Migration at the Gate:

The role of Lysosomal Calcium Channel TRPML-1 in Endolysosomal Regulation and Cancer Cell Invasion.

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Abbreviations

CatB: Cathepsin-B.

CatD: Cathepsin-D.

CD63: Cluster of Differentiation 63 antigen protein.

EM: electron microscopy.

ER: Endoplasmic reticulum.

GFP: Green Fluorescent Protein.

HA: Hemagglutinin.

IF: immunofluorescence microscopy.

ILV: Intraluminal vesicle.

kDa: kilodalton.

Knockdown: Kd.

LAMP1: Lysosomal-associated membrane protein 1.

LELYS: Late Endosome/Lysosome.

LYS: Lysosome.

MVB: Multivesicular body.

PI(3, 5)P_2: Phosphatidylinositol 3,5-bisphosphate.

PI(4, 5)P_2: Phosphatidylinositol 4,5-bisphosphate.

V-ATPase: Vacuolar-type H+-ATPase.

Acknowledgements

Abstract:

Correlation between lysosomes and cancer has been demonstrated in many studies. Lysosomal compartments in cancer showed various differences compared to normal conditions, such as their activity, biogenesis, hydrolytic activity and exocytosis. The lysosome itself contains many ionic channels on its membrane which regulate lysosomal activity and functionality, among which TRPML-1, a calcium releasing channel, was shown to play an important role in cancer invasion. In this study we investigated the role TRPML-1 has in lysosomal regulation and in HT1080 cancer invasion specifically. Overexpression of these channels in HT1080 cells caused the creation of enlarged endolysosomal compartments possessing the same acidic range as normal lysosomes, and containing active cathepsin and calcium ions in their intraluminal environments. On the other hand, depletion of TRPML-1 caused slightly smaller and more lysosomal organelles to appear in HT1080 cells. We also showed TRPML-1 alteration not merely alters lysosomal organelles, but also affects other organelles within the cell, with regard to their interaction and distribution. We showed increased association of ER and mitochondria upon TRPML-1 depletion and the appearance of lipid droplets in these cells. We also showed TRPML-1 knockdown increased the level of lysosomal proteins and translocated transcription factor E3 to the nucleus. In line with the link between this channel and invasive cancer we showed that TRPML-1 upregulation significantly increases lysosomal exocytosis, which in turn increases ECM degradation and cell migration.

1.Introduction:

Cancer is one of the most challenging health issues that human beings have faced to date. Beside the fact that cancer causes a lower life expectancy, but also has a reflection on social and economic development. Nonetheless, fighting and solving this challenge will not only overshadow the patient's life but also those of their families, doctors, medical staff and even the researchers who work in this field. According to the last estimation from the World Health Organization (WHO) in 2019, cancer is the most common or second most common cause of death in 112 countries in ages 70 and below. (Bray et al. 2020)

Although the amount of therapeutic targets and types of treatment have increased greatly over the years, cancer remains an ever increasing problem worldwide.

Cancer cells can invade and spread from the initial site of cancer to the different parts of the body and penetrate into neighbouring tissues. The mentioned process is referred to as cancer metastasis. Many types of therapeutic techniques such as chemotherapy, immunotherapy, radiation therapy and hormone therapy are considered for metastatic cancer treatment, which all aim to stop and control its growth. Still, the majority (around 90 percent) of death caused by cancer is from the metastasis. (Dillekås et al., 2019). Therefore understanding the invasion and metastasis mechanism in cancer can help identify the right targets and suitable drugs to reduce cancer mortality rates.

One such therapeutic target is the TRPML-1 channel: a calcium-release channel on the late endosomal/lysosomal membrane. It shows great promise for cancer therapy because of its role in cancer development ((Jung & Venkatachalam, 2019)).(Xu et al., 2019)(Grimm et al., 2018)

The development of cancer in human beings consists of various steps of cellular transformation which slowly alters cellular metabolism over time, inducing tumorigenesis and in some cases malignancy.
Alterations in lysosomes (LYS), the degradation centres within cells, is one of the established traits of most cancer cells. Functional changes in the Lys and its spatial distribution are previously linked to cancer development and progression. (Tang et al., 2020)

Many lysosomal alterations have been reported in cancer, such as their distribution, the level of their exocytosis, different secreted compositions to the extracellular environment, hydrolytic activity, intraluminal content and ionic channel activity. (Tang et al., 2020)

One of the alterations regarding the lysosomal Ca^{2+} channel TRPML-1 which has recently been reported in which TRPML-1, is specifically activated in invasive cancers (M. Xu et al. 2019). Due to these roles in cancer progression, the LYS and its TRPML-1 channel are emerging as promising therapeutic targets in cancer treatment strategies.

This report addresses the role of lysosomal calcium channel TRPML-1 in endolysosomal regulation and cancer cell invasion. In this chapter we will first describe the current state of knowledge on the link between the TRPML-1 channel and cancer. To achieve this, we first summarize the current scientific knowledge on Lyss and their relation to cancer, then provide a description of TRP channel superclass, and then TRPML-1 channel of Introduction is concluded by a discussion of the most recent insights into the relation between TRPML-1 and cancer progression. Subsequent chapters will deal with the methodology, results, conclusion and discussion, in this particular order.

1.1 Lysosomes

LYSs are terminal degradation stations within the endo-lysosomal system. Endo-lysosomal system is a complex network of lipid bilayer bound organelles, containing early, recycling, late endosomes, and LYSs. Endocytosis and autophagy pathways dynamically transport/deliver extracellular cargos and intracellular components to LYSs, respectively. Delivered cargos are then broken down into their building blocks (e.g., amino acids and fatty acids) by LYSs through hydrolysis (Faris et al. 2019) (Geisslinger et al. 2020)(Ballabio and Bonifacino 2020).The LYS carries different hydrolytic enzymes such as proteases and lipases, which function in the acidic intraluminal environment of the LYSs. With an acidic range of 4.5 to 5, LYSs are the most acidic organelle within the endo-lysosomal system (Ishida et al. 2013) (Perera and Zoncu 2016) (Ballabio and Bonifacino 2020).

In addition to being the degradative endpoints, LYSs are also the cellular signalling hubs that are involved in the regulation of cell growth, proliferation and differentiation. Based on the availability of nutrients, growth factors..etc.

 Lysosomal degradation and signalling tightly depend on the establishment of the luminal ionic homeostasis. The activity of lysosomal enzymes is influenced

by the homeostasis of Ca^{2+}, Na^+, K^+, Cl^- ions within the LYS lumen. LYSs with a calcium content of 0.5 mM are considered to be one of the cell's main centres of calcium storage.(The other intracellular calcium storage are the endoplasmic reticulum(ER) with 0.1–0.8 mM and mitochondria with 100 to 200 nmol/L calcium content concentration.)

Ca^{2+} plays an important role in regulating intracellular signalling activation, fusion/fission event between endosomal organelles (Christensen, Myers, and Swanson 2002) (Dong, Wang, and Xu 2010) (Patel and Cai 2015) (Sun et al. 2018). The efflux of this ion is regulated by Ca^{2+}permeable channels located in the lysosomal membrane, including TRP Mucolipins (TRPMLs, TRPML1-3), Two Pore Channels (TPCs, TPC1-2), TRP Melastatin 2 (TRPM2), TRP Ankyrin 1 (TRPA1), P2X4 purinoceptor, and Voltage-Gated Ca^{2+} Channel (VGCC) (Patel and Docampo 2010) (Patel and Cai 2015) (H. Xu and Ren 2015).(*Figure1.A*)

All of these properties give LYS an major role in nutrient regulation, metabolic signalling, homeostasis, apoptosis, autophagy and membrane trafficking in a cell(Ballabio and Bonifacino 2020) (Santoni et al. 2020).

1.2 Lysosomes In Cancer:

In many studies focusing on cancer, LYSs have been considered an interesting target in the recent years. (Davidson and Vander Heiden 2017) Ever since the relation between cancer and LYSs was discovered decades ago, many researches have shown that LYS biogenesis in cancer tissues significantly differs from that of normal tissues. (Berkeley 2013) (Davidson and Vander Heiden 2017) (Geisslinger et al. 2020)

Considering the dire need of cancer cells for nutrients and energy due to their continuous and abundant proliferation, and since nutrient availability is mainly regulated by LYSs, in cancer cells LYSs undergo changes to meet the elevated demands.(Davidson and Vander Heiden 2017) These alterations can be used to increase catabolic activity so it provides more nutrients, and energy for the cancer cells by digesting and recycling the endo- and exogenous macromolecules. (Davidson and Vander Heiden 2017)(M. Xu and Dong 2021). In confirmation of the above mentioned information, various studies have shown an increase in the activity and amount of lysosomal enzymes in cancer cells compared to normal cells (Sukhai et al. 2013). Also, various research works demonstrated that there is an increased lysosomal exocytosis in cancer cells (Berkeley 2013) (Nomura and Katunuma 2005). This may be due to the fact that an increased release of lysosomal enzymes into the ECM environment

during the increased lysosomal exocytosis may contribute to degradation of the extracellular matrix. This in turn promotes detachment from the degrading ECM, so the cancerous tumor can metastasize more easily. In a study on sarcoma cells it was shown that inhibiting LYS exocytosis could reduce cancer invasion and progression. (Nomura and Katunuma 2005) (Machado et al. 2015) (M. Xu and Dong 2021) In lysosomal exocytosis, LYSs first migrate along microtubes using kinesin proteins from the perinuclear area to the plasma membrane (PM) region. Once there, specific molecular mechanisms facilitate the fusion procedure of the lysosomal membrane with PM. This process requires N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complexes which pull the membranes closer to each other. The mentioned step requires the release of calcium and is orchestrated by the membrane protein SytVII, which has two calcium binding domains. Calcium ions can be provided by lysosomal TRPML-1 and once bound to SytVII, can increase phosphoinositide binding with the SNARE, which at the end leads to bilayer mixing and membrane fusion. (Martinez et al. 2000) (Reddy, Caler, and Andrews 2001) (Tancini et al., 2020)

Considering that the lysosomal calcium channel TRPML-1 plays an important role in Ca^{2+} regulation, many studies have shown that cancer cells have a higher expression for this channel than normal cells. Furthermore, research has shown that inhibiting this channel can disrupt cancer development. (M. Xu et al. 2019). (Sun et al. 2018)

In the following sections, more details about the TRPML-1 channel and its role in cancer development will be discussed.

1.3 The TRP Superfamily

In 1960, a study of behavioural mutations in Drosophila led to the discovery of the TRP family. Most of the TRP members located to PM. Six subfamilies of this family have been identified by gene mapping: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin), TRPA (Ankyrin) and (Mucolipin) TRPMLs. (Nilius et al. 2007). These subfamilies may be further divided into 28 different mammalian channel subtypes (*Figure1.B*). The shared characteristic between all the channels of TRP family is the presence of six trans-membrane domains (Nilius et al. 2007) (Wang et al. 2014)(Xu and Dong 2021)

The various TRP channels belonging to these families are mostly non-selective/non-permeable to ions, and any cation, including calcium, is allowed to pass through these channels. (Nilius et al. 2007)

TRPML-1, as mentioned above, is one of the channels in the Mucolipin subfamily. Many studies have examined the importance and role of this channel in a variety of disorders and pathologies. Type IV mucolipidosis (MLIV) is one of the diseases caused by mutations in this channel. (Nilius et al. 2007) (Dong et al. 2008).

1.4 TRPML-1 Structure And Properties

In addition to TRPML-1; the Transient Receptor Potential Mucolipin (TRPML) channels group consists of TRPML- 2 and TRPML-3 and are located on endolysosomal organelles. As mentioned before, this group belongs to the Transient Receptor Potential (TRP) super family. About 75% of the amino acid sequencing similarity is seen in this group of channels (Di Paola, Scotto-Rosato, and Medina 2018). The genes that encode these three channels are: MCOLN1, MCOLN2 and MCOLN3, located on chromosomes 19(19p13.2-13.3), Chromosome1 (1p22) and Chromosome1 (1p22.3) respectively. (Sun et al. 2000) (Bargal et al. 2001) (Nilius et al., 2007) (Samie et al. 2009)

Unlike TRPML-1, only few studies have been conducted on TRPML-2 and TRPML-3. However, both have been shown to be involved in endocytosis activity. (Chen et al. 2017) TRPML-2 is located in early and recycling endosomes as well as on the lysosomal membranes. TRPML-3 beside localizing to the early and late endosomal compartments localize to the PM as well as LYSs. (Karacsonyi, San Miguel, and Puertollano 2007) (Kim et al. 2009) (Remis et al. 2014)(Sun et al. 2015) TRPML-2 is traceable in most mammalian organs, specifically those containing a high amount of immune cells and tissue. TRPML-3, by contrast, can be found in lung, kidney, thymus and skin tissues. (Kim et al. 2009) (Remis et al. 2014) (Di Palma et al. 2002) Although no specific disease related to TRPML-2 has been reported yet, a mutation in mice that is associated with hair loss and circling behaviour has been attributed to TRPML-3. (Di Palma et al. 2002)(Xu et al. 2007).

Due to the fact that TRPML-1 has been the object of extensive study over the years, our knowledge of this protein channel contrasts sharply with its brother channels. It has a mass of about 65 kDa and 580 amino acids, and can be

expressed in all mammal tissues. (Wang et al. 2014) In a cell, TRPML-1 is located on the late endosomes and LYSs within the endosomal system. Its localization in the mentioned compartments has been seen not only in mammalians but also in other animals such as Drosophila or C.elegans. (Pryor et al. 2006)(Fares and Greenwald 2001)(LaPlante et al. 2002) In a previous study using GFP-TRPML1, this channel was observed to be about 80% percent colocalized with lysosome-associated membrane proteins 1, 2, and 3 (Lamp1–3).(Manzoni et al. 2004)

Using standard voltage-clamp techniques in the initial observations on TRPML-1, it has been determined that this channel, like the other channels of the TRP superfamily function as a non-selective channels and different cations including Ca^{2+}, Na^+, K^+ are able to pass through it. (LaPlante et al. 2002)(LaPlante et al. 2004) (Movie1). The structural studies on this channel revealed that it consists of six transmembrane domains(S1-S6). There is a large intraluminal loop between the first and second transmembrane domain (S1 and S2) which is 25% of the channel's lengths and contains 75% of histidine residues. This loop can be cleaved by a protease sensitive to cathepsin B (CatB) inhibitors. The other loop, which is known as he "pore-region", is located between the fifth and the sixth transmembrane domains (S5 and S6). This loop acts as the channel gate for late endosome/ lysosome intraluminal ion transportation across their membranes to the cytosolic environment. Both loops face the intraluminal side of the compartments. (Kiselyov et al. 2005)(Costello, Fenselau, and Franklin 2011)(Milon et al. 2006) (*Figure1.C*)

TRPML-1, like the other TRP channels family, contains an N-terminus (NH2) and C-terminus (COOH) tails, both of which are oriented toward the cytosol. (*Figure1.C*). Each tail contains a di-leucin motif which mediates the cannel to localize on the late endosome and LYS compartments.(Vergarajauregui & Puertollano, 2006) It has been shown that when these motifs have been deleted, TRPML-1 accumulates on the plasma membrane. This observation suggests that this protein can be delivered to the LYS via the plasma membrane or reaches the LYS from Trans-Golgi without any intermediate transport.

Pryor et al. 2006)(Manzoni et al. 2004)(Pryor et al. 2006) Furthermore, plasma membrane localization of 1va showed a significant increase compared to wild-type TRPML-1. This may be mediated by the release of luminal cations (most likely Ca^{2+}) via lysosomal exocytosis. (Miedel et al. 2006)

There are two PKA sites (Ser557, Ser559) in the C terminal, which associate with the late endosomal/lysosomal membrane. In the polybasic domain in the

amino-terminal on the other hand, multiple positively charged amino acid residues form a site which is evolutionarily an extremely conserved domain. The lysosomal lipid marker Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) directly binds to this site (*Figure1.C – Movie1*) and activates the gating in the pore region of the channel. PiKfyve kinase can phosphorylate Pi3P which generates PI(3,5)P2. Its dephosphorylation by Sac3/FIG4 and MTME lipid phosphatase can convert it to PI3 and PI5. This has been shown by overexpressing the PI3-Phosphatase MTM1, which leads to decreased TRPML-1 channel activity. The inhibition of channel activity occurs through binding the plasma-membrane-localized lipid PI(4,5)P2 in a site within the same polybasic domain. (Puertollano and Kiselyov 2009)(Grimm et al. 2012)(Wang et al. 2014)

PI(4,5)P2 binding keeps the TRPML-1 from being activated before arriving at the LYS environment containing PI(3,5)P2. PI(4,5)P2 in turn limits channel activity following lysosomal exocytosis, which is mediatedby TRPML-1 channel activity. (Venkatachalam and Kiselyov 2015)

All of these properties, which cause late endosome/lysosome (LELYS) intraluminal cation release, most importantly the universal second messenger Ca^{2+}, allow TRPML-1 to play important roles in various cell processes. Among these are endocytic membrane trafficking regulation, signal transduction, lysosomal exocytosis and ion homostasis, both in LLy lumen and cell cytosol. Due to its involvement in these processes, mutations in TRPML-1 cause lysosomal storage diseases and impairment. (Wang et al. 2014) Therefore therapeutic strategies focusing on lysosomal function warrant the investigation of TRPML1 as a drug target.

1.5 TRPML-1 and cancer:

In recent years, the relationship between increased TRPML-1 expression and cancer proliferation, development and migration has received much attention. These observations have been reported in many research papers on different tumor types, including head & neck, bladder, melanoma, Non-small-cell lung carcinoma (NSCLC) , Pancreatic ductal adenocarcinoma (PDAC) and Triple-negative Breast Cancer (TNBC). (Jung et al. 2019) (M. Xu et al. 2019)(Yin et al. 2019) (Hu et al. 2019) (Kasitinon et al. 2019)(Morelli et al. 2019). Also an increased expression of TRPML-1 has been shown to correlate with the presence of HRAS-driven cancer, while its downregulation or inhibition decreases proliferation of this type of cancer cell. (Jung et al. 2019)
In another study on breast cancer, the report states that this channel is highly expressed in Triple-negative cells   compared to nontumorigenic and

nonmetastatic cells. As in the aforementioned paper on HRAS-driven cancer, down-regulation, either using genetic modification or drug inhibitors, decreases the growth of these cells significantly. This study has also shown cancer development is related to mTORC1 channel activity and lysosomal ATP release regulation. (M. Xu et al. 2019)

A study on melanoma cells on the other hand, has shown that elevated TRPML-1 expression is also associated with cell progression and proliferation, but in a negative regulatory of mTORC1 signaling. (Kasitinon et al. 2019) In a study on NSCLC, increased TRPML-1 expression was shown as well, although a lower expression of the channel occurred in advanced cancer stages, when compared to the normal lung tissue. The authors suggest that oxidative stress and genetic instability, caused by MCOLN1 gene deletion, contribute to tumorigenesis, whereas advanced tumors are aided in their survival by a surplus of TRPML-1. (Yin et al. 2019)

An in vitro and in vivo study on PDAC associates increased expression of TRPML-1 with PDAC growth and formation. On the other hand, a study on glioblastoma reported a reduction of cell viability due to the application of a TRPML-1 agonist and its resulting activation. In contrast, TRPML-1 silencing or inhibition had the opposite effect. (Hu et al. 2019)

Overall, although these studies show that high TRPML-1 expression correlates with cancer proliferation, progression and invasion, the consequences of TRPML-1 misregulation may differ based on the cancer type and its state. (Santoni et al. 2020)(Yang, Zhai, and El Hiani 2020)(Grimm et al. 2018).
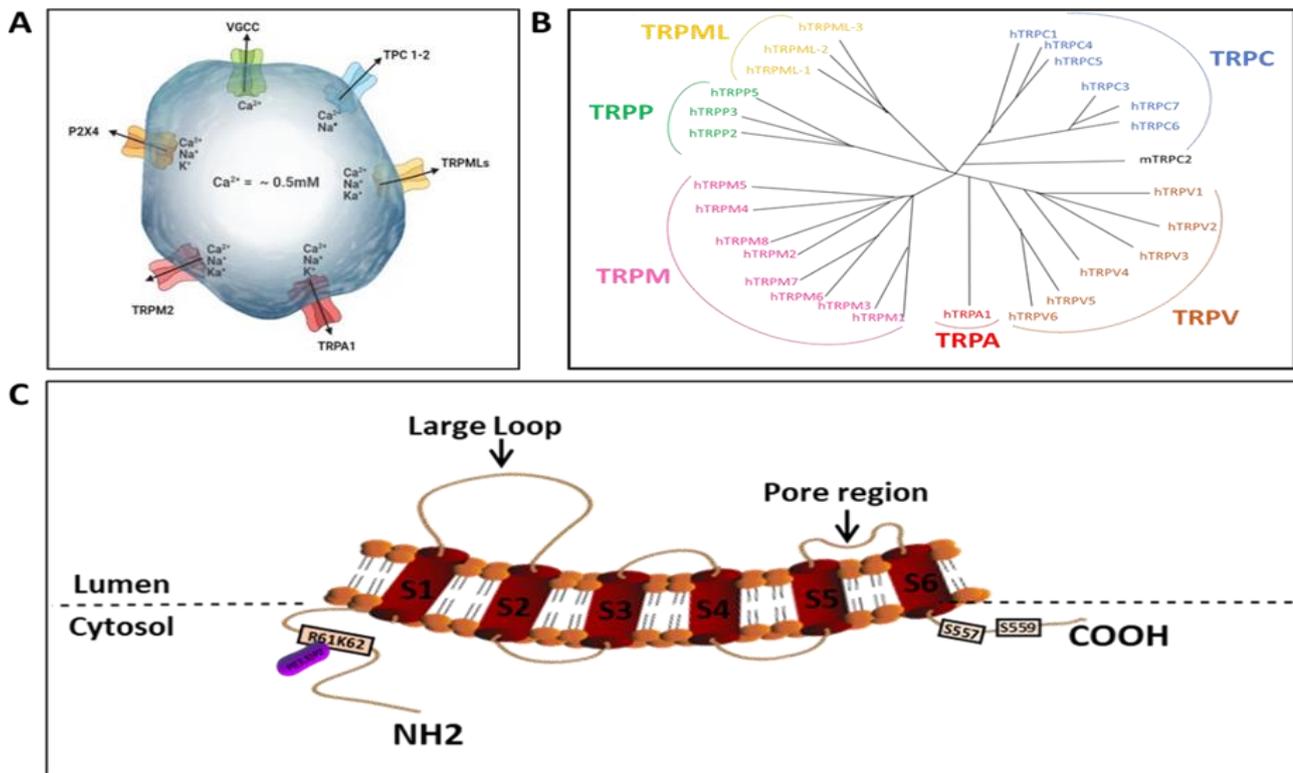
Figure 1. TRPLML-1: a lysosomal calcium release channel belonging to the TRP superfamily. (A) Ca2+permeable channels located on the lysosomal membrane. **(B)** The phylogenetic tree of the transient receptor potential (TRP) superfamily. As shown in the image, the 28 mammalian TRP channels are subdivided into six main subfamilies: TRPC , TRPM ,TRPV ,TRPA , TRPP and TRPML. **(C)** TRPML-1 consists of six transmembrane domains (S1-S6), its amino-terminal and carboxyl-terminal tails facing the cytosol. On the lumen side, there are two loops, one of which acts as the channel gating (the pore region). Pi(3,5)p2 is a late endosomal/lysosomal-localized phosphoinositide which directly binds to the R61K62 site located in the NH2 tail, activating the ca2+ channel running from the lumen toward the cytosol through the pore region.

2.Materials and Methods:

Cell culture:

HT1080 cells were cultured in Sigma Dulbecco's Modified Eagle's Medium (DMEM) high glucose (3,151 g/L) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS), 1% penicillin(100 U/mL) streptomycin (0.1 mg/mL) and (?)2mM L-glutamine. Growing cell lines were maintained according to standard procedures at 37°C and a humidified atmosphere of a 5% CO2 incubator. For the immunofluorescence labelling assay, cells were cultured on coverslips in 6 well plates with 0.3×10^6 cell density per well. For Western blotting, cells were grown with 2.2×10^6 density in a 10 cm dish. In live-cell imaging, the cells were seeded with 10×10^3 density in a µ-Slide 8 Well ibidi dish.

Immunofluorescence labelling:

HT1080 cells grown on coverslips were washed with 1XPBS and fixed under a foam hood using 4% paraformaldehyde (PFA) for 30 to-60 minutes. After 3-step washing with 1XPBS, the cells were placed at room temperature (RT) and were permeabilized with 0.1% Tritone for 10 minutes. All the following procedures have been done at RT. Considering that aldehyde fixatives such as PFA react with amines and lead to autofluorescence, it would be beneficial to reduce this effect using common quenching steps such as adding Sodium Boride, Tris or Glycine. In line with this, 0.15% Glycine (20 mM)/PBS suffices to cover the coverslips added to the cells for 10 minutes. Next, 1% BSA added to cells for 10 minutes to block non-specific antibody binding. By placing a parafilm on the bench, diluted primary antibodies in 1%BSA were then added as drops on its surface. Coverslips were then transferred on the parafilm containing the antibody droplets, in such a way that the fixed cells faced down towards them. Coverslips were then incubated for 1 hour. Next the coverslips were washed 3 times with 1X PBS for 10 minutes each. Later the secondary antibodies diluted in 1%BSA were added in the same fashion as the primary ones, and incubated for 30 minutes. Later, the coverslips were washed with 1X PBS 3 times again for 10 minutes each time. Later the coverslips were rinsed with MiliQ for a few seconds and dried out using their edges dabbing in a tissue paper. Finally the desired amount of glass slides were prepared and 5 µL of prolonged gold dapi was added onto the glass slides in droplets for each coverslip. The coverslips were then placed on the droplets in such a way that the cells faced down the prolonged gold. They were subsequently incubated

overnight and then imaged using a DeltaVision ultra high-resolution microscope. The glass slides with coverslips could be kept for a long duration at 4 °C.

Immunoblotting

Cells were grown in 10 cm dishes and scraped on ice at 80% confluency using a lysis buffer containing 1uM DTT and 1x protease inhibitor. The harvested cells were then transferred into 1.5 ml tubes and rotated at 4°C for 15 minutes. To get rid of the membrane and DNA, the tubes were then centrifuged at high speed for 15 minutes. The supernatants were transferred to new 1.5 ml tubes and the remaining pallets were discarded. To determine protein concentration in each sample, 1ml of Bradford solution mixed with 2 µl of the supernatants and then measured with a spectrophotometer. Subsequently they were adjusted for equalization using the remaining lysis buffer and mixed with a sample buffer of ratio 1 to 5. The mixture was boiled for 5 minutes at around 100°C and spun down shortly. Afterwards, the samples were loaded on a 4-15% gradient gel (BIO-RAD Mini-PROTEAN TGX™ precast gels). The gel was run at 100V for 1 hour. To activate the cellulose membrane, 98% methanol was used for 5 minutes and transferred into a blocking buffer(Intercept™ Blocking Buffer, diluted 1:1 in PBS) by carrying out a Blotting Cassette (BIO-RAD). It was then incubated for 1 hour, followed by overnight incubation of the membrane with primary antibodies at 4°C on a rocker(Supporting data, table 3). It was washed using secondary antibodies with 1xTBS-Tween 0.1% before and after incubation. The details of antibodies used are described in the corresponding table. (Supporting data, table 4). At the end, the membrane was visualized using a Typhoon™ Biomolecular Imager.

Overexpression using Effectene (Qiagen)

Cells were seeded in 6 well plates and incubated until approximately 50-70% confluency. To apply the transfection procedure, approximately 600 ng DNA plasmid was diluted in 150 µl EC buffer per well. The buffer and DNA complex was then mixed with 4.8 µl enhancer and incubated for 5 minutes at room temperature. Next, to form the Effectene-DNA complex, 12 µl of effectene reagent was added to the mixture and incubated for 10 minutes at RT. At the end, the final effectene complex was mixed with 800 µl of medium and added dropwise to the well. The transfected cells were then incubated at 37 °C until evaluation (7-24 hours) prior to fixation and further analysis.

Gene knock-down by RNA interference using Hiperfect (Qiagen)

For siRNA transfection, cells were seeded in a 6-well plate and transfected using siRNA, to target specific sequences in the human MCOLN1 gene. Per each well, 2.3 µl of the siRNA was added to 800 µl of serum-free medium with Hiperfect reagent and incubated 10 minutes at RT. Later, the mixture was added dropwise to each well using filtered tips and maintained at 37 °C in an incubator for 72 hours. The knock-down cells were then fixed and used for further visualisation and analysis.

Plasmids

HT1080 cells were transfected using TRPML-1- pEGFP C3 , TRPML-1-HA, mEGFP-PI35P2 and Gcamp-GFP all from addgene. We constructed TRPML-1-mCherry using using PCR of TRPML-1-GFP adding two attB forward and reverse sequences. later using BP reaction we added TRPMl-1 in pDONAR plasmid and with LR reaction we insert TRPML-1 in pDest-mCheery N1 plasmid.
(*Supplementary data S1*)

Using different probes

LysoTracker™ Green DND-26 Invitrogen was used for lysosomal compartment detection with a final concentration of 100nM in media in 6-well plates for 30 minutes. To detect active cathepsin in endolysosomal organelles, cells were incubated with 500nM Invitrogen SiR-lysosome SPY™ 647 for 3 hours. To examine the degradative ability of endolysosomal organelles, cells were incubated with Invitrogen™ DQ™ Green BSA for 30-60 minutes with the final concentration of 10 µg/ml. To reveal the endocytic efficiency in desired organelles the cells were incubated with 250 µg/ml of Dextran, Texas Red™Invitrogen for 3 hours. To visualize calcium content within the endosomal compartments, 250 µg/ml of Invitrogen Oregon Green™ 488 BAPTA-1 dextran, was used in the cells and incubated for 3 hours prior to live cell imaging.

Live-cell imaging

Both the knock-down-TRPML-1, as well as overexpressed TRPML-1 and WT/Scrambled HT1080 cells were seeded in ibid µ-Slide 8 wells with a density of 10 x 103 per well. The cells were then imaged as a time lapse in live

conditions using Thunder (?) for a two hour duration at a 2 minutes interval. Later, the time lapse images were exported in video format for further analysis.

Electron microscopy

Cells cultured in 6 cm dishes were grown up to approximately 80% confluency and consequently fixed under a foam hood at RT for 30 minutes with 2.5%GA + 2% PFA and 0.1M PB. Later, the cells were harvested using a scraper, after rinsing with 0.1M PB twice for 5 minutes. Next the cells were centrifuged quickly after a fast resuspension in 2% low melting point agarose (in MiliQ). The obtained pellet was placed on Ice for 30 minutes. The tip of the eppendorf tube was then cut to facilitate the removal of the agarose, after which the extracted agarose was cut into 5 to 6 blocks. Blocks thus obtained were maintained in a postfix solution containing 1% OsO_4, 1.5% K3FE(III) in 0.065M PB at 4°C for 2 hours. After rinsing three times with Aq.d, they were kept in 0.5% uranyl acetate for 1 hour at 4°C. The blocks then were then rinsed once more with Aq.d prior to being kept in 70% acetone. Subsequently, they were kept in the following concentrations of acetone: 90% and 96% for 15 minutes and three times 30 minutes in 100% acetone. Next, collected blocks were incubated in three ratios of epon and acetone mixture. The ratio and incubation time were as follows : 1:3 , 1:1 and 3:1 for 30,30 and 45 minutes respectively. The blocks were then placed in pure and fresh epon for 30 minutes. At the end, the blocks were embedded in pure epon together with paper labels and were kept at 60C° for three days. After these days, the blocks were ready for sectioning. The sections were obtained using a microtome machine with 60nm thickness.. Each section was placed on a carbon-formvar coated grid and visualised with Tecnai 12 Transmission Electron Microscope (Thermo Fisher) at 80 kV.

Immuno-Electron Microscopy

Cells were cultured as described and fixed for immuno-EM upon reaching a confluency of approximately 70%. Fixation was achieved by adding 4% wt/vol paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH = 7.4) to an equal volume of culture medium for 15 minutes at room temperature (RT). Subsequent post-fixation with 4% wt/vol PFA in 0.1 M PB was for >2 h at RT. Embedding for immuno-EM, preparation of ultrathin cryosections and immunogold labeling was performed as previously

described(Slot JW, Geuze HJ). Cryo sectioning and immunolabeling. Nat Protocol 2007; 2:2480-91; PMID:17947990; http://dx.doi.org/10.1038/nprot.2007.365). To detect TRPML-GFP ultrathin cryosections were immunolabeled with a mouse anti-GFP antibody (1/300, Biolegend). Labelled sections were analyzed in a Tecnai 12 Transmission Electron Microscope (Thermo Fisher) at 80 kV.

Wound healing assay

For the wound healing assay, cells were seeded in a 6-well plate containing coverslips. To simulate a wound, a plastic pipette tip was used to mechanically damage the cell monolayer by scratching. The images were taken by a revolve microscope at different points in time: 0 before transfection and at the time of transfection, as well as 8, 16, and 32 hours after transfection. Each time point belongs to one coverslip being fixed and stained for Dapi before imaging.

Transwell invasion assay

To perform a Transwell invasion assay, a 24-well Transwell insert (? μm pore size; ?) was used with 100 μl of serum-free DMEM and 0.5mg matrix and 500 μl medium containing FBS. To obtain the matrix, 3.78mg/ml collagen was used mixed with NAOH, PBS, and MiliQ to bring the final concentration to 0.5mg/ml. All the steps were done on ice. Next, 500 μl of complete medium was added to the lower chambers. The prepared collagen mixture was then added to the upper surface of the Transwell chamber and reconstituted with 100 μl serum-free medium and incubated for 1 hour. Then 3×103 Cells were seeded in the upper chamber and were allowed to migrate for 16 hours while being kept in an incubator. Later the cells remaining on the upper membrane were removed using cotton soap. The cells that invaded through the matrix were fixed using 4% PFA and stained for the nucleus using ProLong™ Gold with dapi and visualized and counted under a revolve microscope.

Analysis:

To analyse different colocalizations, particle detection was done using the ComDet plugin in ImageJ with the desired adjustment in max distance between colocalized spots for each channel. Exported quantified data was then plotted using ttps://huygens.science.uva.nl/PlotsOfData/. For the lysosomal size analysis, a data set of electron microscopy images was imported in ImageJ and the observed LYSs in each image were measured using the "freehand line" tool in the software. The obtained calculated sizes from the software were based

on the perimeter sizes. The quantified data was then imported into the aforementioned website and the corresponding plots were obtained. To analyse Western blotting data, the intensity of each protein band was calculated by image J and later the outcome measurements used into a graph in excel.

Software:

All the immunofluorescence, electron microscopy and western blot data were analysed using ImageJ and Microsoft Excel. The schematics were made by using Adobe Photoshop, Illustrator and BioRender. The animated video was made by importing the illustrated images into Moho Anime Studio Pro 13.

3. Results

Previous studies have reported an association between TRPML-1 and cancer, but the direct relation and the underlying mechanisms remained elusive. In their report, Xu et al. observed differences in the expression of this channel in metastatic cell lines (MDA-MB-231, Hs 578 T and SUM159PT) compared to non-tumorigenic cells (MCF-10 A) and non-metastatic cells (MCF-7), among which the invasive cancer cells (SUM159PT) showed the highest level of TRPML-1 expression. (M. Xu et al. 2019) Previous research in our lab also showed a higher expression of TRPML-1 channel in HT1080 cells, human derived invasive fibrosarcoma cancer cells, compared to non-cancerous and non-metastatic cell lines *(Supplementary data S2)*. Therefore, in this report, HT1080 cells have been used as an in vitro model system to address our question regarding the role of TRPML-1 in lysosomal function in invasive cancer cells.

3.1. TRPML-1 localization within the endo-lysosomal system.

Previous studies have shown that the TRPML-1 localizes on the late endosomal and lysosomal organelles in the endolysosomal system. (Manzoni et al. 2004) To detect the localization of TRPML-1 within the organelles in this system in HT1080 cells, we exogenously overexpressed this channel using GFP and HA-tagged TRPML-1 plasmid. This decision was made based on the lack of antibodies able to detect endogenous TRPML-1. We applied immunofluorescence labelling to the overexpressed TRPML-1 HT1080 cells, using different antibodies acting as endosomal markers. The cells overexpressed with TRPML-1-GFP were labelled for late endosomal/ lysosomal compartment using LAMP-1 antibodies *(Figure2.A)*. Labelled cells on coverslips have been visualized using immunofluorescence microscopy (IF).The results showed that TRPML-1-GFP was located on LAMP-1 positive compartments *(Figure2.A, White Arrow)*, but also on compartments not showing the LAMP-1 signal, which suggests the channels are also located on other organelles such as earlier endosomes *(Figure2.A, Zoomed-in Box, Red Arrow)*. Later, we used cells that overexpressed TRPML-1-HA, labelled for HA-tag and EEA1, the latter of which is known as an early endosomal marker *(Figure2.B)*. Visualizing the cells under a IF revealed partial TRPML-1 localization on early endosomes *(Figure2.B, White Arrow)*, which is interesting considering that the bulk of TRPML-1 is usually located in late endosomes and lysosomal organelles. This suggests either we are detecting the TRPML-1 being trafficked from plasma membrane

to LELYS, or EEA1 is also present on later organelles in addition to earlier endosomes. Interestingly we also observed enlarged organelles which were only positive for TRPML-1 *(Figure2.B, Zoomed-in Box, Red Arrow)*.

For further colocalization, HT1080 cells transfected with TRPML-1-GPP plasmid were labelled to detect late endosomes with CD63 antibodies *(Figure2.C)*. later we also used HT1080 overexpressing TRPML-1-GFP CathepsinD monoclonal antibodies, which detect lysosomal protease CatD *(Figure2.D)*. This enzyme plays important roles in degradation and normally localizes in LYSs in nearly all mammals. The data shows that TRPML-1 colocalizes with both CD63 *(Figure2.C, White Arrow)* and CatD *(Figure2.D, White Arrow)*. We also observed that both CD63*(Figure2.C I,II Red Arrow)* and CatD *(Figure2.D I, Red Arrow)* were clustered in specific subdomains within enlarged TRPML-1-positive organelles in HT1080 cells

All the collected images using IF have been processed with Fiji to obtain quantitative colocalization analysis. The results show that TRPML-1 localizes ~ 10% , 50% , 30% and 25% with EEA1, LAMP1, CD63, CatD respectively. *(Figure2.E)* The numerical results show the highest colocalization of TRPML-1 with LAMP-1, despite the fact that the visual interpretation of the images below shows more colocalization of TRPML-1 with CD63 compared to other endosomal markers. The discrepancy may be a result of the limitations of object detection using imageJ. As we showed, TRPML-1 overexpression causes the enlarged organelles, which were found positive for CD63, although only on one side. Most of the TRPML-1 was however concentrated on the opposite side of these organelles, causing the ComDet colocalization option in ImageJ to detect both signal clusters as separate locations. A plugin for ImageJ would therefore be needed that groups signal cluster according to the organelle from which they stem.

To investigate the ultrastructural localization of TRPML-1, we also prepared HT1080 cells overexpressing TRPML-1-GFP for immuno-electron microscopy (immuno-EM). For this purpose, after the transfection process, we have fixed and prepared the cells for EM, prepared 70nm thin sections, immuno-labelled the cells for GFP in conjugation with 10 nm gold particles, and visualized under electron microscopy(EM). As we saw in immunofluorescence data, we could also detect the TRPML-1 localization on the limiting membrane of late endosomes *(Figure2.F,I White Arrow)* and LYSs. *(Figure2.F II, Red Arrow)*

Another interesting observation that caught our attention was the high presence of label on the membranes of intraluminal vesicles (ILVs). *(Figure2.F III, White Arrow)* Overall, based on our immuno-EM observation, TRPML-1

channels are localized on the limiting membrane of late endosome and lysosomal compartments, and also in some intraluminal vesicles in multi-vesicular bodies (MVBs).

3.2. Endogenous PI(3,5)P2 colocalizes with TRPML-1

As mentioned in section 1.4 and shown in the animation *(Movie1)*, there is a binding site (R61K62) in the N-terminus tail of TRPML-1. The lysosomal lipid marker Phosphatidylinositol 3,5-bisphosphate (Pi (3.5)P2) can bind directly to and active the TRPML-1 channels by opening its gating in the pore region between the S5 and S6 transmembrane domains. To detect the localization of this lipid we transfected HT1080 cells with a genetically encoded GFP fluorescent probe in parallel with TRPML-1-HA plasmid. The mentioned probe is obtained by fusion of fluorescent tags with TRPML-1 binding site (R61K62). Considering that this site has a high affinity to endogenous Pi(3,5)P2, it could be used as a sensor to visualise its subcellular localization.(Li et al., 2013) (*Supplementary data S3*). The cells transfected with the probe and TRPMl-1-HA were later fixed and labelled for HA and LAMp-1 to detect TRPML-1 and late endosomal/lysosomal compartments respectively. The fixed cells were then observed using IF. Pi(3,5)P2 probes were detected with the microscope using 488 filters for GFP signals visualization. Obtained images, as expected, showed that Pi (3,5)P2s colocalize with TRPMl-1 and they both colocalize with LAMP-1 positive compartments. *(Figure2.G)*

## 3.3. Overexpression of TRPML-1 causes enlarged organelles

In the experiments where we transfected the HT1080 cells with TRPML-1 plasmids, we observed the appearance of large peculiar organelle profiles in overexpressing cells and as showed in *(Figure2.B, Zoomed-in Box, Red Arrow) they were negative for EEA1 (Figure 2.B), but were positive for later endoplasm/lysosomal markers such as LAMP-1. (Supplementary data. S4 , Figure 2.A).* On the other hand, we did not observe the same profile upon overexpression TRPML-1 in Hela cells. *(Supplementary data. S5).*

The observation of the enlarged TRPML-1-positive organelles, encouraged us to investigate and identify the characteristics of these organelles. .Because these organelles have always labelled positive for the TRPML-1 signal, the notion that they were created by higher TRPML-1 expression was reinforced. Observation of enlarged organelles due to TRPML-1 alteration has been reported before, although alteration was due to channel downregulation. Our

study, on the other hand, shows aberrant organelles while the HT1080 cells overexpress the channels.

In order to confirm the immunofluorescence data, we transfected HT1080 cells with a TRPML-1-GFP construct, fixed, and embedded for Resin-EM. After ultra-thin sectioning using a microtome, they were visualized under EM. In accordance with earlier data, we observed enlarged organelles in the sections containing HT1080 overexpressing TRPML-1 *(Figure3.A, ,White Arrow)*. This observation, when compared to the LELYS HT1080 wild-type cells normally have*(Figure3.B, Red Arrow)*, demonstrates that these enlarged compartments are caused by TRPML-1 overexpression.
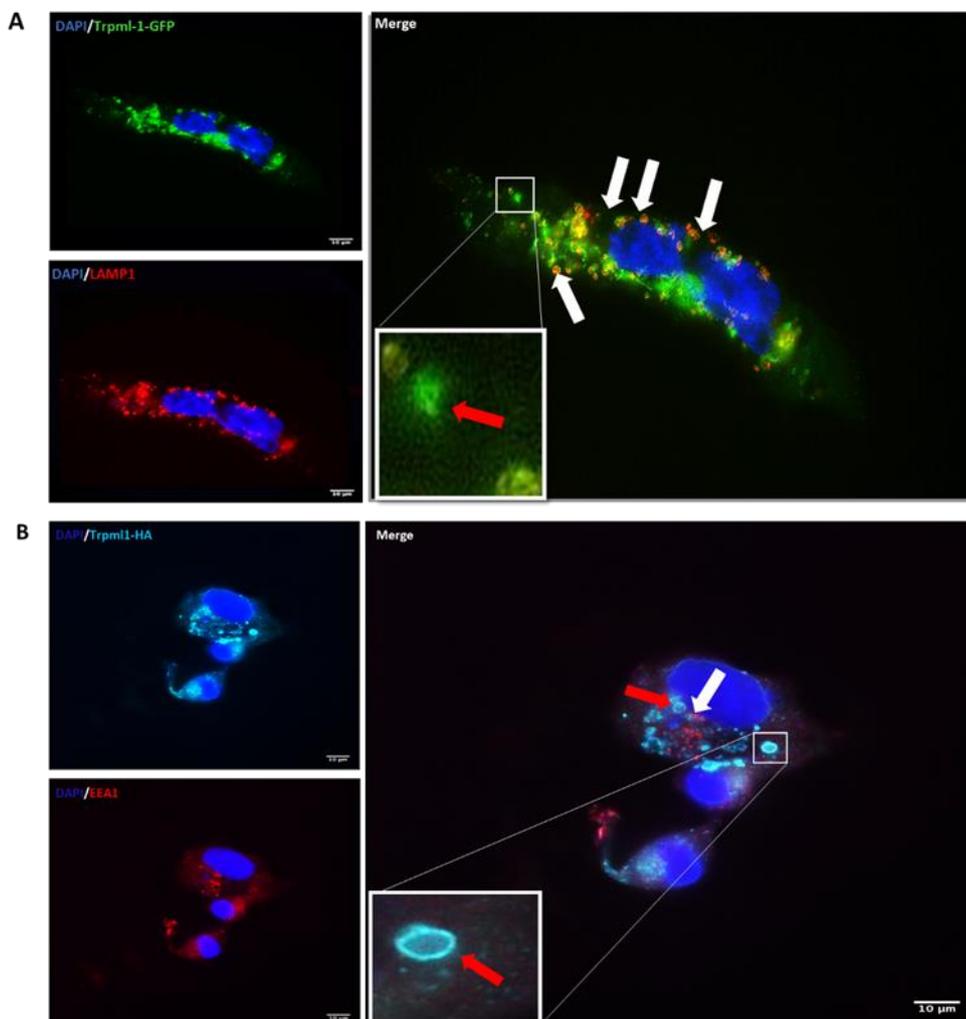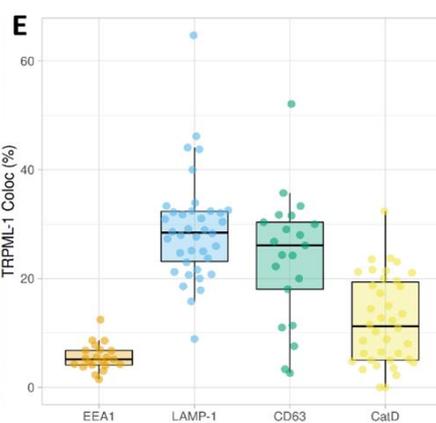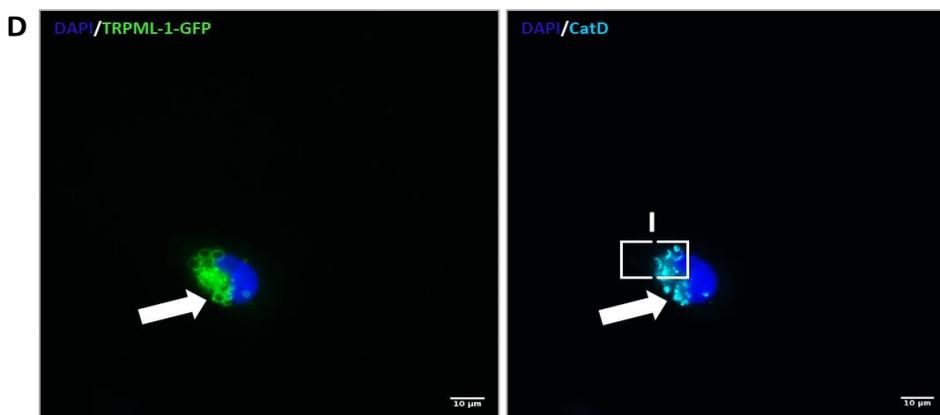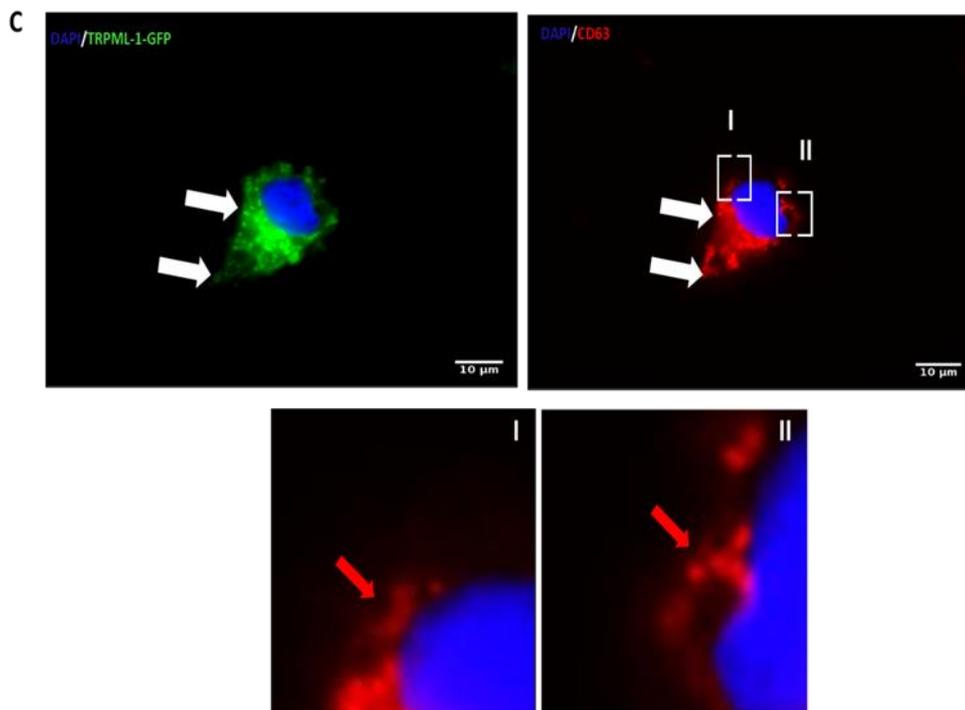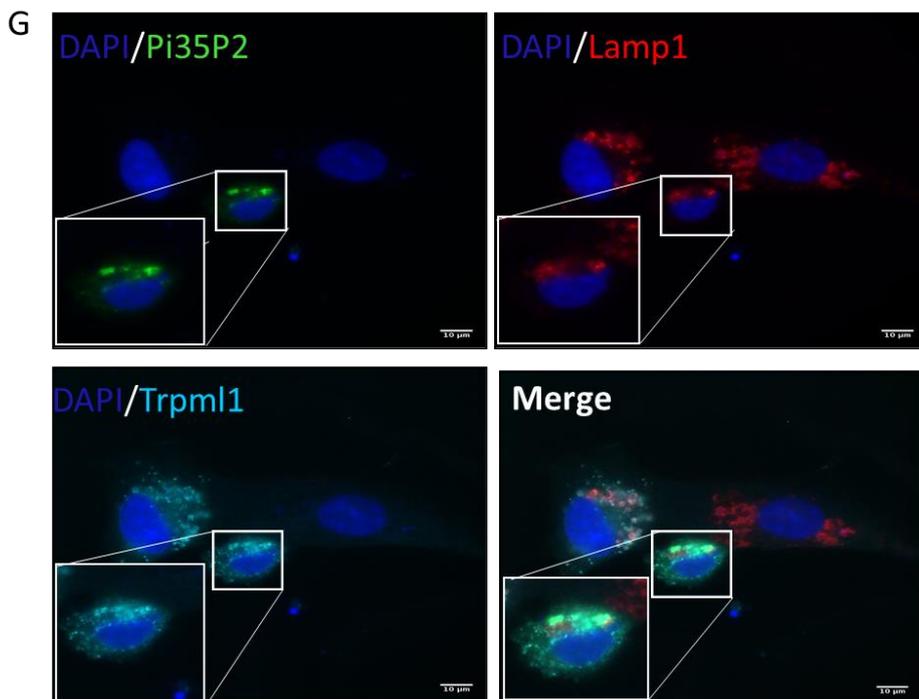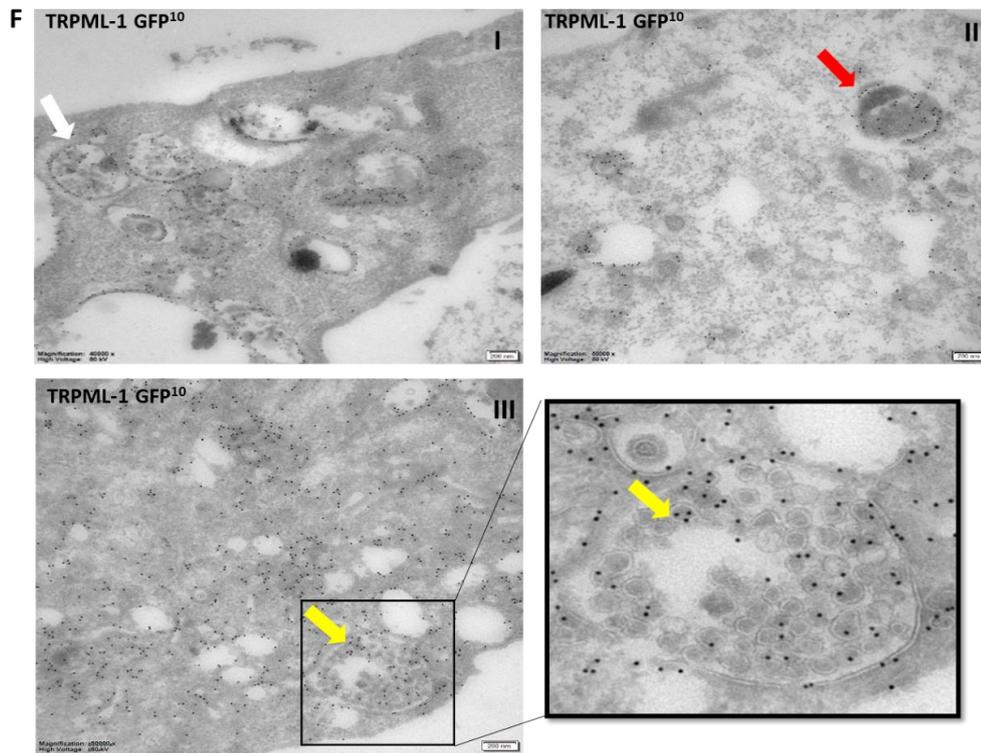
Figure 2. TRPML-1 and Pi(3,5)P2 localization within the endolysosomal system. (A) Immunofluorescence observation of TRPML-1 overexpressing HT1080 cells showed TRPML-1 (Green) colocalizes with late endosomal/ lysosomal compartments (White Arrows). LAMP-1 antibody (Red) was used for late endosomal/lysosomal detection. The data also showed some compartments showing TRPML-1 signal but not LAMP-1(Red Arrow). **(B)** EEA1 (Red) an early endosomal marker partially localizes with TRPML-1 (White Arrow) . Enlarged organelles were observed upon overexpression of TRPML-1 (Red Arrow). **(C,D)** TRPML-1 (Green) colocalize with CD63 (Red) / CatD (Cyan) (late endosomal marker/Lysosomal marker) and both signals for CD63 and CatD have been observed on one side of the enlarged organelles.(C I,II & D I, Red Arrows). **(E)** Quantitative TRPML-1 colocalization analysis shows TRPML-1 mostly colocalizes with LAMP-1 and CD63. **(F)** TRPML-1 localization detection using electron microscopy (in I , II, III) shows that the channel localizes within the late endosomal (white Arrow) and lysosomal (Red Arrow) membrane, as well as in multivesicular bodies on the intraluminal vesicles (Yellow Arrow) respectively. **(G)** Phosphatidylinositol 3,5-bisphosphate (Pi (3.5)P2) was detected using a Pi(3,5)P2 genetically encoded GFP fluorescence probe and showed Pi(3,5)P2 (Green) localizes with TRPML-1 (Cyan) and LAMP-1 positive compartments (Red).

We previously saw that parts of these enlarged compartments were positive for CatD and CD63 signals in the immuno-fluorescence microscopy data *(Figure 2.C I,II Red Arrow; Figure 2.D I, Red Arrow)*. Interestingly the morphological visualization by electron microscopy also showed accumulated content in one of the sides of these structures *(Figure 3.A, Yellow Arrow)*.This suggests these specific parts of the structures may include the enzymes and proteins carried by these organelles, and represent the CatD and CD63 positive domains in these organelles.

Later, a set of EM images was collected for quantitative calculations; the organelles observed in each image were measured individually using ImageJ software and its "freehand line" tool. The sizes thus obtained were then compared between wild-type and overexpressed-TRPML-1 HT1080 cells. The data showed significant increase in lysosomal size in HT1080 overexpressing TRPML-1 cells comparing to the Wild Type. *(Figure 3.C)*.

To investigate the enlarged organelles with immuno-EM, we again prepared overexpressed TRPML-1-GFP HT1080 cells, , labelled the cells for GFP conjugation with 10 nm gold particles, and visualized under the TEM. As expected, the enlarged organelles were positive for gold particles targeting GFP*(Figure 3.D, Red Arrow)*.

3.4. Luminal Characteristics of Enlarged Organelles.

3.4.1. Endocytosis efficiency within the enlarged compartments.

To investigate whether the cargo entering through endocytic pathways in the cells can reach these organelles, we used the overexpressing cells which were treated with BSA-coated gold particles for 3 hours. Owing to their small size, biocompatibility and binding affinity for negatively charged lipids on the cell surface , these gold particles can be taken up into cells and pass-through endosomal system compartments from early endosomes to late endosomes

and LYSs. All these properties make them good candidates for studying endocytic pathways within the cells. (Griffith and Reggiori 2009)(Purohit and Singh 2018) .After fixation and embedding in resin, they were observed under a TEM.

Obtained data from the experiment, showed that although some of the mentioned compartments had taken up the external gold particles *(Figure 4.A, Yellow Arrow)*, the amount of gold particles in the organelles was low, and majority of them did not have the particles *(Figure 4.B, Red Arrow)*.To further study the fluid-phase endocytosis in the transfected cells using TRPML-1-mCherry plasmid *(Supplementary data, S1)*, we treated the cells with a endocytic dextran probe and incubated them for 3 hours .This probe can be taken up by micropinocytosis and it's a common probe to study the properties of endocytic pathways within the endolysosomal system. (Li et al. 2015)
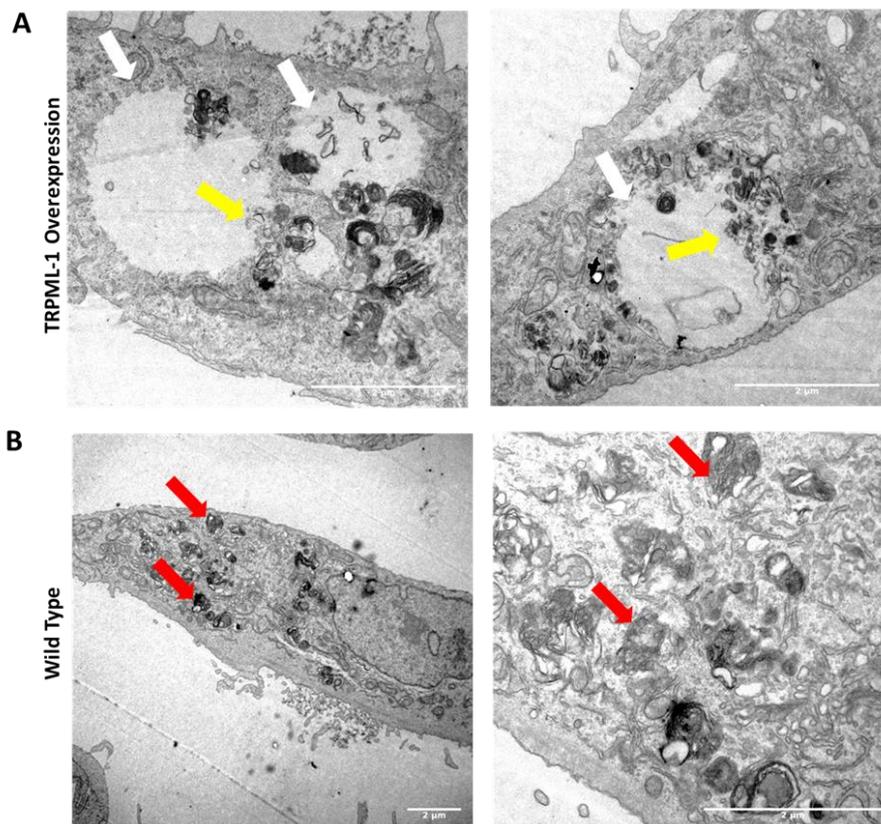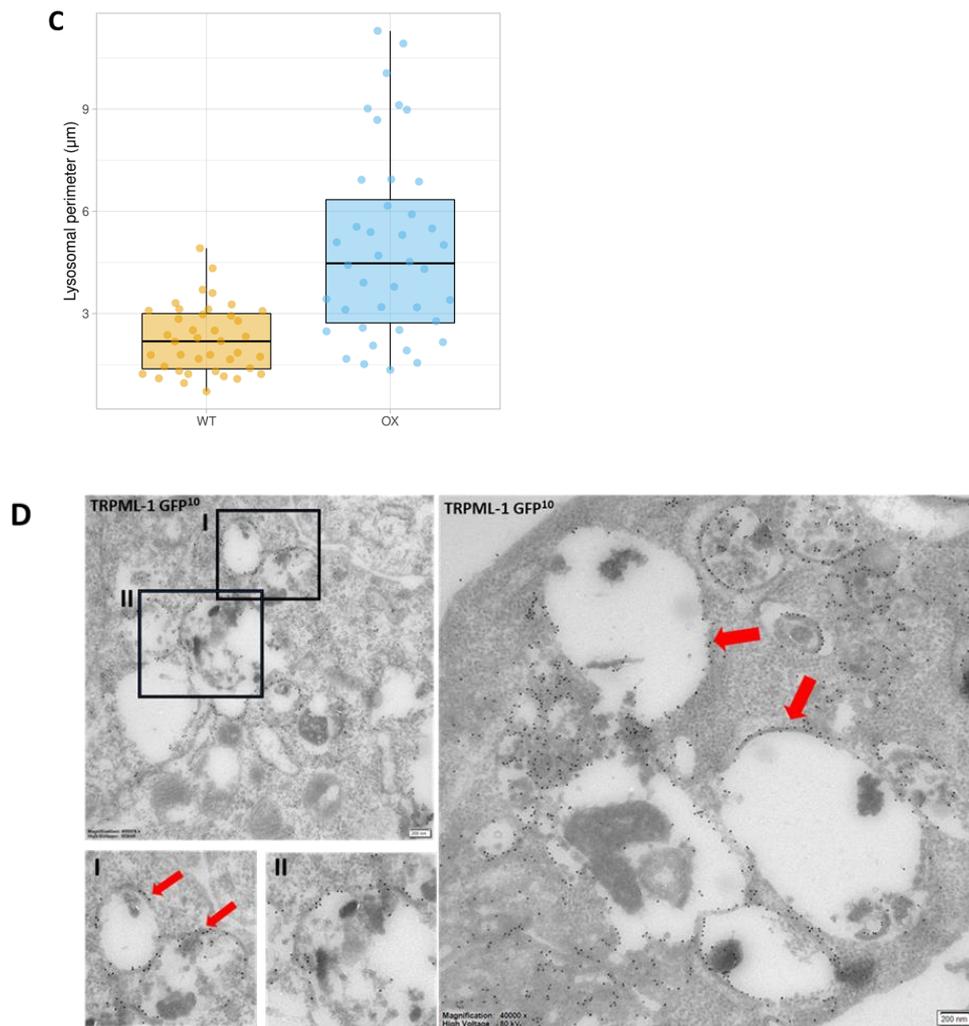
Figure 3. Appearance of enlarged organelles upon TRPML-1 overexpression. (A) Transmission electron microscopy visualization of HT1080 cells shows enlarged organelles upon overexpression of TRPML-1(White Arrows) and also accumulated content were observed in in once side of the enlarged organelles (Yellow Arrows). (B) Heterogeneous and smaller lysosomal compartment size in wild type HT1080 cells (Red Arrows). (C) Quantitative size analysis in HT1080 wild type and overexpressed TRPML-1, showing the increased lysosomal organelle size upon TRPML-1 overexpression. (D) Immune electron microscopy visualization of HT1080 cells overexpressing TRPML-1 GFP, showing TRPML-1 localization on the enlarged organelles (Red Arrows), which confirms the enlarged organelles were caused by TRPMl-1 overexpression.

After 3 hours and further fixation, the cells were labelled for LAMP-1 by the immunolabeling procedure for late endosomal/lysosomal compartment detection, and visualized using immunofluorescence microscopy. In accordance with previous data, the images showed that although some of the enlarged TRPML-1/LAMP-1-positive organelles reached by the dextran probe in 3 hours*(Figure4.C, I-I , I-II, white Arrow),* but most of the these organelles in overexpressed cells can't be efficiently reached by the probe *(Figure4.C, I-I , I-II, Yellow Arrow),* while in the none overexpressing cells *(Figure4.C, II-I , II-II, white Arrow)*

all the LAMP-1 positive late endosomal/lysosomal compartments were reached by the endocytosed materials.

### 3.4.2. Luminal pH of the enlarged organelles

LYSs have an acidic intraluminal environment compared to the cytosol of cells. This acidity ranges from ~4.5 to 5.0 and can be generated and maintained by the activity of lysosomal proton-pumping V-type ATPase. The rest of the cells can be protected from this acidity and degradative enzymes by the lysosomal membrane. (Mindell 2012). It has already been demonstrated that TRPML-1 alteration can affect lysosomal pH (Soyombo et al. 2006). We therefore wanted to show whether or not these enlarged organelles are acidified compartments. To this end we transfected cells with TRPML-1-mCherry construct, and stained them with lysotracker: a fluorescent acidotropic probe able to detect acidic organelles within the cell after 30 minutes of exposure. After fixation, we labelled with LAMP-1 antibodies for detection of lysosomal compartments. Data shows lysotracker colocalizes in the mentioned organelles*(Figure 5.A, I-I , I-II)*, which means they have an intraluminal acidity in the range of ~4.5 to 5.0.

3.4.3. Hydrolase activity within the enlarged organelles

LYSs contain more than 50 enzymes used for lipid degradation: phospholipids, glycolipids, proteins, nucleic acids and sugars. Cathepsins are one of these enzymes which act in hydrolytic activities inside the LYS. The activity and functionality of these enzymes can be triggered by and maintained in the aforementioned acidic environment of LYSs. Earlier, using CatD antibodies that detect this enzyme within the late endosomal/lysosomal compartments, we could see the enlarged organelles caused by overexpressing TRPML-1 contain CatD. Here we wanted to address the question whether these detected enzymes are active and functional. For this purpose, the cells were transfected with a TRPML1-mCherry construct and treated with SiR-lysosome for three hours. This probe is cell-permeable, highly specific for LYSs and able to bind to the active forms of catD enzymes. Three hours later the cells were fixed and, after LAMP-1 immunolabeling, visualized under a fluorescence microscope. Obtained images showed that the organelles which are positive for both TRPML-1 and LAMP-1 contain active CatD and interestingly, these enzymes were accumulated on one side of the organelle (similar to as observed for CD63). *(Figure 5.B,White Arrow)* This finding is in accordance with the immunofluorescent images and electron microscopy images which were showing presence of luminal.
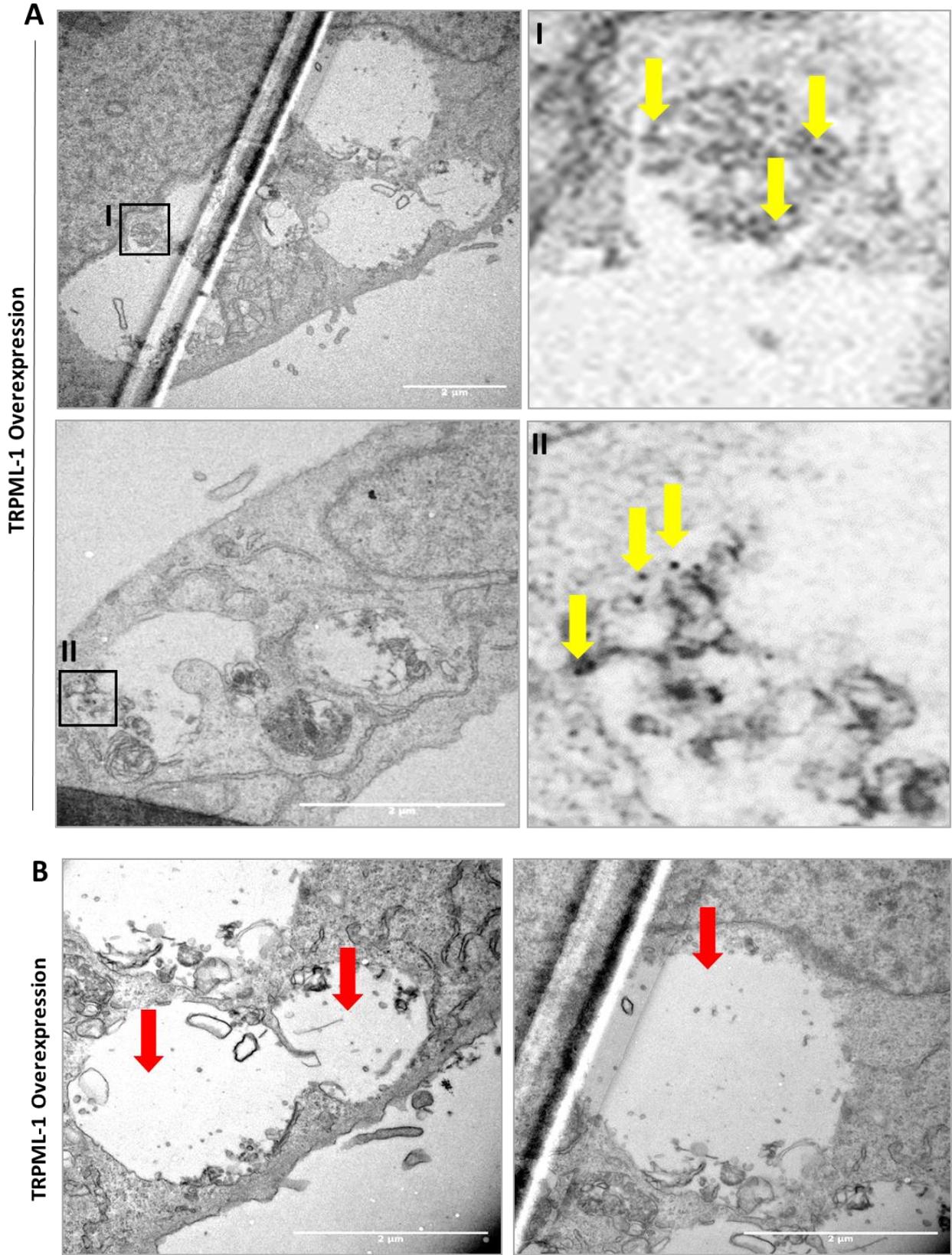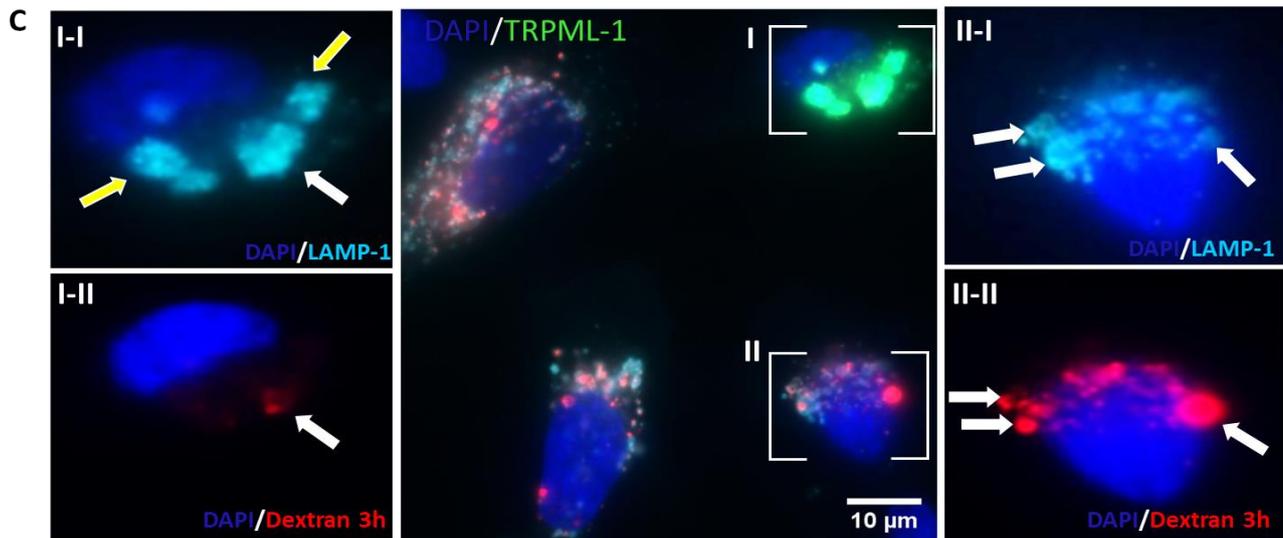
Figure.4. Enlarged compartment endocytosis efficiency. (A,B) Electron microscopy data of HT1080 wild type and overexpressed TRPML-1 cells, treated with BSA-coated gold particles. This data indicates that most of the internal compartments don't show internal cargo (Red Arrow), even though low amounts of gold particles were observed in a few enlarged compartments (Yellow Arrows). **(C)** Immunofluorescent observation of fluid-phase endocytosis in HT1080 transfected TRPML-1 cells using a dextran probe for 3 hours. Lysosomal compartments were detected using LAMP-1 antibodies. The data showed although some of the enlarged organelles reached by fluid material (I-I,I-II white Arrows) most of LAMP-1-positive enlarged organelles cannot be efficiently reached by endocytosed fluid materials (I-I, I-II, Yellow Arrow). On the other hand, in none overexpressing cells (II) all the LAMP-1-positive compartments were reached by the probe. (II-I,II-II, White Arrows)

Another point that can be seen in these pictures is that not all the TRPML-1 positive organelles are LAMP-1 positive or Sir-lysosome positive, which reveals they are possibly earlier endosomes with a less acidic environment and subsequently less active cathepsins. This is in accordance with the previous findings about the TRPML-1 location within the endosomal system. Overall, this data shows that the enlarged lysosomal compartments caused by TRPML-1 upregulation contain active cathepsin.

Due to the fact that the activity of these enzymes within LYSs normally leads to the degradation of endocytosed materials in these organelles, we examined whether endocytosed cargo can break down in these enlarged organelles. After the transfection process using the TRPML-1-mCherry construct, we added a DQ-BSA probe in the growth medium for 30 minutes. DQ-BSA is a fluorogenic substrate for hydrolysis enzymes such as CatD. This substance turns fluorescent when cleaved by these proteases. Therefore they are commonly used to detect active cathepsins.

Observing the data, these organelles, show DQBSA signal and degraded materials are stored on one particular side of the enlarged LYSs. A similar positioning has been shown for CD63 and CatD in immunofluorescence images in the previous sections. *(Figure 5.C, White Arrow)*

3.4.4. Calcium content of the enlarged organelles

Ca^{2+} is known as a universal second messenger and it plays important roles in many signalling events within the cell. These events constitute essential processes in the cell, such as gene expression, exocytosis, cell growth, proliferation, differentiation, cell motility and cell death. LYSs containing 0.4–0.6 mmol/L of calcium ions are considered to be the main intracellular storage for Ca^{2+}. To track the calcium content in the lumen of these big LYSs, TRPML-1 overexpressed cells are treated with dextran and a calcium indicator (BAPTA-dextran) for 3 hours. They were then visualized under live-cell imaging conditions using Thunder FM. BAPTA-dextran can translocate to intracellular compartments and once bound to calcium, turns fluorescent. It is therefore a good candidate for investigating intraluminal calcium content of endo-lysosomal organelles. It is combined with normal dextran, which is used as an internal control for investigating endocytosis. This combination reveals if the presence or absence of calcium is because the organelles lack calcium or because the calcium indicator probes could not reach them by endocytosis. In this case if we see a dextran signal but no calcium, it means these organelles do not contain calcium, whereas if we don't see any signals, dextran couldn't be taken up by the oganelles during the endocytic process. These can provide us accurate observation judging the intralaminar calcium content of the intracellular compartments.

Collected images show strong signals of the calcium indicator within the big LYSs, revealing they contain this ion in their intralaminar environment. *(Figure 5.D, White Arrow)*

This data also shows us some of the TRPML-1 positive compartments are not reached by dextran *(Figure 5.D, Red Arrow)*, this is in accordance with endocytosis efficiency upon TRPML-1 overexpression, which has been shown in detail earlier. *(Section 3.4.1)*.
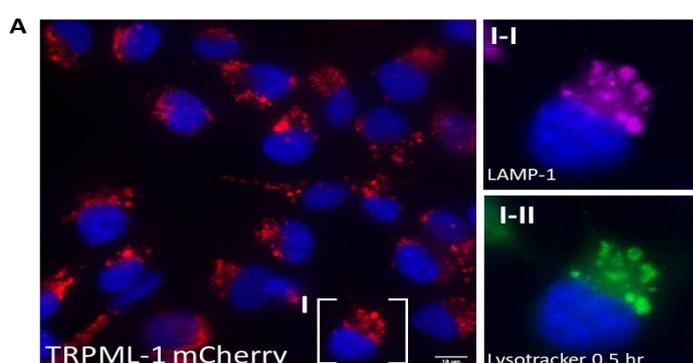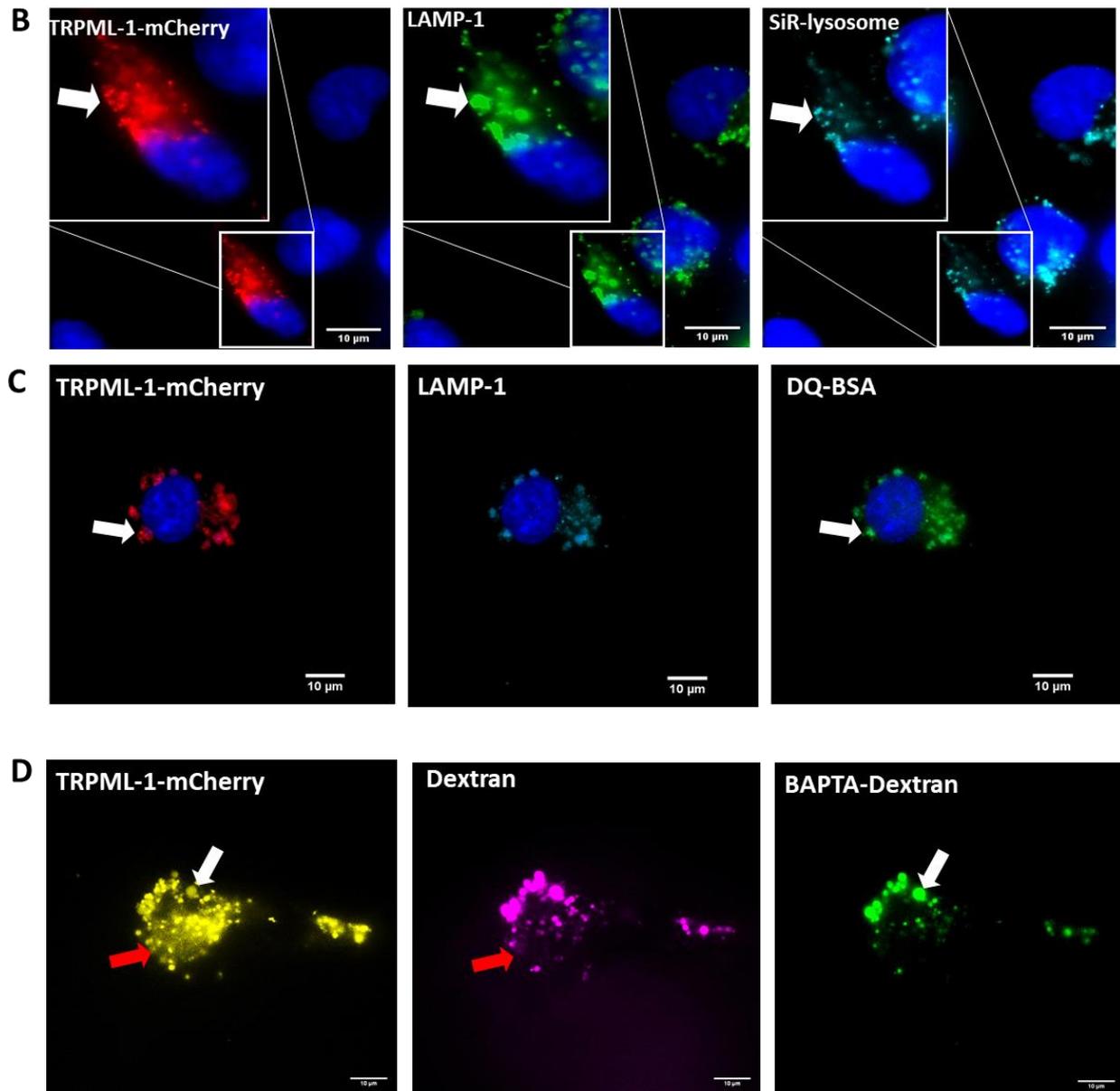
Figure 5. Luminal characteristics of enlarged organelles. (A) Using lysotracker (Green) and LAMP-1 (Magenta) for lysosomal organelle detection in HT1080 overexpression TRPML_1mCherrry (Red) , the acidity of the enlarged organelles was revealed to range from 4.5 to 5.0. **(B,C)** Enlarged organelles in HT1080 TRPML-1-mCherry (Red) overexpressing cells showed active cathepsin and degradation ability using SiR-Lysosome (Cyan) and DQ-BSA probe (Green). **(D)** Using BAPTA-Dextran (Green) as a calcium indicator and live cell imaging of HT1080 TRPML-1-mCherry (Yellow) overexpressed cells showed the enlarged organelles have intraluminal calcium.

3.5. Depletion of TRPML-1 results in more lysosomes

To resolve the effect of TRPML-1 depletion in lysosomal compartments in HT1080 cells, the cells were transfected with siRNA-TRPML-1 to knock-down (Kd) the channel in these cells. Later, the fixed Kd and control cells (scrambled) cells on coverslips were stained for lysosomal proteins LAMP-1 and CatD following the immunofluorescence labelling procedure. The samples were visualised using an Delta vision FM . The data showed strong differences in lysosomal numbers between control and kd cells. Compared to the control (scrambled), a higher number of lysosomal organelles was observed in Kd cells , the LYSs of which also showed slightly smaller sizes *(Figure 6.A)*. These results corresponded with the transmission electron microscopy data from the ultrathin sections of resin embedded Kd cells *(Figure 6.B Circulated in Red)*. Later each lysosomal compartment in the TEM data sets were identified, and their sizes were measured using the "freehand line" tool in ImageJ software. The lysosomal perimeter sizes were compared between TRPML-1-Kd and control samples. As shown in the plot, lysosomal sizes in control are slightly bigger than the ones in the kd samples *(Figure 6.C)*.
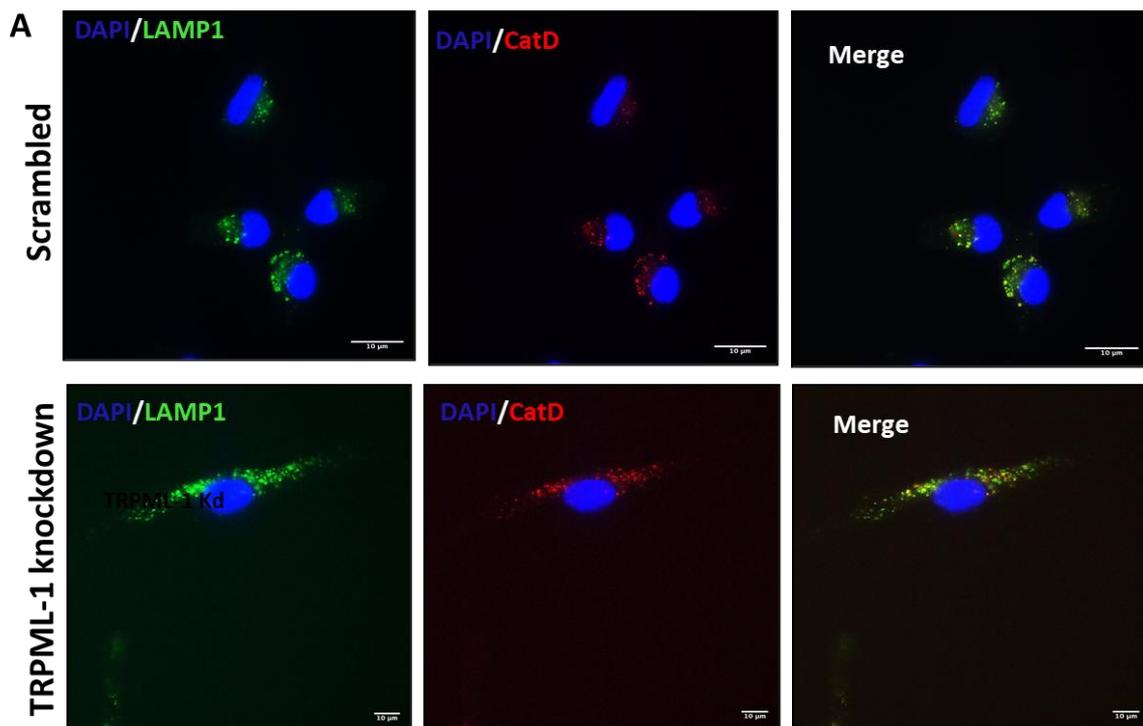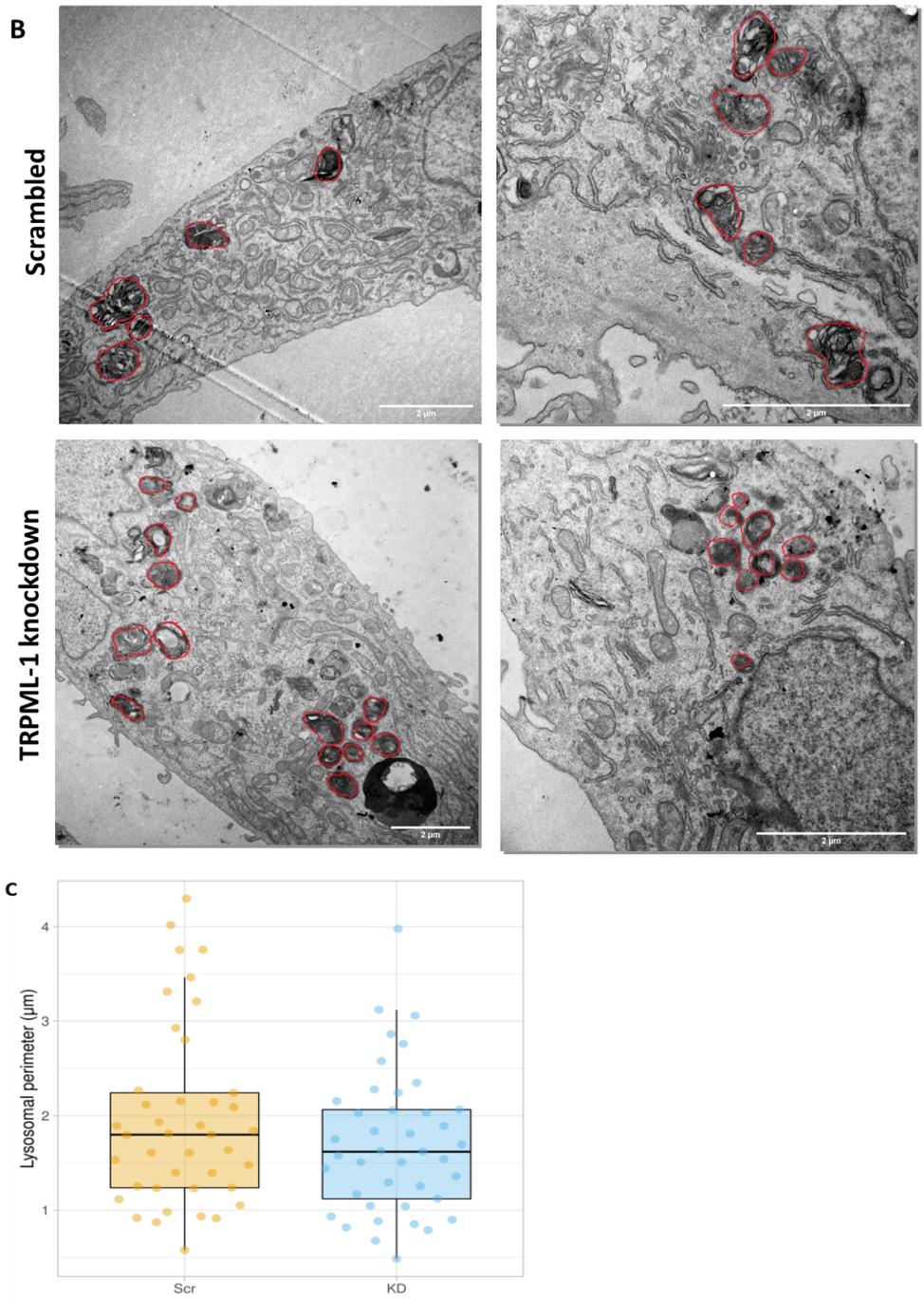
Figure 6. Depletion of TRPML-1 results in more and slightly smaller lysosomes. (A) Lysosomal compartments were detected using LAMP-1 (Green) and CatD (Red) in scrambled and TRPML-1 knockdown cells. TRPML-1 knockdown cells showed more lysosomal organelles compared to the scrambled ones and were slightly smaller as well.**(B)** EM data obtained from both scrambled and TRPML-1 knockdown confirmed previously obtained immunofluorescence data. Lysosomal compartments are circulated in red. **(B)** Size analysis comparison in scrambled and knockdown cells showing lysosomes in knockdown TRPML-1 cells are slightly smaller.

3.6. TRPML-1 expression level affect other organelles in HT1080 cells

Two distinct organelles can communicate by coming into close apposition in a way that their plasma membranes contact each other and can therefore exchange different molecules and ions. Previous studies on contact sites have shown their important roles in cellular homeostasis regulation and transformation of different ions such as calcium. It has already been demonstrated that TRPML-1 activity regulates mitochondrial calcium dynamics by tethering mitochondrial-lysosomal contact sites. (Peng et al., 2020).

Analysing data obtained during our study on HT1080 cells, we observed TRPML-1 expression to affect other organelles within the cells. Our observation showed ER contact deficiency around endolysosomal compartments (*Figure 7A,Red Arrow*), whereas under normal conditions these organelles are surrounded by an intense population of ER tubules. (Fermie et al., 2018; *Supplementary data S6*). Furthermore, we observed increased association between ER and mitochondria (*Figure 7B*), as well the appearance of a significant amount of lipid droplets upon TRPML-1 depletion, which was not seen in the wild type HT1080 cells. (*Figure 7C, Orange Stars*) Although this preliminary data needs more investigation and study, it removes any doubt that TRPML-1 expression levels alter and otherwise affect other organelles.
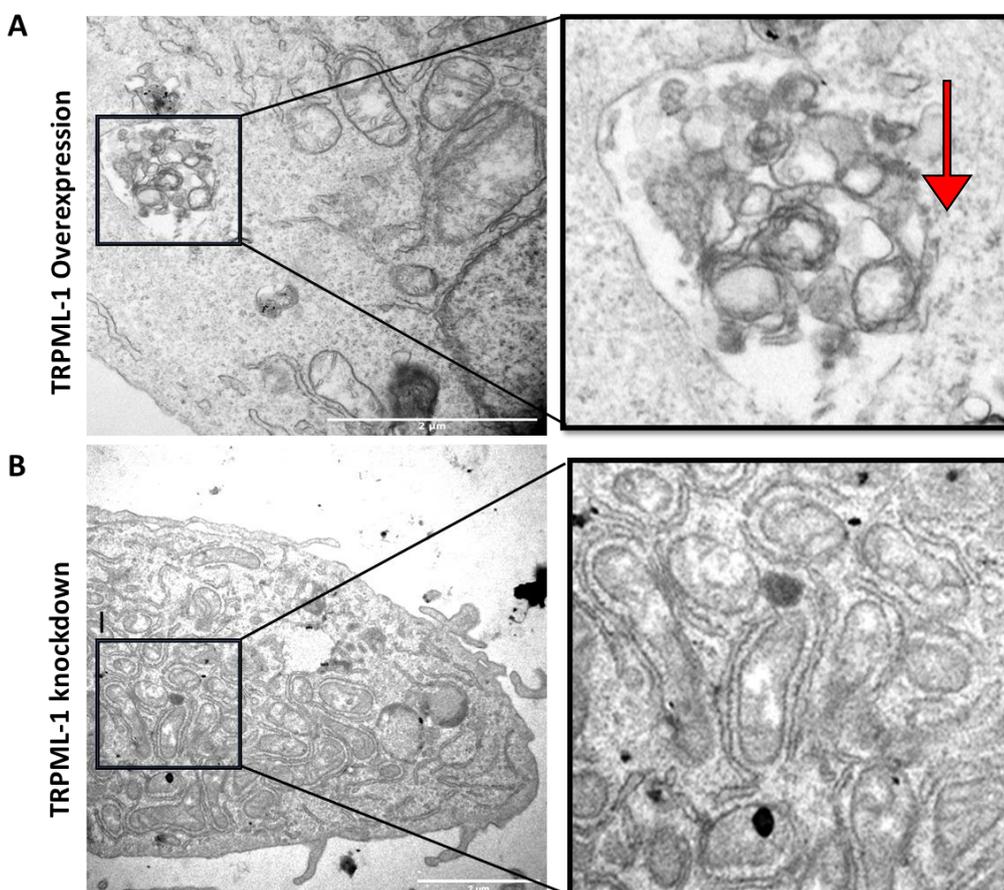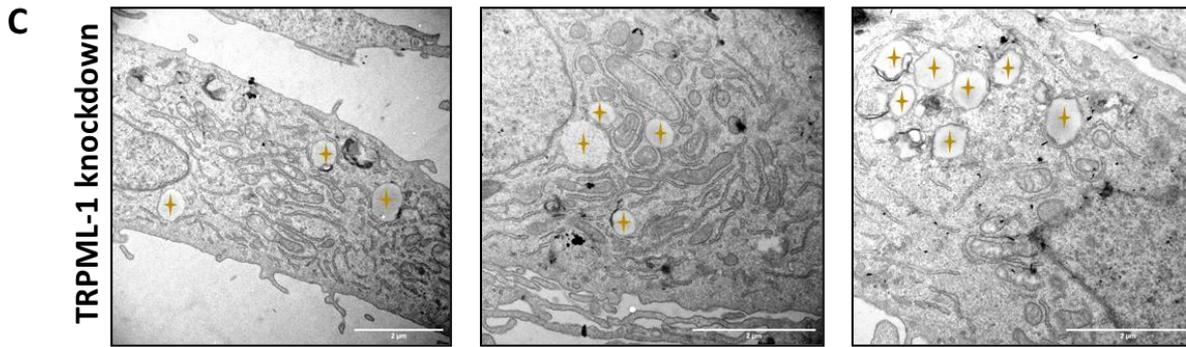
Figure 7. TRPMl-1 overexpression and remodelling of other organelles. (A) Lysosomal compartments surrounding HT1080-overexpressed cells showed no presence of any endoplasmic reticulum (Red Arrow). These organelles are normally surrounded by intense populations of ER. This data showed TRPML-1 overexpression in HT1080 causes a deficiency in lysosome-ER interactions. **(B)** Intense distribution between ER and mitochondria observed in HT1080 cells upon TRPML-1 depletion. **(C)** HT1080 cells showed a lot of lipid droplets (Stars) upon TRPML-1 depletion.

3.7. TRPML-1 expression affects TFE3 signalling

Previous studies have already showed a link between TRPMl-1 activity and TFEB/TFE3 transcription factors regulations (Wang et al., 2015). To investigate whether the expression level of this channel affects the level of late endosomal and lysosomal proteins in invasive HT1080 cells, the TRPML-1 Kd cells, TRPML-1 overexpressing cells and the control HT1080 cells were prepared for western blotting. The following proteins were detected in the blot: Cathepsin B and D, CD63 and LAMP-1. The obtained blot revealed that kD of TRPML-1 caused elevation of expression level of lysosomal proteins, exceeding both the control (scrambled), the TRPML-1 overexpression and wild-type samples *(Figure 8.A)*.

Among the aforementioned proteins, especially the level of Cathepsin B in Kd cells showed a significant difference compared to the other samples. *(Figure 8.B)*

Concluding the above observation, we questioned whether TRPML-1 may act as a negative regulator of TFE3/TFEB signalling. TFE3 is a master transcriptional regulatory gene for lysosomal function, metabolism and biogenesis. Activation

and deactivation of TFE3 upon its de-phosphorylation and phosphorylation regulates its intracellular distribution and its transcriptional program. Inactive TFE3 accumulates in cytoplasm and de-phosphorylated TFE3 translocates into the nucleus to drive a transcriptional program and therefore more lysosomal proteins are transcribed to further expression. To examine this hypothesis, using siRNA, TRPML-1 was depleted t in HT1080 cells, fixed, and labelled for

TFE3 in comparison with its control. The data obtained by the Delta vision FM showed in the kd cells, TFE3 highly translocated from the cytoplasm to the nucleus compared to the control. This indicates in kd cells TFE3 is active in the nucleus transcribing the lysosomal genes, matching our WB data which indeed shows an upregulation of e.g. Cathepsin B upon kd *(Figure 8.C)*.
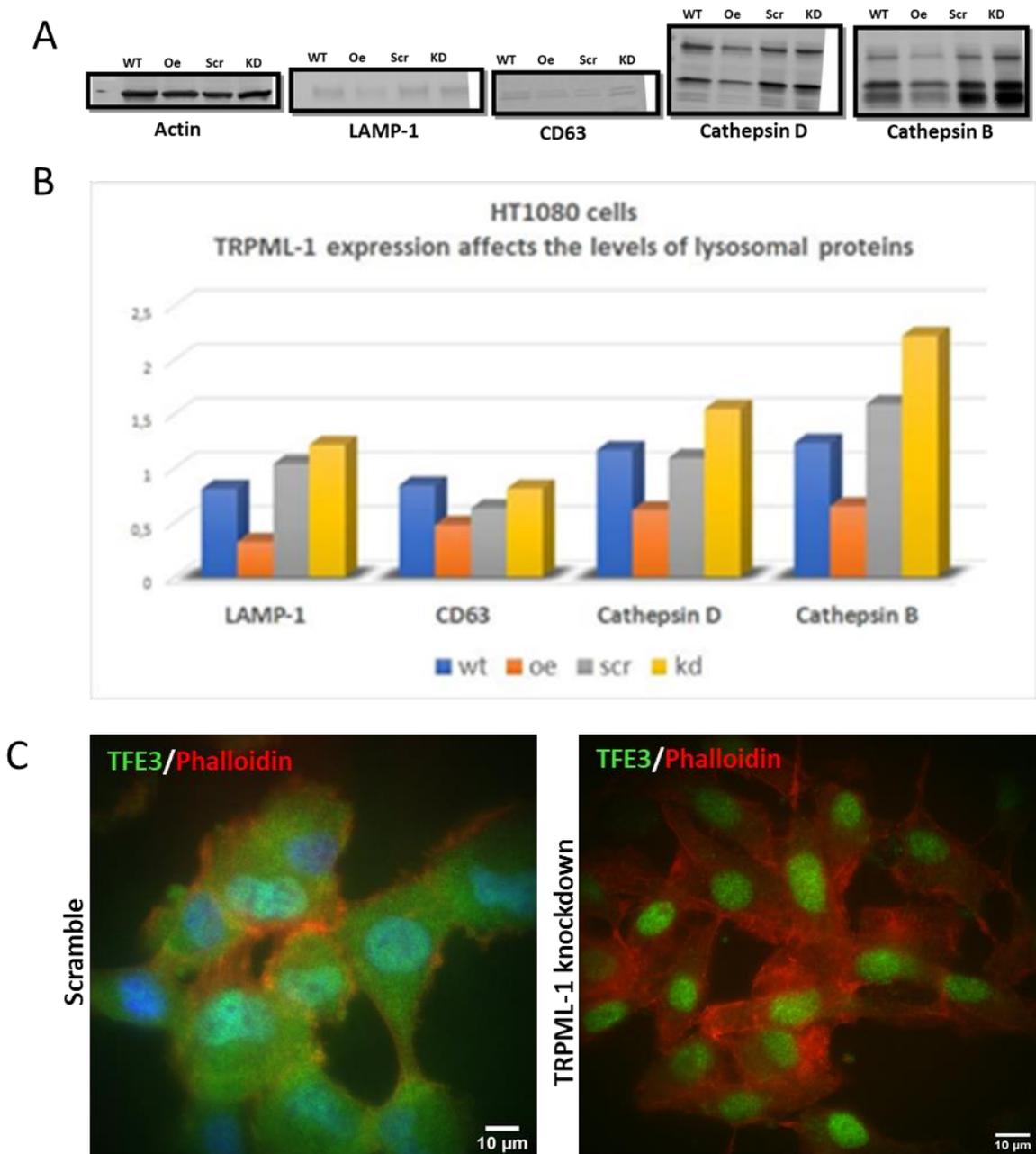
Figure 8. TRPML-1 affects the level of lysosomal proteins and TFE3 signalling. (A,B) WB data of TRPML-1 overexpression and knockdown and wildtype cells, detected for Cathepsin D (CatD), Cathepsin B (CatB), LAMP-1 and CD63 as late endosomal/lysosomal organelles. TRPML-1 depletion showed higher levels of the mentioned proteins in HT1080 cells, CatB generally surpassing even the other three. **(C)** Transcription factors E3 (TFE3 : Green) were translocated to the nucleus in TRPML-1 knockdown cells, whereas the scrambled cells showed cytoplasmic localization of TFE3.

3.7. TRPML-1 and cancer cells migration:
3.7.1. HT1080 overexpressed TRPML-1 increases lysosomal exocytosis

In contrast to most cells with a dominant perinuclear localization, LYSs in cancer cells often have a peripheral localization . This distribution is in line with their fusion with plasma membrane and liberation of their intraluminal components to the extracellular matrix (ECM), which increases the acidity and causes ECM degradation by releasing hydrolases. This ultimately contributes to cancer development and invasion. (Kikuta and Ishii 2013) It has already been shown that TRPML-1 upregulation elevates lysosomal exocytosis in triple negative breast cancer, and stimulates the creation of tubular protrusions on the cell surface caused bij LE/Lys exocytosis.(Xu et al. 2019) (LaPlante et al. 2011). To investigate the positioning and changes in the level of lysosomal exocytosis in HT1080 cancer cells upon alteration of TRPML-1, we transfected the cells with a TRPML-1-GFP construct and fixed on coverslips after 24 hours. They were subsequently prepared for immunolabelling with LAMP-1 and CatD markers. To measure the amount of the exocytosed LYS (LAMP-1 content) on the plasma membrane of the cells, the cells were not permeabilized by triton during the labelling steps. The coverslips were observed by the Delta vison FM. The data showed an outstanding increase in the presence of LAMP-1-positive compartments on/around the plasma membrane upon overexpression of TRPML-1 *(Figure 9.A , White Arrow)*, while the cells which were not overexpressing the channel showed a lower percentage of these exocytosis organelles on their plasma membrane *(Figure 9.A, Red Arrow).* On the other hand, there were no significant signals showing CatD enzymes on the membrane, which suggests these enzymes may already have been released into the ECM environment *(Figure 9.A,CatD).*

Taken together the data shows the significant increase in lysosomal exocytosis upon overexpression of TRPML-1 in HT1080 cells. Also some expressing cells showed a ring shaped compartment around the plasma membrane which suggesting the ring shaped organelles which we often observed upon TRPML-1 overexpression could be the exocytotic profile of the organelles (*Figure 9.B,White Arrow).*

3.7.2. TRPML-1 expression level does not significantly affect HT1080 cell migration in 2D

As a consequence of lysosomal exocytosis the intraluminal hydrolases such as cathepsins can be secreted outside the cells and consequently degrade the extracellular matrix causing faster cancer cell migration and invasion.(Morgan

et al. 2018). To study 2D migration of HT1080 cells upon TRPML-1 alteration, we performed a wound healing assay. The cells were cultured on coverslips and later depleted for the channel using siRNA-TRPML-1. To induce wounds in the 2D cell sheet, the cell growth surface was mechanically scratched by using pipette tips, and later imaged at different time points from 0 to 32 hours. The data did not show a significant difference in Kd TRPML-1 cells compared to the control (*Figure 9.C*). In parallel, overexpressed TRPML-1 and wild type HT1080 cells were observed for a duration of two hours, using live-cell imaging capturing an image sequence at regular intervals of 2 minutes. This data as well as the previous wound healing data did not show a significant difference in 2D cell movement or displacement in either condition. (*Movie 2 and 3*).

### 3.7.3.TRPML-1 expression level affects HT1080 cell migration in 3D

To further investigate the role of TRPML-1 in cancer cell migration, HT1080 cells were cultured in a 3D collagen environment , and their migration was studied through transwell assays. Transwell migration is a commonly used technique to study 3D cell migration. During the procedure growth medium lacking FBS and medium containing FBS should be added to the upper and lower chambers of the transwell system, respectively (*Figure 9.D,I*). This provides chemical attraction for the cells to move toward the nutrients inducing 3D migration (*Figure 9.D,II*). It is also common to add collagen as ECM material in the upper chamber to mimic a 3D environment for the cells. The invaded cells can then degrade the collagen and move t towards the lower chamber (*Figure 9.D, III*).Then, cells can be fixed and stained for observation with a microscope. Using this approach in our study, HT1080 cells were transfected with TRPML-1-mcherry plasmid and later seeded in a trans well assay containing the ECM. After 16 hours the overexpressing cells were fixed and stained for the nucleus using prolong-gold DAPI and visualized under Revolve microscope. Interestingly, unlike the cell migration in 2D, the transwell assay showed a significant difference in the 3D migration capacity of HT1080 cells overexpressing TRPML-1 and wild-type cells. The HT1080 cell overexpressing TRPML-1 showed a significantly higher amount of cells which have migrated towards the lower chambers (*Figure 9.E*). This observation is in accordance with elevated lysosomal exocytosis upon TRPML-1 overexpression meaning that TRPML-1 mediates thelysosomal exocytosis and in consequence more intraluminal hydrolases are secreted, which degrades the ECM, w, causing a more efficient 3D cell movement and possibly invasion during cancer development. (*Movie 4*).

C

| | 0 | 8 hrs. | 16 hrs. | 32 hrs. |

Scrambled

TRPML-1 knockdown

D

I

Medium(-FBS)

ECM

Medium(+FBS)

II

III

E

Wild Type

TRPML-1 Overexpression
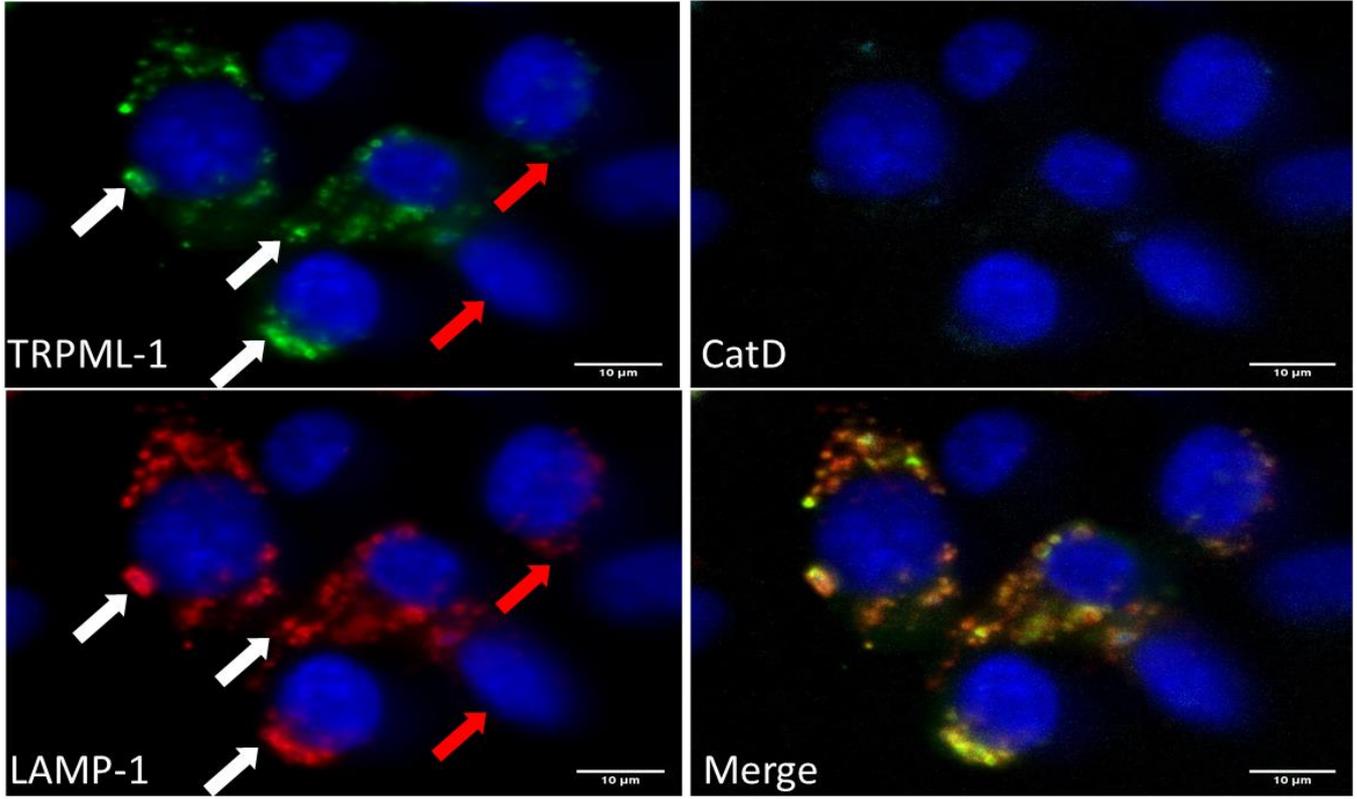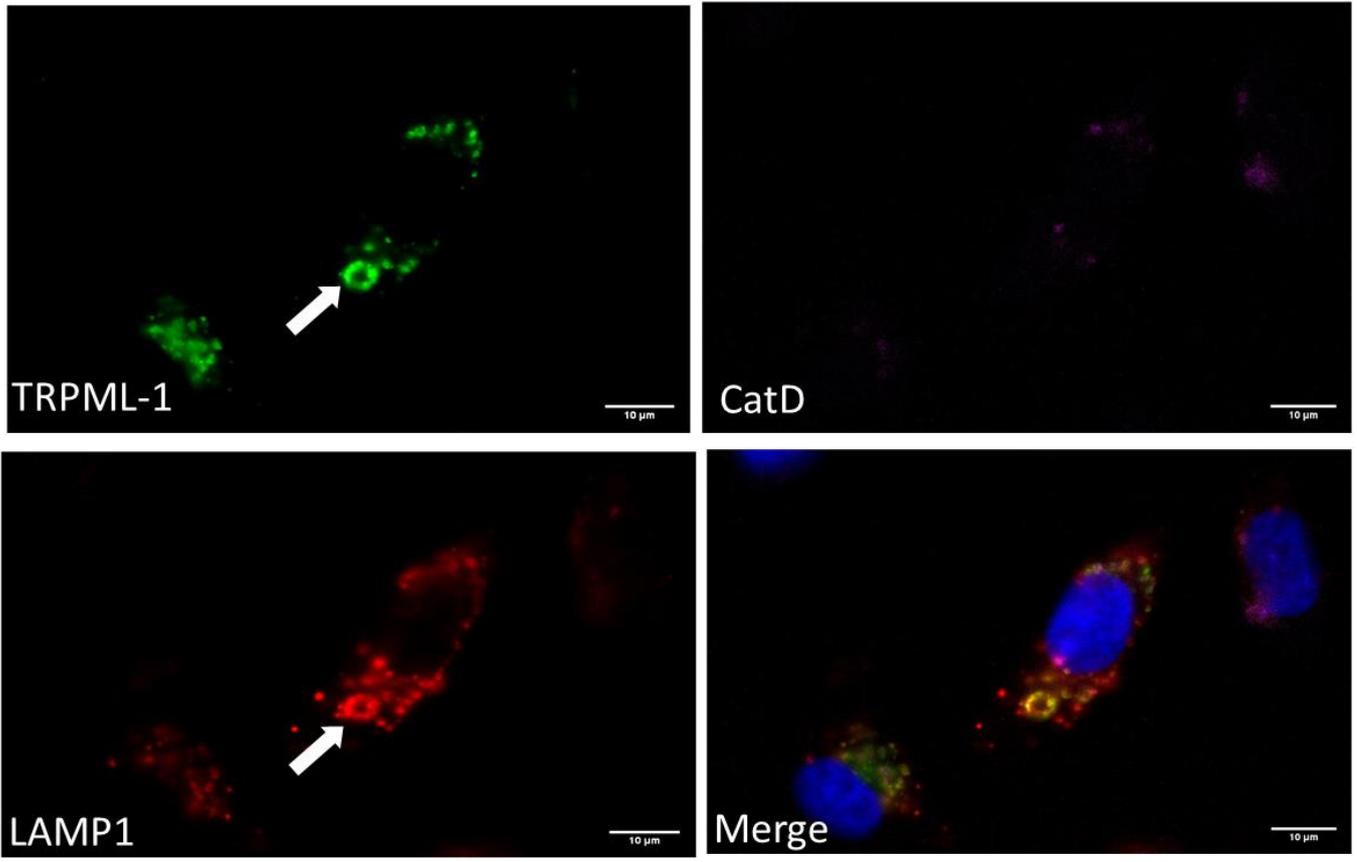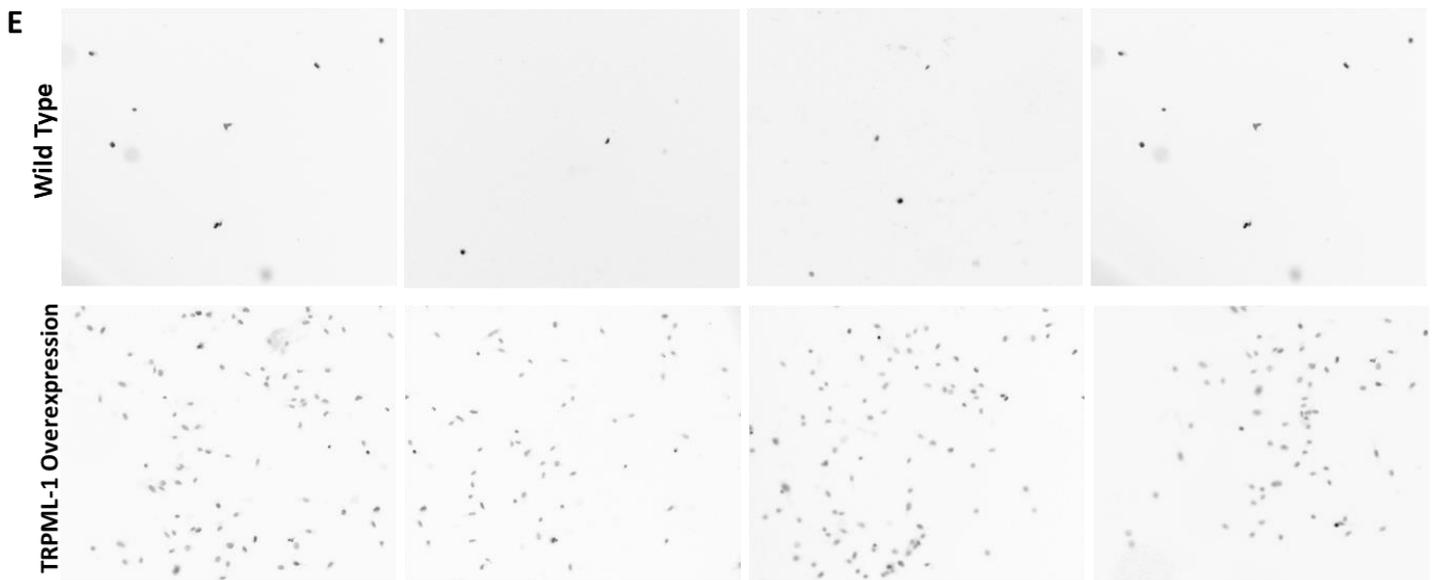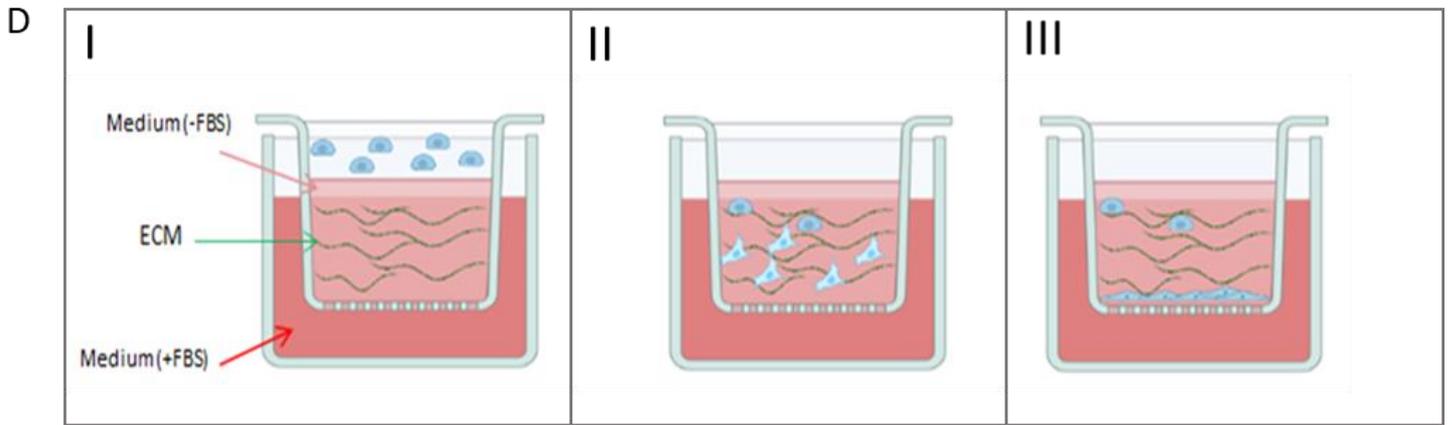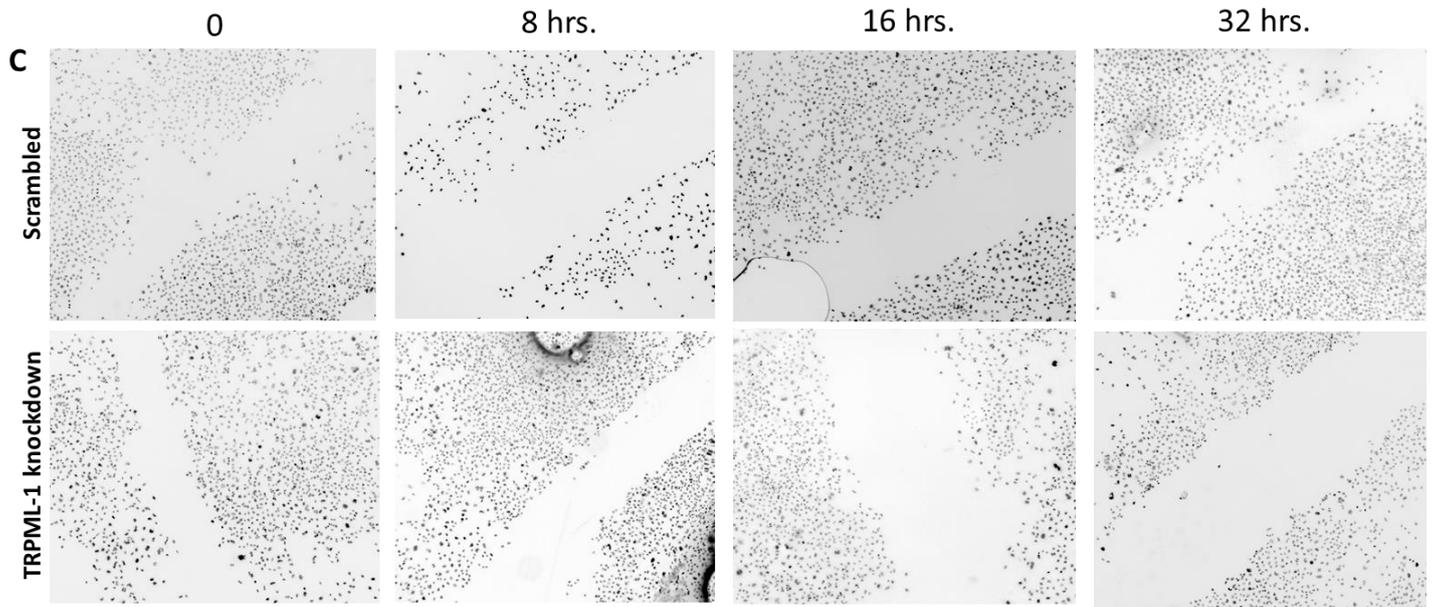
Figure 9. TRPML-1 and cancer cell migration. (A) TRPMl-1-overexpressed cells showed an increase in LAMP-1(Red) lysosomal compartments on/ around the plasma membrane (White Arrow). On the other hand, none of the overexpressing cells showed a lower percentage of lysosomal compartments on the plasma membrane (Red Arrows). The data demonstrates overexpression of TRPML-1 induces lysosomal exocytosis from the plasma membrane to the extracellular environment, which in turn increases cancer cell movement and migration, due to ECM degradation. **(B)** Ring-shaped LAMP-1 positive lysosomal organelles were observed on/around the plasma membrane in some overexpressing cells (white Arrows) , suggesting it could be an exocytosis profile for these organelles. **(C)** 2D cell migration assays induced by scratching coverslip cell surface using pipet tips. The coverslips were imaged in different time points from 0 to 32 hours. The data did not show a significant difference in TRPML-1 knockdown cells compared to scrambled. **(D)** Schematic of 3D cell migration assays using transwell. Transwell consists of two chambers, the upper one of which contains medium without nutrients and ECM, whereas the lower chamber does have nutrients. (I) This provides chemical attraction for the cultured cells in the upper chamber to migrate toward the chamber with the nutrients. (II) The invaded cells can degrade the extracellular matrix and move through it during their migration(III) .**(E)** Invasion comparison between wildtype and overexpressed TRPML-1 cells cultured in transwells. The cells were stained using dapi and observed using a revolve microscope. The data shows a significant difference in the amount of invaded cells upon TRPML-1 overexpression. TRPML-1 overexpression increases the migration of the cell toward the lower chamber, in consequence of increased ECM degradation induced by invaded cells.

4.Disscusion

Alteration of LYSs in cancer cells has been demonstrated before .Previous studies have shown a higher activity in LYSs in cancer. A lot of these alterations and changes in LYSs are associated in Ca^{2+} homeostasis in cells. (Davidson and Van der Heiden 2017) (Geisslinger et al. 2020) LYSs are one of the main storages for this ion and its current is regulated by the calcium channels on the lysosomal membranes(Dong, Wang, and Xu 2010). TRPML-1 is one the ionic channels on the lysosomal membrane which releases the calcium from the intraluminal to the cytosolic environment. Previous studies showed a strong correlation between this channel and tumorigenesis and cancer migration (Yin et al. 2019) (Hu et al. 2019) (Kasitinon et al. 2019)(Morelli et al. 2019). It has already been demonstrated that TRPML-1 is upregulated in invasive cancer cells (Jung et al. 2019) (M. Xu et al. 2019). In this study we showed the role of these channels in endolysosomal regulation and cancer invasive migration. In (*section 3.1*) we showed the localization of the channels within the endosomal system and our data showed the channel is present mainly on late endosomal and lysosomal compartments *(Figure 2.A,C,D,E)*. We also found localization of TRPML-1 in intraluminal vesicles *(Figure 2.F,II)*, considering that the intraluminal vesicles will release as exosomes to the extracellular environment, and previous studies have showed different roles of exosomes in cancer progression, for example, it has been shown cancer-derived exosomes promotes cancer cell growth and invasive behaviour in different types of stromal cells (Bebelman et al., 2018), but the role of TRPML-1 in cancer-derived exosomes is poorly studied, therefore it would be beneficial to investigate further research on the role of TRPML-1 on these exosomes in cancer.

We later observed the appearance of enlarged compartments (*section 3.1, Figure 3*) upon overexpression of TRPML-1 in HT1080 invasive cancer cell lines using TRPML-1 plasmids. These compartments tested positive for CD63 and LAMP-1 which are late endosomal and lysosomal markers. Hela cells on the other hand, did not show the same enlarged phenotype upon overexpression of TRPML-1 (Supplementary data S4). We can therefore argue that this enlargement can happen explicitly in invasive cancer cell lines. We also observed discrepancies in the expression level of the HT1080 cell line: whereas some cells had enlarged compartments were more pronounced (Supplementary data S2). others were definitely overexpressing TRPML-1 but did not show the enlarged organelles *(Figure 2A)*. It may therefore be relevant to address specific expression levels of the protein causing this size alteration.

Our subsequent experiments were performed to identify the luminal features of these large endo-lysosomal compartments (*section 3.4*). In the initial experiments in (*section 3.4.1*)we showed that the majority of these enlarged endo-lysosomal compartments were not reached by endocytotic material in either solid or fluid phase (*Figure 3*), while all the lysosomal-organelle wildtype samples were reached by internal materials (*Figure 3.C,II-I ,II-II*). Using lysotracker and SirLyso, we later showed (*sections 3.4.2 , 3.4.3*) these organelles to be acidified and to contain active cathepsin. They therefore have the ability to degrade the internal material received by endocytosis. Although the result from (*section 3.4.3*) shows less signals for degraded material in these organelles, the result from (*section 3.4.3*) suggests that this is may these organelles are less easily reached by internal material, and not because they are unable to degrade the delivered materials.

Later we also showed (*section 3.4.5*)these organelles to have intense calcium content (*Figure 5.D*), which, in conjunction with the fact that that overexpressing these channels may not induce more channel activity, causes the question to arise whether or not the aforementioned size alteration takes place by accumulation of this ion in the intraluminal of these compartments. The size alteration by overexpression of this channel may be caused by inactive channels which leads to calcium stock in intraluminal environments. On the other hand, the result of using cytoplasmic calcium reporters (*Supplementary data S5*) showed an intense calcium signal reporter in the cytosolic region around these organelles, demonstrating that these organelles are able to release the calcium from their lumen toward the cytosol. The fact that these organelles are less easily reached by endocytotic material may suggest that there is abnormal alteration upon overexpression of this channel in the fusion or fission process, which leads to an abnormal size. Considering that these enlarged organelles were also observed in the plasma membrane while the cells were not permeabilized, it is also possible these organelles are the exocytotic profile of the lysosomal organelles releasing their lumen into the extracellular environment. This would explain why they have less endocytotic material, having already been released into the ECM environment. It is however equally possible that observation of accumulated proteins on one side of these organelles (*Figure 3.C,D , Figure 5.A*)suggests that we are looking at different steps of the exocytosis event, during which LYSs fuse with plasma membranes, some material not having been released into the ECM environment yet. It would be interesting to study the extracellular medium composition of the overexpressed cells to analyse the released material from the cells to the extracellular environment.

In section (*section 3.5*) we observed smaller but more numerous LYSs upon TRPML-1 depletion. This is also confirmed by WB data (*Figure 8.A*), which suggests that TRPML-1 may act as a negative regulator of transcription factors TFE3/TFEB, also demonstrated in (*section 3.7 , Figure 8.C*),

It has already been shown that TRPML-1 activity induces autophagy independently of TFEB/TFE3 (Wang et al., 2015).It is possible that TRPML-1 knockdown causes a compensating effect in our study which induces autophagy through TFE3/TFEB in TRPML-1's stead.

However, our current result has a discrepancy with literature. Previous research has shown TRPML-1 active TFEB/TFE3 goes to the nucleus(Wang et al., 2015), whereas in our case TRPML-1 depletion actually causes translocation of the transcription factor to the nucleus *(Figure 8.C)*,This discrepancy may depend on cell type or expression level and we should also consider that expression level does not intrinsically mean activity.

In (*section 3.8*) we studied whether TRPML-1 is associated with HT1080 cell migration. Our data confirms previous studies showing that TRPML-1 increases cancer cell migration(Wang et al., 2015)(Xu et al., 2019). We observed significant increases in lysosomal exocytosis upon overexpression of TRPML-1 *(Figure 9.A,B)*, which leads to the liberation of more intraluminal components and thereby to further degradation of the extracellular environment, causing cancer development and invasion (*Movie4*). Although our data from the 2D cell migration assay did not show increased migration in HT1080 overexpressing TRPML-1 cells (*(Figure9.C, Movie 2,3*) there was a significant increase of cell migration in overexpressed cells compared to WT in 3D culture using transwell assays. (*Figure 9.E*)

The mechanical environment for the cells under 2D and 3D conditions are obviously different: in 2D the cells grow on stiff glass or plastic, whereas, in native conditions, they are growing in soft tissues. This stiffness can affect adhesion, spreading, differentiation and migration of the cells. On the other hand, 3D cell culture provides lower stiffness, allowing cells to remodel and degrade the ECM proteins used in the 3D culture and move through them.

These reasons may explain the difference in migration assay results between 2D and 3D samples, the 3D samples representing a more accurate result upon alteration of TRPML-1.

Although our study revealed important roles of TRPML-1 channels in endolysosomal regulation and cancer cell migration, we should consider that

HT1080 is already upregulating these channels and in this case we are actually overexpressing overexpressed cells. It could therefore be best to do further study on knocked-out samples, to open up a new avenue of TRPMl-1 relevant studies.

Future Perspectives

We already shown that TRPML-1 has a strong effect on lysosomal exocytosis, which leads to invading cancer. Total internal reflection fluorescence microscopy (TIRFM) is an imaging technique that could visualise the dynamics of plasma membrane and vesicle trafficking and exocytosis more precisely (Burchfield et al., 2010). Using TIRFM, it is possible to selectively image events on or close to the plasma membrane. In our study we showed that overexpression of TRPML-1 causes an increase of the enlarged lysosomal exocytosis in HT1080 cells. It would therefore be beneficial to track this exocytosis event selectively to study each step more precisely during fusion of the lysosomal organelles with the plasma membrane. It would be relevant to combine this with a lysosomal fluorescent construct, such as for example lysosomal protein conjugated with a GFP-fusion molecule, while the lysosomal organelles are fusing with the plasma membrane. This would only image the GFP molecules in the vicinity of the plasma membrane. Compared to standard IF, this approach offers a significant reduction in background noise (with obvious advantages for studying the later stages of exocytotic trafficking).

We showed that overexpression of TRPMl-1 causes a significant increase in HT1080 cell migration. Although this result was obtained using a 3D environment for the cells, performing a 2D migration assay on the overexpressed TRPML-1 cells did show a significant difference in their migration upon comparison to wild type HT1080. This demonstrates the importance of applying a 3D environment to more closely mimic a native situation for cells. Nowadays many 3D culturing approaches are available to be used both in vitro and in vivo,. among which are organoids. Many studies have demonstrated malignant cancer tissue heterogeneity (both genetic and morphological) to be closely mimicked by organoids (Ji & Wu, 2020) It is already shown that TRPMl-1 has a crucial role in Ras-driven cancer cells, where its inhibition or downregulation alters cell proliferation. (Jung & Venkatachalam, 2019). Colorectal cancer (CRC) is the third most fatal cancer in the world, and 45 percent of this type of cancer derives from RAS family cancers. In previous studies human tumor organoids from colon have already successfully been generated(Ji & Wu, 2020). It would therefore be applicable

to use these organoids as a cancer model to study the role of TRPML-1 upon its alteration in CRC.

Furthermore, the effects caused by alterations in TRPML-1 functionality - such as the appearance of lipid droplets, intense association between mitochondria and the ER, as well as ER deficiency around the lysosomal compartments – point to the pivotal role this ion channel plays within the cell. Further investigation of its interaction with other organelles, may provide exciting new insights into the inner workings of subcellular processes.

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