mM EGTA and 250 mM sucrose)
- 155kDa TRITC-dextran (25mg/ml in permeabilization buffer)
- 20x Energy mix (200mM creatine phosphate, 10mM GTP, and 10mM ATP in 50mM HEPES pH 7.0; add 1mg/ml creatine kinase and 250mM sucrose; snap freeze and store in small aliquots at -80°C)
- NEBD reaction mix (for 1 well: 20µl mitotic extract, 2.75µl permeabilization buffer, 1µl 155kDa TRITC-dextran, and 1.25 µl 20x Energy mix)

Procedure

Work on ice during the entire procedure.

1. Grow Hela cells on coverslips and perform siRNA and LBR-GFP transfection prior to NEBD assay.
2. Place cells on a live-imaging microscope chamber.
3. Wash cells with ice cold PBS.
4. Permeabilize cells in ice cold permeabilization buffer containing 40µg/ml digitonin for 10min at RT.
5. Wash cells 3 times with ice-cold permeabilization buffer (without digitonin) for 2, 5 and 10 minutes. Use cells for disassembly reaction within the next 10 min.
6. Prepare NEBD reaction mix.
7. After the final washing step, remove permeabilization buffer and add 25µl NEBD reaction mix. Mount a coverslip on top of the other coverslip.
8. Perform laser-scanning microscopy of in vitro disassembly reaction, using a confocal microscope, scanning at 20sec intervals. Excitation: GFP at 488 nm and TRITC at 561 nm.

5. EdU assay

To study the effect of the *SMPD4* knockdown on the proliferation of NSCs, an EdU assay will be performed. The Click-iT Plus EdU Imaging Kit (Life Technologies) will be used for this procedure.

1. Plate NSCs on a 12-well plate ($1.0x10^5$ cells/well). Grow cells on sterilized PDL + Geltrex coated coverslips.
2. Perform siRNA transfection procedure to knockdown *SMPD4* and allow cells to recover overnight.
3. Add 1ml of 10µM EdU solution and incubate cells for 24hrs at 37°C and 5% CO2. (Add 1µl 10mM EdU to 1ml medium)
4. After incubation, fix the cells in 4% PFA in PBS for 15min at RT.
5. Wash cells twice with 1ml 3% Bovine serum albumin (BSA) in PBS.
6. Permeabilize cells by adding 1ml of 0.1% Triton in PBS and incubate for 20min at RT.
7. In the meanwhile, prepare reaction mixes:
    I. Dilute the EdU buffer additive 1:10 in demi water. For 2 coverslips, dilute 10µl 10x buffer additive (solution F) in 90µl demi water)
    II. Prepare 1x Clicl-iT reaction buffer by diluting component D 1:10. For 2 coverslips, Dilute 100µl component D in 900µl demi water.
8. Prepare Click-iT Plus reaction cocktail mix. Add the following solutions in the order listed below:

I. 860µl 1x Click-iT reaction buffer.
II. 40µl Copper protectant (Component E)
III. 3.25µl Alexa Fluor 647 picolyl azide (Component B)
IV. 100µl reaction buffer additive

You need to use reaction cocktail mix within 15min of preparation!

9. Wash cells with 3% BSA in PBS after permeabilization.
10. Add 0.5ml Click-iT Plus reaction cocktail mix to each well. Protect plate from light and rock plate lightly for 30 minutes at RT.
11. Remove the reaction cocktail and wash cells once with 3% BSA in PBS. Remove wash solution.
12. Mount coverslips on microscopy slides using 20 µl ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific) to stain nuclei.
13. Image cells by means of confocal microscopy to measure the number of NSCs that proliferated during EdU incubation. Alexa Fluor 647 excitation: 650nm; emission: 670nm.
14. Count the number of EdU+ and DAPI+ cells using the Fiji Software:
    I. Split channels
    II. Z-project: max intensity.
    III. Threshold: default.
    IV. Analyze particles: size: 10 – infinity.

6. TUNEL apoptosis assay

To detect cell apoptosis in *SMPD4* knockdown NSCs a TUNEL Assay will be performed.

Perform siRNA transfection on NSCs ($1.5x10^5$ cells/well on 12-well plate) prior to apoptosis induction and TUNEL assay.

1. Dilute 1µl 30% H_2O_2 in 99µl Milli-Q for a final concentration of 100mM. Add 1µl to cells (in 1ml medium) for 0.1mM.
2. Incubate cells at 37°C and 5% CO_2 for 20 hours.
3. After incubation, fix the cells in 1ml 4% PFA in PBS for 15min at RT.
4. Wash cells once with 1ml 3% Bovine serum albumin (BSA) in PBS.
5. Permeabilize cells in 1ml 0.1% Triton X-100 in PBS for 20min at RT.
6. Wash cells twice with 3% BSA in PBS.
7. Add 100 µl TdT reaction buffer to coverslip and incubated for 10min at RT.
8. In the meanwhile, prepare the TdT reaction buffers by adding the following (for 2 coverslips (18mm)):
    I. 94 µl TdT reaction buffer
    II. 2 µl EdUTP
    III. 4 µl TdT enzyme.
9. Add 50 µl of the TdT reaction cocktail to each coverslip and incubate for 1hr at 37°C in a humidified chamber.
10. Wash cells were twice with 3% BSA in PBS for 2min.
11. Prepare the Click-iT reaction cocktail by adding the following (for 2 coverslips (18mm)):
    I. 64.35 µl Click-iT reaction buffer (containing Alexa Fluor 647 azide)
    II. 1.65 µl Click-iT reaction buffer additive.
12. Add immediately 33 µl of the Click-iT reaction cocktail to the cells and incubate for 30 min at RT in the dark.
13. Afterwards, wash cells with 3% BSA in PBS for 5min.

14. Finally, mount coverslips on microscopy slides using 10 µl ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, USA) to stain nuclei.
15. Cells were imaged by means of confocal microscopy and the Fiji software was used to quantify the number of cells that present apoptotic DNA fragmentation (TUNEL+ foci) and the number of DAPI+ cells. To determine if there is a significant increase in apoptotic cells in the *SMPD4* knockdown group, GraphPad Prism Software was used to perform an unpaired t-test.
16. Image cells by means of confocal microscopy to measure the number of apoptotic NSCs. Alexa Fluor 647 excitation: 650nm; emission: 670nm.
17. Count the number of TUNEL+ and DAPI+ cells using the Fiji Software:
    I. Split channels
    II. Z-project: max intensity.
    III. Adjust threshold to default and analyze particles (size: 10 – infinity) to count DAPI+ cells (total cell count)
    IV. Use the multi-point tool to mark and count the cells that present apoptotic DNA fragmentation.

7. Mutant SMPD4 expression in Hek293T cells

7.1 Site-Directed Mutagenesis (SDM)

To create the patient specific mutations in wild-type *SMPD4* plasmid, the Quick-Lightning Site-Directed Mutagenesis (SDM) Kit (Agilent Technologies, USA) was used.

1. Prepare reaction mix by adding the following:
    I. 5 µl 10x reaction buffer
    II. 0.5 µl Wild-type human *SMPD4* Myc-DKK-tagged ORF plasmid (100ng, Origene)
    III. 1.25 µl fw primer (125ng, (IDT; Leuven, Belgium))
    IV. 1.25 µl rv primer (125ng, (IDT; Leuven, Belgium))
    V. 1 µl dNTP mix, 1.5 µl QuickSolution reagent
    VI. 1 µl QuickChange Lightning Enzyme
    VII. 38.5 µl ddH_2O (for a total volume of 50 µl).
2. Use the following thermal cycling program:
    I. 1x 95°C for 2min
    II. 30x 95°C for 20sec, 60°C for 10sec, 68°C for 3min
    III. 1x 68°C for 5 sec.

7.2 Transformation

1. Thaw 70 µl XL10-Gold *Escherichia coli Bacteria* ultracompetent cells (Agilent Technologies, USA) on ice and add 5 µl SDM PCR.
2. Incubate on ice for 15 min.
3. Perform transformation by means of heat shock at 42°C for 45 sec subsequently with 2min incubation on ice.
4. Add 500 µl LB broth (EZ Mix, Lennox) to bacterial cells and incubate under agitation (200rpm) at 37°C for 1h.
5. Add 100 µl of transformation mix to LB-Ampicillin (0.1% Ampicillin) agar plates and incubated overnight at 37°C.
6. Select transformed bacterial colonies and grow colonies to 50ml midiprep.
7. Isolate mutated *SMPD4* plasmid with the Plasmid Plus Midi kit (Qiagen; Hilden, Germany).
8. To determine if the SDM procedure was successful, sequence isolated plasmid DNA by means of Sanger Sequencing method.

7.3 DNA isolation

For the isolation of the mutated plasmid, use the NucleoBond Xtra Maxi kit for transfection-grade plasmid DNA (Macherey-Nagel; Düren, Germany).

7.4 Sanger sequencing

PCR reaction

1. Create a PCR reaction mix by adding the following substances (volumes shown below are for 1 sample):
    - 13.75 µl H_2O
    - 2 µl 10x Fast Start Buffer
    - 1.6 µl dNTPs (10mM)
    - 1 µl fw primer
    - 1 µl rv primer
    - 0.1 µl Fast Start Taq polymerase
2. Use 20 µl of the PCR master mix for each sample and add 0.5 µl of the isolated DNA sample.
3. Place the samples in a thermocycler machine, C1000 Touch (Bio-Rad) and run the following program:
    - 95°C for 5 minutes
    - 25 cycles:
        i. 95°C for 30 seconds
        ii. 58°C for 30 seconds
        iii. 72°C for 2 min
    - 72°C for 5 minutes
4. Run the PCR product through an agarose gel to confirm the PCR reaction was successful.

Exo-Sap

1. Add 1 µl Exo-Sap-it® Affymetrix (Thermo Fisher) to 5 µl DNA from PCR and 5 µl Milli-Q. Exo-Sap-it hydrolyses excess primers and nucleotides from the previous PCR reaction. (ExoSAP-IT™ PCR Product Cleanup Reagent sd)
2. Place the sample in a thermocycler machine, C1000 Touch (Bio-Rad) and run the following program:
    - 37°C for 45 min
    - 80°C for 15 min to deactivate the Exo-Sap-it.

BDT reaction

1. Create a BDT reaction mix by adding the following substances (volumes shown below are for 1 sample):
    - 3.5 µl 5x Sequence buffer (400mM Tris-HCl pH 9.0, 10mM $MgCl_2$, Applied Biosystems®)
    - 1 µl either fw or rv primer (10µM, IDT)
    - 0.5 µl Big Dye Terminator (BDT) v3.1 (Applied Biosystems®)
2. Add 5 µl BDT mix to 5 µl DNA from Exo-Sap.
3. Place the sample in a thermocycler machine, C1000 Touch (Bio-Rad) and run the following program:
    - 96°C for 45 sec
    - 24 cycles:
        i. 96°C for 10 sec
        ii. 58°C for 5 sec
        iii. 60°C for 2 min

Sephadex purification of BDT product

1. Add Sephadex powder to black metal plate mould. Sephadex is a gel filtration medium that desalts, removes contaminants and exchanges buffers. (Sephadex® G-50 sd)
2. Transfer Sephadex powder from mould to a filtration plate. And place filtration plate on a transparent collector plate.
3. Pipet 300µl demi water into Sephadex and wait for 20-30 min at RT.
4. Spin plate for 5 min at 910 rcf and discard the flow-through afterwards.
5. Pipet 10µl of your BDT sample into the middle of your Sephadex.
6. Get a purple sample collector plate and place it under the filtration plate.
7. Spin plate for another 5 min at 910 rcf to collect purified BDT product in collector plate.
8. Check whether all well have ~10 µl and add 10 µl Milli-Q water to each well for a total volume of 20 µl.
9. Place a plastic cover on the top of the purple plate and bring the samples to the diagnostics department where the samples will be run through an ABI 3730 genetic analyzer (Applied Biosystems®) or store samples in a freezer at -20°C until further use.

7.5 Transfection

1. Plate HEK293T cells on a 6-well plate (surface 9,6 cm^2) at 5x10^4 cells/cm^2 on a 24mm coverslip in +/+ DMEM.
2. One day after plating the cells, add 1.6ml -/-DMEM medium to cells.
3. For the DNA mix, add 1µg isolated mutated *SMPD4* plasmid to 200 µl DMEM -/-.
4. Prepare transfection reagent mix (for 1 DNA mix) by adding 200µl medium to 3µl Lipofectamine 2000 Reagent.
5. Incubate mixes for 5min at RT before adding 200µl of the Lipofectamine mix to the DNA mix.
6. Incubate reaction mix for 20min at RT.
7. After incubation, add 400µl of reaction mix to well for a total volume of 2ml.
8. Incubate plates at 37°C and 5% CO2 for at least 24hours before immunofluorescence staining. After 3hrs of incubation, add 10% FCS (200 µl) and 1% PS (20 µl) to cells.

7.6 Immunofluorescence staining

1. Fix HEK293T cells transfected with mutated Myc-SMPD4 with 4% PFA in PBS for 20min on ice.
2. Wash cells once with PBS
3. Block and permeabilize cells in blocking buffer (50 mM Tris HCl [pH 7.4], 0.9% NaCl, 0.25% gelatin, 0.5% Triton X-100) for 1hr on ice.
4. Add primary antibodies dissolved in blocking buffer to cells and incubate overnight at 4°C.
    a. polyclonal anti-human SMPD4 (1:300) was used to stain SMPD4, when simultaneously staining nuclear pores with mouse monoclonal anti-414 covans MMS-120P (1:500).
    b. mouse monoclonal anti-Myc (1:1000) was used to stain Myc-tagged SMPD4 when simultaneously staining the ER with monoclonal rabbit anti-human Calnexin (1:50)
5. The next day, wash coverslips once with PBS and add the secondary antibodies dissolved in blocking buffer to cells: Red Cy3 affiniPure Donkey Anti-Mouse IgG (1:100) and goat anti-Rabbit (IgG) Alexa fluor 488 (1:300).
6. Incubate coverslips in the dark at RT for 1hr.
7. Mount coverslips with ProLong Gold antifade reagent with DAPI and let the coverslips dey at 37°C before analyzing through confocal microscopy.
8. Use the Fiji software was to determine colocalization of SMPD4 mutants with the ER and nuclear pores.
    a. Select the area and slice of interest (transfected cell)

b. Split the colour channels
c. Adjust brightness
d. Use the JACoP plugin to adjust the threshold and to determine the Pearson's coefficient.

Appendix III. Supplementary figures

1. qPCR *SMPD4* knockdown

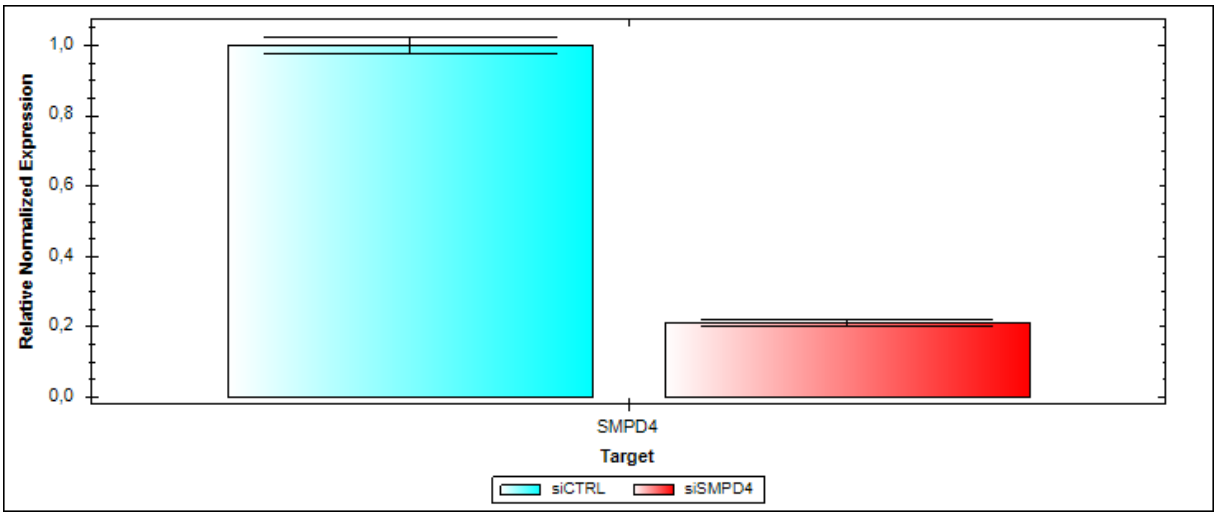

Figure 1. qPCR expression data of SMPD4 knockdown in Hela cells*. The expression of SMPD4 was determined 24hours after the siCTRL (blue bar) and siSMPD4 (red bar) transfection in Hela cells. These results show a SMPD4 knockdown of approximately 80% in Hela cells.*

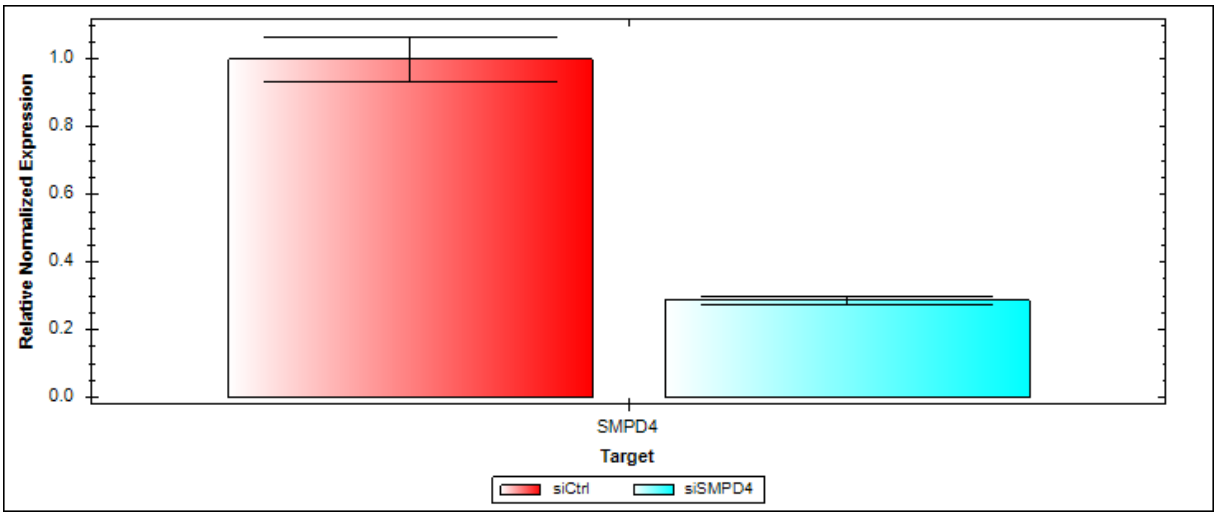

Figure 2. qPCR expression data of SMPD4 knockdown in NSCs. *The expression of SMPD4 was determined 24hours after the siCTRL (red bar) and siSMPD4 (blue bar) transfection, which was performed twice during two consecutive days in Hela cells. These results show a SMPD4 knockdown of approximately 70% in NSCs.*

2. Sequencing PCR primers

Table 1. Primers used to sequence entire SMPD4 plasmid after SDM procedure.

SMPD4 primer		**Sequence (5'-3')**
SMPD4 1	FW	GACGCAAATGGGCGGTAGG
	RV	CTGCTGCAGGTGAGGGAACG
SMPD4 2	FW	GGCGGCTTCCATCTCTGAGG
	RV	TGGATGGACGCCTTCACAGG
SMPD4 3	FW	TGGATGGACGCCTTCACAGG
	RV	TGGTGGAGGCCATAGGAAGC
SMPD4 4	FW	GTGCCCCCACCACTCTCC
	RV	GCGCTGGAGACACTGAGTCG
SMPD4 5	FW	GAGATCTGGAGGTCAGAAACTCTGC
	RV	AGGGTGAGGCCTGCTCTGG
SMPD4 6	FW	AGGGTGAGGCCTGCTCTGG
	RV	AGGGTGAGGCCTGCTCTGG
SMPD4 7	FW	CACCCTTTGTCCAGGAGAACC
	RV	GGCCAGGGTGACAGGAAGC
SMPD4 8	FW	GCTGAGATGATTCAGAAAGGTGAGC
	RV	GCCCAGCCATGAGAGGAAGG
SMPD4 9	FW	TCGCTCAGCTCATCACACAGG
	RV	CAGGGGCGTAAGGATGAGTCC
SMPD4 10	FW	GCAGTTCACACTCGCCTTGG
	RV	CCAGCCCAGGTTCTGTGAGG
SMPD4 11	FW	TGGTCCGCACACTCTTTAGGC
	RV	CCCGACGCAGAACAGAGAGG
SMPD4 12	FW	CACCTGCTGAGCCCTGTGG
	RV	GGCACCTTCCAGGGTCAAGG

3. EdU proliferation assay

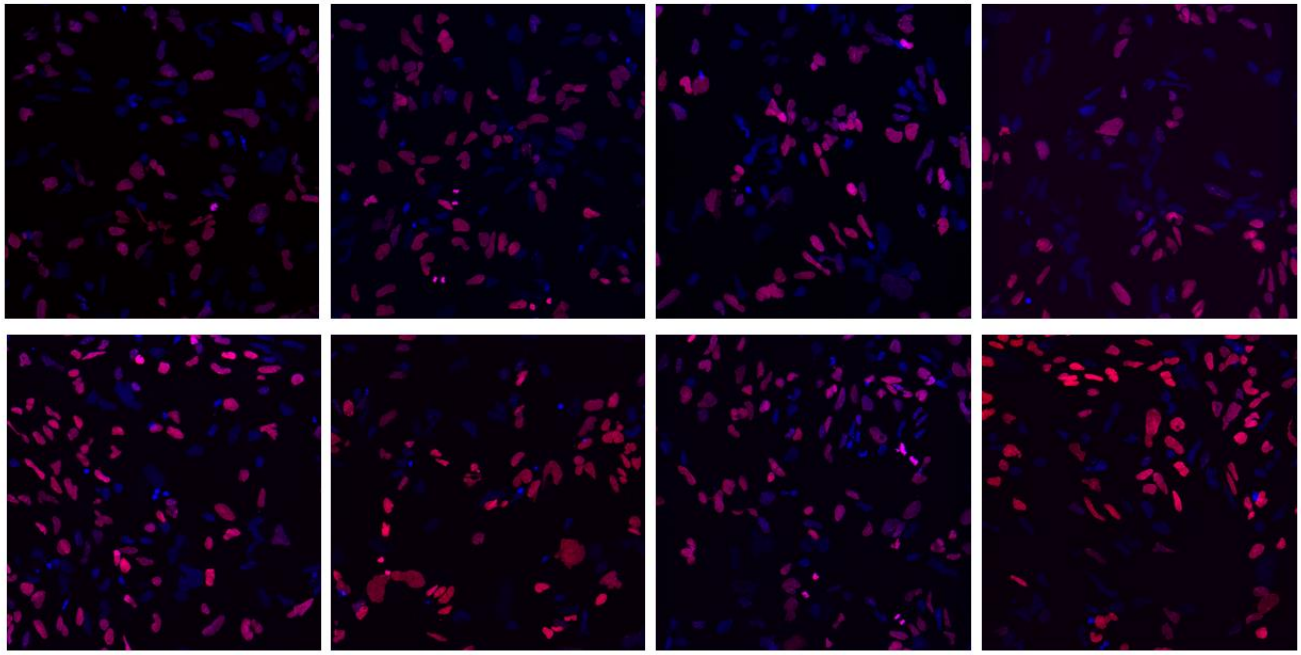

Figure 3. EdU proliferation assay of control knockdown NSCs.
Confocal microscopy imaging of siCTRL NSCs after EdU proliferation assay. Proliferated cells (EdU positive cells) are shown in red, and the cell nuclei stained with DAPI are shown in blue.

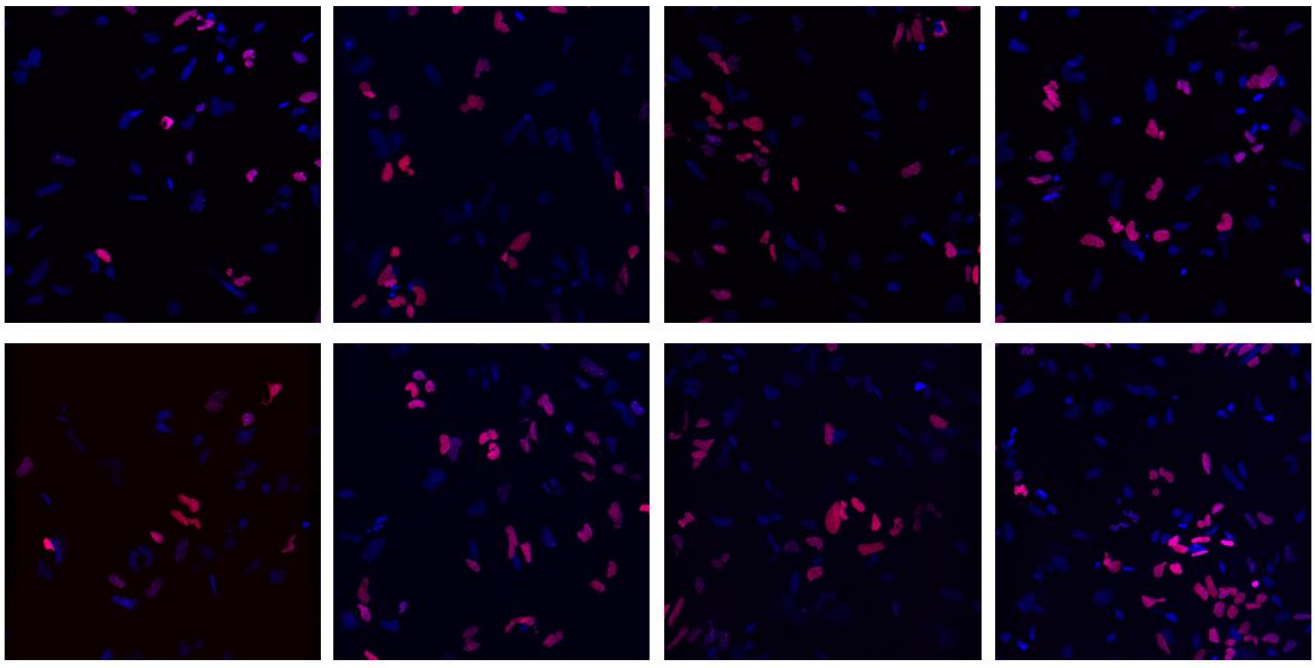

Figure 4. EdU proliferation assay of SMPD4 knockdown NSCs
Confocal microscopy imaging of siSMPD4 NSCs after EdU proliferation assay. Proliferated cells (EdU positive cells) are shown in red, and the cell nuclei stained with DAPI are shown in blue.

4. TUNEL apoptosis assay

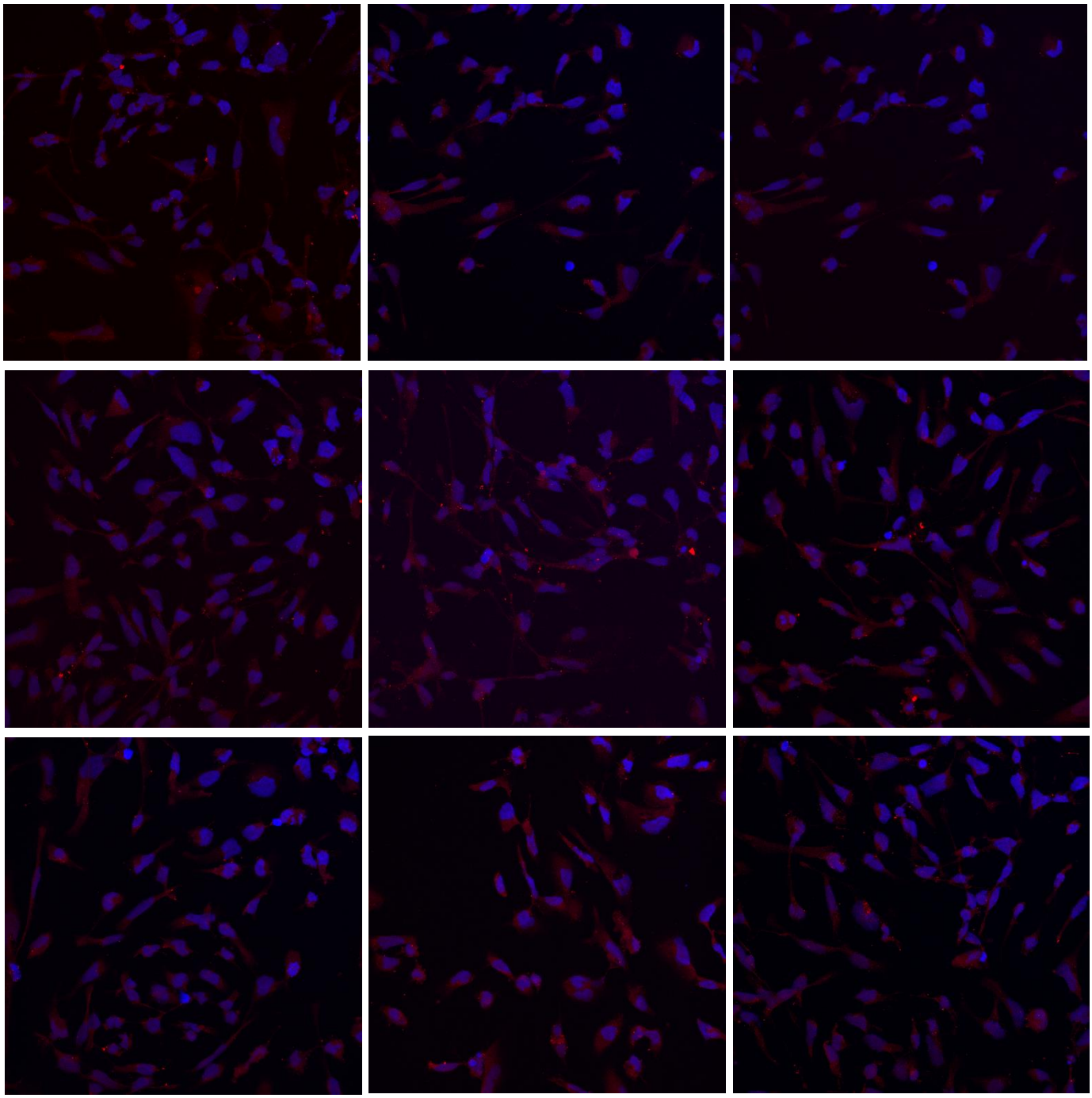

Figure 5. TUNEL apoptosis assay** of **control knockdown NSCs.
Confocal microscopy imaging of siCTRL NSCs after TUNEL apoptosis assay. Apoptotic cells are presented with apoptotic DNA fragmentation (shown in red fluorescence foci), and the cell nuclei stained with DAPI are shown in blue.

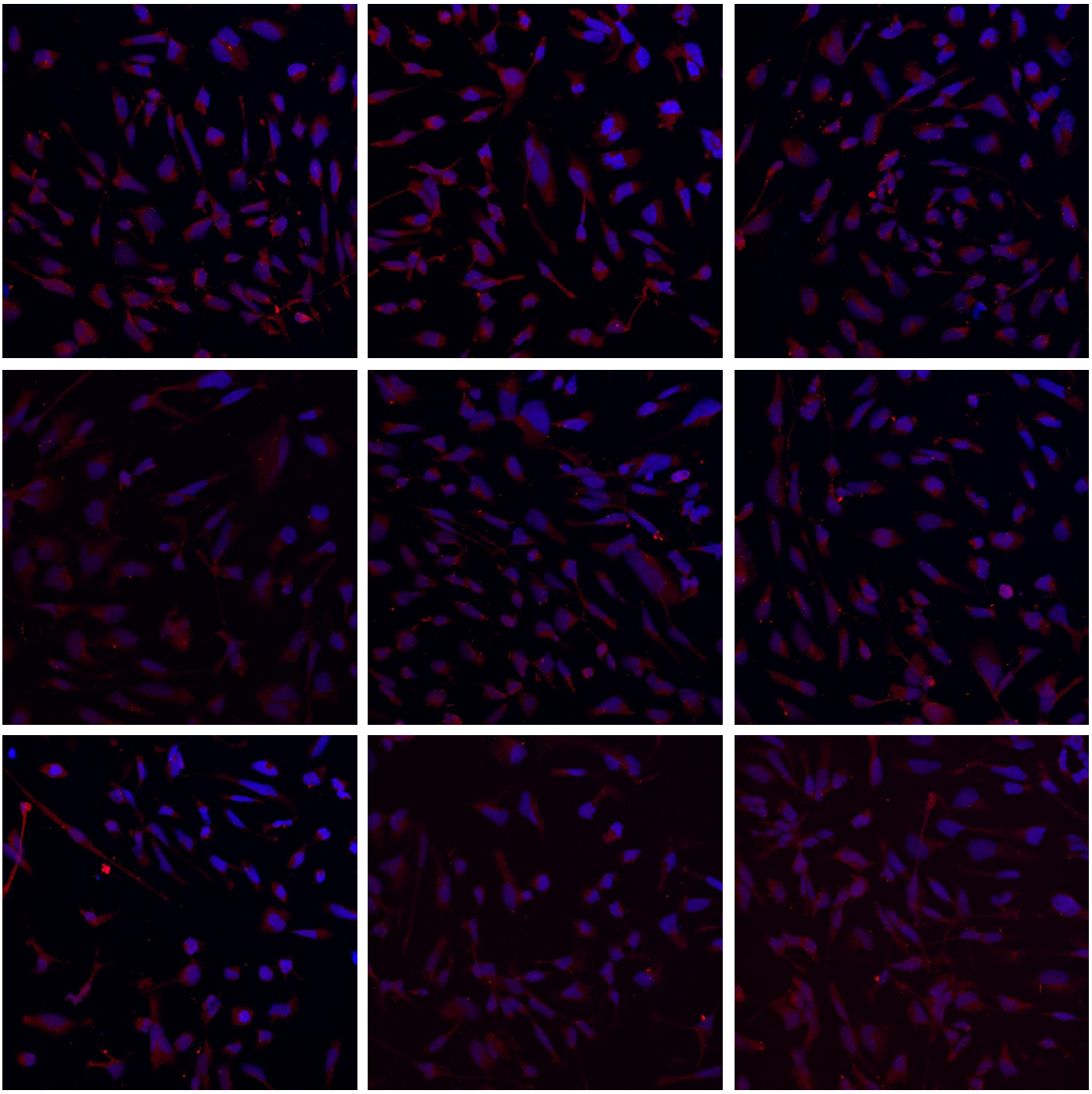

Figure 6. TUNEL apoptosis assay of SMPD4 knockdown NSCs.
Confocal microscopy imaging of siSMPD4 NSCs after TUNEL apoptosis assay. Apoptotic cells are presented with apoptotic DNA fragmentation (shown in red fluorescence foci), and the cell nuclei stained with DAPI are shown in blue.

5. Colocalization with the ER

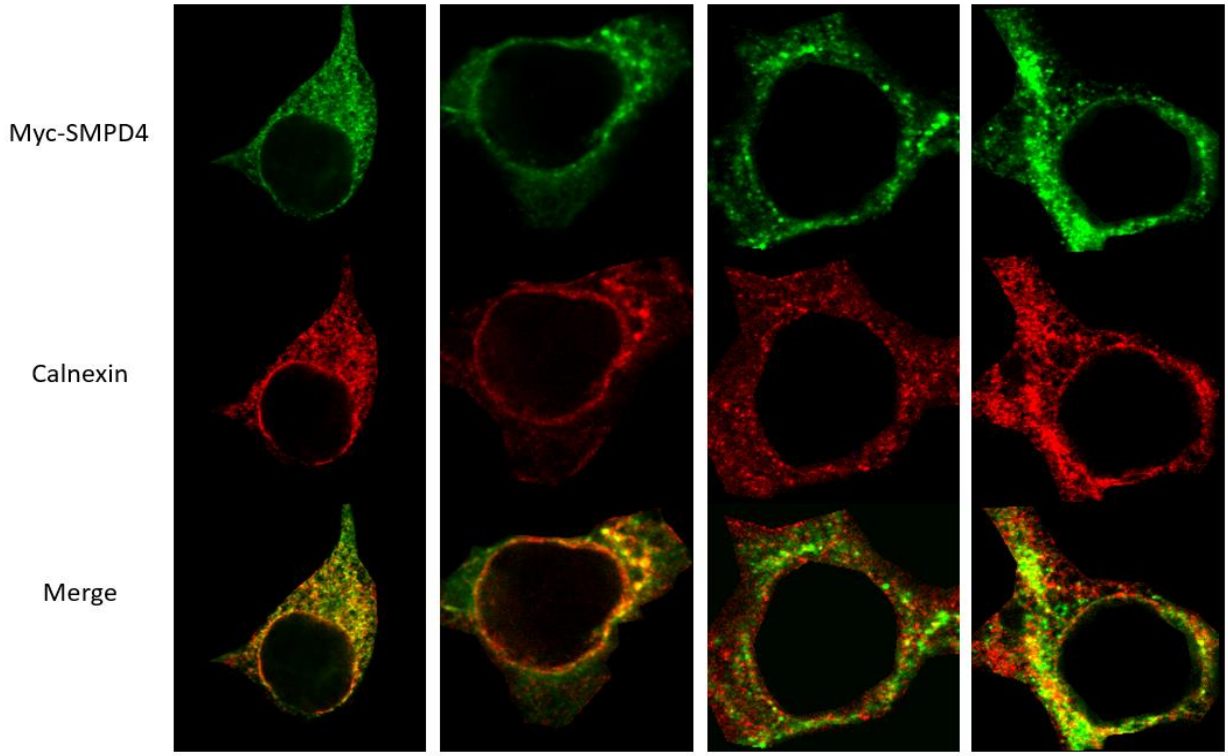

Figure 7. Colocalization of overexpressed Myc-tagged wild-type SMPD4 with the ER. *HEK293T cells transfected with wild-type Myc-tagged SMPD4 were fixed and stained with anti-Myc and calnexin antibodies and analysed through confocal microscopy. Colocalization of the ER showed with calnexin (red) and the Myc-tagged SMPD4 (green) was analysed with the Fiji Software. Colocalization can be observed in the lower panel with the merged images (shown in yellow).*

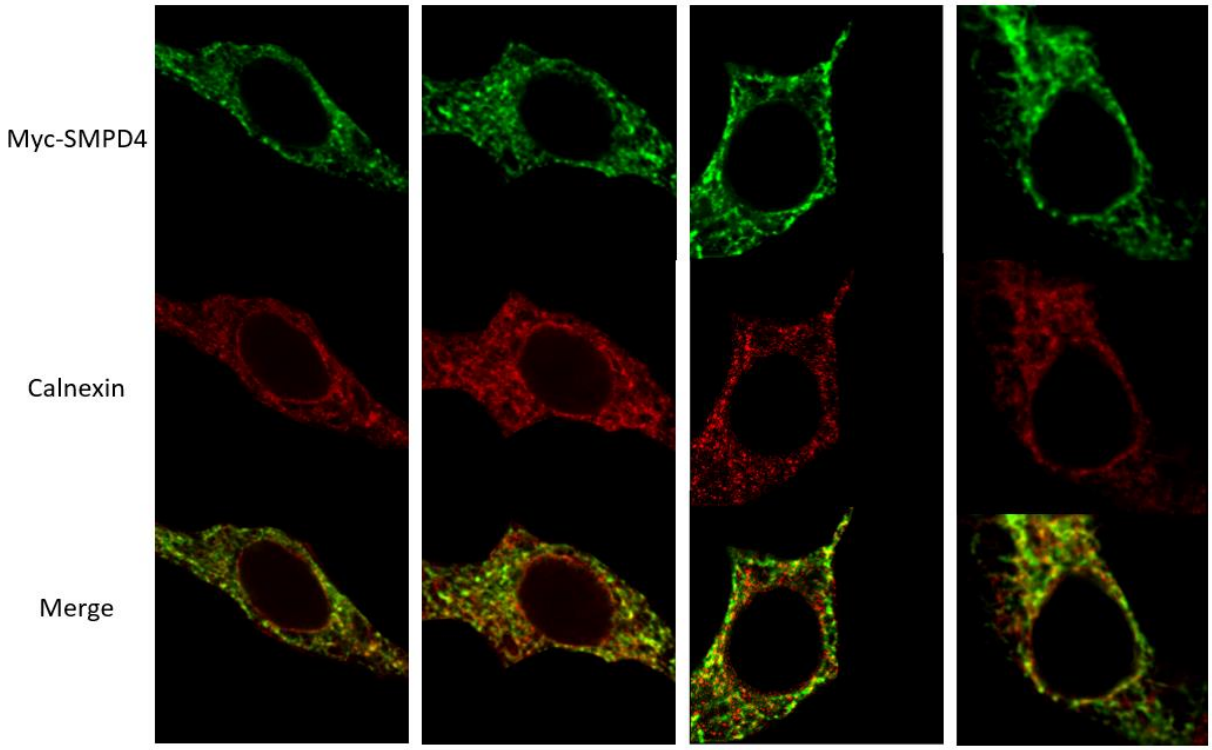

Figure 8. Colocalization of overexpressed Myc-tagged Pro446Leu SMPD4 mutant with the ER. *HEK293T cells transfected with Myc-tagged Pro446Leu SMPD4 were fixed and stained with anti-Myc and calnexin antibodies and analysed through confocal microscopy. Colocalization of the ER showed with calnexin (red) and the Myc-tagged SMPD4 (green) was analysed with the Fiji Software. Colocalization can be observed in the lower panel with the merged images (shown in yellow).*

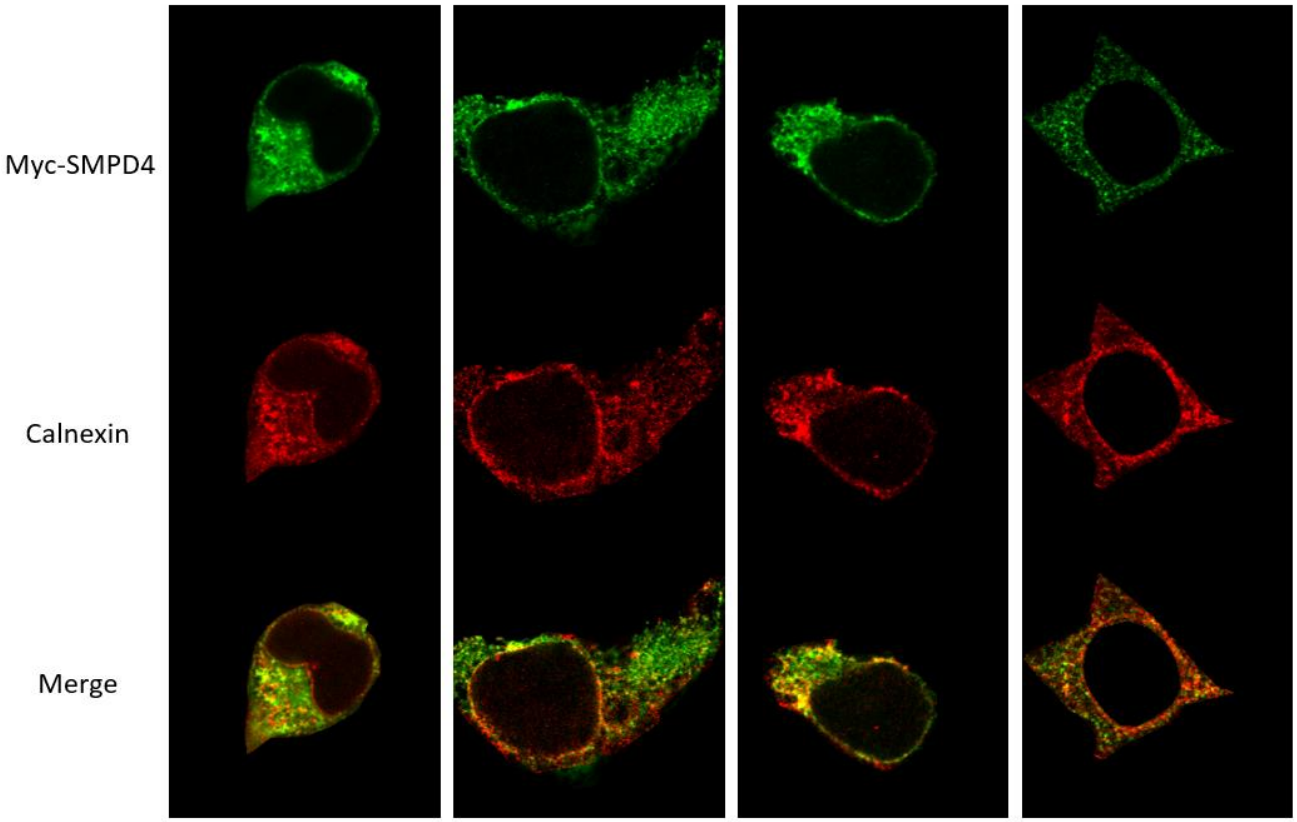

Figure 9. Colocalization of overexpressed Myc-tagged Leu231Pro SMPD4 mutant with the ER. *HEK293T cells transfected with Myc-tagged Leu231Pro SMPD4 were fixed and stained with anti-Myc and calnexin antibodies and analysed through confocal microscopy. Colocalization of the ER showed with calnexin (red) and the Myc-tagged SMPD4 (green) was analysed with the Fiji Software. Colocalization can be observed in the lower panel with the merged images (shown in yellow).*

5. Colocalization with nuclear pores

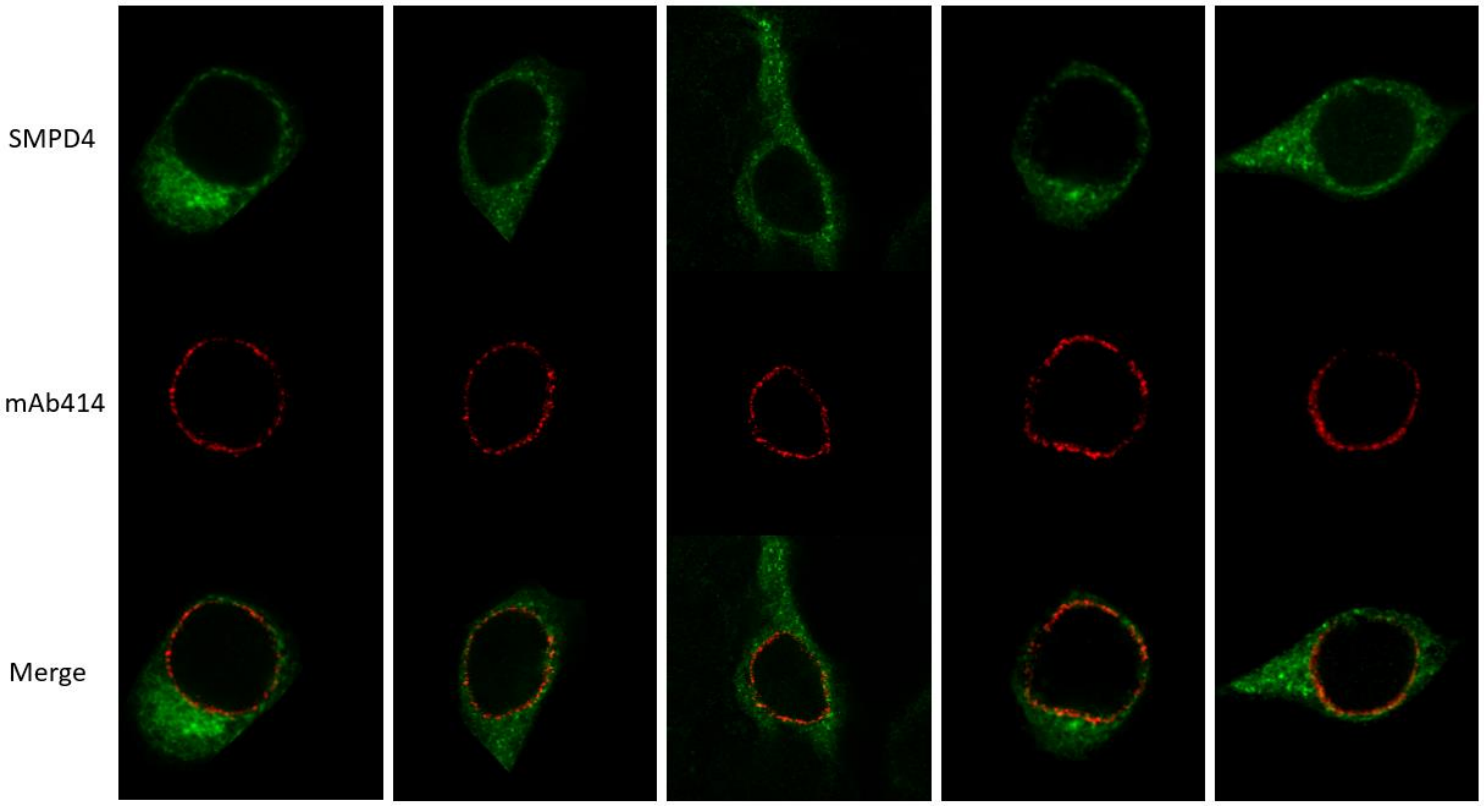

Figure 10. Colocalization of overexpressed exogenous wild-type SMPD4 with the nuclear pores. *HEK293T cells transfected with wild-type Myc-tagged SMPD4 were fixed and stained with an anti-SMPD4 and anti-414 antibodies and analysed through confocal microscopy. Panel shows colocalization of SMPD4 (in green) and the nuclear pore marker (mAb414), along with a merged image.*

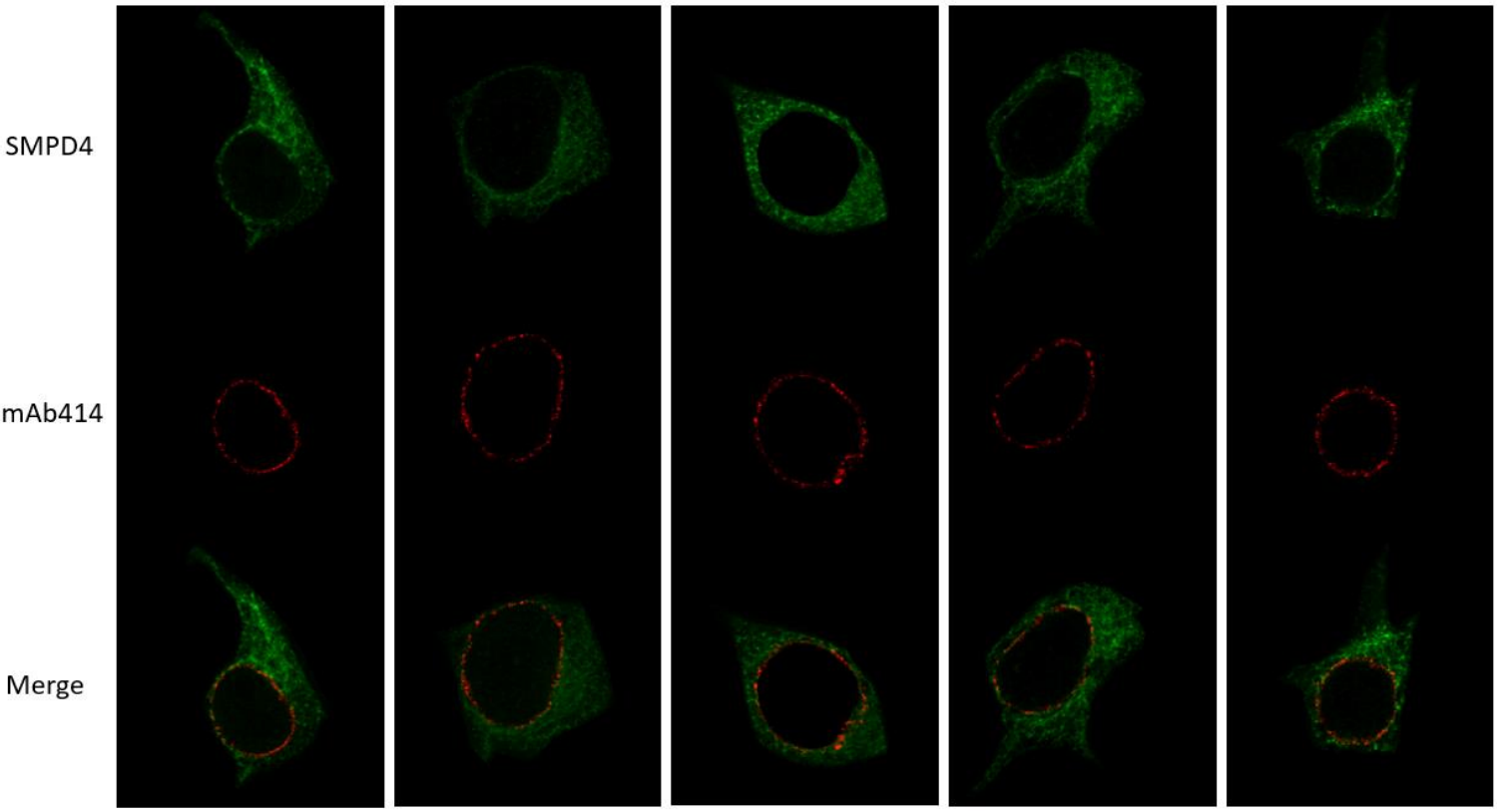

Figure 11. Colocalization of overexpressed exogenous Pro446Leu SMPD4 mutant with the nuclear pores. *HEK293T cells transfected with Pro446Leu Myc-tagged SMPD4 mutant were fixed and stained with an anti-SMPD4 and anti-414 antibodies and analysed through confocal microscopy. Panel shows colocalization of SMPD4 (in green) and the nuclear pore marker (mAb414), along with a merged image.*

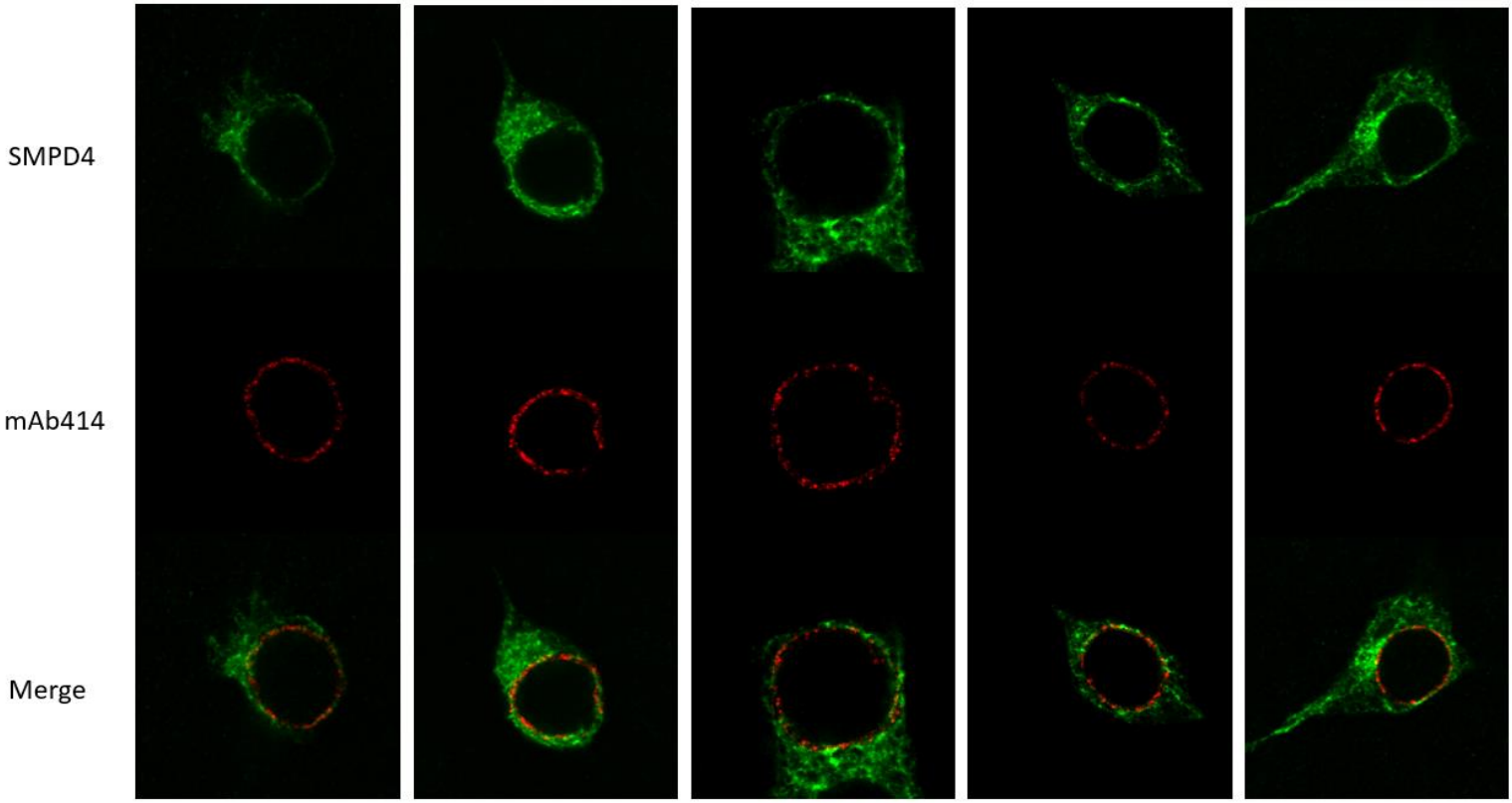

Figure 12. Colocalization of overexpressed exogenous Leu231Pro SMPD4 mutant with the nuclear pores. *HEK293T cells transfected with Leu231Pro Myc-tagged SMPD4 mutant were fixed and stained with an anti-SMPD4 and anti-414 antibodies and analysed through confocal microscopy. Panel shows colocalization of SMPD4 (in green) and the nuclear pore marker (mAb414), along with a merged image.*