

**Determining the mechanism of pathogenesis of Mucopolidosis
type IV and related lysosomal storage disorders for
development of novel therapies**

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Abstract

Mucopolidosis type IV (MLIV) is a rare, autosomal recessive, neurodegenerative, lysosomal storage disorder. MLIV is caused by mutations in a gene (*MCOLN1*) encoding a TRP channel family member known as Mucolipin 1 or TRPML1. TRPML1 is a lysosomal transmembrane protein that appears to be required for normal lysosomal pH regulation, recycling of molecules and membrane reorganisation including lysosomal biogenesis, fusion and exocytosis. The exact function of the channel is unknown but it is permeable to multiple ions including Ca^{2+} , Na^{+} and K^{+} , possibly also Fe^{2+} and Zn^{2+} . How normal TRPML1 function regulates lysosomal processes is not clearly understood. Mutations in the *MCOLN1* gene can lead to complete loss of TRPML1 function, partial loss of function or mislocalisation, all of which lead to lysosomal dysfunction, lysosomal lipid storage and ultimately neurodegeneration. The disease processes that lead to neurodegeneration are poorly understood and at present no therapy exists for MLIV.

We have discovered that TRPML1 results in regulating lysosomal Ca^{2+} homeostasis that is the opposite of the Ca^{2+} dysregulation associated with Niemann-Pick type C disease (NPC). Our findings indicate that disrupted function of TRPML1 leads to enhanced Ca^{2+} release via the NAADP receptor, recently shown to be the lysosomal two-pore channel TPC2. This indicates that TRPML1 is not the NAADP receptor as suggested by others, indeed NAADP mediated Ca^{2+} release is enhanced with multiple NAADP induced lysosomal Ca^{2+} release events occurring in TRPML1 null cells compared to single releases in normal cells. This phenotype appears to be responsible for the cellular dysfunction associated with MLIV disease cells, enhanced lysosomal fusion, defective endocytosis and potentially even altered lysosomal pH. Several of these phenotypes are normalised by the NAADP receptor specific antagonist Ned-19. These findings illustrate that the NAADP receptor is central to MLIV disease pathology and may be a novel candidate for disease therapy.

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Abbreviations

2-AA	Anthranilic Acid
ALG2	Apoptosis Linked Gene 2
AP1	Adaptor Protein 1
ATP	Adenosine Tri Phosphate
BCP	1-Bromo-3-Chloropropane
BMP	Bis(Monoacylglycero)Phosphate
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium
CD-M6PR	Cation Dependent Mannose-6-Phosphate Receptor
CDE	Clathrin Dependent Endocytosis
CDP	Cytidine Diphosphate
Cer	Ceramide
CIE	Independent Endocytosis
CI-M6PR	Cation Independent Mannose-6-Phosphate Receptor
CK	Choline Kinase
DMEM	Dulbecco Modified Eagles Medium
DMT1	Divalent Metal Transporter
EM	Electron Microscopy
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
Fe ²⁺	Iron Ion

g	Gravity
GALC β	Galactosidase
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
GD	Gaucher Disease
GGA	Golgi Localizing, γ Adaptin Ear Domain Homology, ARF–Binding Protein
GlcNAc	N-Acetyl Glucosamine
Gln	Glutamine
GM1	Monocialo Ganglioside GM1
GM130	Monocialo Ganglioside GM130
GPN	Glycyl-L-Phenylalanine 2-Napthalamide
GSL	Glycosphingolipid
HMGR	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
HPLC	High Pressure Liquid Chromatography
LacCer	Lactosyl Ceramide
LAMP	Lysosome-Associated Membrane Protein
LBPA	Lysobisphosphatidic Acid
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LIMP	Lysosome Integral Membrane Protein
LSD	Lysosomal Storage Disease

LRO	Lysosome Related Organelle
M6P	Mannose-6-Phosphate
M6PR	Mannose-6-Phosphate Receptor
MLI	Mucopolipidosis Type I
MLII	Mucopolipidosis Type II
MLIII	Mucopolipidosis Type III
MLIV	Mucopolipidosis Type IV
MLD	Metachromatic Leucodystrophy
NAADP	Nicotinic Acid Adenine Dinucleotide Phosphate
NPA	Niemann Pick A
NPB	Niemann Pick B
NPC1	Niemann Pick Type 1
NRAMP1	Natural Resistance-Associated Macrophage Protein 1
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-Methyl Transferase
PFA	Paraformaldehyde
PIP	Phosphor Inositol Phosphate
PKC	Protein Kinase C
PS	Phosphatidylserine
PTC	Patched

RND	Resistance Nodulation Division
RPM	Rotations Per Minute
RPMI	Roswell Park Memorial
RyR	Ryanodine Receptor
SCAP	SREB Cleavage-Activating Protein
SD	Sandhoff Disease
SERCA	Sarcoplasmic / Endoplasmic Reticulum
SL	Sphingolipids
SLOS	Smith-Lemli-Opitz Syndrome
SM	Sphingomyelin
SPT	Serine Palmitoyl Transferase
SREBP	Sterol Regulatory Element Binding Protein
SSD	Sterol Sensing Domain
TGN	Trans Golgi Network
V-type H-ATPase	Vacuolar Proton ATPase
TFR	Transferrin Receptor
TPC	Two Pore Channel
TRP	Transient Receptor Potential
TRPML1	Transient Receptor Potential Mucolipin 1
β GC	β Glucocerebrosidase

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1 Introduction

1.1 LYSOSOMAL FUNCTION

The lysosome is a cytoplasmic organelle, ranging from 0.3 to 3.0 μm in diameter [1], that is present in all nucleated eukaryotic cells. Lysosomes were first discovered in 1954 by Christian De Duve, who characterized them as lytic bodies that, when ruptured, release acidic hydrolases [2]. Lysosomes were first visualized in 1966 by Alex Novikoff using electron microscopy [3]. The lumen of the lysosome is typically maintained at pH 4.5-5 [4]. The low pH environment is required for enzyme function and is maintained by a multi-protein complex, the vacuolar proton ATPase (V-type H^+ ATPase) [5], which drives protons, H^+ , into the lysosomal lumen against the electrochemical gradient using energy derived from ATP hydrolysis with a stoichiometry of 2-4 H^+ per ATP [6]. Intravesicular acidification is not only important for hydrolase activation, low pH is also necessary for the release of lysosome-targeted ligands from their receptor carriers and rapid recycling of these receptors. By pumping H^+ into the lysosomal lumen, a positive electrochemical gradient is created which can block further H^+ import. The V-type ATPase indirectly drives uptake of other cations such as Na^+ and Ca^{2+} via H^+ driven antiport mechanisms.

Over 60 soluble enzymes (e.g. peptidases, lipases, nucleases, glucosidases) collectively termed acidic hydrolases, and their accessory proteins have been identified in the lysosome to date [7]. Collectively these enzymes are able to break down complex macromolecules into their monomeric constituents, which can then be recycled. The lysosomal membrane is a single phospholipid bilayer with a “gel-like” glycocalyx coat formed by heavily glycosylated membrane proteins the most prominent of which are lysosome-associated membrane protein 1 (LAMP1) and 2 (LAMP2), and lysosome integral protein 1 (LIMP1) and 2 (LIMP2). LAMPs constitute 50% of

lysosomal membrane proteins and are usually used as markers for the lysosomal compartment; LIMPs are the second most abundant class of lysosomal membrane proteins. Protein domains of LAMPs and LIMPs directed towards the lysosomal lumen are heavily glycosylated and constitute most of molecular mass of the protein. Complex glycosylation of these membrane proteins plays a crucial role in maintaining lysosomal membrane integrity and acts to protect the cytosol from the acidic content of the lysosome. The role of the glycocalyx is to protect the lysosomal membrane from self digestion.

1.2 TARGETING PROTEINS TO THE LYSOSOME

Lysosomal proteins are synthesized and correctly folded in the rough endoplasmic reticulum (ER), and glycosylated in the Golgi complex. There are multiple pathways for lysosomal protein exit from the trans-Golgi network (TGN) and targeting to the lysosome.

The mannose-6 phosphate dependent pathway is the major and best-understood pathway for targeting soluble proteins to the lysosome. A mannose-6-phosphate (M6P) tag is added to the carbohydrate chain of lysosomal proteins by N-acetylglucosamine (GlcNAc) phosphotransferase in the Golgi, and acts as a recognition marker for the mannose-6-phosphate receptor (M6PR). There are two types of M6PR, cation-dependent (CD-M6PR) and cation-independent (CI-M6PR), which have complementary functions. M6PR bind to M6P tagged proteins at the membrane of the TGN, and are then packed into clathrin-coated vesicles, which carry ligand-bound M6PR from the TGN into the endosomal system. M6PR are sorted into clathrin-coated vesicles via interaction with adaptor proteins AP-1 or GGA at the TGN membrane. Clathrin coated vesicles either fuse with the endosome directly, or are delivered to the plasma membrane and then endocytosed. The latter pathway allows CI-M6PR to bind to the M6P tag of mistrafficked lysosomal proteins or recombinant enzymes used for

treatment of lysosomal storage disorders. Following fusion of TGN derived vesicles with endosomes, M6P tagged proteins dissociate from M6PR and are transported along the endocytic pathway to the lysosome; M6PR are recycled into the TGN, so are not found in the lysosome. Deficiency in enzymes that add the M6P tag to lysosomal enzymes, as is the case in mucopolipidosis type II, or deficiency in M6PR, results in secretion of lysosomal enzymes into the extracellular milieu and accumulation of undegraded substrates in the lysosome.

An M6PR independent pathway has also been identified; mediated by specific chaperones such as sortilin, which facilitates trafficking of saposins and acid sphingomyelinase to the lysosome. Sortilin interacts with AP-1 and GGA at the membrane of the TGN, similarly to M6PR, and is transported to the endosomal system via the same clathrin-coated vesicles.

In addition, LIMP2 has been shown to target β glucocerebrosidase to the lysosome. Here, however, LIMP2 binds β GC in the ER and dissociates in the lysosome, indicating a pH dependent protein-protein interaction. Both sortilin and LIMP2 interact with their ligand directly and do not require a glycosylated tag.

Lysosomal targeting of membrane proteins into the lysosome is less clear, however there is evidence that suggests an overlap between soluble and membrane protein trafficking to the lysosome. Lysosomal membrane proteins exit the TGN in clathrin coated vesicles and are delivered to the lysosome either directly, or indirectly, whereby they are trafficked to the plasma membrane and are subsequently internalized and transported down the endocytic system to the lysosome[8]. Lysosomal targeting of integral and membrane-associated proteins is reliant on short amino acid sequences present on the cytosolic tails of transmembrane protein, most commonly tyrosine, YXX ϕ or di-leucine, DXLL, motifs [9]. TM proteins are transported using adapter proteins to the lysosome via interaction with the di-leucine/tyrosine motif.

1.3 INTRACELLULAR ROLE OF THE LYSOSOME IN ENDOCYTIC SYSTEM

Endocytic uptake at the cell surface can occur via different mechanisms; clathrin-dependent [10] and clathrin-independent [11] pathways.

Clathrin-dependent endocytosis (CDE) is reliant on adaptor proteins such as adaptor protein 2 (AP2) [12], dynamin 1 [13, 14] and endophilin [15], recognizing the cytoplasmic domains of the plasma membrane proteins and packaging them into clathrin coated vesicles, which are then internalized. Low-density lipoprotein receptor (LDLR) [16] with associated LDL-cholesterol and transferrin receptors (TFR) [17] with associated iron are taken up by CDE.

Clathrin-independent endocytosis (CIE) can occur via different mechanism [11]. Some examples of plasma membrane receptors taken up by CIE are major histocompatibility complex class I (MHC I), interleukin receptors [18], glucose transporters, fluid phase markers; whole bacteria [19] as well as bacterial toxins are also internalized by CIE (e.g. cholera toxin [20], shiga toxin [21]).

Following internalization, plasma membrane receptors and bound ligands pass through morphologically distinct endocytic compartments [22] with varying membrane lipid/protein composition and intraluminal ionic environment. Endocytosed cargo is first delivered to early endosomes (EE), a tubular vesicular compartment located at the periphery of the cell [23] and identified by early endosome antigen 1 (EEA1) and small GTPase Rab5 markers [22, 24]. In EE, cargo is either sorted back to the plasma membrane, directly, or via the recycling endosomes (RE) back to the PM, or, is passed down the degradative pathway to the late endosomes (LE) [25]. LE are spherical organelles positive for Rab7 [26, 27] and M6PR. MVBs are characterized by the presence of LBPA, that is important for generating the inner luminal vesicles, and then

mate into LE. LE then delivers the cargo to the lysosome where it is degraded by lysosomal hydrolases and the products recycled back to the cytosol. Lysosomes have perinuclear localization, and are positive for LAMP 2 [28] and Rab9 protein markers but lack M6PR.

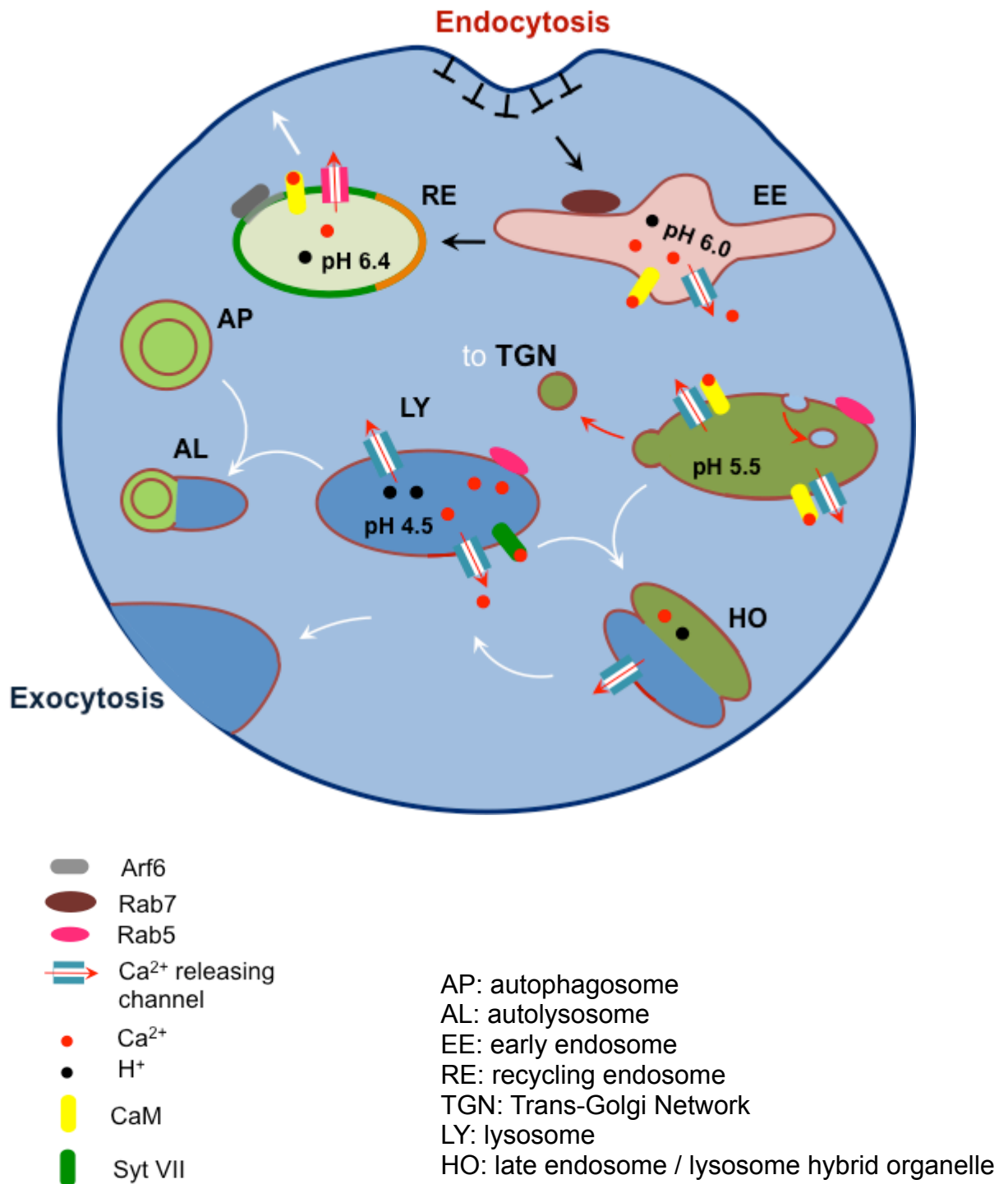


Figure 1.1 Diagrammatic representation of endocytic compartments.

1.3.1 Acidification and Ca^{2+} concentration in different compartments of the endocytic pathway

Endocytosed material encounters decreasing pH as it goes through the endocytic pathway. In fact progressive acidification of the compartments along the endocytic pathway is required for protein sorting and vesicle trafficking [29]. The marginally acidic pH of EE (pH 6.8-5.9) [30] helps dissociation of the receptors from the bound ligand, allowing these receptors to be recycled back to the plasma membrane [31]; whereas the very acidic pH of the lysosome (pH 4-4.5) [30] is required for optimal activity of lysosomal hydrolases [2] and degradation of internalized macromolecules like LDL [32]. The decreasing pH gradient is established by a combination of H^+ pumping by V-ATPase [33], H^+ exchange for counter ions by a family of chloride channels (ClC) [34, 35] that balance the positive charge generated by V-ATPase, and H^+ exchangers [36, 37] that contributes to steady state pH.

In addition, the intraluminal Ca^{2+} concentration also changes as the cargo transits down the endocytic pathway [38]. The exact mechanism through which Ca^{2+} uptake into the endolysosomal compartments occurs is not clear [39]. Extracellular Ca^{2+} does not contribute to the endocytic Ca^{2+} content [40] since most of the 1mM Ca^{2+} in the extracellular fluid is removed at the EE stage by the onset of acidification [40-42]. However, it is evident that the entry of Ca^{2+} into the endocytic compartment occurs through proteins that require ATP and H^+ gradient since inhibition of V-ATPase by bafilomycin [43] depletes the lysosome of Ca^{2+} [40, 44]. The lysosome has been identified as a second major store of Ca^{2+} , with Ca^{2+} levels estimated to be 400-600 μM [40, 45].

Luminal Ca^{2+} release is necessary for both the homotypic fusion of early endosome with late endosome and the heterotypic fusion between late endosome and the lysosome [1]. Mechanism of lysosomal Ca^{2+} release is discussed in more detail below.

1.3.2 Role of the lysosomes in exocytosis

Unlike early endosomes, lysosomes are not recycled back to the plasma membrane [46]. Lysosomal exocytosis occurs only when the plasma membrane is damaged [47]. Influx of extracellular Ca^{2+} into the cytosol following plasma membrane damage causes mislocalization of the lysosomes from a perinuclear localisation to the periphery of the cell; there lysosomes are docked with the plasma membrane at the lesion site, lysosomal membrane can then fuse with the plasma membrane forming a seal and reducing tension of the plasma membrane. This mechanism is very similar to Ca^{2+} regulated exocytosis of lysosome related organelles (LRO) [48] in cells with specialized secretory function.

1.3.3 Role of lysosomes in autophagy and phagocytosis

Lysosomes function to degrade protein and lipids that are trafficked to the lysosome via the endocytic pathway, but also by the autophagic pathway [49]. Autophagy is a catabolic lysosome mediated degradation of intracellular proteins (microautophagy [50]) and whole organelles (macroautophagy [51]) that maintains cellular homeostasis under normal physiological conditions. Autophagy is highly conserved and occurs constitutively in all eukaryotic cells to turn over long-lived proteins and dysfunctional organelles, which is important during development, differentiation, aging and immune response. Autophagy is upregulated in starvation [52] or stress induced [53] conditions in order to supply the components necessary for metabolism or to remove toxic material for cell survival. Reduced autophagy leads to accumulation of aggregate prone proteins [54], which results in altered cell function and ultimately cell death.

Proper lysosomal function is essential for the process of autophagy [49]. It is thought that progressive lipid accumulation in LSDs may cause a defect in fusion between the lysosome and autophagosome; as a result proteins can no longer be degraded and accumulate in the cytosol. Defective autophagy pathway is evident by the

neurodegenerative aspect in most LSDs since neurons are particularly sensitive to protein aggregation [55]. Motor dysfunctions ensue as a result of neurodegeneration.

1.4 LYSOSOMAL STORAGE DISEASE

Since the discovery of the lysosome, the research of lysosomal function has been largely driven by the existence of lysosomal storage diseases, which give insight into the physiological function of lysosomal proteins that otherwise would probably not have been identified. Lysosomal storage disorders (LSDs) are a group of over 50 disorders that arise as a result of a single gene defect [56]. Most LSDs are caused by deficiency in lysosomal hydrolases, lysosomal transmembrane proteins or accessory proteins. In these LSDs, the substrate of the defective hydrolase is the major material that accumulates and is referred to as primary storage material. Therefore, traditionally, LSDs were classified according to primary storage material, such as the mucopolysaccharidoses, sphingolipidoses and mucopolysaccharidoses.

In most LSDs, however, there are multiple types of storage material. This cannot be explained by the underlying enzyme deficiency and is thought to occur as a downstream event of compromised lysosomal function; it is referred to as secondary lipid accumulation.

1.4.1 Drug induced phospholipidosis, an acquired LSD.

A lot of the drugs fall into a group of cationic amphiphilic drugs or CADs; these typically comprise a hydrophobic ring structure and a charged amide group, a halogen attached to the hydrophobic moiety further enhances membrane permeability. Drug induced phospholipidosis is an excessive accumulation of phospholipids induced by short or long term administration of CADs. These accumulate in the lysosome causing swelling of a lysosome and resulting in formation of lamellar bodies within the lysosome, a phenotype shared by most LSDs. CADs affect phospholipid metabolism by binding to

phospholipids making them inaccessible for digestion by phospholipases and by directly affecting the activity of phospholipases.

1.4.2 Lysosomal storage disease treatments.

About 40 of the known LSDs are caused by the absence or reduced activity of lysosomal hydrolases. Therapies currently in use for treatment of LSDs include enzyme replacement therapy, substrate reduction therapy and bone marrow transplantation.

De Duve himself first proposed the concept of enzyme replacement therapy (ERT). However, ERT was initially developed by R. Brady in the mid 1960s and was FDA approved for treatment of LSDs in the early 1990s. Initially, the principles of ERT were developed and tested for safety and effectiveness in treatment of Gaucher disease, where a lysosomal glucocerebrosidase is deficient. The principles of ERT were extended for treatment of mucopolysaccharidoses type I, type II and type VI, Fabry and Pompe disease.

Although effective in treatment of several LSDs, the use of ERT has some limitations. Most LSDs have a severe neurologic involvement, however the recombinant enzymes used in ERT cannot permeate through the blood brain barrier and so have no effect on the brain, and are only effective in reducing storage in visceral organs.

A small molecule imino sugar, *N*-butyl-deoxynojirimycin (NB-DNJ or miglustat), is an inhibitor of glucosylceramide synthase. NB-DNJ competes with ceramide for the enzyme's active site, and in doing so inhibits synthesis downstream of glucosylceramide (all GSLs including GlcCer and gangliosides). Miglustat can cross blood-brain barrier and has been approved by food and drug administration (FDA) and EMA for treatment of type I Gaucher disease and pediatric and adult onset Niemann Pick type C. It has also been tested as a treatment for Tay Sachs disease patients and MPS type III but was not found to have efficacy.

NB-DNJ has proven to be an effective therapy, capable of stabilizing disease progression in patients. The main side effect of miglustat is diarrhea, which can be treated with loperamide or a lactose free diet (caused by inhibition of gut disaccharidases).

Bone marrow transplantation has been used as therapy for treatment of some LSD patients; over the past two decades over 1000 bone marrow transplantations were performed on LSD patients worldwide. Bone marrow grafting provides the patients with macrophages and microglia which secrete functional lysosomal hydrolases.

1.5 LYSOSOMAL STORAGE

1.5.1 Sphingolipids and Glycosphingolipids

Sphingolipids consist of a sphingoid base (a single hydrocarbon chain with an amino alcohol head group), acting as a backbone to which a fatty acid is attached by an amide bond. There are at least three different chain length of sphingoid bases found in mammalian cells, and over twenty different fatty acid species, which can be attached to the sphingoid base backbone. In mammalian cells, the sphingoid backbone of the sphingolipids is usually sphingosine, whereas in plants and yeast it is phytosphingosine.

Ceramide (Cer) is an acylated form of sphingosine that acts as a precursor to more complex sphingolipids such as sphingomyelin (SM) and glycosylated derivatives, glycosphingolipids (GSLs) via the addition of a phosphocholine headgroup or a glucose and more complex glycan headgroups respectively.

Ceramide biosynthesis occurs in the cytosolic leaflet of the membrane of the ER, where all enzymes of Cer biosynthesis are localized [57]. The initial step in the pathway of Cer synthesis is a condensation of L-Serine with palmitoyl-CoA to 3-ketosphinganine in a reaction catalyzed by serine palmitoyl transferase (SPT). SPT

catalyses the rate limiting step of Cer biosynthesis and is therefore the point of regulation: SPT activity has been reported to correlate with relative amounts of sphingolipids [58, 59]. 3-ketosphinganine is then reduced to D-erythro-sphinganine in a reaction catalyzed by 3-ketosphinganine reductase. D-erythrosphinganine is acylated to dihydroceramide, catalyzed by sphinganine N-acyltransferase. Dihydroceramide desaturase catalyses the desaturation of dihydroceramide to ceramide. Following synthesis, Cer is extracted from ER membrane by a chaperone known as CERT and transported to the Golgi [60], where more complex glycosphingolipids are synthesised. Once in the Golgi, carbohydrate moieties are added to ceramide in a stepwise manner to form more complex glycosphingolipids. Sphingolipids (SLs) and their derivative glycosphingolipids (GSLs) are major constituent of cell membranes, where they play a structural and regulatory role. SLs also play a role in biosynthesis of sphingosine-1-P and apoptosis (ceramide) signalling. GSLs are also capable of mobilizing Ca^{2+} from the ER [61].

1.5.2 Phospholipids

Phospholipids can be distinguished from sphingolipids by a phosphate headgroup and are not based on a sphingoid backbone. Phospholipids are major components of cell membranes and precursors of the second messengers, phosphoinositol phosphate (PIPs). They are synthesized in the ER by various mechanisms, outlined below. Examples of phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and lysobisphosphatidic acid (LBPA). Four major groups of phospholipids have been reported to accumulate in Mucopolipidosis type IV (MLIV): LBPA, PC, lysoPC, and PE.

1.5.2.1 Phosphatidylcholine, PC

There are two pathways for PC biosynthesis in mammalian cells. The major pathway for synthesis of PC from choline obtained from the diet is the Cytidine Diphosphate,

CDP, -choline pathway [62]. It is a series of three enzymatic steps: choline is phosphorylated to phosphocholine by cytosolic choline kinase, CK; phosphocholine is converted to CDP-choline in a reaction catalyzed by CTP:phosphocholine cytidyltransferase, CT; CDP is then exchanged for diacylglycerol to form PC in a final reaction catalyzed by CDP-choline:1,2,-diacylglycerol cholinephosphotransferase. PC can also be synthesized endogenously from PE via three sequential methylation reactions catalyzed by phosphatidylethanolamine N-methyl transferase, PEMT [63]. The PEMT pathway is only significant in hepatic PC synthesis [64].

1.5.2.2 *Phosphatidylethanolamine, PE*

There are four pathways for PE synthesis. The major pathway for synthesis is the CDP-ethanolamine pathway [62], which is very similar to CDP-choline pathway for PC synthesis. It is a series of three enzymatic steps: first, ethanolamine is phosphorylated by ethanolamine kinase, one of the two enzyme isoforms is capable of phosphorylating both choline and ethanolamine, in the cytosol; CTP:phosphoethanolamine cytidyltransferase then catalyses the formation of CDP-ethanolamine from phosphoethanolamine; finally CDP-ethanolamine:1,2-diacylglycerol ethanolaminophosphotransferase on the ER membrane catalyses the formation of PE in the final step of CDP-ethanolamine pathway. Similarly to the first enzyme of the pathway, there is an isoform of phosphotransferase that has dual specificity for phosphocholine and phosphoethanolamine as its substrate [65]. Therefore synthesis of PC and PE is parallel and can be catalyzed by the same enzymes.

Although three other pathways for PE synthesis exist, mutations of any of the enzymes of CDP-ethanolamine pathway are embryonically lethal in mice, indicating that it is the major pathway for PE synthesis during development. PE can also be generated through PS decarboxylation.

BMP is more commonly referred to as lysobisphosphatidic acid, LBPA. LBPA is found in the membranes of late endosomes and lysosomes, and is often used as a marker for these organelles. It is stored in NPC disease. LBPA regulates the levels of cholesterol in the late endosome. The pathway of LBPA biosynthesis is not clear, however in mammalian cells phosphatidylglycerol has been shown to be the precursor of LBPA [66].

1.5.3 Cholesterol

Cholesterol is a sterol molecule essential for regulating membrane fluidity [67]; either obtained from the diet or generated in a stepwise manner from HMG [68] at the ER it acts as a precursor for steroid hormones, such as glucocorticoids, oestrogen and progesterone, bile acids and Vitamin D.

1.5.3.1 Biosynthesis

Cholesterol is synthesized in all mammalian cells, however due its precursor properties, cholesterol synthesis is particularly prominent in reproductive tissues, adrenal cortex and the liver. Cholesterol biosynthesis is a complex process involving 30 enzymatic steps that takes place in the ER .

Biosynthesis is regulated at the transcriptional level, as well as at the level of HMG reductase. Sterol regulatory element binding proteins (SREBPs) are transcriptional factors that directly activate the expression of genes required for synthesis and uptake of cholesterol. SREBPs are found in the ER in an inactive state, bound to the sterol-sensing protein, SREBP cleavage activating protein (SCAP). In presence of cholesterol, Insig protein binds the SREBP-SCAP complex, stabilizing it and anchoring SREBP in the ER. However when cholesterol levels are low, SREBP is transported to the Golgi where it is cleaved into an active transcription factor that then migrates to the nucleus and activates transcription of all genes required for cholesterol synthesis [69] and the low-density lipoprotein (LDL) receptor gene. Interestingly, SREBP is also

regulated by ceramide, which highlights the crosstalk between sphingolipid and cholesterol metabolism. Cells that are unable to synthesise sphingolipids cannot activate SREBPs in response to lipid depletion. There is an important association between sterols and sphingolipids, particularly, sphingomyelin that form lipid rafts that are thought to be dynamic signaling platforms in the membrane.

1.5.3.2 Disturbances in cholesterol homeostasis in disease

Cholesterol accumulation causes disturbances in glycosphingolipid trafficking and is associated with Niemann-Pick type C disease [70]. This will be discussed in more detail in section 1.8.

A reduction in cellular cholesterol can also lead to severe health problems [71, 72]. Smith-Lemli-Opitz syndrome (SLOS) is caused by a mutation in the final enzyme of cholesterol biosynthesis, 3 β -hydroxysterol Δ^7 reductase, which catalyses the conversion of 7 dihydrocholesterol into cholesterol. SLOS patients present with severe congenital defects [73]. Therefore, the ability of the cell to synthesise cholesterol de novo appears to be critical in foetal development.

1.5.4 Lipofuscin

Lipofuscin is a generic term used to describe insoluble aggregates consisting of cross-linked proteins, phospholipids which are normally components of cells membranes [74], neutral lipids, carbohydrates and metal ions. This heterogeneous material is resistant to proteolytic degradation; it is not exocytosed, but accumulates within lysosomes. The composition of lipofuscin is varied depending on cell type, with protein content ranging from 30 to 50% and lipid content from 19 to 51% [75].

Lipofuscin is autofluorescent, with a broad emission spectrum that changes with different compositions. This means that the use of fluorescence microscopy is complicated in cells with a high lipofuscin burden. The rate of lipofuscin formation increases over time and lipofuscin can be used as an aging marker.

Although lipofuscin has been well researched for over 100 years, the mechanisms of lipofuscin accumulation remain speculative. Lipid peroxidation theory of lipofuscin formation has been supported by a number of studies. Lipid peroxidation is damage to lipids by reactive oxygen species (ROS) generated in reaction catalyzed by redox active iron. During degradation of iron containing proteins in the lysosome, the iron is released and can react with hydrogen peroxide forming free radicals (Fenton reaction). These ROS can cross-link the material in the lysosome leading to lipofuscin formation. Block in protein degradation can contribute towards lipofuscin formation. Functionally impaired proteins are normally targeted for proteosomal degradation where they are degraded into short chain oligopeptides. Cross-linked proteins are poor substrates for proteosomal degradation and more over can inhibit proteasome function, instead these proteins are directed to lysosomes for degradation; this leads to intralysosomal accumulation of material that cannot be degraded and contributes towards lipofuscinogenesis. This has been confirmed by infusing rat brain with protease inhibitor results in formation of lipofuscin like pigment.

1.5.4.1 Role of lipofuscin in pathology.

Lipofuscin, also referred to as ceroid lipopigment, has been shown to accumulate in a group of LSDs known as the ceroid lipofuscinoses. There are eight distinct forms of lipofuscinosis found in humans (CLN1-8) with different underlying genetic defects. Another lysosomal storage disease that presents with large autofluorescent lysosomal inclusions visible by EM is mucopolipidosis type IV (MLIV). Autofluorescent aggregates seen in skin biopsies were used for diagnosis of MLIV before gene has been mapped. Until recently, NCL and MLIV were the only diseases associated with storage of lipofuscin.

1.6 MUCOLIPIDOSIS TYPE IV

1.6.1 *Disease etiology*

Mucopolipidosis type IV (MLIV) is an autosomal recessive lysosomal storage disease first reported in 1974 [76]. This disease is rare even within lysosomal storage diseases and mostly affects the Ashkenazi Jewish (AJ) population. Although within this population disease incidence has been estimated to be 1 in 40,000, carrier frequency has been reported to be 1 in 100 [77]. MLIV can arise in other ethnic groups with much lower frequency [78]. Age of symptom onset is 2-24 months however diagnosis is often made much later once the symptoms have progressed [79]. Disease progression is rapid in the juvenile years but stabilizes into adulthood with no current typical age of death known.

Patients present with severe visual impairments due to corneal clouding and retinal degeneration [80], muscle hypotonia and spasticity [81]. Anemia and reduced gastric acid secretion, or achlorhydria/hypochlorhydria, which corresponds to increased gastrin hormone levels in the blood are also documented in MLIV patients [82]; elevated gastrin serves as one of the clinical markers for MLIV [83]. Psychomotor retardation seen in patients indicates that neurologic development is also impaired in these patients [79]. Although neurological deficits typically present in early childhood, progression of neurodegeneration is much slower compared to other LSDs [83]. Patients have been reported to reach a steady state without further deterioration for 2-3 decades of life. MRI findings suggest deterioration of the corpus callosum of MLIV patients [84]. Hepatosplenomegaly seen in patients with sphingolipidoses, and facial dysmorphisms or skeletal abnormalities typical for patients with mucopolysaccharidoses have not been reported in MLIV patients.

Variation in age of symptom onset and degree of severity often lead to misdiagnosis. Late or misdiagnosis of patients can cause further family burden since subsequent

siblings may also be affected. Before the gene defect causing MLIV had been identified, definitive diagnosis was made based on tissue biopsy. Currently there are no available therapies for MLIV.

1.6.2 Gene defect

MLIV is caused by a single gene defect. The gene mutated in MLIV is *MCOLN1* (GenBank accession number NM_053177). It has been mapped to human chromosome 19p13.2-13.3 [85]. The gene consists of 14 coding regions spanning about 14kb of genomic DNA [86]. Two MLIV causing mutations account for 95% of all mutations found in Ashkenazi Jewish patients: major haplotype, found in 75% of affected individuals, is an A → G substitution between coding regions 3 and 4, which results in deletion of exon 4 and slicing of mRNA into two fragments; minor haplotype, found in 20% of affected individuals, causes a deletion of a region upstream from exon 1 to exon 7, as a result no functional mRNA is produced. No splicing variants have been reported in humans however the gene is alternatively spliced in the mouse model.

Homozygous patients with either of the two mutations, and compound heterozygotes, present with similar symptom severity. Two other genes *MCOLN2* and *MCOLN3* have been identified and cloned; both localize to chromosome 1p22.3. Unlike *MCOLN1*, *MCOLN2* and *MCOLN3* are not associated with human disease. However, mutations in *Mcoln3* cause deafness and abnormal pigmentation in mice. The MLIV patient fibroblast line used in this study, GM02527, is homozygous for an A to G substitution at 3' acceptor site at intron 3 (486-2 A → G mutation) resulting in deletion of exon 4 in the mRNA. This mutation is associated with the major AJ haplotype. No protein is expressed.

1.6.3 *TRPML1* protein

Mucolipin1 is the 580 amino acid protein encoded by *MCOLN1*, with a predicted molecular weight of ~65 kDa. Protein topology analysis of the amino acid sequence of mucolipin1 shows the protein to have six trans membrane (TM) domains, with cytosolic N- and C- termini.

Comparison against proteins with known motifs reveals transient receptor potential, TRP [87], channel domain spanning TM domains 3-6 and a putative pore forming loop between 5th and 6th TM domains. Mucolipin1 has since become known as TRPML1. Two di-leucine motifs (L-L-X-X) located at C-terminus regulate trafficking of TRPML1 to the lysosome [88].

A large extracellular loop between 1st and 2nd TM domains contains conserved sites for N-glycosylation that have been suggested as potential sites for protein regulation.

TRPML1 is the founding member of the TRML subfamily of TRP channels, two other members of the TRPML protein subfamily have also been identified, TRPML2 and TRPML3. All three TRPML proteins localize to the endolysosomal system. In addition TRPML1 is the only isoform that does not have a tissue specific expression pattern but is ubiquitously expressed [89]. However TRPML1 is the best characterised member of the TRPML family and the only one implicated in human disease. TRPML orthologues have been identified in *Caenorhabditis elegans* and *Drosophila melanogaster*, Cup-5 [90] and CG874 [91] respectively; both share 30% amino acid homology to human TRPML1. Unlike in mammalian organisms, only one isoform of TRPML protein is expressed in *C.elegans* and *Drosophila*.

Although the predicted molecular weight of TRPML1 is ~65 kDa, there is also a cleaved form of the protein with a lower molecular mass of 37 kDa. TRPML1 undergoes proteolytic cleavage, inhibited by the inhibitors of cathepsin B [92]. Since it

is not currently known how TRPML1 function is activated under physiological conditions, proteolytic cleavage may act as a means of protein regulation.

1.6.4 Putative function of TRPML1

Although the *TRPML1* gene was mapped in 1999 [85], the function of the TRPML1 protein is still debated. The endogenous ion channel function of TRPML1 is difficult to study due to the endolysosomal localization of the protein. TRPML1 channel function has been explored either in studies where the protein has been overexpressed and mislocalized to the plasma membrane [93], protein that has been reconstituted into a lipid bilayer or studied in the pharmacologically enlarged and isolated lysosomes [94]. Several studies have found the protein to be permeable to monovalent (H^+ [95]) and divalent (Ca^{2+} [93, 96] and Fe^{2+} [94]) cations. These studies suggest that TRPML1 is thought to act as an inwardly rectifying channel, moving ions from the lysosomal lumen into the cytosol. Several theories with regards to TRPML1 function have been put forward.

1.6.4.1 TRPML1 is involved in regulating endolysosomal trafficking

MLIV patient fibroblasts have an abnormal accumulation of phospholipids, sphingolipids and mucopolysaccharides within LE/lysosomes [97]. Moreover EM images show that the LE/lysosomal compartment or hybrid organelle is enlarged. *C.elegans* [90], *Drosophila* [91] and mouse [98] models of MLIV also show an enlarged lipid laden LE / lysosomal compartment. Since no underlying enzyme defect is observed in MLIV, it was suggested that TRPML1 is involved in the late endocytic pathway, more specifically in facilitating lipid trafficking out of the hybrid organelle and to the trans Golgi network (TGN) [99]. TRPML1 therefore must be required for fission of LE/lysosomes into separate compartments and lysosomal biogenesis from hybrid organelle [1].

There is however another way to explain lipid accumulation. Ca^{2+} permeability of TRPML1, the potential ability of TRPML1 to transport Ca^{2+} ions taken together with the delays in recycling out of LE/lysosome gave rise to a theory that TRPML1 may function as a lysosomal Ca^{2+} release or a Ca^{2+} leak channel [96]. If TRPML1 was to function as a Ca^{2+} release through TRPML1 may be required for fission of LE and lysosomal compartments from the fused intermediates. In absence of a functional TRPML1, Ca^{2+} release does not occur and the LE is unable to separate from the lysosome, endocytosed material can not escape the fused late endosome-lysosome hybrid organelle and consequently can not be degraded causing a build up of undigested material within the endocytic system. TRPML1 function is suggested to be required for both fusion between LE and lysosome and formation of the hybrid organelle, as well as fission of the hybrid organelle and separation of LE from lysosome.

Ca^{2+} release by TRPML1 is thought to be regulated by phosphoinositol phosphates (PIPs) [93], which are known activators of other members of TRP family channels.

Although lipid trafficking defects are observed both in human patient cells [99] and in *cup5*^{-/-} *C.elegans* [100], they are not found in *Drosophila* model of MLIV but its unclear if this reflects biological differences or different assays used to study different model organisms. Moreover, it is difficult to say whether trafficking defect happens as a direct consequence of TRPML1 loss, or whether it is the accumulation of lipids within the endocytic system that causes a delay in trafficking.

Association of TRPML1 with other proteins may shed some light onto its function within the endocytic system. Interaction between TRPML1 and apoptosis linked gene 2 (ALG-2) protein, an intracellular Ca^{2+} binding protein [101], which binds to TRPML1 in a Ca^{2+} dependent manner [102]. Mutation of the ALG2 binding domain on TRPML1 results in accumulation of enlarged endolysosomes suggesting that ALG2 acts to modulate the function of TRMPL1 [103].

TRPML1 has been found to co-immunoprecipitate with endolysosomal two pore channels (TPCs) 1 and 2 [104]. These channels mediate lysosomal Ca^{2+} release in response to NAADP [105]. TPC expression does not affect single channel current through TRPML1, so does not regulate the activity of TRPML1, however the effect of TRPML1 on NAADP single channel current has not been studied. The functional significance of TRPML1 co-immunoprecipitating with TPC1 and TPC2 remains to be elucidated.

1.6.4.2 TRPML1 functions as a lysosomal Fe^{2+} channel

Single channel conductance studies in pharmacologically enlarged isolated lysosomes have found that TRPML1 is permeable to divalent cations, including Fe^{2+} [94]. This study shows iron homeostasis to be perturbed in TRPML1^{-/-} cells, with reduced cytosolic and increased intra-lysosomal levels of Fe^{2+} . The authors have suggested a potential role of TRPML1 as a lysosomal Fe^{2+} release channel, preventing Fe^{2+} build up within the lysosome which otherwise may lead to lipofuscin accumulation. In addition, Fe^{2+} accumulation is capable of inhibiting cathepsin activity, which may lead to lipid storage downstream of Fe^{2+} dyshomeostasis.

However, Fe^{2+} transport out of the lysosome is known to occur through a divalent metal transporter (DMT1) otherwise known as natural resistance associated macrophage protein 2 (NRAMP2). The mechanism of Fe^{2+} efflux via DMT1 is well studied; it is therefore not clear why it would be necessary to have an alternative way of Fe^{2+} export out of the lysosome. Furthermore, Fe^{2+} accumulation within the lysosomes would be expected to lead to a large increase in membrane damage, perhaps this accounts for the pH defect that is occasionally reported but would probably cause significant cell death, which is not seen in the disease.

1.6.4.3 Other potential functions of TRPML1

Autophagosome accumulation and defects in clearing dysfunctional mitochondria have been reported in MLIV patient fibroblasts [106]. *Drosophila* MLIV model also shows impaired autophagy and mitochondrial function [91]. This phenotype most likely happens as a downstream event following the buildup of lipofuscin that has been linked to defects in autophagy and mitophagy in aging cells.

TRPML1 has been implicated in regulating lysosomal pH. As mentioned previously, lysosomal pH regulation is important for the correct function of hydrolases and, subsequently, degradation of internalized material. One study suggested that TRPML1 functions as a H⁺ channel, and in doing so prevents over acidification of the lysosome [95]. However, pH recordings in TRPML1 null cells are conflicting, with other groups reporting elevated lysosomal pH in these cells [107, 108].

TRPML1 was also proposed to function as a mediator of lysosomal exocytosis as reduced lysosomal exocytosis has been reported in MLIV [109]. The serine lipase binding motif, GYSDG, of TRPML1- membrane remodeling capability- regulates formation of tubulovesicular structures and lysosomal exocytosis [110]. Reduced exocytosis may explain the involvement of the parietal cells, stomach epithelial cells with exocrine function and account for hypochlorhydria seen in MLIV patients; alternatively this could happen as a result of parietal cell degeneration.

In summary, structural analysis of TRPML1 suggests that it functions as an ion channel within the endocytic system. However, it is difficult to determine what ion dysregulation is a direct effect of TRPML1 loss and what is a downstream effect.

1.6.5 Role of TRPML1 in cellular phenotypes of MLIV

MLIV has been classed as a mucopolysaccharidosis. All mucopolysaccharidoses are characterized by 'heterogeneous' storage, where both lipid and water-soluble material is stored. Storage material includes water-soluble mucopolysaccharides and glycoproteins, and lipids

such as phospholipids, gangliosides and neutral lipids. Other mucopolidoses include MLI or Sialidosis, MLII or I-cell disease, and MLIII also known as pseudo-Hurler polydystrophy. These types of mucopolidoses are caused by lysosomal enzyme deficiency; sialidase in MLI, and N-acetylglucosamine-1- phosphotransferase in MLII and MLIII. Similarly to patients with MLIV, patients with MLI, II and III present with delays in cognitive and motor functions, as well as visual impairments.

EM studies reveal presence of electron dense multi lamellar bodies in all tissues of MLIV patients. These storage bodies are autofluorescent in human fibroblasts suggesting accumulation of lipofuscin [78]. Overall, lysosomal lipid storage is found in all tissues [111], however the lipid profile is different depending on the type of tissue. Glycosaminoglycans are stored in neural tissues, whereas phospholipids are the prominent lipid group stored in visceral organs [111]. The lipid storage profile of MLIV is similar to other mucopolidoses, however defects in lysosomal enzyme activity have not been reported [112]. *In vitro* enzyme activity assays of various phospholipases in MLIV were found to be normal [97].

1.7 NIEMANN PICK C DISEASE TYPE 1

1.7.1 Disease etiology

At the cellular level, NPC is classically characterized by aberrant cholesterol trafficking and processing [113]. NPC has a complex storage pattern. Unesterified cholesterol, sphingomyelin, sphingolipids, glycosphingolipids and gangliosides constitute the stored material in peripheral tissues. Cholesterol is the major substrate by mass that accumulates in the visceral organs of NPC patients. Interestingly, in the brain, it is not cholesterol but the GSLs [114], GlcCer and LacCer, and gangliosides GM2 and GM3, that accumulate as well as sphingosine [45]. Levels of both cholesterol and

sphingomyelin are not elevated in neurons or glia [115], but these lipids are mislocalized.

Patients are characterized by CNS involvement – ataxia due to cerebellar atrophy with selective loss of Purkinje neurons [116], epilepsy, and corpus callosum damage. NPC patients have the earliest recorded onset Alzheimer's [117], with neurofibrillary tangles found in postmortem biopsies of NPC patients' brains. Life expectancy of NPC patients is variable with disease onset from early childhood, all the way through to adulthood [118]. Patients with severe early onset forms of NPC disease do not live through the second decade of life, whereas late onset form of the disease tend to be less aggressive [119]. Patients also have visceral involvement and organomegally .

1.7.2 Gene defect

Most LSDs are monogenic. However, NPC is caused by a mutation in either *NPC1* or *NPC2* genes [120]. Mutation in the *NPC1* gene accounts for 95% of all reported NPC cases. *NPC1* (Gen bank accession number NM_000271) has been mapped onto chromosome 18q11-12 [121] [122]. The gene consists of 25 exons spanning 47 kb of genomic DNA, and encodes a 4.9 kb mRNA message [123]. Over 200 different mutations have been reported to arise in patients [124]. There is a cluster of mutations that occur in the cysteine rich luminal loop of *NPC1* [125]; these mutations are associated with adult onset of NPC. Otherwise disease-causing mutations occur throughout the gene suggesting that there is not one domain essential for protein function. Mutation in *NPC2* accounts for remaining 5% of NPC cases [126]. *NPC2* (Gen bank accession number NM_006432) is localized to chromosome 14q24.3. The gene spans 0.9 kb DNA. Mutations in either protein result in identical disease progression [118], which implies that these proteins act in a concerted fashion.

1.7.3 NPC1 protein

The NPC1 protein is predicted to be 1274 amino acids in length, with a predicted molecular weight of 170-190 kDa [127]. Topological analysis predicts the protein to have 13 transmembrane domains, with three long heavily glycosylated loops on the luminal side of the membrane, smaller cytoplasmic loops and a cytoplasmic C-terminal tail [127]. A di-leucine motif at the C-terminus targets the protein to the endocytic system [128]; mutations in this motif lead to protein mislocalization to the ER. NPC1 is found in LAMP positive late endosomes and lysosomes [129].

NPC1 shares a weak sequence homology with several proteins. Transmembrane domains 3-7 of NPC1 are homologous to sterol sensing domains (SSDs) of two of the enzymes involved in cholesterol metabolism: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and sterol regulatory element binding protein cleavage activating protein (SCAP) [122]; and to the morphogen receptor Patched. Patched interacts with Sonic Hedgehog [130], which when active has a cholesterol moiety attached. The function of the SSD in NPC1 remains unknown.

There is a weak homology in the entire sequence to the resistance-nodulation-division (RND) family of prokaryotic permeases [131], including *Escherichia coli* AcrB and *Pseudomonas aeruginosa* MexD. Although sequence homology is weak, topologically NPC1 and RND permeases are very similar ; RND permeases have 12 transmembrane spans, consisting of duplicated domains, each with six transmembrane domains, repeated between loop 1 on the first transmembrane domain and loop 2 on the seventh transmembrane domain. Similarly, NPC1 is arranged in a way that transmembrane domains 2 to 8 are duplicated, with an additional N-terminal transmembrane domain that is not found in RND permeases. In prokaryotes RND permeases function to pump multiple substrates including fatty acids, lipid detergents and heavy metals out of the cell.

NPC2, also known as human epididymal protein 1 (HE1), is a 151 amino acid soluble protein. NPC2 co-localises with lysosomal markers [120]. NPC2 has been crystallised and its structure has been resolved [132]. A hydrophobic pocket for sterol binding has been identified from the structure. Point mutation of amino acids around the sterol-binding pocket leads to cholesterol accumulation.

1.7.4 NPC1 protein function

The function of NPC2 is inferred from its structure. The lipid binding domain of NPC2 is specific for binding cholesterol, and not any other lipids. Mutations in the lipid binding domain of NPC2 alter its function. However its interaction with NPC1 is not necessary for cholesterol transport out of the endosomal lumen [133]. It is hypothesized that NPC1 and NPC2 act in concert, with NPC2 binding cholesterol in the lumen of the lysosome then transferring that cholesterol to the N-terminal loop of NPC1.

NPC1 is classically primarily characterized by perturbation of cholesterol homeostasis and accumulation of unesterified cholesterol [134], with cholesterol being the major storage substrate in human patients and animal models. For a long time it has been hypothesized that NPC1 itself acts as a cholesterol transporter. It was proposed that NPC1 interacts with cholesterol via its SSD and transports cholesterol out of the lysosome, therefore the absence of a functional protein leads to perturbed cholesterol trafficking out of the lysosome. The SSD on HMGR acts as a 'sensor' of cholesterol levels and activates cholesterol degradation when the levels are high. Similarly, the SSD on SCAP acts to sense low levels of cholesterol and initiate cleavage and activation of SREB transcription factors, and re-localization of these from the cytosol into the nucleus where they regulate transcription of several enzymes involved in cholesterol biosynthesis. The SSD of NPC1 could also function as a cholesterol 'sensor'.

However, there are also several streams of evidence to suggest that NPC1 does not directly transport cholesterol. The yeast orthologue of NPC1, *Ncr1*, has 35% identity to human NPC1 and structural similarity to PATCHED and SSD of SCAP. *Ncr1* is localized to the vacuole, which is equivalent to the endo-lysosomal system of mammalian cells, an organelle in yeast that does not process endocytosed LDL or cholesterol. *Ncr1* null yeast mutants do not have sterol redistribution or accumulation. However if SSD of NCR1 is mutated sphingolipid transport is altered and sphingolipids accumulate in the vacuole, causing secondary increase in the levels of sterols [135]. The authors suggest that *Ncr1* therefore is not a sterol transporter itself, and that sterol accumulation occurs as a result of sphingolipid mistrafficking. They report that cholesterol is normal in both completely null cells and if you mutate the sterol sensing domain, suggesting NCR1 is not a cholesterol transporter. Sphingolipids accumulate though. NCR1 can rescue NPC1 null human cells indicating a conserved function even though NCR1 homology is as weak as NPC1 to RND permeases.

Cholesterol accumulation is a downstream event in a pharmacologically (U18666a) induced model of NPC; sphingosine is the first substrate to accumulate [45]. This is followed by a reduction in lysosomal Ca^{2+} content and nicotinic acid adenine dinucleotide phosphate (NAADP)-induced Ca^{2+} signalling. The cause of the Ca^{2+} defect remains unknown. Correcting the lysosomal Ca^{2+} defect was shown to improve NPC storage phenotypes.

A new treatment for NPC is cyclodextrin, which reduces the lipid storage burden and improves life span in the NPC mouse model. Due to cyclodextrin's structural nature, it was thought to bind to and remove cholesterol. It has been shown to improve storage in neurons and glia as well as in affected peripheral organs. However cyclodextrin cannot cross the blood-brain barrier, therefore the improvement seen in CNS is not

due to lowering of cholesterol. The mechanism of action of cyclodextrin remains to be elucidated.

Lowering plasma cholesterol does not ameliorate the lipid storage phenotype. However reducing GSL biosynthesis using small molecule inhibitor, NB-DNJ, has proven to be an effective therapy that has been FDA approved and in use in treating patients for the past several years. Recent work [136] has indicated that miglustat can reduce sphingosine levels in NPC cat tissues suggesting that this may contribute to the mechanism of action of this therapy in NPC patients.

Although cholesterol storage is the most prominent phenotype in both NPC patients, pharmacologically induced and the mouse model of NPC, the proposed function of NPC1 in cholesterol transport out of the lysosome does not explain the disease pathogenesis.

1.8 LYSOSOMAL Ca^{2+} HOMEOSTASIS

1.8.1 Mechanism of Ca^{2+} uptake and sequestration

The lysosome has recently been identified as a major intracellular Ca^{2+} store.

In order for the lysosome to function as a long term Ca^{2+} store the mechanism for Ca^{2+} uptake and sequestration must be present. Although the mechanism of Ca^{2+} uptake into the lysosome is not well understood, filling of lysosomes with Ca^{2+} is most likely dependent on proton motive force or ATPase activity. Disrupting the H^+ gradient, either indirectly by inhibiting V-type ATPase using either by using specific inhibitors such as bafilomycin A1, concanomycin, or more directly by raising the pH using H^+ ionophores, such as monensin or nigericin, leads to release of Ca^{2+} from the lysosomal lumen. Therefore collapsing the proton gradient leads to emptying of the store and prevents re-filling.

1.8.2 Ca^{2+} releasing second messenger

NAADP, nicotinic acid adenine dinucleotide phosphate, is a potent second messenger that induces lysosomal calcium release. The Ca^{2+} mobilizing properties of NAADP were first discovered in sea urchin egg homogenate. Resting levels of NAADP are quite low. NAADP is produced when a cell is stimulated with Ca^{2+} mobilizing agonists at the plasma membrane. The ADP ribosyl cyclase, CD38, that synthesizes cADPR is also linked to NAADP synthesis. NAADP releases Ca^{2+} from vesicles insensitive to IP_3 and cADPR. The release is not blocked by IP_3 and ryanodine receptor inhibitors. Moreover, depleting the ER Ca^{2+} store using the SERCA inhibitor thapsigargin abolishes IP_3 and ryanodine, but not the NAADP evoked release in intact cells. NAADP release was instead found to evoke Ca^{2+} release from acidic Ca^{2+} stores, distinct from ER Ca^{2+} stores. NAADP sensitive Ca^{2+} release is ubiquitous, and is much smaller than Ca^{2+} release from the ER. However, in some cell types with a preponderance of acidic stores the release is more sizeable. Studies in intact sea urchin egg using caged NAADP show that the initial Ca^{2+} response occurs in the cortical region of the egg but subsequently spreads to the center of the cell. Subsequent data from mammalian systems confirmed that although the NAADP evoked Ca^{2+} release itself is small and localized, it triggers a 'global' Ca^{2+} response from the ER by recruiting IP_3 and ryanodine receptors via calcium induced calcium release.

1.8.3 Lysosomal Ca^{2+} release receptor

Two-pore channels, or TPCs, are the proposed molecular targets of NAADP. TPCs were initially identified by screening cDNA libraries based on sequences of known voltage-gated channels. Subsequently, vertebrate and invertebrate isoforms of TPCs have been cloned and their NAADP affinity and Ca^{2+} permeability have been examined. Similarly to other voltage gated Ca^{2+} channels, TPCs have been predicted to have a 6 transmembrane domain topology with a pore-forming loop between

domains 5 and 6. TPCs are predicted to form homodimers in order to act as functional channels. Their molecular mass ranges from 80 to 100kDa depending on the degree of protein glycosylation. Three isoforms are found in vertebrates: TPC1, TPC2 and TPC3. TPC3 is not found in rodents or humans [137]. TPC isoforms are sufficiently distinct from each other with conserved 30% amino acid identity in the transmembrane domain, and conserved N-terminal di-leucine motifs. All three TPCs have a punctate intracellular distribution indicating endolysosomal localization, with TPC1 localising to endosomes and lysosomes, and TPC2 preferentially co-localising with lysosomal markers [138]. TPC3 is localized to recycling endosomes. Although both TPC1 and TPC2 are NAADP-sensitive Ca^{2+} -permeable channels, signal amplification from the ER is specifically linked to TPC2, as mislocalizing TPC2 to the plasma membrane dissociates Ca^{2+} release by NAADP from subsequent amplification by the ER. Overexpression of any of the isoforms enhances NAADP-mediated Ca^{2+} release. However, NAADP response is abolished in *tpc2* knock out mice therefore the TPC2 isoform of the receptor has been most investigated in terms of Ca^{2+} release from the acidic compartment.

Studying the channel conductance properties of lysosomal transmembrane proteins by the 'classical' patch clamp technique is complicated by the fact that the average lysosomal diameter is $0.5\mu\text{m}$ and the diameter of a pipette tip is $1\mu\text{m}$. Therefore, the conductance properties of TPC channels were studied by patch clamp electrophysiology of immuno-precipitated TPCs reconstituted into a lipid bilayer. These experiments also looked at pH, at less acidic pH the NAADP receptor does not release NAADP.

Channel conductance properties were also studied in purified pharmacologically enlarged lysosomes.

1.8.3.1 NAADP affinity for lysosomal Ca^{2+} release receptor

There is an interesting relationship between NAADP concentration and Ca^{2+} release. In mammalian cells, at very low and very high concentrations of NAADP, TPC does not release Ca^{2+} . This evidence suggests existence of two binding sites on TPC receptors. A high affinity-binding site to which NAADP binds when it is present at low concentration and in doing so inhibits Ca^{2+} release. As concentration of NAADP increases, it also binds to a low affinity-binding site and activates the receptor. At an optimal concentration of NAADP most Ca^{2+} is released, beyond this concentration of NAADP the amount of Ca^{2+} released decreases. Ca^{2+} release in response to NAADP concentrations in mammalian cells can therefore be described as a bell shaped response curve. Churchill *et al.* proposed a model to explain NAADP binding and receptor activation. The receptor is thought to exist in an open and locked states. When the concentrations of NAADP are low, the molecule binds to a high affinity allosteric site on the luminal side of the lysosomal membrane stabilizing the 'locked' conformational state of the receptor preventing Ca^{2+} release thereby desensitizing the receptor to signal. However when the concentration is high it binds to a different low affinity allosteric site on the receptor, opening the receptor to allow Ca^{2+} release. Although there are several lines of evidence linking TPCs to NAADP induced Ca^{2+} release, the mechanism of Ca^{2+} release following NAADP binding to the channel remains to be elucidated.

1.8.4 Role of lysosomal calcium in LSD pathogenesis

Aberrant Ca^{2+} signalling has been linked to pathogenesis of a number of LSDs [139]. An increase in the ER Ca^{2+} pool has been reported in Gaucher disease (GD), both in intact neurons and in microsomes derived from brain tissue with a positive correlation between the increase in Ca^{2+} release and the amount of stored GlcCer. Aberrations in ER Ca^{2+} signaling was also reported in Sandhoff disease (SD) where the major stored

substrate GM2 was shown to inhibit SERCA activity and reduce uptake of Ca^{2+} into the ER. In both GD and SD stored sphingolipids affect Ca^{2+} homeostasis by modulating the activity of Ca^{2+} release (RyR) and Ca^{2+} re-uptake (SERCA) channels on the ER membrane respectively.

Niemann Pick A (NPA) is a disease characterized by a reduced activity of acid sphingomyelinase; the major accumulating substrate in this disease is sphingomyelin. Here however, it is not the direct interaction of the primary storage sphingolipid with the proteins regulating Ca^{2+} homeostasis, but a reduced expression pattern of these proteins that was observed [139]. In the mouse model of NPA, expression of both SERCA and IP3R is strikingly reduced prior to onset of neurological symptoms [139].

In recent years however the importance of Ca^{2+} signaling from acidic stores as an important step in LSD pathogenesis has emerged. As discussed above, lysosomal Ca^{2+} is reduced in NPC. Sphingosine, one of the major substrates that accumulates in NPC, causes a reduction in lysosomal Ca^{2+} [45]; the mechanism through which it does so is unknown. It has been speculated that sphingosine affects the lysosomal Ca^{2+} transporters either directly or by inhibiting protein kinase C (PKC) [140] causing a downregulation of Ca^{2+} re-uptake pumps. This reduction in the lysosomal Ca^{2+} pool leads to a reduced Ca^{2+} release in response to NAADP signal.

As discussed above, aberrant lysosomal Ca^{2+} signaling is also observed in MLIV. In contrast to NPC, there is an increased lysosomal Ca^{2+} release in response to NAADP, lending to speculation the role of TRPML1 in regulating NAADP receptor function.

For a long time the lysosome was thought to solely act as an end point for the endocytic pathway where degradation and recycling of macromolecules and whole organelles occurs. However, it has proven to be a more dynamic organelle with many functions, which, when perturbed, result in disease with an increasing evidence of

lysosomal dysfunction as an underlying cause of neurodegenerative disorders such Alzheimer's, Huntington's, Parkinson's disorder.

2 Comparison of MLIV and NPC cellular phenotypes

2.1 INTRODUCTION

There has been ample discussion in the lysosomal disease field about potential similarities between NPC and MLIV at the cellular level including reports that indicate loss of function of TRPML1 in NPC disease [96] and the possibility of a shared loss of function in NAADP signalling. Considering the clear clinical differences that exist between the two disorders this is an area that certainly warrants further investigation. For the first time, these similarities will be discussed and tested in this study.

2.1.1 Endocytosis, vesicular fusion and lipid storage in NPC1 and MLIV

Niemann Pick type C1 and Mucopolysaccharidosis type IV both belong to a sub group of LSDs caused by a defect in lysosomal transmembrane proteins, NPC1 and TRPML1 respectively. Trafficking defects in the late endocytic system out of late endosome/lysosome to the Golgi, and lysosomal storage, albeit of different macromolecules, have been previously reported in both NPC and MLIV [87, 99]. Both membrane traffic defects and lysosomal lipid accumulation are common to other sphingolipidoses including Niemann Pick type A and B [141]. Despite these similarities a number of differences are apparent. First, the endocytosis defect in NPC disease constitutes a retardation in transport along the endocytic system including delayed transport of cargo between early and late endosomes as a result of Annexin A2 mislocalisation [142]. Furthermore, transport out of the endocytic system is also retarded [143]. In contrast, Pagano and co-workers have shown that transport of BODIPY-lactosylceramide through the endocytic system is accelerated in fibroblasts from MLIV patients [144] suggesting that here the two diseases have clearly opposing effects on endocytosis. Second, we have previously shown that in NPC disease there

is a retardation in vesicular fusion between late endosomes and lysosomes [45], in MLIV it has been suggested that there is an acceleration in vesicular fusion [1] and a defect in separation of the fused intermediate late endosome-lysosome heterotypic organelle. Again, here it would appear that the phenotypes are opposed between NPC and MLIV. Finally, we would expect that if the two diseases shared similar cellular abnormalities that the stored macromolecules within the endolysosomal system would be similar, however this is not the case. In NPC there is an accumulation of multiple lipids – sphingosine [45], sphingomyelin [145], cholesterol [146], the majority of glycosphingolipids [147], with no reported accumulation of water soluble material such as glycosaminoglycans or proteins, although a recent report was the first to suggest a higher level of autofluorescence in NPC patient cells [96]. This report however does not identify the precise source of the autofluorescence. In MLIV the storage pattern is quite different, MLIV cells are reported to store some gangliosides [148], phospholipids [97, 149], water soluble complex glycosaminoglycans [150] and autofluorescent protein based lipofuscin [151]. The accumulation of cholesterol has been reported from one group [152], suggesting a potential connection to NPC disease but secondary cholesterol storage is common amongst most of the lysosomal storage diseases as a result of the tight interactions between cholesterol and the accumulating glycosphingolipids [153, 154].

2.1.2 Alterations in lysosomal Ca^{2+} signaling in NPC1 and MLIV

Our group recently identified a lysosomal Ca^{2+} homeostasis defect in NPC patient fibroblasts and pharmacologically induced model of NPC cells, namely a reduction in lysosomal Ca^{2+} content and a concomitant reduction in NAADP mediated lysosomal Ca^{2+} release [45]. This defect accounts for the endocytic transport abnormalities and downstream lipid storage discussed in section 1.9.1. Interestingly, as discussed earlier TRPML1 has been suggested by a number of groups [155] to potentially be the

lysosomal target of NAADP. This is discussed in more detail in the next chapter. However, should TRPML1 genuinely function as a NAADP responsive lysosomal Ca^{2+} channel it is logical to consider that its function may also be perturbed in NPC disease where lysosomal Ca^{2+} levels are reduced and NAADP mediated Ca^{2+} release is also reduced. Considering the clear effects of alterations in lysosomal Ca^{2+} release on endocytosis [45, 156] it would be likely that if TRPML1 were indeed working as an NAADP mediated Ca^{2+} release channel that the phenotypes in TRPML1 null cells would resemble those of NPC disease cells. Recently, this logic has been questioned by the study from Shen *et al.* [96]. Here the authors do not report any changes in lysosomal Ca^{2+} in NPC cells and suggest that the defects in lysosomal Ca^{2+} release in NPC cells are in fact caused by the accumulating sphingomyelin inhibiting the function of TRPML1. No mention is made of how the sphingomyelin accumulates in NPC cells and neither is any mention made of the fact that Niemann-Pick A cells, where acid sphingomyelinase is deficient and the cells accumulate far more sphingomyelin than NPC [145], should therefore have far worse MLIV-like cellular phenotypes but do not. No effort was made to reduce the levels of sphingomyelin within NPC cells in this study by using a specific inhibitor of sphingomyelin synthase, D609 [157], to see if they could rescue the cellular phenotypes. Finally, perhaps the most damning set of results that are critical of this study of Shen *et al.* [96] is that miglustat, the approved therapy for NPC disease in the EU, actually elevates sphingomyelin levels within the lysosomes in the treated NPC cells yet leads to a correction in the endocytic trafficking abnormalities [158, 159] and a partial correction in the lysosomal Ca^{2+} release defect (Haslett and Lloyd-Evans, unpublished), both results indicate that this study needs to be revisited.

2.1.3 Aims of this chapter

The aim of this chapter is to compare the cellular phenotypes typically found in cells with NPC1 null/reduced function to TRPML1 null MLIV patient fibroblasts. Lipid storage

profile and distribution of the stored material will be explored. Lysosomal Ca^{2+} homeostasis in NPC will be compared to that in MLIV.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

Human patient fibroblasts used in this study were obtained from the cell repository at the Coriell Institute for Medical Research, New Jersey. Cells used for the study were as follows: healthy control lines, GM05399 and GM05659; NPC1 patient fibroblasts GM03123; MLIV patient fibroblasts, GM02527. Additional lines of MLIV patients fibroblasts used, patient WG909 and HET WG988, were kindly donated by Prof. Paul Luzio (Cambridge University).

Cells were cultured in Dulbecco Modified Eagles Medium, DMEM, (Sigma, Gillingham, UK) supplemented with 10% foetal calf serum, FCS (Lonza) and 1% L-Glutamine, Gln (Invitrogen, Paisley, UK). Cells were maintained as a confluent monolayer in tissue culture coated flasks (Fisher, Loughborough, UK) in 5% CO_2 37°C humidified incubator.

2.2.2 Preparation of cells and coverslips

Round coverslips (VWR, Lutterworth, UK) 13mm² and 16mm² in diameter were washed in 2 M HNO_3 acid (Sigma, Gillingham, UK) for 30 min followed by 5 washes in 5 l double distilled water, ddH₂O, and left in 5 l ddH₂O overnight. Acid washed coverslips were stored in absolute ethanol (Sigma, Gillingham, UK).

Confluent cell monolayers, used for experiments, were washed once with PBS (Sigma, Gillingham, UK) and cells were detached from the surface of the flask using 0.025% trypsin (Sigma, Gillingham, UK) for 5 min. Cells were re-suspended in supplemented medium, counted and seeded onto coverslips at a density of 20,000-30,000 unless

stated otherwise. Cells were allowed to adhere onto the surface of the coverslip overnight.

2.2.3 Protein quantification

The amount of protein in the sample was quantified using a Pierce Bicinchoninic Acid, BCA, protein assay kit (Fisher, Loughborough, UK) in a 96 well format, according to manufacturer's instructions. The assay was read using a Tecan Infinite F50 absorbance plate reader at 570nm absorbance.

Bovine serum albumin, BSA, 1 mg / ml standard solution (Sigma, Gillingham, UK) was diluted to a series of known concentrations in order to obtain a standard curve of absorbance values. This standard curve of absorbance values for known protein concentrations was used to calculate the amount of protein in unknown samples.

Protein concentration in the sample was determined using a Pierce BCA assay kit (Fisher, Loughborough, UK) as directed by the manufacturer.

2.2.4 Evaluating the distribution of unesterified cholesterol

Cells were grown on 13mm² coverslips overnight to allow cells to adhere. Cells were washed twice with 1 ml PBS and fixed using 4% Paraformaldehyde, PFA (Sigma, Gillingham, UK) for 15 min at room temperature. Following fixation cells were washed twice with 1 ml PBS and once in 10% FCS/ 1% Gln DMEM for 10 min to quench residual PFA. Cells were incubated with 187.5 µg / ml filipin (Polysciences, Eppelheim, Germany) in 10% FCS/ 1% Gln DMEM for 30 min at room temperature, 250 µl per coverslip. Filipin solution was removed and cells were washed twice with 1 ml 10% FCS/ 1% Gln DMEM, followed by two washes in PBS. Coverslips were mounted in Mowiol (Calbiochem, Feltham, UK) onto glass slides (VWR, Lutterworth, UK). Coverslips were visualized using a Zeiss Axiovert microscope fitted with Colibri LED

light system 24 h following mounting to allow Mowiol mounting medium to harden ($\lambda_{\text{excitation}}/\lambda_{\text{emission}} = 380 / 460 \text{ nm}$).

2.2.5 Quantifying the levels of unesterified cholesterol

The Amplex Red cholesterol assay kit (Invitrogen, Paisley, UK) was used to quantify the level of cholesterol. Cholesterol esterase was omitted from this assay, only free cholesterol has been measured. Fluorescence was measured using a NOVOSTAR fluorescence plate reader and analysed using OPTIMA software. A series of cholesterol concentrations were used to obtain a standard curve and estimate a linear regression equation. Sample cholesterol concentration was determined using the regression equation and normalised to protein concentration.

2.2.6 Evaluating distribution of sphingomyelin

Sphingomyelin levels were measured using lysenin, a toxin found in coelomic fluid of earthworms that binds specifically to sphingomyelin [160]. Cells were grown on 13mm² coverslips overnight in 10% FCS/ 1% Gln DMEM to allow the cells to adhere. Cells were washed twice with PBS and fixed using 4% PFA for 15 min at room temperature. Cells were incubated in blocking buffer, 1% bovine serum albumin, BSA, (Sigma, Gillingham, UK) / 0.1% saponin (Sigma, Gillingham, UK) in PBS for 1 h at room temperature. Lysenin toxin (Peptides International, Louisville, USA), 5 µg / ml in blocking buffer was added and incubated overnight at 4°C. Lysenin solution was removed and cells were washed three times in 1 ml PBS, 10min per wash. Cells were then incubated with lysenin anti serum (Peptides International, Louisville, USA) raised in rabbit, at a 1:1000 dilution in blocking buffer for 1 h at room temperature. Following the incubation the blocking buffer was removed and the cells were washed three times in PBS, 10 min each wash. DyLight 594 goat anti-rabbit secondary antibody (Vector labs, Peterborough, UK) used at a 1:200 dilution in blocking buffer, was applied for 30

min at room temperature followed by three 5 min washes in PBS. Cells were incubated with 5 μg / ml Hoescht for 5 min, and washed three times with PBS. Coverslips were mounted onto glass slides in Mowiol. Coverslips were visualized using a Zeiss Axiovert microscope fitted with Colibri LED light system 24 h following mounting to allow Mowiol mounting medium to harden ($\lambda_{\text{excitation}}/\lambda_{\text{emission}} = 593 / 618 \text{ nm}$).

2.2.7 Quantifying GSL levels by HPLC analysis

Patient fibroblasts grown in tissue culture coated T175 cm^2 flasks were trypsinized, harvested and precipitated by centrifugation at 1,500 rpm for 5 min. Supernatant was removed and cell pellet was washed in 10 ml PBS, cells were precipitated again by centrifugation at 1,500 rpm for 5 min. The pellet was resuspended in 0.5 ml Mili-Q water and cells were lysed by 3 rounds of freeze/thaw cycles. The cell suspension was then homogenized using Dounce homogenizer and GSLs were extracted using the Svennerholm method of extraction [161]. Prior to extraction, the protein concentration in the cell suspension was determined as described in section 2.2.3.

The cell suspension was mixed with 4 volumes of chloroform/methanol (1:2, v/v) overnight at 4°C. Phase separation of the sample into organic (lipid) and aqueous (protein) phases was achieved by adding 1 ml chloroform and 1 ml PBS followed by centrifuging the sample at 1,000 g for 10 min. Lower organic phase (lipid extract) was taken, dried down at 40°C under N_2 flow and redissolved in 50 μl chloroform/methanol (1:3, v/v). GSLs were purified from the total lipid extract using pre-equilibrated SepPak C18 column (Supelco, Poole, UK). GSL-derived oligosaccharides were released from GSLs by ceramide glycanase (Calbiochem, Nottingham, UK) digestion; these oligosaccharides were labeled using fluorescent 2-anthranilic acid, 2-AA (Sigma, Gillingham, UK) and analysed using high-performance liquid chromatography, HPLC. Ceramide glycanase digestion, 2-AA labelling and HPLC analysis were performed as described in Neville *et al.* [162].

2.2.8 Lysotracker probe loading

Lysotracker (Molecular probes, Paisley, UK) is loaded into cells in 1X HBSS (Invitrogen, Paisley, UK) containing 1 mM CaCl_2 (Sigma Aldrich, Poole, UK), 1 mM MgCl_2 (Sigma Aldrich, Poole, UK) and 20 mM HEPES pH 7.4 (Invitrogen, Paisley, UK) pre-warmed to 37°C at a concentration of 200 nM Lysotracker Green DND-26 or 300 nM LysoTracker Red DND-99, for 15 min at 37°C. Lysotracker solution was removed and the cells were washed three times with PBS. Cells were incubated with 5 μg / ml Hoechst for 5 min, then washed three times with PBS. Coverslips were visualized live using a Zeiss Axiovert microscope fitted with Colibri LED light system. Lysotracker Green DND-26 ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$ = 504 / 511 nm). Lysotracker Red DND-99 ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$ = 570 / 590 nm).

2.2.9 Determining content of charged phospholipids using LipidTOX Red probe

HCS LipidTOX Red phospholipidosis detection reagent (Molecular Probes, Paisley, UK) is a fluorescent phospholipid analogue probe, the accumulation of which within cells is indicative of a defect in lysosomal processing of phospholipids [163]. Cells were grown on 13mm² coverslips overnight in 10% FCS/ 1% Gln DMEM to allow the cells to adhere. 1 μg / ml HSC LipidTOX Red probe in pre-warmed 10% FCS/ 1% Gln DMEM was added to cells grown on coverslips and incubated for 4 h at 37°C. DMEM medium was removed, cells were washed twice with 10% FCS/ 1% Gln DMEM and incubated for further 20 h. Cells were then washed twice in PBS and incubated in 5 μg / ml Hoechst (Invitrogen, Paisley, UK) in PBS for 10 min at room temperature. Coverslips were visualized live using a Zeiss Axiovert microscope fitted with Colibri LED light system ($\lambda_{\text{excitation}}/\lambda_{\text{emission}}$ = 595 / 615 nm).

2.2.10 Cholera toxin transport assay

Cells were grown on 13mm² coverslips overnight in 10% FCS/ 1% Gln DMEM to allow the cells to adhere. Cells were incubated with 2 µg / ml Cholera Toxin AF488 (Invitrogen, Paisley, UK) in 1% BSA/ 10% FCS/ 1% Gln DMEM for 30 min at room temperature, washed with pre-warmed 10% FCS/ 1% Gln DMEM, and incubated in DMEM for 2 h at 37°C. Cells were subsequently placed on ice and washed with pre-chilled 2% BSA DMEM medium three times, 10 min per wash. Cells were then washed with cold PBS and fixed using 4% PFA for 15 min, and washed three times with PBS. Coverslips were mounted onto glass slides in Mowiol and visualized after 24 h using a Zeiss Axiovert microscope fitted with Colibri LED light system at 40X magnification ($\lambda_{\text{excitation}}/\lambda_{\text{emission}} = 495 / 519 \text{ nm}$).

2.2.11 Measuring lipofuscin storage using Sudan Black

(2,2-dimethyl-1,3-dihydroperimidin-6-yl)-(4-phenylazo-1-naphthyl) diazene also referred to as Sudan Black (SB) is a lipophilic diazo dye probe that has been previously used to eliminate lipofuscin-associated autofluorescence in tissue histology by binding to lipids [164]. We have used Sudan black as a marker for lipofuscin in methanol (MeOH) fixed patient fibroblasts.

Cells were grown on 13mm² coverslips overnight in 10% FCS/ 1% Gln DMEM to allow the cells to adhere. Cells were fixed with cold MeOH for 5 min. Cells were then washed three times with PBS followed by two washes in ethanol. Cells were incubated in 0.25% Sudan Black powder (Sigma, Gillingham, UK) in ethanol for 5 min. Sudan Black solution was removed and cells were washed under running water for 20 min. Sudan Black staining was visualised by light microscopy.

2.2.12 Loading the cells with Ca^{2+} indicators

In order to measure intracellular Ca^{2+} cells were plated into chambers of an 8-well chamber slide (Ibidi, Germany) in 10% FCS / 1% Glc DMEM at 20,000-30,000-cell density and allowed to adhere overnight.

Indicators used in these study were Fura2- AM, Calcium Green-1 AM and Fura Red all obtained from Molecular Probes, Paisley, UK. A stock solution of Ca^{2+} indicators, was prepared to 1 mM concentration using anhydrous DMSO (Sigma, Gillingham, UK). Pluronic F127 (Sigma, Gillingham, UK) was used to prevent Ca^{2+} indicators aggregation and aid dispersal. 5% Pluronic F127 in DMSO, was added to an equal volume of Ca^{2+} probe and vortexed vigorously to mix. Working concentration of 5 μM probe was prepared by adding probe/ Pluronic F127 to unsupplemented DMEM containing 1% BSA. Cells were incubated with Ca^{2+} probe in the dark for 1 h at room temperature to allow the probe to cross the plasma membrane and be loaded in the cytosol. Solution was removed and cells were washed twice with cold 1% BSA DMEM, and incubated for 10 min at room temperature to de-esterify the AM ester. DMEM solution was removed and the cells were washed twice with 1X HBSS containing 1 mM MgCl_2 (Sigma, Gillingham, UK), 20 mM HEPES (Invitrogen, Paisley, UK).

2.2.13 Lysosomal Ca^{2+} release measurements

Ca^{2+} release in fibroblasts pre-loaded with fluorescent Ca^{2+} indicators was visualized using a Zeiss Axiovert microscope fitted with a Colibri LED illumination system. Ca^{2+} transience over time was recorded using the Axiovision physiology suite. Regions of interest, ROIs, were placed over an entire cell. Baseline value of fluorescence was measured for 60 s prior to adding pharmacological releasing agents to the chamber slide with adherent cells. Ca^{2+} releasing agents were added carefully to the without disturbing the cells in the chamber slide. Ca^{2+} releasing agents remained in the chamber slide following the addition.

The amount of Ca^{2+} release in relative fluorescence units, in response to various pharmacological agents (200 μM GPN (Sigma, Gillingham, UK) in this chapter) was measured as a difference between the baseline and peak values on a minimum of 10-15 cells in a minimum of three individual experiments with minimum of two technical repeats. Traces shown are a representative Ca^{2+} response of cells.

2.3 RESULTS

2.3.1 Cholesterol levels and distribution is different between MLIV and NPC1

Cellular distribution of cholesterol was visualized using filipin (Fig. 2.1 A), a fluorescent probe that binds to unesterified cholesterol at a 1 to 16 ratio [165]; cells were counted according to cholesterol localisation punctate late endosomal/lysosomal versus plasma membrane (Fig. 2.1 B). Both WT and MLIV fibroblasts show diffuse plasma membrane and focal peri-nuclear punctate staining with filipin indicating that cholesterol is distributed in the plasma membrane and endocytic recycling compartment (Fig. 2.1). In contrast, NPC fibroblasts show widely distributed punctate staining with filipin (Fig. 2.1 A) indicating that cholesterol is primarily sequestered into late endosomes/lysosomes with little cholesterol found at the plasma membrane. In addition, the intensity of filipin staining in NPC fibroblasts goes up compared to WT and MLIV fibroblasts indicating an increase in cholesterol storage in the acidic compartment of NPC cells.

This data was confirmed biochemically utilizing the Amplex Red cholesterol assay (data not shown) that indicates no elevation in free cholesterol levels in MLIV patient fibroblasts compared to healthy controls.

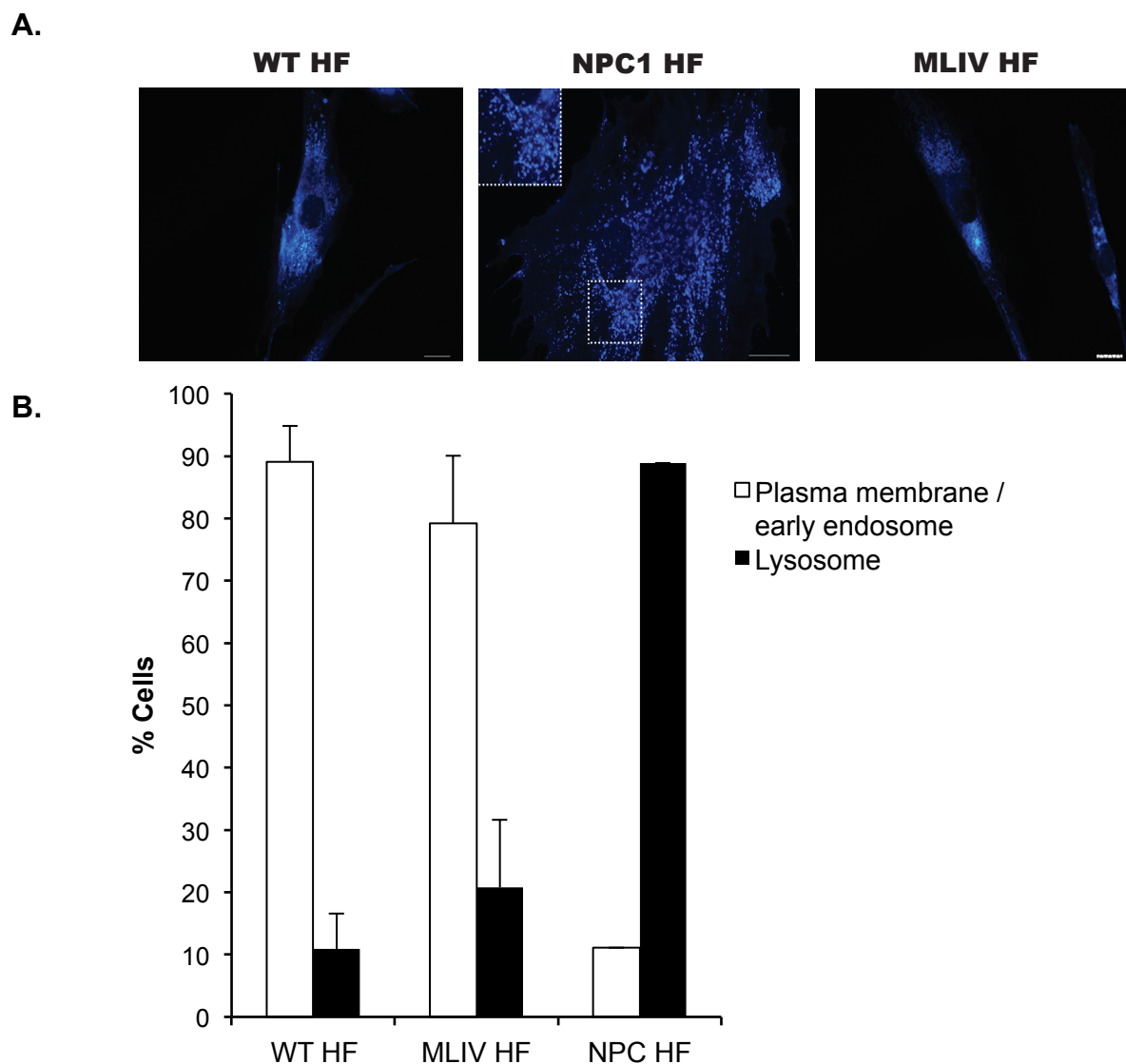


Figure 2.1 Cholesterol cellular distribution and levels in patient fibroblasts. A. Cholesterol was detected in patient fibroblasts using fluorescent filipin in fixed cells. Insert in NPC1 HF picture is zoomed in image of the selection shown in the picture. Scale bar represent 5 mm. **B.** Cholesterol distribution in filipin stained fibroblasts was analysed according to intracellular localisation of filipin. Statistical significance was assessed using one-way ANOVA. Error bars represent S.D.; n=3.

2.3.2 *Sphingomyelin localisation is different between MLIV and NPC1*

The levels of sphingomyelin were measured using lysenin toxin in 4% PFA fixed patient fibroblasts (Fig. 2.2); lysenin toxin staining was visualized by staining the cells with lysenin anti-serum followed by a fluorescent secondary antibody as described in section 2.2.6. We found that MLIV fibroblasts do not have elevated levels of sphingomyelin compared to WT fibroblasts (Fig. 2.2 A), in fact we find levels of lysenin fluorescence to be marginally reduced in MLIV compared to WT fibroblasts, as the fluorescence intensity is lower. In NPC1 fibroblasts, however there is a clear and expected increase in lysenin fluorescence indicating that levels of sphingomyelin are elevated, as previously reported [145].

We have also counted the cells according to localisation of the lysenin stained sphingomyelin- plasma membrane versus endosomal/recycling versus lysosomal compartment (Fig. 2.2 B). Counts were performed blind. We found that there is also a marked difference in distribution of sphingomyelin between WT, MLIV and NPC1 fibroblasts. In WT fibroblasts sphingomyelin is localized primarily at the plasma membrane ($83.2 \pm 3\%$ of the cells), with very little being found in early endosomes ($6.7 \pm 4.8\%$ of the cells) and lysosomes ($10 \pm 4.8\%$ of the cells). In MLIV cells, localisation of sphingomyelin shifts from the plasma membrane ($56.7 \pm 6\%$ of the cells) towards early endosomal structures that aggregate close to the plasma membrane ($36.2 \pm 3\%$ of the cells) ($p < 0.001$). Similarly to WT fibroblasts very little sphingomyelin is found in lysosomes ($7.1 \pm 7\%$ of the cells) ($p = 1$). In NPC1 fibroblasts however, sphingomyelin is primarily found in the late endosome/lysosomal system ($81.7 \pm 2.3\%$ of the cells) ($p < 0.001$). Very little sphingomyelin is found on the plasma membrane ($15 \pm 2.8\%$ of the cells) ($p < 0.001$). We have also found that lysenin to co-localise with antibody markers for late endosomes and lysosomes in NPC fibroblasts (data not shown).

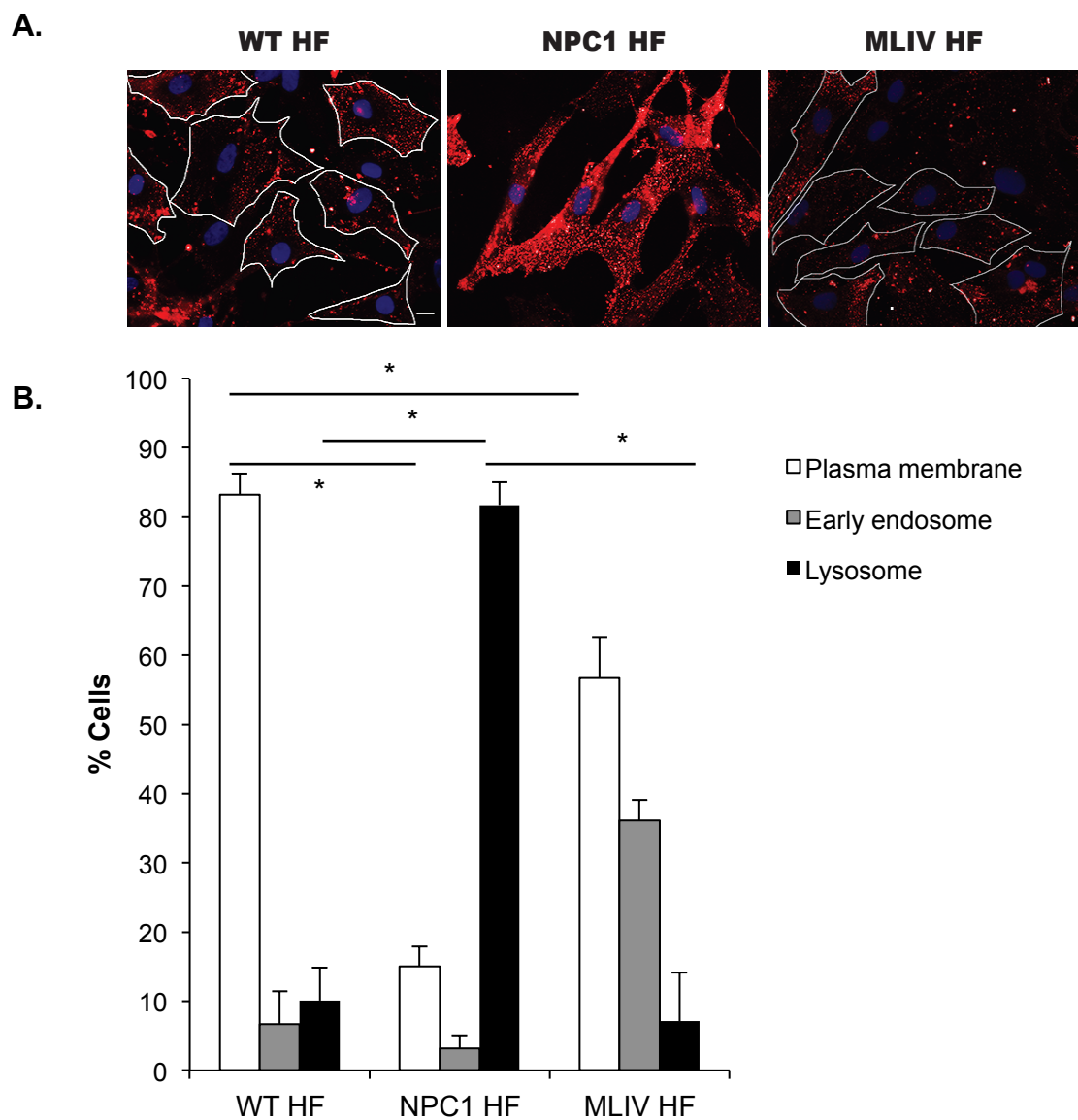


Figure 2.2 Sphingomyelin cellular distribution and levels are altered in NPC and MLIV patient fibroblasts. A. Sphingomyelin was detected in fixed HFs using lysenin toxin as described in section 2.2.6. Scale bar represent 5 μ m. **B.** Sphingomyelin distribution in lysenin stained fibroblasts was analysed according to intracellular localisation. Statistical significance was assessed using one-way ANOVA; * $p < 0.001$. Error bars represent S.D.; $n=5$.

2.3.3 *Glycosphingolipid storage in MLIV is lower compared to NPC1*

We have compared levels of GSLs in NPC1 and MLIV patient fibroblasts using HPLC analysis. Please note the n number is 2 as 1-2 mg protein is required per single run of HPLC and as a cell type, fibroblasts yield little amount of protein the cell pellets from 8-10 flasks were pulled to acquire the necessary amount of protein.

Lipids were extracted from harvested patient fibroblasts and GSLs were purified from the total lipid extract. GSLs were then cleaved by ceramide glycanase, and the GSL-derived oligosaccharides were labeled using a fluorescent 2-AA probe, as described in section 2.2.7. HPLC was used to detect and quantify the levels of labeled oligosaccharides as described in Neville *et al.* [162].

Preliminary results from HPLC analysis of lipids extracted from patient fibroblast has revealed that GSL levels are elevated in NPC1 fibroblasts compared to WT fibroblasts (Fig. 2.3 B-C) as was previously reported for brain tissue of *Npc1*^{-/-} mice [136, 158]. We observed at least 1.5 fold elevation in GSL levels in NPC1 fibroblast compared to WT fibroblasts. In contrast, MLIV fibroblasts show reduced total levels of GSL compared to both NPC1 and WT fibroblasts (Fig. 2.3 B-C). An almost 2 fold reduction in the levels of GSLs is observed in MLIV compared to WT fibroblasts: 211,608 fmol GSL-derived oligosaccharides /mg protein in WT fibroblasts compared to 110,713 fmol GSL-derived oligosaccharides /mg protein in MIV fibroblasts. Preliminary HPLC analysis showed that globoside series GSLs, globotriaosylceramide, Gb3, and globotetraosylceramide, Gb4, are elevated in both MLIV and NPC1 fibroblasts. In contrast, a-series gangliosides such as GM3, GM2 and GM1a are reduced. Reduction in GM2 and GM1a can be explained by reduction in GM3, which feeds into the synthesis of both GM2 and GM1a. B-series ganglioiside, GD3, is also reduced in NPC1 fibroblasts. Overall levels of gangliosides are reduced in NPC1 patient fibroblasts, which is in contrast to previously reported elevation in level of gangliosides

in *Npc1*^{-/-} mouse brain this may be explained by the difference in tissue being analysed in that brain tissue has higher expression of gangliosides compared to other GSLs and in fibroblasts globosides are the main GSL species.

In contrast to NPC1 fibroblasts, GD3 is elevated in MLIV fibroblasts. In fact it is the only GSL species that is elevated in MLIV compared to WT: 9,453 fmol GSL-derived oligosaccharides /mg protein in WT fibroblasts compared to 11,287 fmol GSL-derived oligosaccharides /mg protein in MIV fibroblasts. The activity of ganglioside sialidase, the enzyme that hydrolyses GD3 into GM3 and LacCer, was previously reported to be reduced in MLIV fibroblasts [166]. This may explain why the levels of GD3 are elevated and the levels of both LacCer and GM3 are reduced. GD3 was previously reported to inhibit Ca²⁺ reuptake into the ER by inhibiting SERCA [167]. In order to investigate the subcellular localisation of this particular ganglioside species we used a specific antibody to o-acetylated-GD3, Jones (data not shown). Staining was poor suggesting that either the levels of GD3 being too low in patient fibroblasts or that the antibody was raised against o-acetylated GD3 instead of non acetylated GD3 which may have compromised this experiment; antibodies are now available against non acetylated GD3 and should be tested to confirm the difference between MLIV and the other cells. Overall, there is an increase in GSL levels in NPC fibroblasts, which is supported by studies with previously reported increases in GSL species in other cell types of NPC and brain of NPC mouse model [158]. GSL levels of MLIV cells are lower compared to WT fibroblasts which could be due to a defect in GSL biosynthesis, there will need to be further testing in the same MLIV patient cell line as well as from cell lines from other patients to confirm the altered glycolipid expression that we observe here.

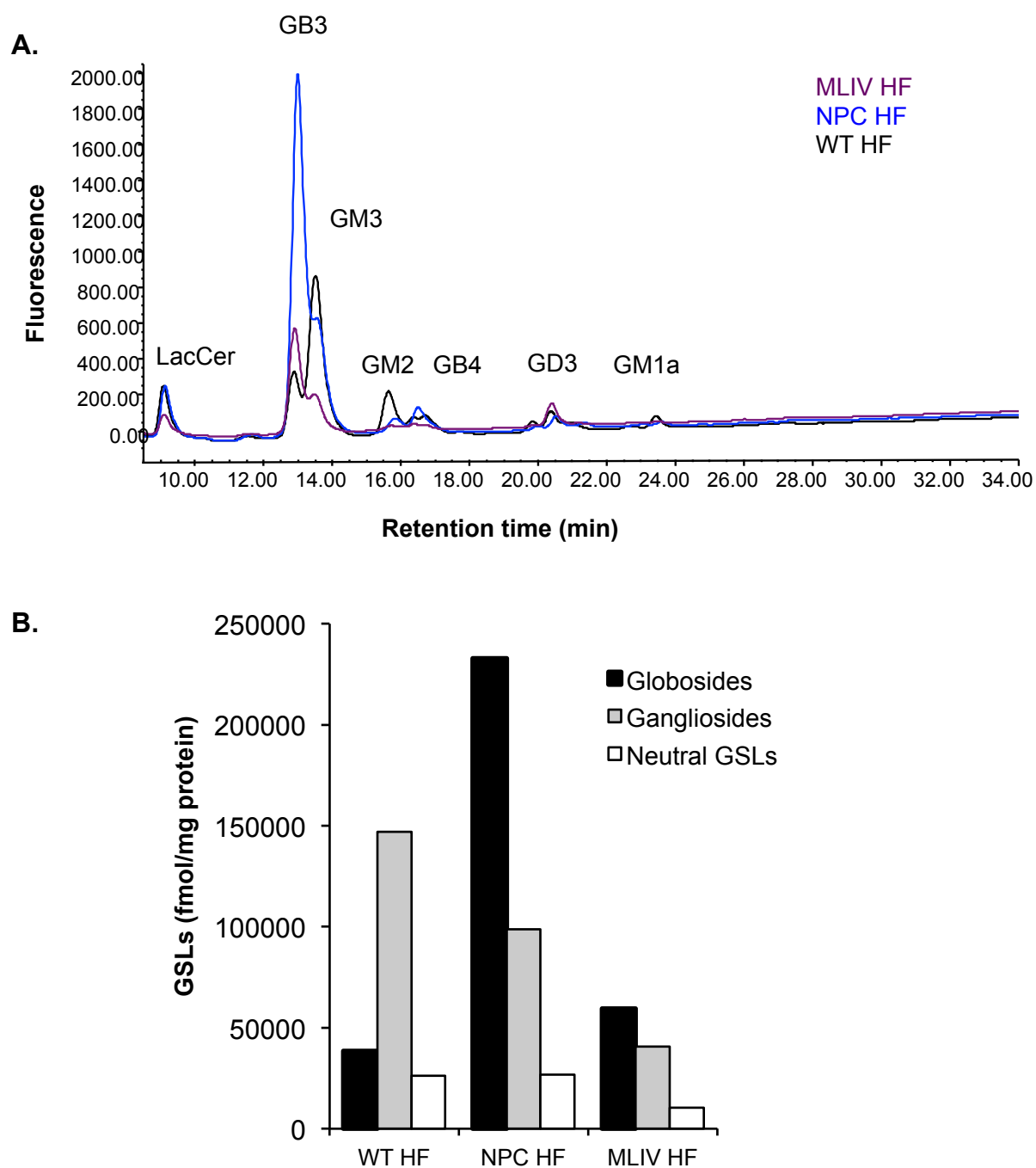


Figure 2.3 Altered GSL profiles in fibroblasts from patients with NPC1 and MLIV. A. Scheme showing GSL biosynthesis pathway adapted from McDonald et al. with some of the enzymes of GSL biosynthesis. Lipids were isolated from 1 NPC patient line, 1 MLIV patient line and 1 patient control cell line using Svennerholm method of extraction, GSLs were isolated by ceramide glycanase digestion and analysed by HPLC. **B.** Representative qualitative HPLC plots showing the positions of GSL species. **C.** Cumulative quantitative data for total GSLs separated into species identified from HPLC plots.

2.3.4 Lysosomal expansion occurs in both MLIV and NPC1

Overall lysosomal storage was examined using Lysotracker probe Fig. 2.4. The Lysotracker probe is a fluorophore linked to a weak base that is only partially protonated at neutral pH. Once Lysotracker reaches the acidic compartment it is further protonated becoming highly fluorescent. Lysotracker probe selectively accumulates in the acidic compartment and can be used as a marker for late endosomes/lysosomes.

We have loaded the patient fibroblasts with Lysotracker, as described in section 2.2.8, and examined the staining by fluorescence microscopy. We find that Lysotracker gives punctate staining pattern (Fig. 2.4) indicating that the probe loads into the endocytic compartment. Lysotracker fluorescence goes up in MLIV and NPC patient fibroblasts compared to WT indicating an expansion of the late endosomal and lysosomal compartments. Moreover the staining in the WT fibroblasts are more finely punctate compared to NPC and MLIV fibroblasts where the punctae look bigger as well as brighter, indicating expansion of late endosomal / lysosomal compartment as well as storage within the compartment. We detect lysosomal storage and expansion in MLIV and NPC patient fibroblasts with both Green and Red Lysotracker (Fig. 2.4). It should be noted that Lysotracker does not discriminate between lysosomes or late endosomes and as such it is of limited value when attempting to dissect the differences between lysosomal diseases, however it is an excellent tool for determining whether lysosomal storage is present. Conditions for lysotracker loading and the use of the probe to detect storage in LSDs will be discussed in more detail in Chapter 5.

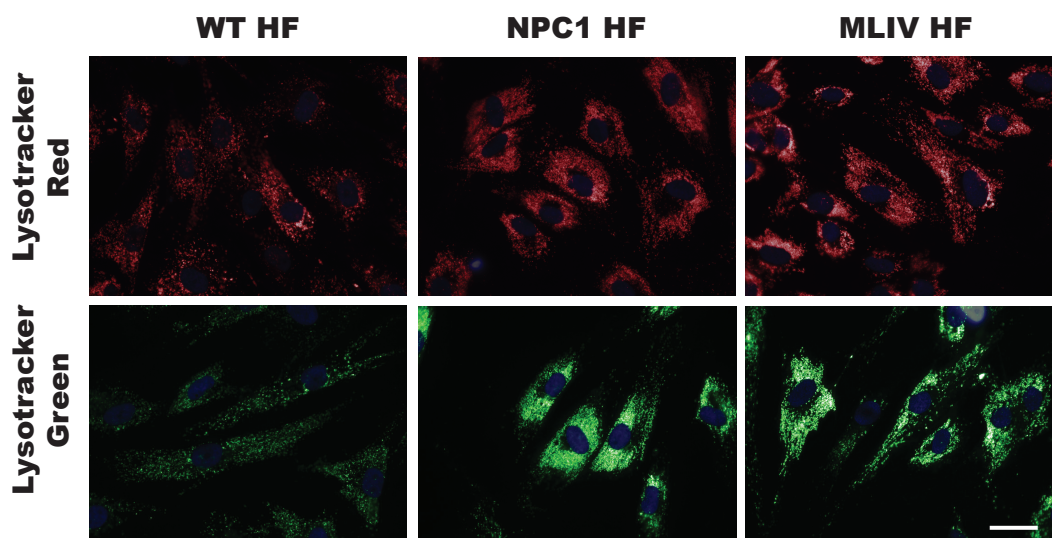


Figure 2.4 Lysosomal expansion and lipid storage detected in NPC and MLIV patient fibroblasts. Living WT HFs grown on glass coverslips were loaded with 300nM Lysotracker Red DND-99 or 200 nM Lysotracker Green DND-26 as described in section 2.2.8 and visualized live. Scale bar represent 5 μ m. n= 5-10.

2.3.5 Phospholipid storage

Phospholipid storage was assessed using a novel fluorescent probe, LipidTox Red, which detects charged phospholipid species. Fibroblasts were loaded with the phospholipid detection probe for 4 h, then washed and incubated with fresh medium for further 20 h as described in section 2.2.9. Following 4 h pulse, 20 h chase cells were visualized live (Fig. 2.5 a). MLIV and NPC1 patient fibroblasts as well as control fibroblasts show punctate staining pattern indicating that the probe is localized to endocytic compartments. We have counted cells with visibly bright punctae versus cells without bright punctae (Fig. 2.5 b); cell counts were performed blind.

We found that both WT and NPC1 fibroblasts have fewer cells with bright punctate staining: $46.9 \pm 2.5\%$ WT cells and $47.5 \pm 1.4\%$ NPC cells have bright punctate staining, indicating that phospholipids are cleared from lysosomal compartment. Whereas over 70% ($73 \pm 1.5\%$) of MLIV fibroblasts have a much brighter punctate staining compared to both WT and NPC cells ($p < 0.01$) indicating a reduced rate of

clearance of phospholipids from the lysosome that probably occurs as a result of phospholipid storage.

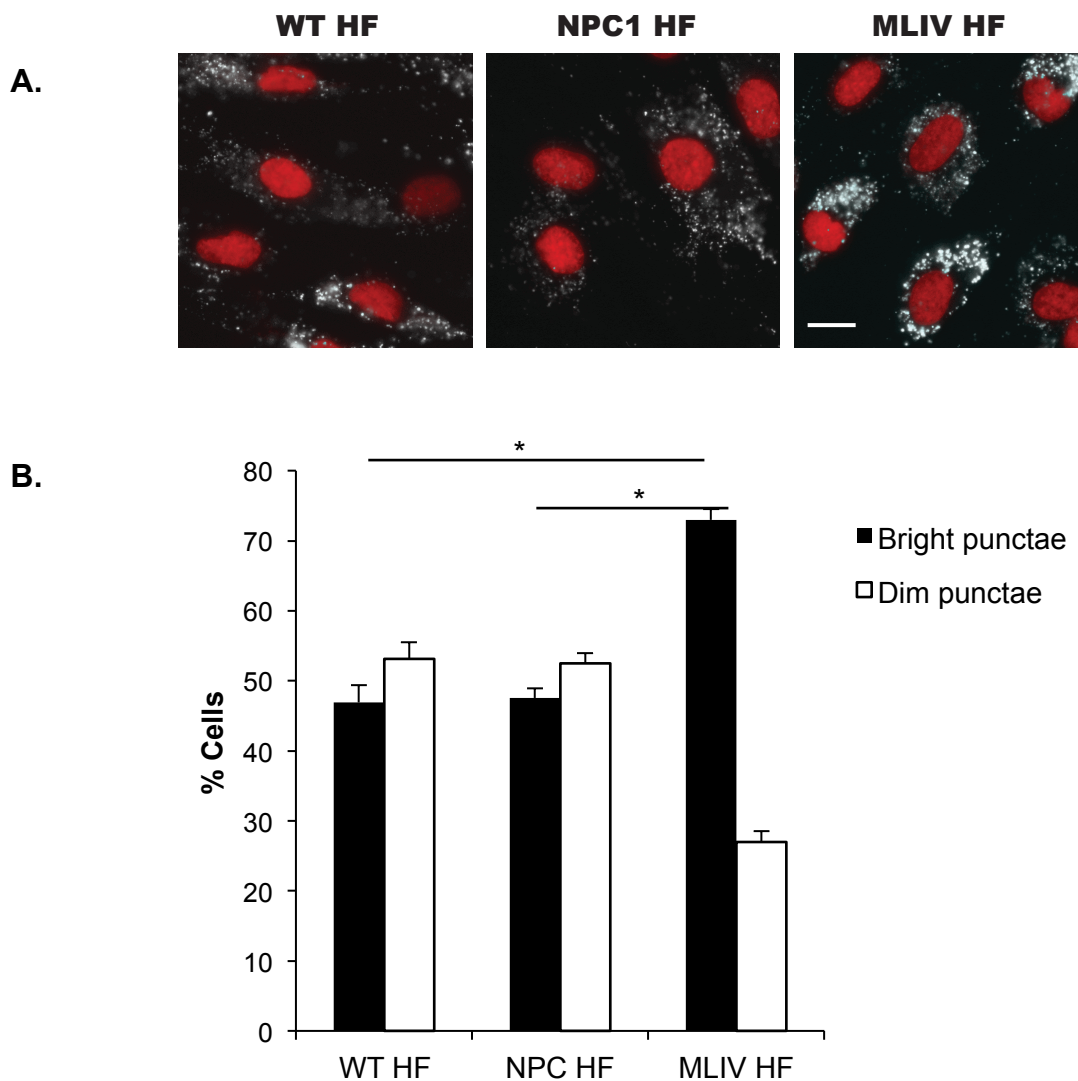


Figure 2.5 Phospholipid storage is elevated in MLIV patient fibroblasts. A. Amount of phospholipids was detected using a fluorescent phospholipid analogue LipidTOX in living cells as described in section 2.2.9. Scale bar represents 5 μ M. **B.** LipidTOX levels were assessed by counting the number of bright vs. dim punctae. Statistical significance was assessed using one-way ANOVA; * $p < 0.01$. Error bars represent S.D. $n=3$.

2.3.6 Lipofuscin storage can be detected only in MLIV

Lipofuscin-like fluorescence was previously reported in MLIV fibroblasts [94], until recently [96] there were no previous reports of autofluorescence in NPC cells. We have assessed autofluorescence as the fluorescence in unstained, living and PFA fixed patient fibroblasts using fluorescent microscopy at various $\lambda_{\text{excitation}}$ since, as discussed in Chapter 1, the fluorescent spectrum of lipofuscin is variable depending on the material being stored. We found that only ~30% of WT ($29.2 \pm 11.9\%$) and NPC ($30.2 \pm 13.9\%$) fibroblasts are autofluorescent (Fig. 2.6). We have examined lipofuscin-like fluorescence in unstained MLIV fibroblasts in all channels to see which channel the lipofuscin attributed autofluorescence of MLIV patient fibroblast line used in this study is most prominent in. We found that MLIV fibroblasts show autofluorescent punctate staining in green ($\lambda_{\text{excitation}} = 470 \text{ nm}$) and blue ($\lambda_{\text{excitation}} = 380 \text{ nm}$) channels with no autofluorescence in the red channel ($\lambda_{\text{excitation}} = 570 \text{ nm}$). In contrast to WT and NPC fibroblasts, > 80% of MLIV fibroblasts ($86.7 \pm 7.4\%$) are autofluorescent ($p < 0.05$) (Fig. 2.6).

Unfortunately SB can only be used in MeOH fixed cells and so is of limited use for the probes that need to be visualized in living cells. It is for this reason that we have predominantly utilized red fluorophores in our MLIV studies.

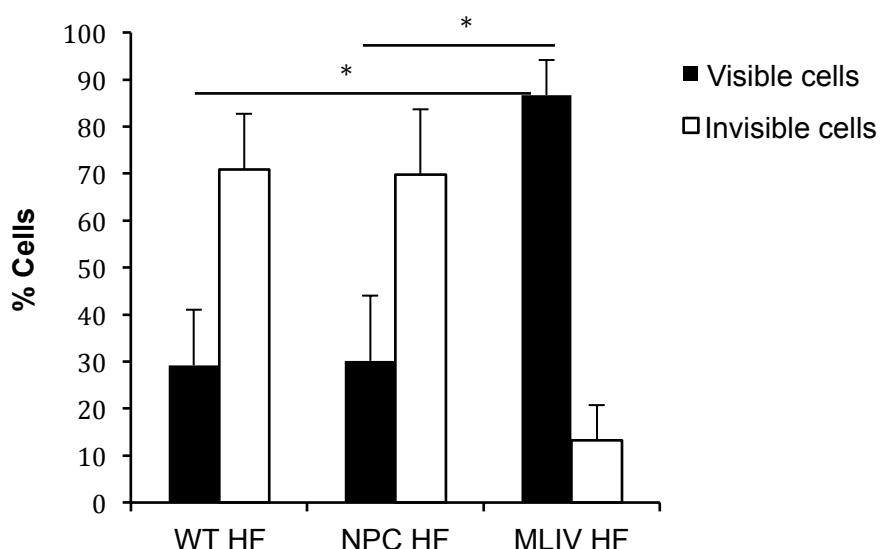


Figure 2.6 Autofluorescence is observed only in MLIV fibroblasts. Living cells grown on coverslips were stained only with nuclear counter stain Hoesht. Cells clearly visible at $\lambda_{\text{excitation}} = 470 \text{ nm}$ were counted as “visible”, cells where only nucleus is visible were counted as “invisible”. Statistical significance was assessed using one-way ANOVA; * $p < 0.05$. Error bars represent mean % $\pm$ S.D. $n=4$.

2.3.7 Lipofuscin-like autofluorescence in MLIV storage can be masked using Sudan Black

(2,2-dimethyl-1,3-dihydroperimidin-6-yl)-(4-phenylazo-1-naphthyl) diazene also referred to as Sudan Black (SB) is widely used in histology to mask lipofuscin autofluorescence in paraffin embedded tissue sections [164]. We have used SB to mask autofluorescence in human fibroblasts grown on coverslips and fixed using MeOH. We found that in cells fixed with MeOH and unstained, MLIV fibroblasts exhibit punctate staining pattern (Fig. 2.7 A., right panel). In contrast, WT and NPC1 fibroblasts do not have bright autofluorescent punctae that we see in MLIV cells (Fig. 2.7 A.). MLIV Cells fixed with MeOH then stained with SB lack the autofluorescent punctae (Fig. 2.7 B.). If these cells are examined by we found black punctate staining indicating that SB binds to lipofuscin masking the fluorescence in green channel completely (Fig. 2.7 C., right panel).

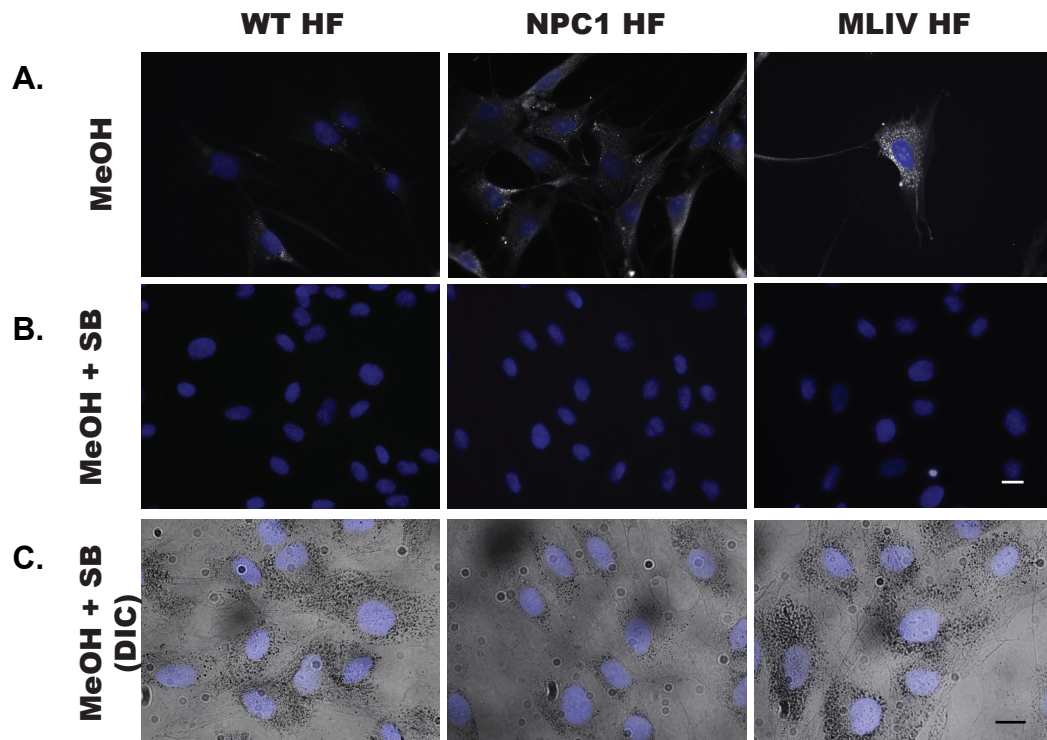


Figure 2.7 Autofluorescence observed in MLIV fibroblasts is masked using Sudan Black. **A.** Patient fibroblast fixed with MeOH and stained with Hoesht nuclear counterstain. **B.** Patient fibroblasts fixed with MeOH then stained with Sudan Black (SB) as described in section 2.2.11. followed by staining with Hoesht. **C.** Same cells as in B. with image taken by DIC. SB bound to lipofuscin appears as black punctae. Scale bar represents 5 μm . $n=3$.

2.3.8 Retrograde trafficking delay occurs in both MLIV and NPC1

Cholera Toxin (Ctx) secreted by *Vibrio cholerae* is a dimeric toxin consisting of non covalently linked subunits A and B; subunit B binds to the ganglioside GM1 on plasma membrane of the host cell allowing the internalization of the toxin. Upon binding of CtxB to GM1, CtxB-GM1 complex is trafficked down the endocytic pathway to the lysosome and then recycled to the Golgi compartment shown by perinuclear localisation. WT fibroblasts have CtxB staining indicative of the Golgi (Fig. 2.8 A, left panel) - perinuclear single or tethered structures that co-localise with GM130 antibody that specifically labels Golgi (data not shown), whereas both NPC and MLIV fibroblasts show a broad punctate distribution of CtxB throughout the cell (Fig. 2.8 A, middle and

right panels) indicative of a defect in transport of CtxB through and out of the endolysosomal system, as has been described previously by several other groups [141, 144].

Using a counting system developed in Lloyd-Evans lab we have counted the % of cells in a population where we could see clear Golgi staining versus the cells with punctate distribution of CtxB indicating endocytic localisation. We found that only ~20% of WT fibroblasts show punctate distribution of CtxB, with 80% ($81.7 \pm 4.2\%$) of the cells showing Golgi localisation of CtxB and therefore the correct trafficking of the CtxB-GM1 complex (Fig. 2.8 B).

In contrast, $73.9 \pm 11\%$ of MLIV fibroblasts and $83.5 \pm 6\%$ of NPC1 fibroblasts show punctate distribution of CtxB stain (Fig. 2. B) and therefore indicating that in the majority of the MLIV and NPC1 fibroblasts CtxB-GM1 complex is mistrafficked compared to WT fibroblasts ($p < 0.05$).

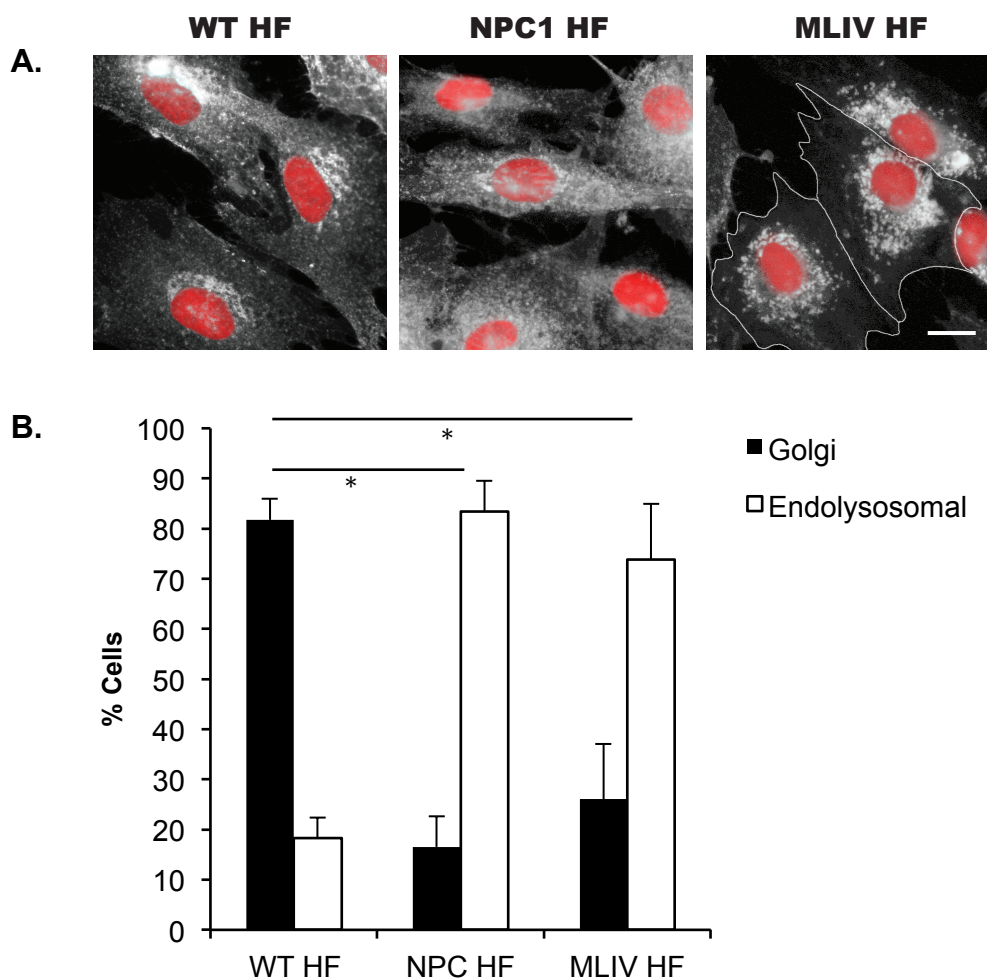


Figure 2.8 Delay in GM1 transport in NPC1 and MLIV fibroblasts. A. GM1 trafficking was followed by pulse labelling living fibroblasts with fluorescent CtxB as described in section 2.2.10. Scale bar represents 5 μ m. B. GM1 trafficking delay was assessed by intracellular distribution of GM1-CtxB complex. Statistical significance was assessed using one-way ANOVA with Tamhane Post Hoc test; * $p < 0.05$. Error bars represent % of cells $\pm$ S.D. $n=3$.

2.3.9 Reduction in lysosomal Ca^{2+} release in NPC1 fibroblasts in not observed in MLIV fibroblasts

Ca^{2+} release in MLIV and NPC1 patient fibroblasts was measured using glycyl-L-phenylalanine 2-naphthalamide, GPN. GPN a cathepsin C substrate, that is cleaved in the lysosome, the hydrolysis products of GPN breakdown cause osmotic lysis of up to 90% of the lysosomes [168].

Fluorescent Ca^{2+} indicator, Calcium Green 1- AM was loaded into the cytosol of patient fibroblasts as described in section 2.2.12. The amount of Ca^{2+} release in relative fluorescence units, in response to 2 μM ionomycin acid free solution and 200 μM GPN (Sigma, Gillingham, UK) was measured as a difference between the baseline and peak values on a minimum of 10-15 cells in a minimum of three individual experiments with minimum of two technical repeats. Traces shown are a representative Ca^{2+} response of cells.

We have clamped all intracellular compartments using ionomycin, so that none of the other Ca^{2+} stores interfere with GPN mediated lysosomal Ca^{2+} release.

We observe a reduction in lysosomal Ca^{2+} release in NPC1 patient fibroblasts compared to WT; this is in agreement with previously reported reduction in lysosomal Ca^{2+} release in NPC1 [45]. Reduced lysosomal Ca^{2+} release probably occurs due to reduced lysosomal Ca^{2+} levels [45]. Although there is one study that reports normal lysosomal Ca^{2+} release in NPC fibroblasts; it should be noted however that the authors utilized GPN alone. In our experiments use of GPN alone leads to a much smaller difference in lysosomal Ca^{2+} release in NPC cells compared to WT cells. The ionomycin pre-treatment done here and thapsigargin pre-treatment experiments previously reported clearly indicate that the reason for this reduced difference is the contribution of the ER Ca^{2+} store masking the reduction in Ca^{2+} release from the lysosome.

However, in MLIV fibroblasts, appear to be elevated.

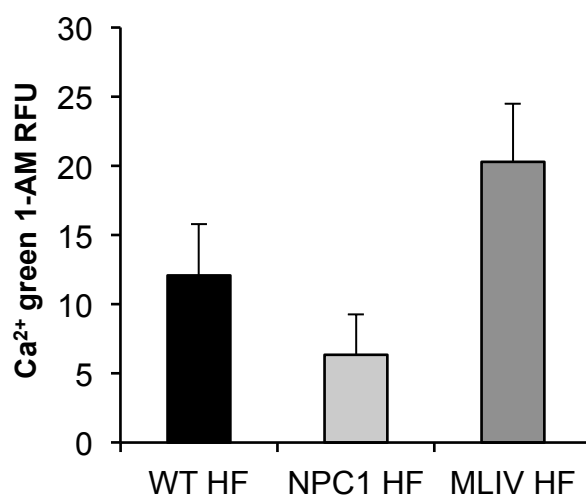


Figure 2.9 GPN induced Lysosomal Ca²⁺ release. HFs grown in chambers of an 8 chamber slide were loaded with Ca²⁺ Green 1-AM as described in section 2.2.12. Ca²⁺ release following 200 μ M GPN addition was measured as peak height. Error bars represent mean peak height S.D., n=5.

2.4 DISCUSSION

Niemann Pick type C1 and Mucopolysaccharidosis type IV both belong to a sub group of LSDs caused by a defect in lysosomal transmembrane proteins, NPC1 and TRPML1 respectively. Both these disorders have a misregulation in trafficking in the late endolysosomal pathway. As previously discussed, fusion/fission between late endosome and lysosome requires Ca^{2+} release which was shown to be reduced in NPC1 disease. Over the past few years TRPML1 has been suggested to be the NAADP sensitive lysosomal Ca^{2+} release channel owing to its localisation and permeability to Ca^{2+} . It is easy to see the parallels could be drawn between these two disorders. But perhaps the tools used to explore similarities between these two disorders have not been well chosen. Our study definitively shows that at cellular levels similarities between the two disorders are a few. This is further supported by completely different phenotypic manifestations of these two diseases and the life span of human patients [150, 169] and mouse models [98, 116].

As previously discussed Shen *et al.* [96] have approached the reduced lysosomal Ca^{2+} release in NPC disease as a misregulation of the lysosomal Ca^{2+} release via TRPML1 thereby attributing the lysosomal Ca^{2+} release function to TRPML1 protein. It should be noted that this group have measured lysosomal Ca^{2+} release by using a synthetic agonist to TRPML1, MLSA1, and monitoring changes in cytosolic Ca^{2+} using a genetically expressed Ca^{2+} sensor.

In order to explore whether or not increased storage of sphingomyelin is affecting activity of TRPML1 Shen *et al.* [96] should have perhaps looked at TRPML1 activity in Niemann Pick type A, NPA, caused by a the defect in acid sphingomyelinase which results in accumulation sphingomyelin in the lysosome [145] and where in fact sphingomyelin levels are higher compared to NPC. Another way to investigate whether TRPML1 is the main lysosomal Ca^{2+} release channel is to look at cells where function

of TRPML1 has been reduced or completely abolished as we have done in the current study.

Our data shows that in absence of a functional TRPML1 protein, as is the case for MLIV patient fibroblasts, lysosomal Ca^{2+} release is not inhibited but is in fact elevated (section 2.3.9) indicating that despite its permeability to Ca^{2+} , TRPML1 cannot function as the main lysosomal Ca^{2+} release channel. We found that when using a lysomotropic agent GPN lysosomal Ca^{2+} release in MLIV fibroblasts GPN Ca^{2+} release is increased by ~30-50% compared to WT fibroblasts. Whereas in NPC fibroblasts GPN induced Ca^{2+} release was reduced by ~70% which agrees with previously reported reduction in lysosomal Ca^{2+} release in NPC [45].

These data suggest that NPC1 and MLIV are different disorders in terms of cellular phenotypes. Whilst NPC1 and MLIV have a common defect in endocytic transport out of the lysosome, the underlying cause and the lipid species that accumulate in the acidic compartment and subsequently contribute to lysosomal storage in these disorders are very different. Lysosomal compartment expansion and storage is a phenotype commonly found in all LSDs.

2.4.1 Common phenotypes in MLIV and NPC disease

2.4.1.1 Defective endocytosis

Transport in the late endosome lysosomes is affected in both MLIV and NPC1. As mentioned in the section 2.1.1, in NPC1 deficient cells the defect in macromolecule trafficking occurs between late endosome and lysosome. This may be explained by reduction in lysosomal content: localized Ca^{2+} release from the lysosome has to occur for the lysosome to fuse with the late endosome to form a homotypic organelle in order to allow the content of the late endosome to be accessed and degraded by lysosomal hydrolases. Reduced content in NPC1 deficient fibroblasts means that the late endosome fuses with the lysosome very slowly, causing a delay in trafficking.

Again as discussed in section 2.1.1 membrane trafficking is also affected in MLIV fibroblasts. Here, however, lysosomal Ca^{2+} release is enhanced, therefore the late endosome is able to fuse with the lysosome to form a homotypic organelle. In order for the lysosome and the late endosome to segregate into separate compartments, localised Ca^{2+} reuptake has to occur. If, however, lysosomal release is enhanced, with the lysosome constantly releasing Ca^{2+} causing excessive fusion between late endosomes and lysosomes. Ca^{2+} reuptake has to compete with excessive Ca^{2+} release meaning that the lysosome persists as a homotypic organelle for longer, so that membrane trafficking out of the lysosome and recycling to the Golgi is also delayed.

Together with our data it may suggest that membrane trafficking to the lysosome is enhanced, however trafficking out of the lysosome is retarded.

2.4.1.2 Lysosomal expansion

The volume of the lysosomal compartment is increased due to storage of macromolecules. LysoTracker staining detects an elevation in storage in both MLIV and NPC1 patient fibroblasts. However the accumulated macromolecules are different between NPC1 and MLIV.

2.4.2 Differences in cellular phenotypes between MLIV and NPC diseases

2.4.2.1 Accumulated lipid species vary between NPC and MLIV

We found that, in accordance with previously reported findings for NPC, NPC1 patient fibroblasts show storage of GSLs (Fig. 2.3), cholesterol (Fig. 2.1) and sphingomyelin (Fig. 2.2). In contrast, preliminary HPLC analysis of MLIV patient fibroblasts suggest that GSL levels are reduced (Fig. 2.3), perhaps indicating a dysfunction in GSL biosynthetic pathway. HPLC analysis of MLIV fibroblasts will need to be repeated to confirm the reduction in GSL levels. Both cholesterol and sphingomyelin levels appear

to be lower in MLIV compared to both NPC and WT. Cholesterol levels appear to be reduced across different cell lines of MLIV fibroblasts. Similarly to cholesterol, sphingomyelin levels in MLIV fibroblasts could also be quantified either by sphingomyelinase plate assay or a thin layer chromatography, TLC, assay to confirm whether or not sphingomyelin levels are reduced. Furthermore, LipidTOX probe revealed storage of charged phospholipids in MLIV fibroblasts, confirming previous reports of phospholipid storage measured biochemically.

Thus far our data suggests that membrane trafficking is affected in both MLIV and NPC1, albeit in different ways, with the trafficking out of the late endosome and into the lysosome being retarded in NPC1, and trafficking out of the homotypic organelle into the lysosome (and then to the Golgi) is delayed in MLIV. Our data also suggests that lysosomal release is affected differently although strongly pointing to TRPML1 not being the lysosomal release channel. The only common phenotype of NPC1 and MLIV is lysosomal expansion due to storage (although again of different macromolecules), as will become apparent in Chapter 5 a phenotype common to most LSDs.

3 Uncovering the order of pathogenic events in MLIV using siRNA

3.1 INTRODUCTION

3.1.1 *Cellular phenotypes*

Results from the previous chapter have demonstrated that lysosomal Ca^{2+} homeostasis defects in MLIV are the opposite of those observed in NPC human patient fibroblasts. Where there is reduced lysosomal Ca^{2+} content in NPC disease, we find an elevation in MLIV which more than likely accounts for the differences in endocytosis between these diseases, namely accelerated endocytosis in MLIV potentially caused by elevated Ca^{2+} release and retardation of endocytosis in NPC1 previously shown to be caused by reduced lysosomal Ca^{2+} release [45]. In the case of NPC disease, we discovered that lysosomal Ca^{2+} defects occurred early in the order of pathogenic events, second only to the primary accumulation of sphingosine, and constituted a druggable therapeutic target for this disease [45]. This approach of inducing the disease by inhibiting NPC1 protein function and following the phenotypes as they emerged proved to be a useful approach to determine the cellular order of disease events in NPC [45]. It was only possible to do such a study as the cellular phenotypes that are present in NPC disease are well characterized. Having completed a comprehensive analysis of all the phenotypes that occur in MLIV in the previous chapter, we are now also in a position to do this kind of experiment for MLIV. As there is currently no known inhibitor of TRPML1 function we used siRNA in order to induce the disease and follow the emergent phenotypes. The group of Kiselyov have previously reported efficient knock down of TRPML1 in murine macrophages [170], which is the cell type we utilized for our NPC diseases studies [45]. We therefore

utilized this cell type for the siRNA study owing to the ease by which their protein expression can be affected by siRNA compared to the primary fibroblasts that we have utilized in the rest of this study.

3.1.2 Aims of this chapter

In this chapter we aimed to investigate the consequences of acute TRPML1 knockdown in murine macrophage cells using a pre-designed siRNA to *Mcoln1* in order to identify the sequence of cellular events in the development of MLIV pathogenesis.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

RAW 264.7 macrophages, a semi-transformed mouse tumour cell line, were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). Cells were cultured in Roswell Park Memorial Institute, RPMI (Invitrogen, Paisley, UK), supplemented with 10% FCS / 1% Gl. RAW cells were maintained as a confluent semi-adherent monolayer on bacteriological Petri dishes (Fisher, Loughborough, UK) in a humidified 5% CO₂ 37°C incubator.

3.2.2 siRNA mediated *Trpml1* knockdown

Pre-designed TRMPL1 siRNA was obtained from Life Technologies Ltd., Paisley, UK. The siRNA probe targets specifically exon 2 of the *Mcoln1* transcript. RAW cells were seeded into the chambers of the 8 well chamber slides (5,000 cell per well) and allowed to adhere prior to transfecting with 0.4 µg siRNA. Transfection was carried out using jet PEI transfection reagent (Polyplus transfection, Illkirch, France) according to manufacturer's instruction. Cells were transfected for 24 h, 48 h, 72 h, and 96 h; cells not transfected with siRNA were used as a negative control. We have also treated cells with jetPEI transfection reagent alone to test whether the effect of *TRPML1* knock down can be attributed to siRNA and not a non-specific event.

3.2.3 Western Blotting of siRNA treated cells samples

3.2.3.1 Protein preparation

TRPML1 protein expression following siRNA treatment was measured and knock down confirmed by Western blotting. RAW cells were grown in wells of a 6 well plate. Following siRNA treatment timepoints (0h, 24h, 48h, 72h and 96h), the medium was removed and cells were washed twice using ice-cold buffer. The cells were then scraped off the surface of the plate in the same buffer, transferred into sterile eppendorf tubes and centrifuged at 1500 RPM for 5 min to pellet down the cells.

3.2.3.2 Protein quantification

The amount of protein in the sample was quantified using Pierce Bicinchoninic Acid, BCA, Protein Assay (Fisher, Loughborough, UK) as described in section 2.2.3.

3.2.3.3 Western Blotting

Protein (5 µg) was denatured in 25 µl 1X Sample buffer (62.5 mM Tris·HCl containing 10% glycerol, 2% sodium dodecyl sulphate (SDS), 2% β-mercaptoethanol and 2 mg / ml bromophenol blue) for 1 h at 60 °C. Samples and ColorPlus pre-stained protein ladder (New England Biolabs, Hitchin) were loaded onto a 10% acrylamide gel (375 mM Tris·HCl pH 8.8 containing 10% acrylamide:bis-acrylamide (37.5:1), 0.1% SDS, 0.1% ammonium persulphate and 0.01% N,N,N',N'-tetramethylethylenediamine (TEMED)). The gel was run for 2 h in running buffer (25 mM Tris·HCl containing 200 mM glycine and 0.1% SDS) at 100 V.

Protein was transferred onto an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Watford, UK), activated by immersion in methanol for 60 s followed by immersion in transfer buffer (48 mM Tris·HCl containing 39 mM glycine,

1.3 mM SDS and 20% methanol). Samples were transferred in transfer buffer using a semi-dry blotter (VWR, Lutterworth, UK) at 40 mA for 3 h.

Following transfer, membranes were blocked for 1 h in TBS-T (Tris buffered saline containing 0.1% Tween 20) containing 5% Marvel milk powder, at room temperature with rocking. Membranes were washed 3 times for 10 min in TBS-T at room temperature with rocking and incubated overnight in Rabbit anti-TRPML1 (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1 in 1,000 in TBS-T, at 4 °C with rocking. Membranes were washed 3 times for 10 min in TBS-T at room temperature with rocking and incubated for 1 h in horseradish peroxidase (HRP) conjugated Donkey anti-Rabbit IgG (GE Healthcare, purchased from Fisher, Loughborough, UK) diluted 1 in 10,000 in TBS-T at room temperature with rocking. Membranes were washed 3 times for 10 min in TBS-T at room temperature with rocking and incubated for 1 min in Pierce ECL reagent (Fisher, Loughborough, UK). Membranes were placed in a cassette with UltraCruz autoradiography film (Santa Cruz Biotechnology, Heidelberg, Germany) and left for 4 h before the film was developed.

3.2.4 Loading cells with Ca^{2+} indicators and intracellular Ca^{2+} measurement

siRNA pre-treated RAW cells grown in chambers of an 8-well chamber slide (Ibidi, Germany) in 10% FCS / 1% Gln RPMI were loaded with fluorescent indicator Fura2-AM as described in section 2.2.12. Loading of Fura2-AM was carried out after loading these cells with CtxB AF488 as described in section 2.2.6.

3.2.5 Lysosomal Ca^{2+} release measurement

Ca^{2+} release in these cells was visualized using a Zeiss Axiovert microscope fitted with a Colibri LED illumination system. Ca^{2+} transience over time was recorded as described in section 2.2.13. Ca^{2+} transience was recorded after imaging cells for LysoTracker Red DND-99 and CtxB AF488 staining.

The amount of Ca^{2+} release in relative fluorescence units, in response to GPN was measured as a difference between the baseline and peak values on a minimum of 15-30 cells in a minimum of three individual experiments with of two technical repeats per experiment. Traces shown are a representative Ca^{2+} response of cells.

3.2.6 *Cholera toxin transport assay*

Cholera toxin B, CtxB, AF488 (Molecular Probes, Paisley, UK) transport assay in siRNA treated RAW cells grown in 8 well chamber slides was performed as described in section 2.2.10. CtxB staining of siRNA pre-treated cells was visualized prior to recording Ca^{2+} release in these cells.

3.2.7 *Lysotracker staining*

siRNA treated RAW cells, pre-loaded with CtxB AF594 followed by loading of Fura2-AM Ca^{2+} probe, were finally loaded with 300 nM Lysotracker Red DND-99 (Molecular Probes, Paisley, UK) as described in section 2.2.8. Lysotracker staining of siRNA pre-treated cells was visualized prior to recording Ca^{2+} release in these cells.

3.3 RESULTS

Based on the results discussed in the previous chapter, lysosomal Ca^{2+} levels are potentially a major difference between NPC1 and MLIV. As lysosomal Ca^{2+} is essential for endocytosis and this process is affected in both diseases we decided to further investigate the relationship between changes in lysosomal Ca^{2+} homeostasis in the absence of functional *TRPML1* and defective endocytic transport of macromolecules by silencing *Mcoln1* and observing the subsequent onset of disease phenotypes in murine macrophages.

3.3.1 siRNA mediated downregulation of Mcoln1 results in reduced TRPML1 protein levels

Following siRNA treatment RAW cells were harvested and *TRPML1* knockdown was verified using Western Blot analysis. Please note that the grey scale intensity of the band corresponding to *TRPML1* protein would typically be normalised to the grey scale intensity of the band corresponding to a product of a house keeping gene such as *GAPDH* to account for any inaccuracies in loading the sample onto the gel. In these experiments however *GAPDH* did not give sensible results, so the intensity of the band corresponding to *TRPML1* (Fig. 3.2 A) was normalised to the greyscale intensity of the band corresponding to another protein (Fig. 3.2 A).

TRPML1 expression of siRNA treated cells was standardised to untreated control (Fig. 3.2 b). Following 24 h and 48 h siRNA treatment the amount of *TRPML1* protein is not reduced significantly (0.62 ± 0.4 24 h, and 0.64 ± 0.4 48 h post treatment). Knockdown of *TRPML1* is more prominent at 72 h (0.22 ± 0.07) and even more so at 96 h (0.07 ± 0.04) post transfection.

Treating the cells with transfection reagent alone (jetPEI without siRNA) does not affect *TRPML1* expression (1.03 ± 0 96h post treatment).

These results validate our use of siRNA to inhibit the function of *TRPML1* and are in agreement with a previous study [90] indicating 72-96 h transfection is required to silence *TRPML1* expression in over 60% of the cells (Fig. 3.2 b).

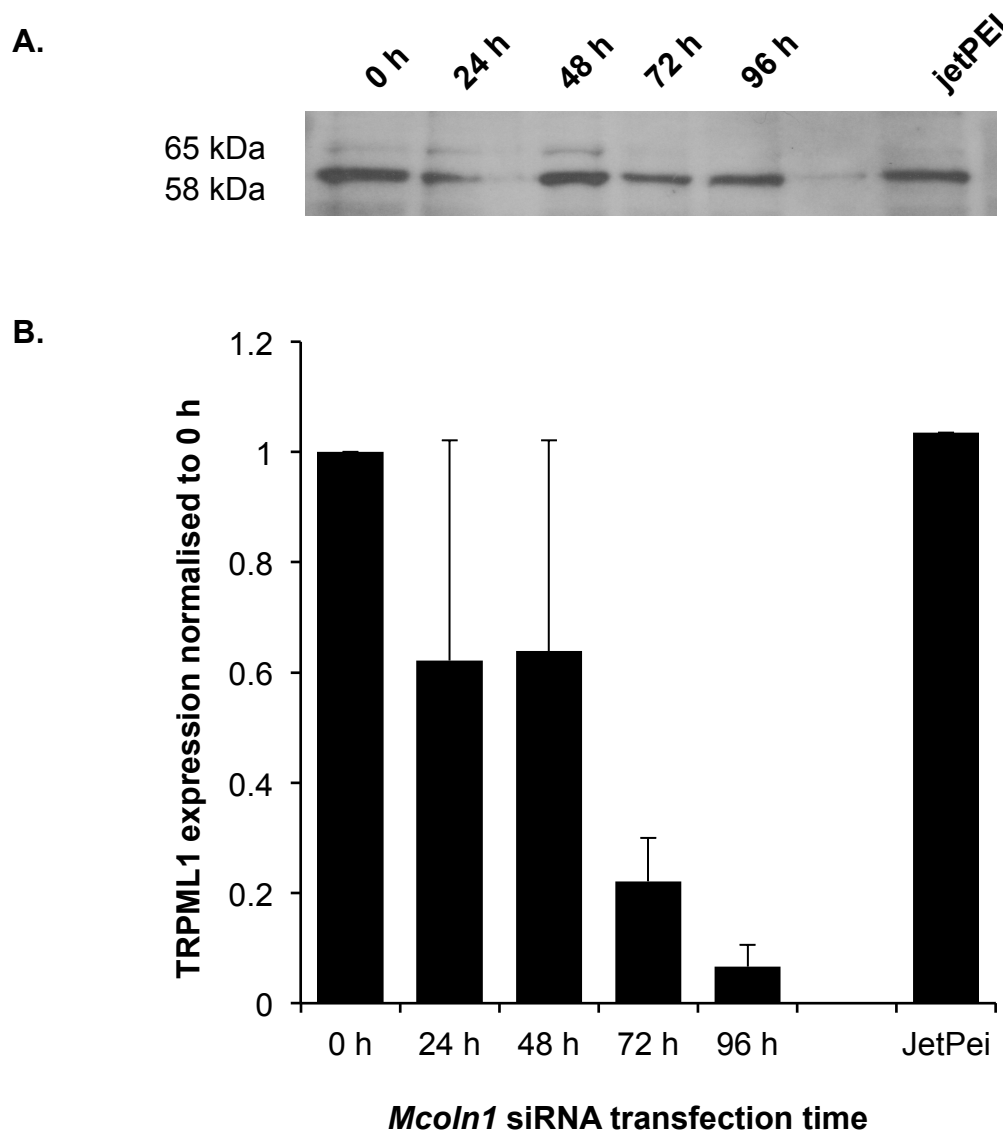


Figure 3.1 *Mcoln1* siRNA mediated reduction of *TRPML1* expression. a. RAW cells were transfected with *Mcoln1* siRNA for 24, 48, 72 and 96 h. Whole cell lysates were harvested and subjected to Western Blot analysis, probing for *TRPML1*. b. Quantification of *TRPML1* expression. *TRPML1* protein levels (65 kD band) were normalized to a 58 kD protein and standardized to non-transfected cells (0 h transfection). Bars represent mean relative *TRPML1* expression $\pm$ SD, n = 2.

Following siRNA treatment of RAW cells with siRNA directed against *Mcoln1*, the MLIV phenotypes that we identified in Chapter 2 which include lysosomal Ca^{2+} dyshomeostasis (section 2.3.9), endocytic trafficking of lipids assessed by CtxB (section 2.3.8) and lysosomal storage assessed using lysotracker (section 2.3.4) were explored. As mentioned in sections 3.2.5 – 3.2.7, all three phenotypes, lysosomal Ca^{2+} release (Fig. 3.5), CtxB trafficking (Fig. 3.3) and lysosomal storage phenotype (Fig. 3.4) were looked at simultaneously in the same population of siRNA transfected RAW cells we therefore could not use a separate marker such as *TRPML1* antibody for these siRNA transfected cells. Instead knockdown of *TRPML1* was confirmed by Western Blot analysis.

Cells were first pulse chased with CtxB AF488 as described in section 3.2.6, followed by the loading of the cells with 5 μM Fura2- AM as described in section 3.2.4, then labeling the cells with 300 nM LysoTracker Red as described in section 3.2.7. Potential fields of view including silenced and non-silenced cells were chosen based on the extent of the endocytic trafficking defect, typically, fields of view that included both correctly transported CtxB (localized in the crescent shaped Golgi) and abnormally transported CtxB (punctate staining) were chosen. Furthermore, the transfection reagent (a lipid vector) used to deliver the siRNA, jetPEI, had no effects by itself and in our hands did not alter lysosomal function such as endocytic trafficking and lipid storage (not shown) as has been suggested for other siRNA delivery vectors such as Eugene [171].

As the experiment progressed, it was found that the number of cells presenting with correctly localized Golgi staining was dramatically reduced suggesting that a large number of the cells had indeed been silenced and were phenotypic. When compared with the Western Blotting analysis, this would indeed appear to be the case as there is substantial knockdown of *Trpml1* levels at 72 and 96 h. For this reason, we have taken

48 h post siRNA to represent an intermediate point in gene silencing (50% silencing and an early cellular disease point), 72 h to be an intermediate point in cellular disease progress and 96 h as the late point at which the majority of cells are phenotypic, and we would expect all phenotypes to be present.

3.3.2 siRNA-mediated reduction of TRPML1 expression affects GM1 endocytic trafficking

Trafficking of AF488 labelled CtxB was used to determine the emergence of endocytic transport defects following knock down of TRPML1. Although for the majority of the experiments involving MLIV cells we decided not to utilize green fluorescent probes because of potential overlap with the autofluorescent lipofuscin we found that CtxB AF488 was always brighter than background autofluorescence in MLIV patient fibroblasts and we did not anticipate or see any accumulation of lipofuscin in these *Mcoln1* silenced RAW cells (not shown). Using a counting system developed in the Lloyd-Evans lab we determined the % of cells in which we could see clear Golgi staining, a combination of Golgi and endocytic staining and only endocytic (punctate) staining, as the % of Golgi staining decreases the severity of the endocytic trafficking defect increases. In cells not transfected with siRNA and cells transfected with jetPEI alone, 44.7% ($\pm 8.2\%$) of the RAW cells have Golgi localisation, 43% ($\pm 9\%$) show both lysosomal and endocytic localisation and 12.3% ($\pm 3.1\%$) of the cells have completely lysosomal localisation of CtxB. This indicates that in the vast majority of cells the CtxB is correctly transported to the Golgi or is on its way.

Following 48 h of siRNA treatment, CtxB localisation shifts from Golgi distribution toward a Golgi/endosomal distribution, with only 16% ($\pm 6.3\%$) of the cells having a clear Golgi only distribution with the combined Golgi/endosomal localisation increasing up to 56.6% ($\pm 15.3\%$) indicating that CtxB is beginning to be retained in late endocytic system but is not completely trapped yet.

After 72 h, 60% ($\pm 9.2\%$) of the cells have punctate endosomal CtxB distribution, 34.9% ($\pm 7.6\%$) of the cells have mixed Golgi/endosomal distribution and 5% ($\pm 2.5\%$) of the cells have a clear Golgi localization of CtxB.

Endocytic transport abnormality observed at 48 and 72 h following siRNA transfection, is fully exacerbated at 96 h where negligible percentage of the cell population ($0.6\% \pm 0.8\%$) have Golgi CtxB localisation and 77.5% ($\pm 12\%$) of the cells show only endosomal (punctate) distribution of CtxB with no indication of efflux out of the endocytic system at all. When compared to the Western Blot data it is clear that the endocytic transport defect does occur early in the *Mcoln1* silencing process.

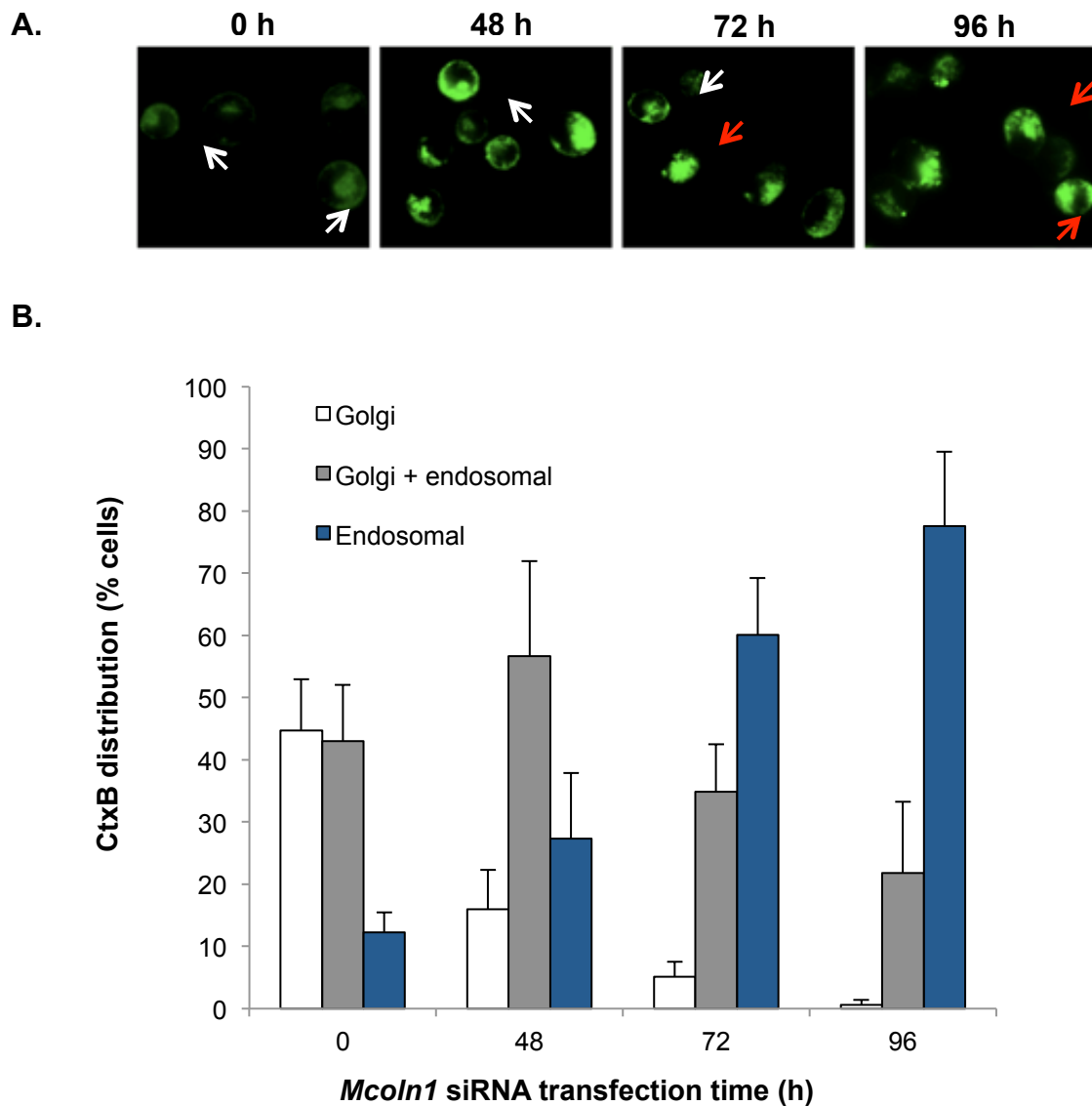


Figure 3.2 *Mcoln1* siRNA mediated disruption of GM1 endocytic trafficking. **a.** RAW cells were transfected with *Mcoln1* siRNA for 48, 72 and 96 h. GM1 trafficking was visualized using CtxB AF488. CtxB transport assay was performed as described in section 3.2.6. White arrow represents Golgi staining; red arrow represents lysosomal staining. Scale bar represents 5 μ m. **b.** At each siRNA transfection timepoint cells were counted according to CtxB distribution. Bars represent CtxB distribution as % of cells $\pm$ SD, n = 4.

3.3.3 *siRNA-mediated reduction of TRPML1 expression affects lysosomal storage*

Following the imaging of CtxB we next recorded the extent of Lysotracker fluorescence prior to measuring lysosomal Ca^{2+} release in response to 200 μM GPN. After 48 h siRNA treatment Lysotracker fluorescence increases by 19%, this gradual increase in lysosomal expansion continues with a 42% increase detected after 72 h treatment, and finally a substantial 84% increase ($p < 0.05$) 96 h after starting siRNA treatment, indicating that lysosomal storage/expansion is a downstream event in MLIV cellular pathogenesis. This increase in Lysotracker signal between control and siRNA treated cells occurs 96 h after treatment start time, indicating that storage material only starts to significantly accumulate within the lysosomal lumen when protein expression has been knocked down to below 20% (72-96 h *Mcoln1* siRNA treatment).

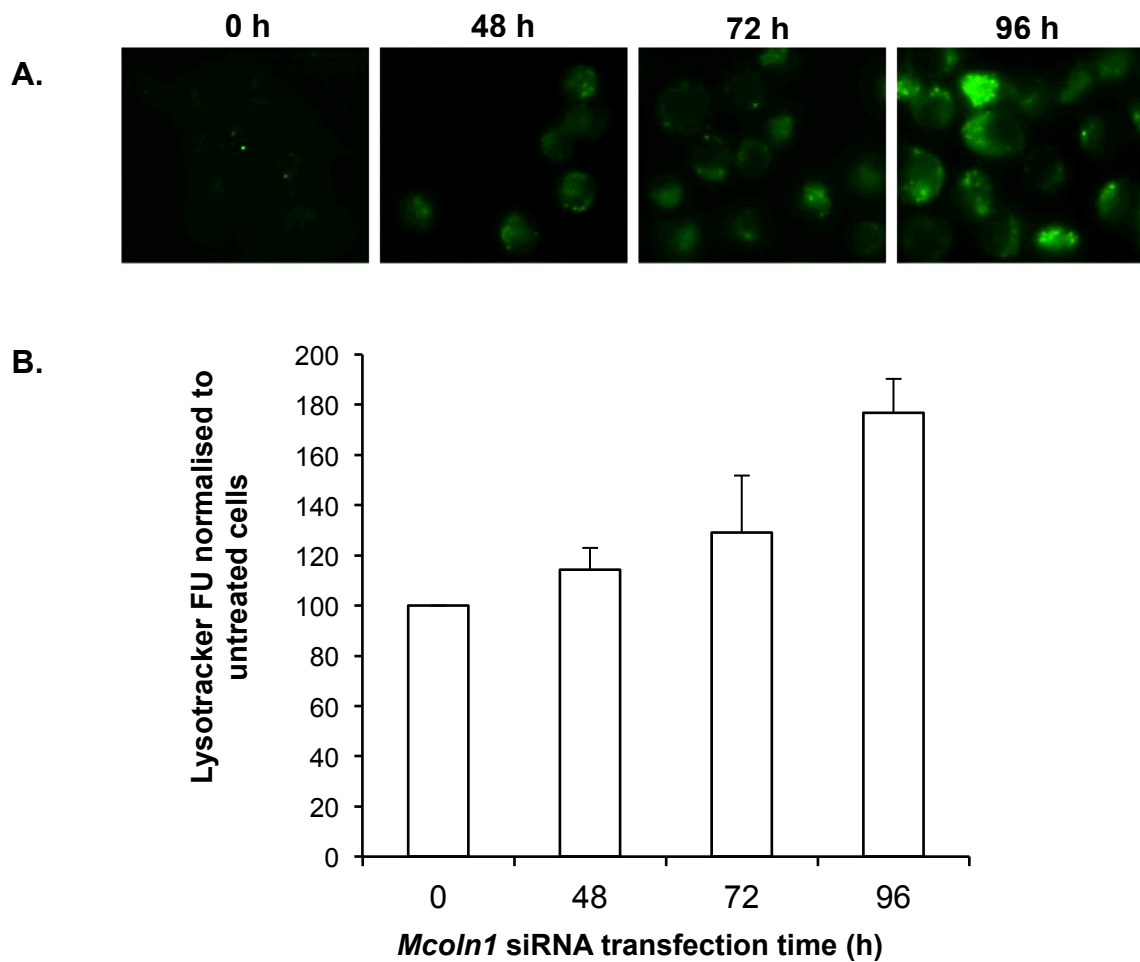


Figure 3.3 *Mcoln1* siRNA mediated lysosomal expansion and storage. **a.** RAW cells were transfected with *Mcoln1* siRNA for 48, 72 and 96 h. Lysosomal storage and expansion was visualized using Lysotracker Red DND-99 and pseudo-coloured green to increase contrast. Lysotracker loading was performed as described in section 3.2.7. Scale bar represents 5 μ m. **b.** Lysotracker fluorescence was quantified using Image J software. Error bars represent the mean $\pm$ SD, n = 4. * p < 0.05.

3.3.4 *siRNA-mediated reduction of TRPML1 expression affects lysosomal Ca^{2+} homeostasis*

Finally, we have quantified the amount of Ca^{2+} release from lysosomes of RAW cells following treatment with siRNA against *Mcoln1*. Interestingly, we obtained a consistent reduction in lysosome Ca^{2+} release mediated by GPN at 24 h post *Mcoln1* silencing, whilst this cannot currently be explained it might correlate with increased protein levels of *TRPML1* that was observed by Western blotting in one of the repeats. Noticeable elevation in lysosomal Ca^{2+} levels as observed in the MLIV human patient fibroblasts were evident at 48 h where there was a small elevation (41%) in lysosomal Ca^{2+} release, this was further elevated at 72 h to 125% increase over normal reaching a plateau at 96 h post silencing where lysosomal Ca^{2+} levels released by GPN were equivalent to 100% higher than control. This data indicates that the alteration in lysosomal Ca^{2+} levels (increased by 55%, in real terms from $\sim 550\mu\text{M}$ to $850\mu\text{M}$) are, together with the endocytic transport defect, the earliest disease events. As the endocytic transport defect worsens with time and as inhibition of lysosomal Ca^{2+} release in later chapters can correct the endocytic transport defect in MLIV fibroblasts it would suggest that the defect in lysosomal Ca^{2+} levels is the earliest disease event in MLIV cellular pathogenesis, potentially caused directly by the loss of function of TRPML1.

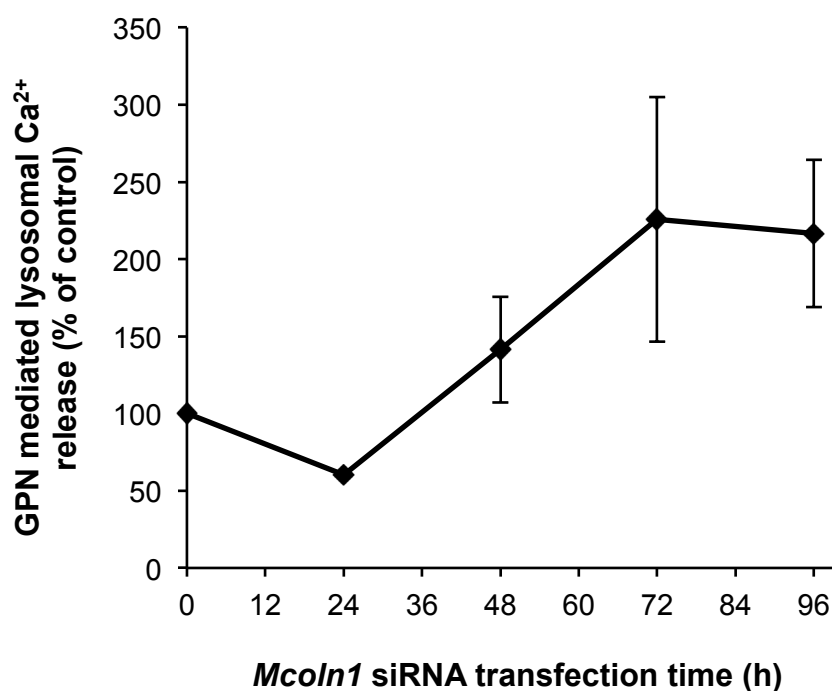


Figure 3.4 *Mcoln1* siRNA mediated lysosomal Ca^{2+} dyshomeostasis. RAW cells were transfected with *Mcoln1* siRNA for 48, 72 and 96 h. Cells were loaded with Ca^{2+} sensitive probe and changes in cytosolic Ca^{2+} in response to GPN were followed as described in section 2.2.13. Lysosomal Ca^{2+} release was measured as a peak height represented by a difference between baseline and peak of the spike, then normalised to Ca^{2+} response in untreated cells. Error bars represent the means $\pm$ SD, n = 3.

3.4 DISCUSSION

For the first time these data show the order of pathogenic events that occur early in MLIV cellular pathogenesis. Our data indicates that alteration in lysosomal Ca^{2+} homeostasis is the earliest measured event in MLIV disease pathogenesis. This defect induces endocytic abnormalities which makes sense when you consider the studies indicating that Ca^{2+} is required in the endocytic system for its maintenance [171]. Endocytic abnormalities clearly lead to accumulation of macromolecules.

Our results indicate that lysosomal Ca^{2+} release is the earliest event to be affected as a result of TRPML1 knockdown and is not a downstream event of lipid accumulation. It suggests that whilst TRPML1 itself is not the lysosomal Ca^{2+} release channel as has been suggested by Shen *et al.* [96], TRPML1 is likely involved in regulating the lysosomal Ca^{2+} release channel.

siRNA data suggests that reducing TRPML1 protein levels down to 50% causes a delay in GM1 (CtxB-GM1 complex) trafficking in over 70% of the cells, this corresponds to retardation in trafficking occurring within the first 48 h of siRNA treatment.

In contrast elevation in storage only occurs when the protein expression is knocked down to below 25% that of control, indicating that storage is not caused by acute knock down of the protein expression but rather is a downstream event.

4 NAADP induced Ca^{2+} release is central to MLIV pathology

4.1 INTRODUCTION

4.1.1 *NAADP signalling and acidic Ca^{2+} stores*

As discussed in Chapter 1, NAADP is the most potent signaling molecule linking the stimulus at the cell surface to Ca^{2+} release from intracellular stores [172]. NAADP is synthesized by CD38, an ADP ribosyl cyclase that is also involved in cADPR synthesis; NAADP synthesis occurs at acidic pH [173]. Experimental evidence from sea urchin egg homogenate [174, 175] and mammalian cells [176] has demonstrated that NAADP targets a novel Ca^{2+} channel on an acidic Ca^{2+} store. NAADP is causing a dual Ca^{2+} response, a small, localized response followed by a more global response Ca^{2+} induced Ca^{2+} release [177, 178] recently identified to be the ER [179]. Disruption in NAADP mediated Ca^{2+} signalling found in NPC lysosomal storage disorders and neurodegenerative diseases [104] highlights the importance of acidic Ca^{2+} stores in cellular function [180].

Novel chemical tools developed by Dr. Grant Churchill (Oxford University), Ned19 [181] and NAADP-AM [182], provided more insight into NAADP mediated Ca^{2+} signalling in intact cells/lysosomes. Membrane permeant NAADP is a major step forward for NAADP research by allowing the assessment of changes in cellular or organ function in response to NAADP-AM in cells that would be otherwise impossible to analyse, such as cells sensitive to microinjection or cells where there is currently no known extracellular messenger that triggers intracellular elevation in NAADP [183, 184].

Early attempt to develop an inhibitor of NAADP signaling produced at best only weak inhibitors BZ194 or non-specific one such as PPADS, that targets P2X channels as

much as TPCs [185]. A breakthrough in this area came from a virtual screening approach against the 3D structure of NAADP against a library of compounds has identified a specific NAADP antagonist, Ned19 [181]. In studies using sea urchin eggs Ned19 was shown to act as an NAADP antagonist and inhibit NAADP mediated Ca^{2+} release. Two mechanistic models of Ned19 inhibiting NAADP release have been proposed: Ned19 could bind at high affinity binding site and inhibit NAADP binding at this site and subsequent NAADP mediated Ca^{2+} release; alternatively Ned19 could bind at the lower affinity binding site locking NAADP receptor in a closed conformation. Moreover, Ned19 was reported to be fluorescent under UV light and can be used to fluorescently label the NAADP receptor.

4.1.2 Candidate NAADP sensitive Ca^{2+} release channels

Since the discovery of NAADP as a second messenger there has been some controversy as to the molecular target of NAADP. Consensus amongst the groups researching NAADP signalling is that NAADP targets a channel located on the lysosomal membrane. Several lines of evidence suggest that the recently identified endo-lysosomal channels TPC1 and TPC2 are essential for NAADP mediated Ca^{2+} release [105]. TPC proteins co-localize with late endosomal and lysosomal markers giving them correct cellular localisation for a potential NAADP responsive channel [105, 186, 187]. In addition, TPC proteins are structurally similar to other voltage gated Ca^{2+} and Na^{+} release channels [188], with a putative pore-forming loop. Furthermore, it has been shown that beta cells from the TPC2 null mouse have reduced intracellular Ca^{2+} release in response to glucose [176], a key intracellular response mediated by NAADP that is crucial for insulin release, indicating the role of TPCs in mediating NAADP evoked Ca^{2+} release from intracellular acidic stores. Finally, site directed mutagenesis of two highly conserved amino acid residue in a putative pore forming region of TPC1 has been shown to abolish NAADP mediated Ca^{2+} release [105].

Additional evidence for TPCs as NAADP responsive Ca^{2+} channels comes from overexpression studies where elevated levels of either TPC1 [105] or TPC2 [189] has been shown to result in an increase in Ca^{2+} release that is disrupted by de-acidification of lysosomes using the v-ATPase inhibitor bafilomycin A1. Conversely, knockdown of either TPC1 [105] or TPC2 [187] using silencing RNA abolishes NAADP mediated Ca^{2+} release. In addition, TPC2 has been linked to amplification of NAADP mediated Ca^{2+} release by IP_3R [190]. These data not only suggest that TPC proteins are likely to mediate Ca^{2+} release from the lysosomal lumen in response to NAADP but that can also trigger subsequent Ca^{2+} release from other intracellular stores. Please note that Ca^{2+} signalling in these studies have been conducted in intact cells using a cell-permeant form of NAADP, NAADP-AM. There is, however, a complication, as NAADP has only been shown to have very weak binding to the TPC proteins [176]. Subsequent studies from Galione and Patel have shown a number of potential proteins can bind to an azo-derivative of NAADP [186] in particular a protein with molecular weight of 22/23 kDa has been identified as the likely NAADP binding protein, suggesting the possibility that this protein complexes with TPCs in order to release Ca^{2+} from lysosomes. Ultimately, owing to their late endosomal / lysosomal localization, permeability to Ca^{2+} , structural similarity to other voltage gated Ca^{2+} release channels, and the effects of their expression on NAADP mediated intracellular Ca^{2+} signals, TPCs have been suggested as the most likely target for NAADP.

Despite the recent evidence in support of TPCs, a number of groups have continued to suggest that the lysosomal multi-ion permeant channel, TRPML1, and not the TPCs, functions as the principal lysosomal Ca^{2+} releasing channel and may yet constitute an NAADP gated channel. Furthermore, recent evidence has come to question even the very possibility that TPCs are Ca^{2+} channels, let alone NAADP gated channels.

Data from the group of Xu has indicated that TPCs are in fact Na^+ selective channels. This study was done in isolated lysosomes expanded by vacuolin that has been reported to increase their size from $0.5\mu\text{m}$ to $5\mu\text{m}$ to allow patch clamping of lysosome in order to measure the current across lysosomal membrane. Ten-fold increase in the volume of lysosomal compartment most likely impairs ionic gradient across lysosomal membrane, such that the system becomes artificial with currents arising as a result of disrupted ionic composition of the compartment. Furthermore, in none of these studies has the purity of these vacuolin-purified lysosomes been assessed. GPN induced swelling of lysosomes leads to their osmotic lysis, as such it is surprising that vacuolin swollen lysosomes are not leaky, this might suggest fusion with other organelles, but this has never been assessed. As such enlarged lysosomes do not exist within any cell of the body it is still questionable how relevant these observations of the group of Xu really are. This is particularly true when considering their recent findings that TPCs are Na^+ selective channels rather than Ca^{2+} selective, it is not unimaginable that the Na^+ content of such a swollen organelle has changed vastly, TPCs may be permeant to Na^+ and Ca^{2+} but without knowing the intra luminal concentration of both ions (neither of which are measured in their studies) it is clearly very difficult to interpret their findings to normal, functional cells.

Work from Dr. P.L. Li and colleagues (Virginia Commonwealth University, VA, USA) has suggested that TRPML1, known to be a non-selective lysosomal cation channel [155] functions as the NAADP receptor [155]. This group found that in lysosomes isolated by density gradient centrifugation and reconstituted into a planar lipid bilayer inhibiting the TRPML1 receptor using a commercial anti-TRPML1 antibody [191] significantly reduced NAADP mediated Ca^{2+} release hence suggesting that TRPML1 is required for NAADP signalling. Interestingly, no evidence was presented in this study indicating that this antibody is specific for TRPML1, such as a Western blot, or that it is

indeed capable of inhibiting its function, it is also surprising that they utilized such an approach but did not utilise TRPML1 null cells which are widely available and would have been an appropriate control. Our group has found that all of the commercially available TRPML1 antibodies supplied by AbCam, SantaCruz and Sigma, are not specific for TRPML1; Western Blot profiles of these antibodies are highly non-specific (data not shown) showing bands at lower and higher molecular weight than TRPML1 indicating that these antibodies are not specific for TRPML1 but may also bind other TRP channels. Authors of the study linking NAADP induced Ca^{2+} signalling to TRPML1 did not themselves show the Western blot therefore there is no direct evidence that anti TRPML1 antibody used by this group only binds TRPML1 and in doing so specifically inhibiting TRPML1.

This group went further in saying that TRPML1 is NAADP receptor by inhibiting TRPML1 expression using silencing RNA [155]; again reporting abolished response to NAADP and ET1. However, findings from this lab have not been independently verified.

In contrast, data from Dr. Sandip Patel and coworkers (UCL, London, UK) indicated that NAADP mediated Ca^{2+} release is normal in TRPML1^{-/-} acinar cells (albeit from n=1) [104]. Moreover, they have also shown that overexpression of TRPML1 does not enhance NAADP mediated Ca^{2+} release, however, interestingly, TRPML1 was shown to co-immunoprecipitate with TPC2, suggesting a possibility that the findings of Li et al above could be explained by a non-specific effect of the anti-TRPML1 antibody on TPCs in the mixed membrane preparation they utilise.

4.1.3 Aims of this chapter

The aim of this chapter was to investigate whether TRPML1 is the target receptor of NAADP by looking at NAADP signalling in MLIV patient fibroblasts null for TRPML1. We also wanted to assess the resting levels of NAADP and expression of TPC

receptors in TRPML1 null cells. In chapter 3, we have demonstrated that the source of multiple spontaneous Ca^{2+} release is the lysosome. In this chapter, we wanted to investigate whether these Ca^{2+} release events can be triggered by the second messenger NAADP. Finally, we wanted to investigate whether inhibiting excessive lysosomal Ca^{2+} release using NAADP or its antagonist Ned19 had any effect on MLIV phenotypes.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

Human fibroblasts were cultured as described in section 2.2.1.

4.2.2 Loading the cells with fluorescent Ca^{2+} probes

Loading of the patient fibroblasts with fluorescent Ca^{2+} indicators was carried out as described in section 2.2.12. The following Ca^{2+} probes were used for this study: Fura2-AM, Fluo3-AM, Calcium Green 1-AM all obtained from Life Technologies (Paisley, UK).

Ca^{2+} release in response to NAADP-AM, thapsigargin, ET1, PPADS, Ned19 was visualized using the Zeiss Axiovert microscope fitted with Colibri LED light system and a high speed capture CCD camera. The whole cell outline was used as the region of interest (ROI) for analysis. Ca^{2+} transients in the ROI were recorded over time using the Zeiss Axiovision physiology module. Acquired data was exported into Excel, and amount of Ca^{2+} (relative fluorescence units) in response to pharmacological agents was measured as a difference between the peak and the baseline fluorescence values.

4.2.3 Measuring lysosomal Ca^{2+} content

Lysosomal Ca^{2+} content was measured in WT and MLIV patient fibroblasts; quantification was carried out as described by Christensen *et al.* [40] and Lloyd-Evans *et al.* [45]. WT and MLIV fibroblasts were loaded with Ca^{2+} sensitive low affinity Rhod-dextran (Sigma, Gillingham, UK), which is used to record Ca^{2+} concentration and Alexa Fluor 488 dextran used as a loading control. Washed with 1X HBSS, and visualized immediately at $\lambda_{\text{excitation}}/\lambda_{\text{emission}} = 570 / 590$ nm for Rhod Dextran, and $\lambda_{\text{excitation}}/\lambda_{\text{emission}} = 470 / 520$ nm for Alexa Fluor 488 Dextran. The ratio values were then used to calculate actual lysosomal Ca^{2+} concentration using the following equation:

$$[\text{Ca}^{2+}]_{\text{lys}} = K_d \times \frac{R_{\text{max}} - F_{\text{ratio}}}{F_{\text{ratio}} - R_{\text{min}}}$$

where F_{ratio} is the red/green fluorescence ratio, R_{min} is red/green fluorescence ratio in presence of saturating lysosomal Ca^{2+} and R_{max} is red/green fluorescence ratio in absence of Ca^{2+} . K_d value of Alexa Fluor 488 was measured as described in Lloyd-Evans *et al.* [45] in previous experiments.

4.2.4 Chelating lysosomal Ca^{2+} using high affinity Rhod Dextran

Human fibroblasts were seeded onto 13mm² coverslips and allowed to adhere overnight. Cells were then treated with 0.2mg/ml high affinity Rhod Dextran (HARD) (Sigma, Gillingham, UK) for 6h at 37°C.

4.2.5 LysoTracker staining

Cells were washed with HBSS and lysoTracker staining was performed as described in section 2.2.8. Imaging of lysoTracker fluorescence was carried out live using Zeiss Axiovert microscope fitted with Colibri LED light system at 40X magnification.

4.2.6 NAADP-AM synthesis

NAADP-AM synthesis was carried out by Duncan Bloor-Young as described by Parkesh *et al.* [182] (Fig. 4.3 A), and kindly gifted by Dr. Grant Churchill, University of Oxford. Concentration of the esterified form of NAADP, NAADP-AM, varies from batch to batch, therefore the concentration of NAADP giving the optimal Ca^{2+} release was determined for every experiment, prior to treatment as described below.

4.2.7 Determining active concentration of NAADP

Cells were grown overnight in wells of a 96 well plate to allow adhesion to the surface of the well. The cells were subsequently loaded with Calcium Green 1-AM or Fluo 3-AM fluorescent Ca^{2+} probes as described in section 2.2.12. NAADP-AM powder was solubilised in dry DMSO (Sigma, Gillingham, UK) and aliquotted in HBSS to make up

varying concentrations of NAADP-AM. Different concentrations of NAADP-AM were added to the cells and changes in the fluorescence of Ca^{2+} sensitive probes, indicating Ca^{2+} release in response to NAADP-AM, were recorded overtime using either a NOVOstar or POLARstar fluorescence microplate readers (both BMG Labtech, Aylesbury, UK) (Fig. 4.3 B).

Once a Ca^{2+} releasing NAADP-AM concentration was established, it was immediately used for treatments.

4.2.8 [^{32}P] NAADP synthesis and NAADP quantification

Radioactive [^{32}P] NADP and [^{32}P] NAADP synthesis was carried out as previously described [175] using [^{32}P] NAD (GE Healthcare, UK) , 0.5U / ml human NAD kinase (Alexis, UK), 100 mM Hepes (pH 7.5), 5 mM MgATP; 100 mM nicotinic acid and 1 μg / ml ADP-ribosyl cyclase were provided by Dr. H.C. Lee, Hong Kong University.

[^{32}P] NAADP was stored in 10 mM Tris buffer at 4°C and used within 6 weeks of synthesis. Standard curve of NAADP binding and quantification was carried out as previously described by Lewis *et al.* [192]. NAADP concentration from patient fibroblasts cell homogenates was calculated using the standard curve.

Synthesis of [^{32}P] NAADP, NAADP binding and quantification were carried out by Aban Shuaib under the supervision of Dr. Alexander Lewis and Dr. Emyr Lloyd-Evans (Dept. Pharmacology, University of Oxford).

4.2.9 Real Time Quantitative PCR

4.2.9.1 Purifying total RNA using TRI reagent method

Confluent T175 plates of patient fibroblasts were used for this experiment. Cells were washed once with ice-cold PBS. Cells were scraped off the surface of the flask using a cell scraper (Fisher, Loughborough, UK) in Total RNA Isolation, TRI, reagent (Fisher, Paisley, UK), 0.1 ml / cm^2 surface area. Cell samples were incubated in TRI reagent

for 5 min at room temperature to allow nucleoprotein complexes to dissociate. Samples were centrifuged at 4 °C for 10 min at 12,000 g and the supernatant transferred to a new tube. 1-bromo-3-chloropropane, BCP (Sigma, Gillingham, UK), was added to each sample at 0.2 ml BCP / 1 ml starting volume of TRI reagent; sample was vortexed for 15 s and incubated at room temperature for 10 min. Once the cell extract turned viscous, samples were sonicated for 5 min and centrifuged at 15,000 g at 4 °C for 10 min to separate the aqueous and organic phases. The upper (aqueous) phase containing RNA was transferred into a new tube without disrupting the DNA at the interphase. Isopropanol was added to RNA sample, 1/10th of the total volume of the aqueous phase, mixed by pipetting and incubated at room temperature for 5 min. Samples were then centrifuged at 15,000 g at 4 °C for 10 min and the supernatant was transferred into a new tube. Isopropanol was added to the sample, 0.5 ml / 1 ml TRI reagent used for extraction, vortexed and incubated at room temperature for 10 min. The sample was centrifuged at 15,000 g at 4 °C for 10 min and the supernatant discarded. 75% ethanol (Sigma, Gillingham, UK) was added to the pellet, 1 ml / ml TRI reagent, and the last centrifugation step was repeated. The purity of isolated RNA was tested by running 1 µg RNA on a 1% agarose gel (data not shown).

4.2.9.2 cDNA Synthesis

cDNA was synthesized from 1 µg Total RNA using Superscript III (Invitrogen, Paisley, UK) and random hexamer primers (Invitrogen, Paisley, UK) according to manufacturer's instructions.

4.2.9.3 RT-qPCR Reaction

The RT-qPCR reactions were carried out according to Davis *et al.* [193]. For each qPCR reaction, 10 µL reaction mixture contained 5 ng cDNA, 0.1 µM forward and reverse primers, and their corresponding universal probe (Roche Applied Science,

Burgess Hill, UK) in 1X LightCycler 480 Probes Master Mix (Roche Applied Science, Burgess Hill, UK). The primer and probe sets used here have been previously validated by Davis *et al.* [193] and were kindly donated by Prof. Antony Galione (University of Oxford). The probe sequences are:

TPC1 – ACTCCGGCTTGGCATCTAT and ACTACCACCATCAGGGCAAA with probe 1

TPC2 – GGCCACCTACCAGATGACTG and CTCTGTGAGCTCATCCTCCTC with probe 25

GAPDH – ACACCCACTCCTCCACCTTT and TGACAAAGTGGTCGTTGAGG with probe 45

Each qPCR reaction was performed in duplicate on a LightCycler 480 qPCR machine (Roche Applied Science, Burgess Hill, UK). Each 10 µl reaction mixture was pipetted into a separate well of a 384 well qPCR plate (Roche Applied Science, Burgess Hill, UK), which was sealed with film, and spun briefly at 2,500 rpm to ensure that reactions were at the bottom of the wells. The cycling conditions were: 50 °C (2 min), 95 °C (10 min) followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min), followed by 40 °C (30 s). Samples were analysed by the LightCycler® 480 system to attain C(T) values. These experiments were carried out in collaboration with Dr Katja Rietdort (Open University, Milton Keynes).

4.2.9.4 Quantifying expression levels of TPC receptors

Expression levels of TPC1 and TPC2 were quantified using the 2^{(-Delta Delta C(T))} method as described by Livak K.J. and Schmittgen T.D. [194].

4.2.10 Immunohistochemistry

Antibodies against TPC1 and TPC2 raised in rabbit were obtained from Osenses (Keswick, Australia) and kindly donated by Prof. Anthony Galione (University of Oxford). WT and MLIV human fibroblasts were washed three times with PBS, fixed for 10 min in 4% PFA, washed a further three times in PBS and incubated in primary antibodies, diluted 1 in 200 in 1% BSA / 0.1% saponin PBS blocking buffer overnight at 4 C°. The primary antibodies were removed and cells were washed in PBS three times, 5 min per wash. Cells were incubated in Texas Red conjugated Donkey Anti-rabbit secondary antibody (Vector Labs, Peterborough, UK), diluted 1 in 200 in blocking buffer in the dark for 30 min at room temperature. The secondary antibody was removed and cells were washed three times in PBS, 5 min per wash. Cell nuclei were stained with 2 µM Hoescht in PBS for 5 min, Hoescht solution was removed and cells were washed three times in PBS. Coverslips were mounted onto glass slides in Mowiol, and visualized after 24 h to allow Mowiol to solidify. Microscopy was carried out using Axioskop 2-Plus fluorescence microscope at 63X magnification, images were captured using QImaging Retiga SRV charge coupled device camera.

4.2.11 Expression of *TRPML1* and *LAMP1* in MLIV fibroblasts

Human fibroblasts were grown in DMEM supplemented with 10% FCS / 1% Gln on 16 mm² coverslips in wells of a six well plate overnight. Cells were transfected with either 3 µg GFP-*TRPML1* or 3 µg GFP-*LAMP1* in 6 µg JetPEI for 48 h in 5% CO₂ 37 °C incubator. Plasmid containing GFP-tagged *TRPML1* construct were kindly donated by Dr. Rosa Puertollano (NIH, MD, USA) GFP-tagged *LAMP1* construct used as a control was kindly provided by Prof. Shmuel Muallem (NIH, MD, USA) Transfection experiments were carried out in collaboration with Dr. Katja Rietdorf (Open University, Milton Keynes) who has kindly allowed the data to be shown here.

4.2.12 Measuring Ca^{2+} release in response to NAADP-AM in transfected human fibroblasts

Following transfection, cells were loaded with Fura2- AM as described in section 2.2.12. Ca^{2+} release in response to NAADP-AM was recorded by live confocal microscopy, as described in section 2.2.13.

These experiments were carried out in collaboration with Dr. Katja Rietdorf (Open University, Milton Keynes) who has kindly allowed the data to be shown here.

4.2.13 Ned19 treatment

Ned19 was obtained from several sources. Prior to *trans*-Ned19 becoming commercially available, a *cis/trans* racemic mixture and *trans* Ned19 were kindly donated by Dr. Grant Churchill, University of Oxford.

Table 4.1

Ned19 composition	Source	Effective concentration
Cis / trans mixture	Dr. G. Churchill (synthesized by Alinda Chemical Ltd.)	50 μM
trans *	Dr. G. Churchill (synthesized by Prof. A. Ganesan, University of Southampton)	20 μM
trans (>98% purity)	Tocris Biosciences, Bristol, UK	N/A
trans (>97% purity)	Enzo Life Sciences, Exeter, UK	N/A

Ned19 powder was solubilized in DMSO and stored at -20°C. We found that *trans* Ned19 supplied by Tocris did not inhibit NAADP receptor in WT fibroblasts as measured by lysotracker staining intensity. Unless indicated otherwise, *trans* Ned19 supplied by Dr. Grant Churchill marked with * was used to obtain the results shown.

Patient fibroblasts were treated with 20 µM *trans* Ned19* for 24 h.

4.2.14 PPADS treatment

PPADS was used to compare the effect of treatment with Ned19. PPADS was either added immediately before measuring lysosomal Ca²⁺ release as described in section 2.2.13, or used as a 24 h treatment to assess the effect of inhibiting TPC proteins on lipid storage phenotype using Lysotracker as described in section 2.2.8.

4.3 RESULTS

4.3.1 Lysosomal Ca^{2+} content in MLIV fibroblasts is not different from that in WT

The lysosomal Ca^{2+} content in MLIV fibroblasts was determined using a previously described approach [40, 45] (Fig. 4.1 A). Alexa Fluor 488 is not sensitive to intraluminal Ca^{2+} and was used as a loading control. Rhod-dextran is sensitive to Ca^{2+} concentration so acts as a reporter of Ca^{2+} . Pulse-chase times were adjusted such that both probes localized to the lysosome and thus recorded the intralysosomal Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{lys}}$). Red (Rhod dextran) to green (Alexa Fluor 488) fluorescence ratio was calculated using simple PCI software. These values were then used to calculate actual lysosomal Ca^{2+} concentration using the following equation:

$$[\text{Ca}^{2+}]_{\text{lys}} = K_d \times \frac{R_{\text{max}} - F_{\text{ratio}}}{F_{\text{ratio}} - R_{\text{min}}}$$

where F_{ratio} is the red/green fluorescence ratio, R_{min} is red/green fluorescence ratio in presence of saturating lysosomal Ca^{2+} and R_{max} is red/green fluorescence ratio in absence of Ca^{2+} . K_d value of Alexa Fluor 488 was measured in previous experiments described in Lloyd-Evans *et al.* [45].

Our data suggested that overall the acidic compartment of MLIV fibroblasts has a higher Ca content compared to WT HFs. However when the lysosomes were assessed according to size we found that the amount of Ca^{2+} in the lysosomes of MLIV patient fibroblasts is not significantly different compared to lysosomes of WT fibroblasts ($p>0.5$) (Fig. 4.1 B). Therefore the amount of $[\text{Ca}^{2+}]_{\text{lys}}$ in the expanded lysosomes of MLIV fibroblasts is elevated above the $[\text{Ca}^{2+}]_{\text{lys}}$ in non expanded MLIV lysosomes. Interestingly, we found that for every image analysed there are a few enlarged lysosomes per single MLIV cell. This finding is supported by electron microscopy imaging of MLIV fibroblasts, where there are few expanded electron dense lysosomes

in a single TRPML1 deficient cell [180]. Red/green fluorescence ratios of these enlarged endolysosomes was used to calculate a separate $[Ca^{2+}]_{lys}$. The amount of $[Ca^{2+}]_{lys}$ in the expanded lysosomes of MLIV fibroblasts is elevated above the $[Ca^{2+}]_{lys}$ in non expanded MLIV lysosomes.

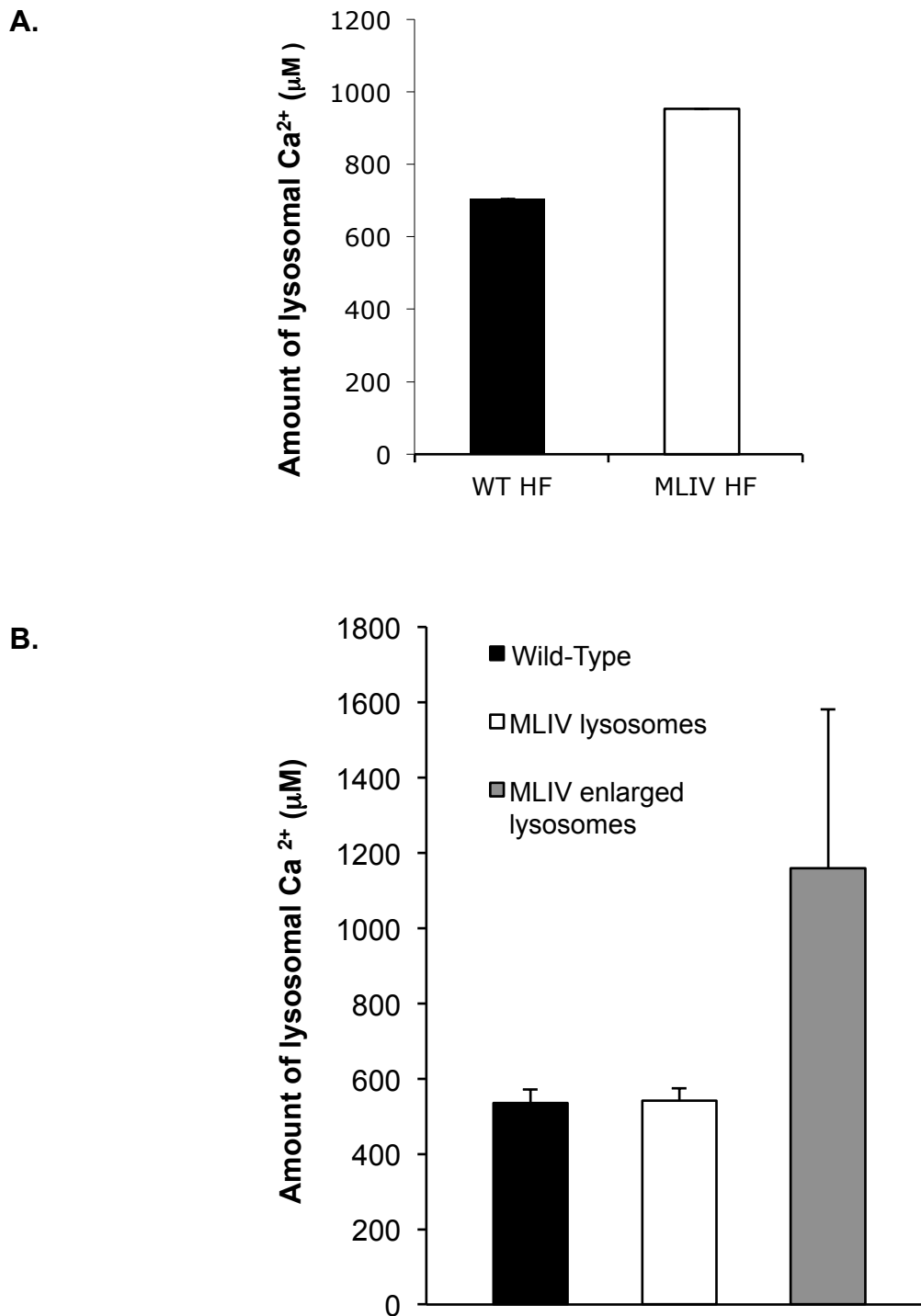


Figure 4.1 Lysosomal Ca^{2+} content of MLIV HF is not significantly different compared to WT HF. Ca^{2+} content was measured using LARD pre-loaded into lysosomes as described in section 4.2.3. LARD records but does not chelate Ca^{2+} within the lysosomal lumen. **A.** Ca^{2+} content in total acidic compartment of MLIV fibroblasts is higher compared to that of WT fibroblasts. **B.** Intralysosomal Ca^{2+} content is higher only in the enlarged lysosomes of MLIV fibroblasts. Error bars represent mean $\pm$ S.D. $n=2$.

4.3.2 Effect of chelating lysosomal Ca^{2+} on lysosomal storage in MLIV patient fibroblasts

Reducing lysosomal Ca^{2+} content using a labelled indicator, Rhod with high affinity for Ca^{2+} coupled to dextran (HARD), a Ca^{2+} chelator active at acidic pH. At acidic pH the affinity of the probe for Ca^{2+} increases from 50 mM to >5 mM, which leads to chelation of Ca^{2+} inside the acidic compartment. HARD probe was used to see whether reducing lysosomal Ca^{2+} has an effect on lipid storage in MLIV cells. WT and MLIV HFs were treated with 0.2 mg/ml HARD for 6 h. Following HARD treatment, changes in lysosomal volume were assessed using Lysotracker probe. Red punctate staining is attributed to HARD fluorescence, whereas green punctate staining is Lysotracker Green fluorescence. Visible reduction in Lysotracker fluorescence indicates that lysosomal lipid storage in MLIV cells is reduced (Fig. 4.2).

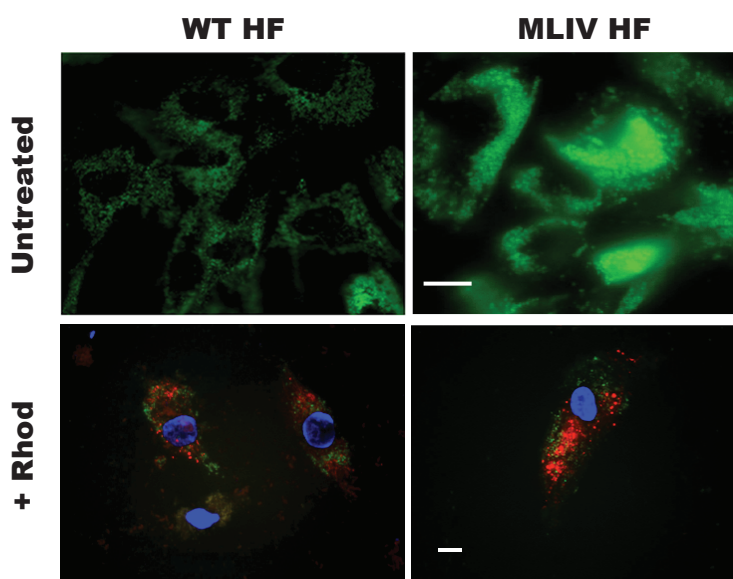


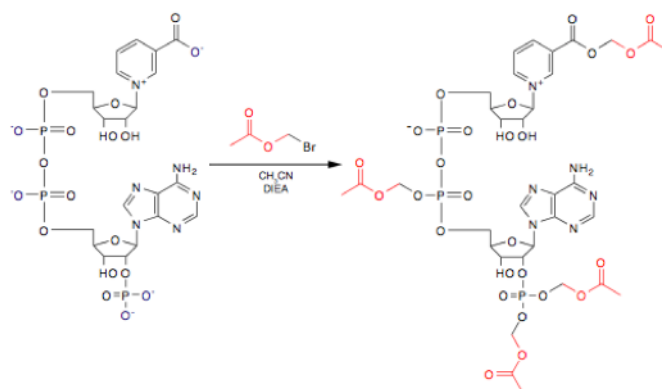
Figure 4.2 Chelating lysosomal Ca^{2+} using HARD corrects lipid storage in MLIV HFs. HARD was pre-loaded into lysosomes as described in section 4.2.3. Lipid storage was verified using Lysotracker Green. Red punctate staining is attributed to HARD; green punctate staining is attributed to Lysotracker Green. Scale bar represents 5 μm ; n=2.

4.3.3 Determining the active dose of NAADP-AM

NAADP-AM crosses the plasma membrane when added outside the cell; the AM group is removed by esterases in the cytosol to release NAADP. This NAADP can then act on the NAADP receptor at the lysosomal membrane. Since the ratio of the esterified NAADP to NAADP without AM group (NAADP-AM : NAADP) varies from batch to batch, the active concentration of NAADP in each batch was determined prior to conducting NAADP treatments as described in section 4.2.6 (Fig. 4.3 B).

Human fibroblasts grown in the wells of 96 well plate were labeled with Ca^{2+} Green 1-AM or Fluo3-AM as described in section 2.2.12. Various concentrations of NAADP-AM were added to the wells, and changes in fluorescence that corresponds to cytosolic Ca^{2+} fluctuations were recorded overtime. In that instance, the greatest increase in fluorescence in the population of cells in response to NAADP-AM stimulus was recorded at 200 nM; generally we find that NAADP-AM concentrations of 100-500 nM gave an optimal Ca^{2+} release; whereas concentrations of NAADP-AM greater than 10 μM were inhibitory (data not shown).

A.



B.

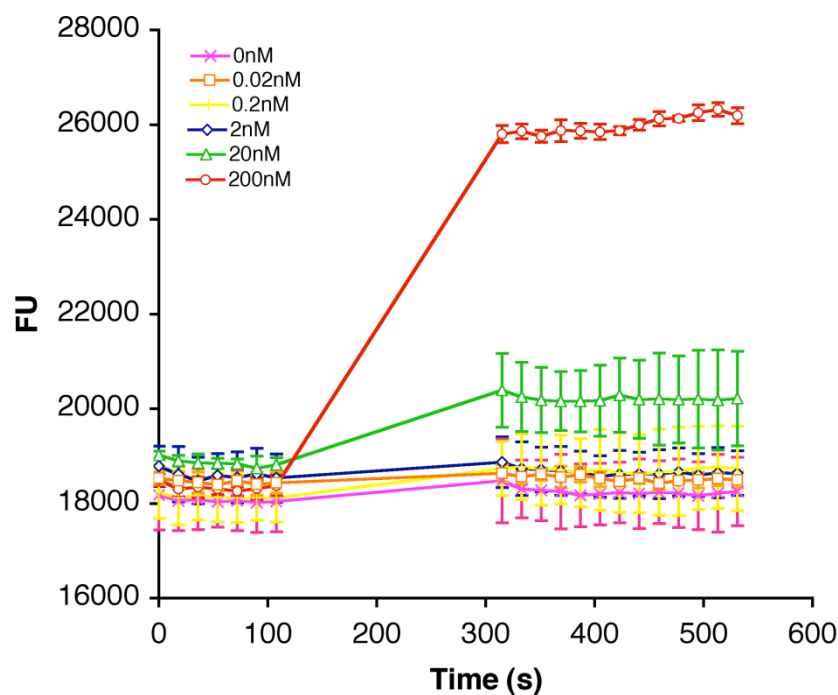


Figure 4.3 NAADP-AM synthesis and active dose determination. A. Pathway for synthesis of cell permeant NAADP analogue. NAADP-AM synthesis was carried out by Duncan Bloor-Young and kindly provided by Dr. Grant Churchill. **B.** Determining the optimum Ca^{2+} releasing concentration of NAADP-AM for treatment. WT HF_s were incubated with Ca^{2+} sensitive probe as described in section 2.2.12 and change in fluorescence in response to NAADP-AM was followed using NOVOstar plate reader. Error bars represent mean fluorescence $\pm$ S.D. $n=4$.

4.3.4 NAADP mediated Ca^{2+} signalling is perturbed in MLIV patient fibroblasts

Patient fibroblasts were plated into wells of an 8 chambers chamber slide and allowed to adhere overnight. Culture medium was removed and cells were loaded with fluorescent Ca^{2+} probe as described in section 2.2.12. Baseline probe fluorescence was recorded prior to adding Ca^{2+} releasing amounts of NAADP-AM. Lysosomal Ca^{2+} release was measured as a function of the change in cytosolic Ca^{2+} in intact human fibroblasts overtime as described in section 2.2.13. We found that NAADP induced a significantly greater Ca^{2+} release in MLIV fibroblasts compared to WT control fibroblasts (Fig. 4.4 A and B). As well as a bigger initial Ca^{2+} release in response to NAADP, MLIV patient fibroblasts have more Ca^{2+} release events after a single addition of NAADP (Fig. 4.4 C).

We also found that pre-treating the cells with 0.5 μM thapsigargin, to prevent Ca^{2+} induced Ca^{2+} release from the ER, did not abolish NAADP induced Ca^{2+} release (Fig. 4.5). However, following thapsigargin treatment, the amount of Ca^{2+} release in response to NAADP was reduced (Fig. 4.5 B). This demonstrates that ER contributes to the NAADP induced spontaneous Ca^{2+} release events following NAADP treatment, it also shows however that multiple Ca^{2+} release events following NAADP receptor activation by NAADP-AM do not originate in ER.

Endothelin 1 (ET1) is a secreted G protein coupled receptor agonist that has been linked to Ca^{2+} release via NAADP receptor signalling in mammalian cells [195] and fibroblasts. In our study, MLIV patient fibroblasts were treated with ET1. This induced multiple lysosomal Ca^{2+} release events, significantly more events than in WT cells following a single addition of ET1 (data not shown) demonstrating that the multiple release events observed in MLIV patient fibroblasts are induced by NAADP.

Moreover, Ned19 was previously shown to attenuate release in response to ET1 [196], confirming that the NAADP receptor mediates ET1 signalling at the cell membrane.

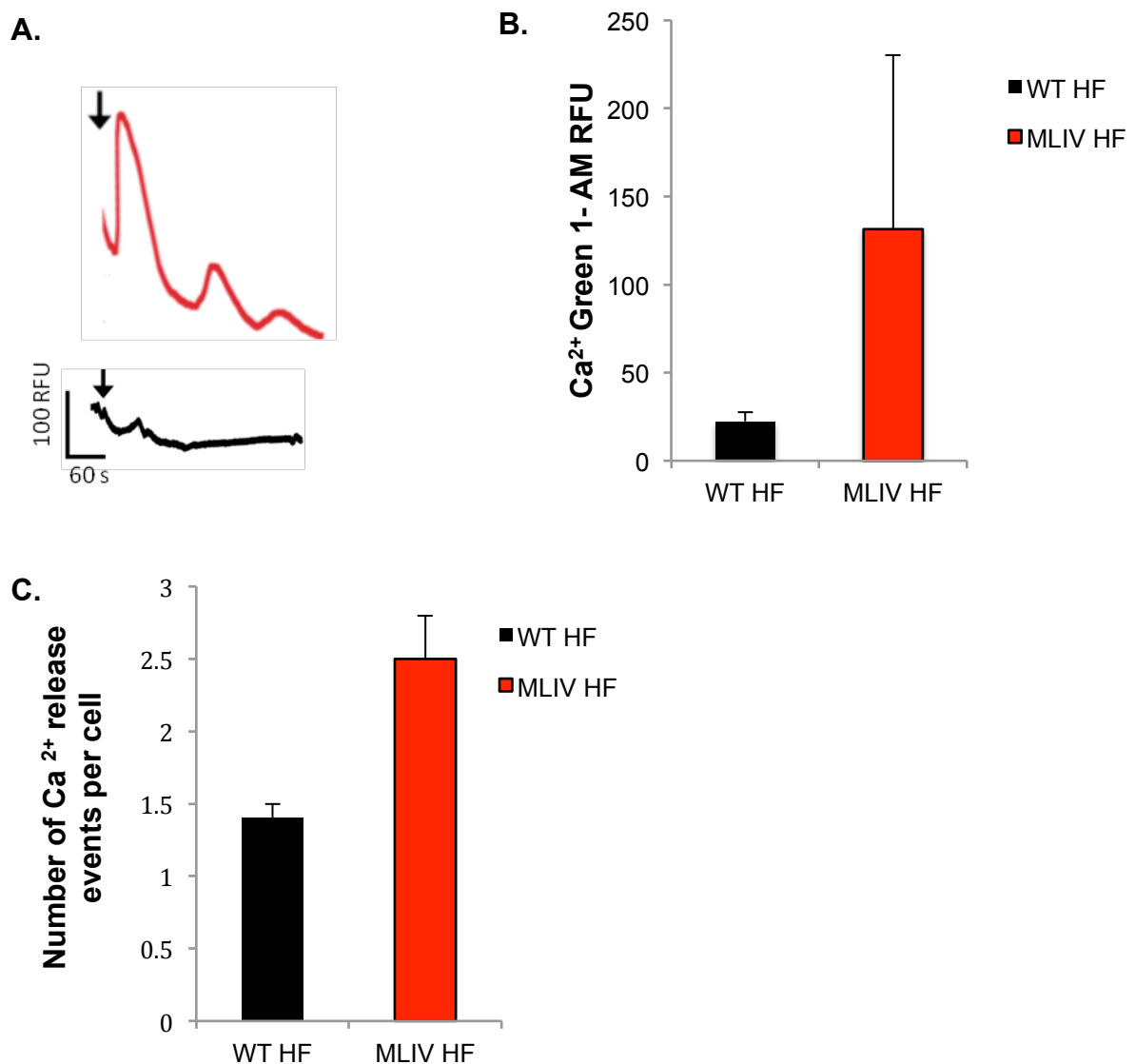


Figure 4.4 NAADP induced lysosomal release in WT and MLIV fibroblasts. **A.** Section of traces of WT (black trace) and MLIV (red trace) HF showing NAADP-AM induced Ca^{2+} release. A black arrow represents 500 nM NAADP-AM addition. **B.** Quantification of NAADP induced Ca^{2+} release expressed peak height of NAADP induced Ca^{2+} release; n=3. **C.** Number of NAADP-AM induced Ca^{2+} release events per cell; n=3. Error bars represent S.D.

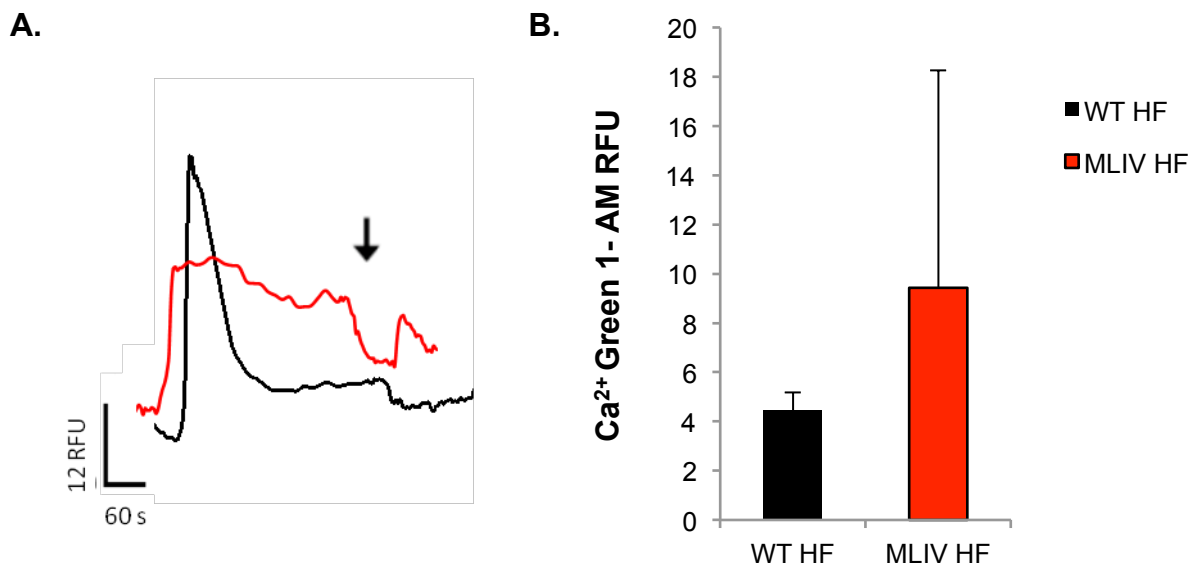


Figure 4.5 NAADP induced lysosomal release in WT and MLIV fibroblasts is reduced when ER store is clamped. A. Section of traces of WT (black trace) and MLIV (red trace) HF showing NAADP-AM induced Ca^{2+} release. A black arrow represents NAADP-AM addition. **B.** Quantification of NAADP induced Ca^{2+} release; $n=3$. Error bars represent S.D.

4.3.5 NAADP and TPC receptor levels and localisation in MLIV patient fibroblasts

NAADP messenger and NAADP receptor levels in MLIV fibroblasts are not elevated above those found in WT fibroblasts

Endogenous NAADP levels in WT and MLIV patient fibroblasts were measured using a competitive radioligand binding assay. [³²P] NAADP synthesis and the NAADP binding assay ($n=2$) were conducted by Aban Shuaib, under the supervision of Dr. Alexander Lewis (University of Oxford) and Dr. Emyr Lloyd-Evans (University of Oxford / Cardiff University) who have kindly allowed the results to be shown here. Basal levels of NAADP were found not to be different between WT and MLIV patient fibroblasts ($p = 0.5$) (Fig. 4.6). Therefore spontaneous Ca^{2+} release events from acid stores are not due to an increase in the level of NAADP.

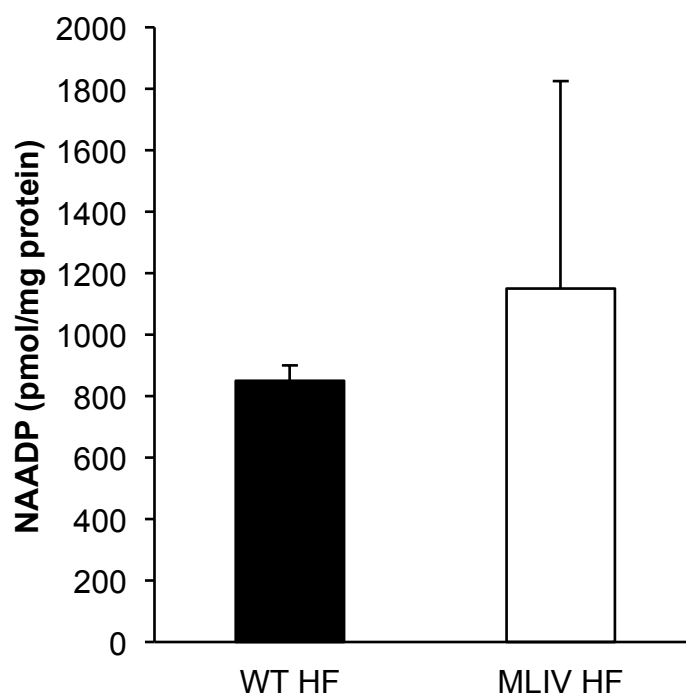
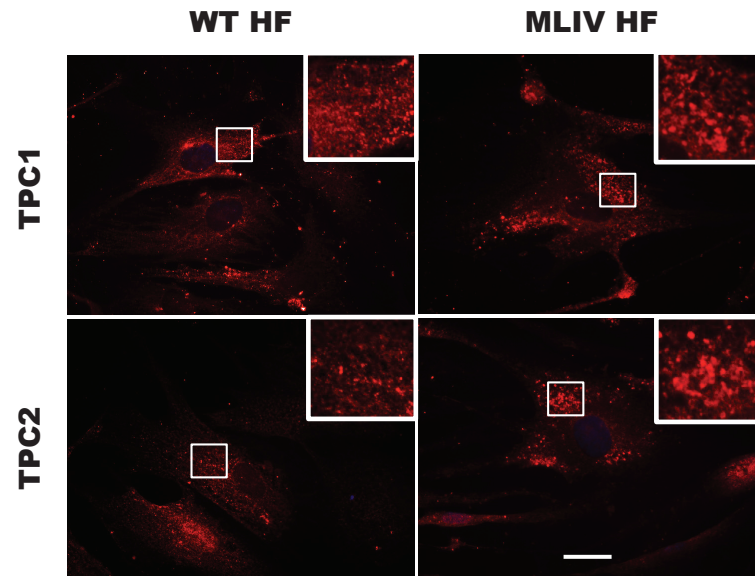


Figure 4.6 NAADP levels in MLIV fibroblasts are not elevated above those found in WT HFs. [^{32}P] NAADP synthesis and intracellular NAADP quantification were carried out by Aban Shuaib, under supervision of Dr. Lewis and Dr. Lloyd-Evans. Error bars represent the mean $\pm$ S.D. n=2.

To assess whether the expression levels of the candidate NAADP receptors, TPC1 and TPC2, contribute to the elevated lysosomal Ca^{2+} release seen in MLIV fibroblasts, we have used immunocytochemistry (Fig. 4.7 A) and qRT-PCR to assess protein and mRNA levels (Fig. 4.7 B). TPC1 and TPC2 immunocytochemistry does differ comparing WT to MLIV patient fibroblasts. Levels of fluorescence of TPC 1 and TPC2 Ab are higher in MLIV HFs, this alteration may be explained by the lysosomal volume expansion in MLIV fibroblasts (Fig. 4.7 A).

Relative expression levels of *TPC1* and *TPC2* in patient fibroblasts was assessed by real time quantitative PCR and analyzed using the 2-(Delta Delta C(T)) method [194] (Fig. 4.7 B). Levels of *TPC1* mRNA in MLIV fibroblasts are not elevated above that of WT fibroblasts ($p>0.5$). Similarly, levels of *TPC2* mRNA in MLIV fibroblasts are not elevated above those of WT fibroblasts ($p>0.5$).

A.



B.

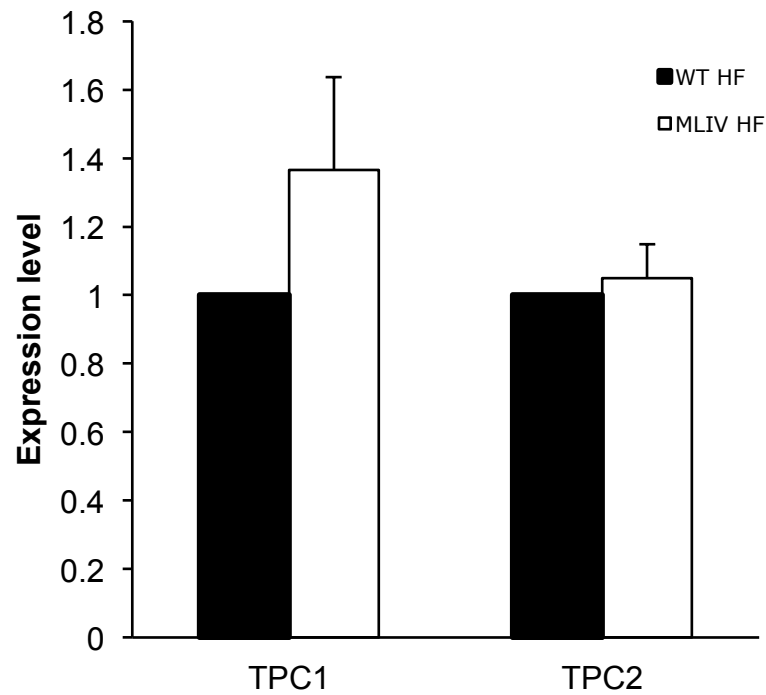


Figure 4.7 TPC expression is not upregulated in MLIV fibroblasts. A. WT and MLIV HF were stained using TPC Abs as described in section 4.2.10. Scale bar represents 5 μ m. n=3. **B.** WT and MLIV HF were harvested and their TPC expression was quantified using qRT-PCR as described in section 4.2.9. Error bars represent the mean expression $\pm$ S.D. n=3.

4.3.6 Ca^{2+} signalling defects in MLIV is the result of loss of TRPML1 activity

To test whether or not the abnormal NAADP induced Ca^{2+} release in TRPML1 null cells can be attributed directly to the absence of TRPML1 or is a downstream event, MLIV patient fibroblasts were transfected with a GFP tagged *MCOLN1* construct (Fig. 4.8 D). MLIV fibroblasts were also treated with a GFP tagged *LAMP1* construct (Fig. 4.8 B) in order to confirm the specificity of lysosomal expression of TRPML1 as the cause of correction in lysosomal Ca^{2+} signaling as opposed to general expression of a lysosomal transmembrane protein LAMP1. As TRPML1 and LAMP1 proteins are tagged with GFP, the transfection was verified by co-localisation of green staining attributed to TRPML1-GFP or LAMP1-GFP staining and LysoTracker Red by fluorescence microscopy (data not shown).

WT and MLIV fibroblasts were transfected with *TRPML1* or *LAMP1* for 48 h as described in section 4.2.3. Following transfection, cells were loaded with Fura2-AM as described in section 2.2.12. Ca^{2+} release in response to NAADP-AM was measured using confocal microscopy. We found that expression of *TRPML1* in MLIV patient fibroblasts (Fig. 4.8 D) reduced the number of Ca^{2+} release events compared to *LAMP1* transfected MLIV patient fibroblasts (Fig. 4.8 B), to levels not significantly different to WT controls (Fig. 4.9 A). Moreover, following TRPML1 expression in MLIV fibroblasts, the numbers of Ca^{2+} release events that follow the initial release were reduced (Fig. 4.9 B). The multiple Ca^{2+} release events following the initial release in response to NAADP are still present in MLIV fibroblasts transfected with *LAMP1* gene construct (Fig. 4.8 B), and the amount of Ca^{2+} released in response to NAADP is not reduced, showing that the perturbed lysosomal Ca^{2+} signalling in MLIV is a direct result of the loss of TRPML1 function. There is also a reduction in LysoTracker staining in the

MLIV cells expressing GFP-TRPML1 (data not shown), which indicates not just correction in Ca^{2+} but also general correction in storage.

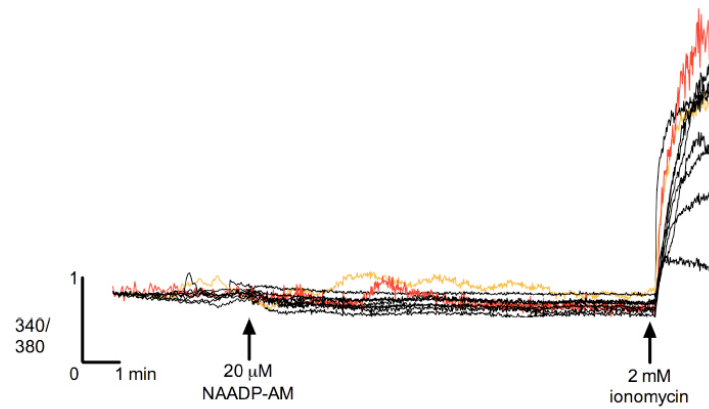
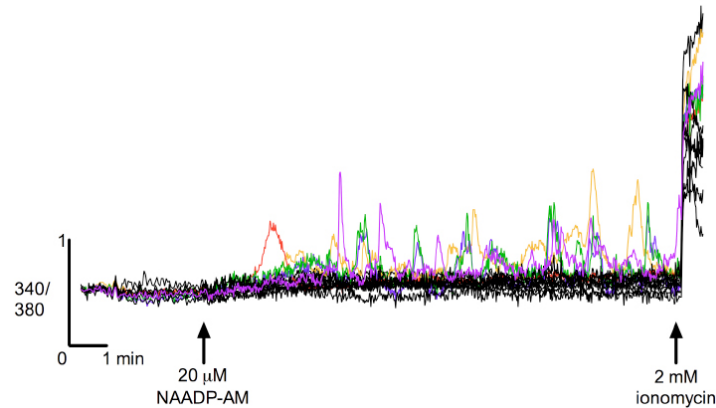
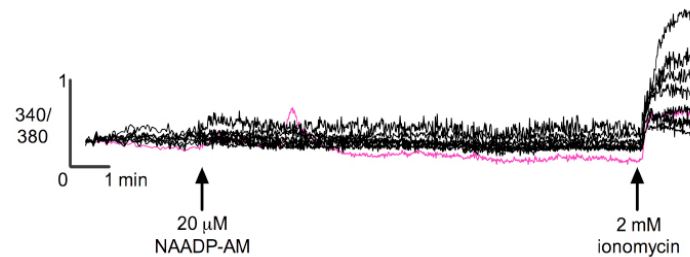
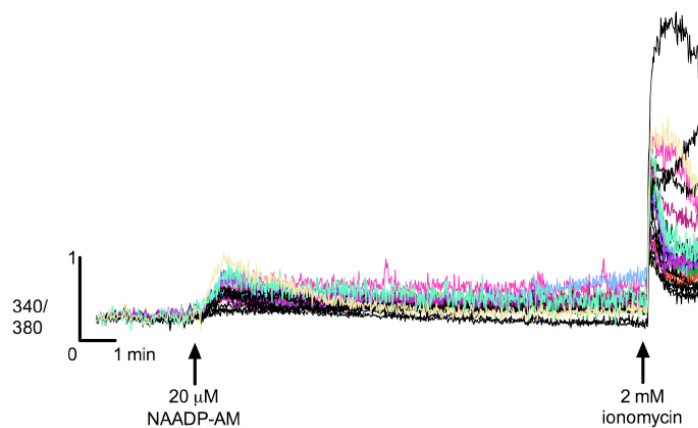
A.**B.****C.****D.**

Figure 4.8 Absence of a functional TRPML1 is the cause of NAADP mediated multiple Ca^{2+} release events in MLIV fibroblasts. A. WT HF transfected with GFP-LAMP-1 construct. B. MLIV fibroblasts transfected with GFP-LAMP-1 construct. C. WT HF transfected with GFP-TRPML1 construct. D. MLIV HF transfected with GFP-TRML1 construct. Transfections were carried out as described in section 4.2.10. Transfected cells were loaded with Ca^{2+} sensitive probe and Ca^{2+} release events in response to 20 μM NAAADP-AM were carried out as described in section 4.2.11.

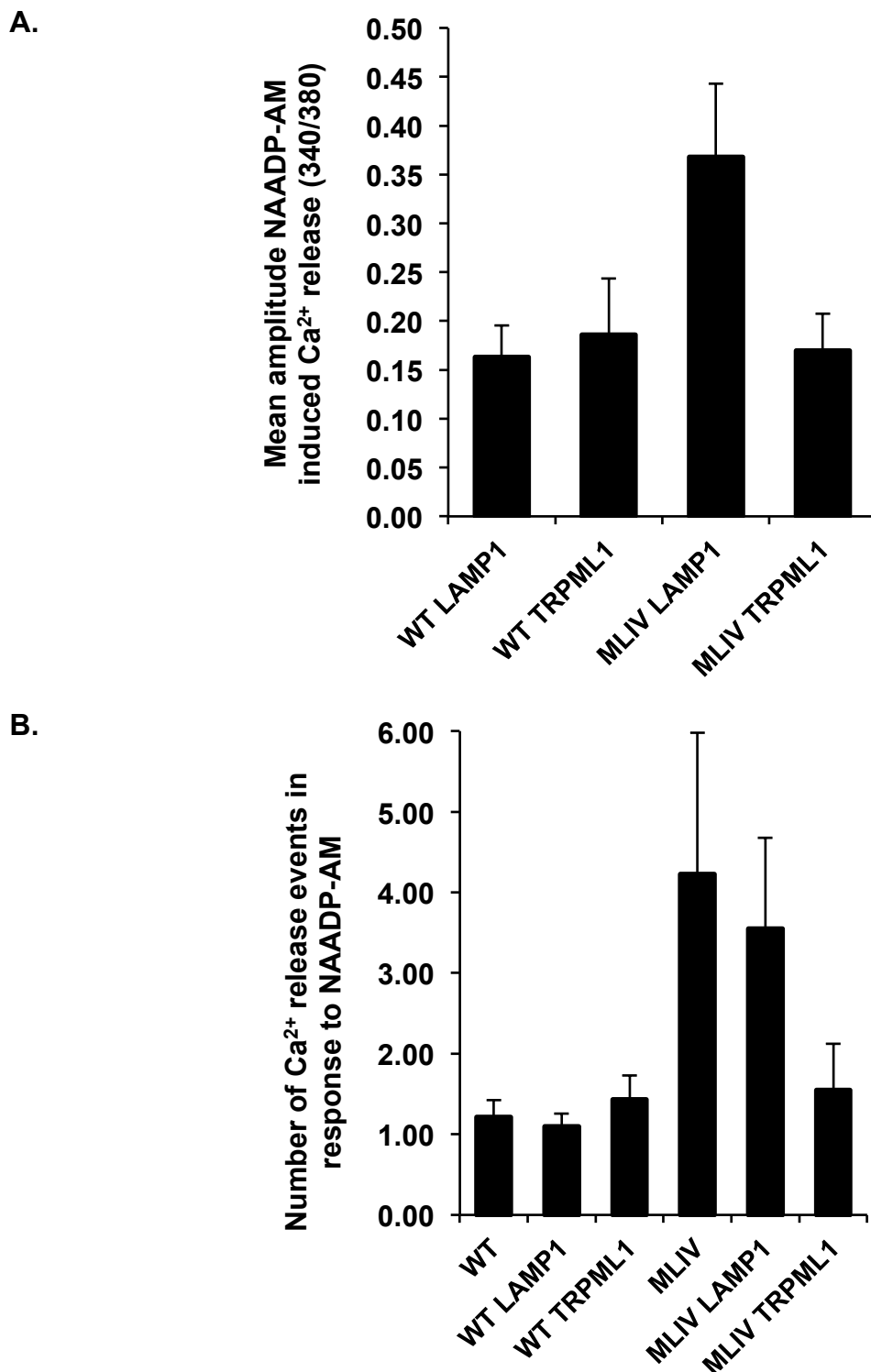


Figure 4.9 Absence of a functional TRPML1 is the direct cause of increased amplitude and number of NAADP mediated multiple Ca^{2+} release events in MLIV fibroblasts. **A.** Amplitude of Ca^{2+} release events in response to 20 μM NAADP-AM in HF transfectants transfected with either *GFP-LAMP1* or *GFP-TRPML1* constructs. **B.** Number of Ca^{2+} release events in response to NAADP is reduced in MLIV fibroblasts transfected with *TRPML1*. Error bars represent mean $\pm$ S.D.

4.3.7 Effect of NAADP treatment on MLIV phenotypes

In mammalian cells, Ca^{2+} release in response to NAADP follows a bell-shaped curve with an increasing amount of Ca^{2+} being released with increasing concentration of NAADP until an optimal NAADP concentration is reached [181]. NAADP concentrations higher than this optimal concentration inhibit further Ca^{2+} release. For every batch of NAADP-AM used we find the optimal Ca^{2+} release concentration of NAADP by applying varying concentrations of NAADP-AM and measuring a change in cytosolic Ca^{2+} in a cell population as above in section 4.3.3.

As mentioned earlier, we determine the Ca^{2+} releasing and Ca^{2+} release inhibiting concentrations of NAADP-AM in a cell population using plate based assay. We then confirm the individual cell response to these NAADP-AM concentrations. Cells grown in chambers of an 8 chambers chamber slide were loaded with Ca^{2+} Green 1- AM as described in section 4.2.2, NAADP-AM was added to the chamberslide and change in fluorescence was recorded over time; Ca^{2+} release in response to 500 nM and 15 μM NAADP was measured as described in section 2.2.13 (Fig. 4.10).

In response to 500 nM NAADP-AM, MLIV fibroblasts have multiple Ca^{2+} release events following initial Ca^{2+} release after NAADP-AM addition, indicating this is an activating concentration of NAADP-AM. Pre-treating MLIV fibroblasts for 15 min using 15 μM NAADP-AM completely inhibited Ca^{2+} release in response to 500 nM NAADP-AM. Ca^{2+} releasing concentration of NAADP-AM (500 nM) and Ca^{2+} release inhibiting (15 μM) concentrations were then used for treatment of human fibroblasts to assess the effect of potentiating and inhibiting Ca^{2+} release on lipid storage in MLIV. From batch to batch we find 100-500 nM NAADP-AM to give the optimal Ca^{2+} release, with NAADP-AM of 10 μM and above inhibiting NAADP induced Ca^{2+} release.

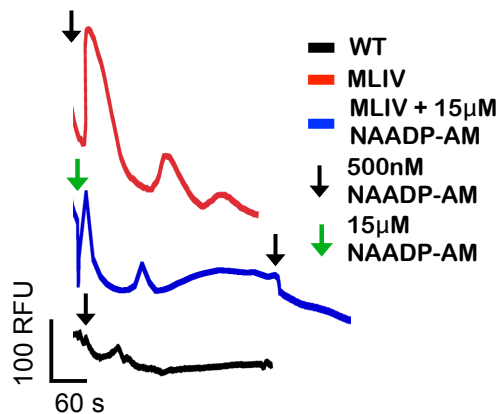


Figure 4.10 Depending on concentration NAADP can potentiate or inhibit lysosomal Ca^{2+} release in WT and MLIV fibroblasts. HF's grown in chambers of an 8 chamber slide were loaded with sensitive probe as described section 4.2.2. Ca^{2+} release in response to NAADP-AM was measured as a described in section 2.2.13. Section of traces of WT (black trace) and MLIV (red and blue traces) HF's showing NAADP-AM induced Ca^{2+} release. Black arrow represents 500 nM NAADP-AM addition, green arrow represents 15 μM NAADP-AM addition. $n=3$.

Patient fibroblasts were treated with Ca^{2+} releasing concentrations of NAADP-AM (100-500 nM) and Ca^{2+} release inhibiting (10-15 μM) NAADP-AM concentrations to determine the effect of lysosomal Ca^{2+} release on storage in MLIV fibroblasts.

Lysosomal storage in MLIV fibroblasts was assessed using LysoTracker Red staining following the treatment with 500 nM and 15 μM NAADP. MLIV fibroblasts treated with 500 nM NAADP for 24 h exhibit up to 30% increase in LysoTracker fluorescence (Fig. 4.11 A, lower middle panel) indicating an increase in lysosomal storage ($p<0.05$). Therefore potentiating Ca^{2+} release in MLIV fibroblasts exacerbates lysosomal storage phenotype in MLIV.

Whereas 24 h treatment of MLIV fibroblasts using 15 μM NAADP has not markedly reduced LysoTracker fluorescence in MLIV cells ($p>0.05$) (Fig. 4.11 A, lower right panel). Perhaps at this concentration of NAADP the NAADP receptor is completely inhibited mimicking conditions observed in NPC1 disease.

The effect of varying NAADP-AM in WT fibroblasts is similar. Both 500 nM and 15 μ M NAADP-AM induced a significant increase in LysoTracker fluorescence ($p < 0.05$). Treating WT fibroblasts with Ca^{2+} releasing concentration of NAADP mimics NAADP induced enhanced Ca^{2+} release observed in MLIV, as a result lysosomal storage is induced in WT cells (Fig. 4.11 A, upper middle panel). However, 15 μ M NAADP may completely inhibit the NAADP receptor in doing so reduce the Ca^{2+} release mimicking conditions observed in NPC1 disease (Fig. 4.11 A, upper right panel).

These results are consistent with previously used NAADP-AM concentrations. Similarly to the lipid storage phenotype, CtxB mistrafficking is exacerbated at the Ca^{2+} releasing (100 nM) concentration of NAADP (Fig. 4.12) and corrected at high inhibiting (10 μ M) concentrations of NAADP (Fig. 4.12).

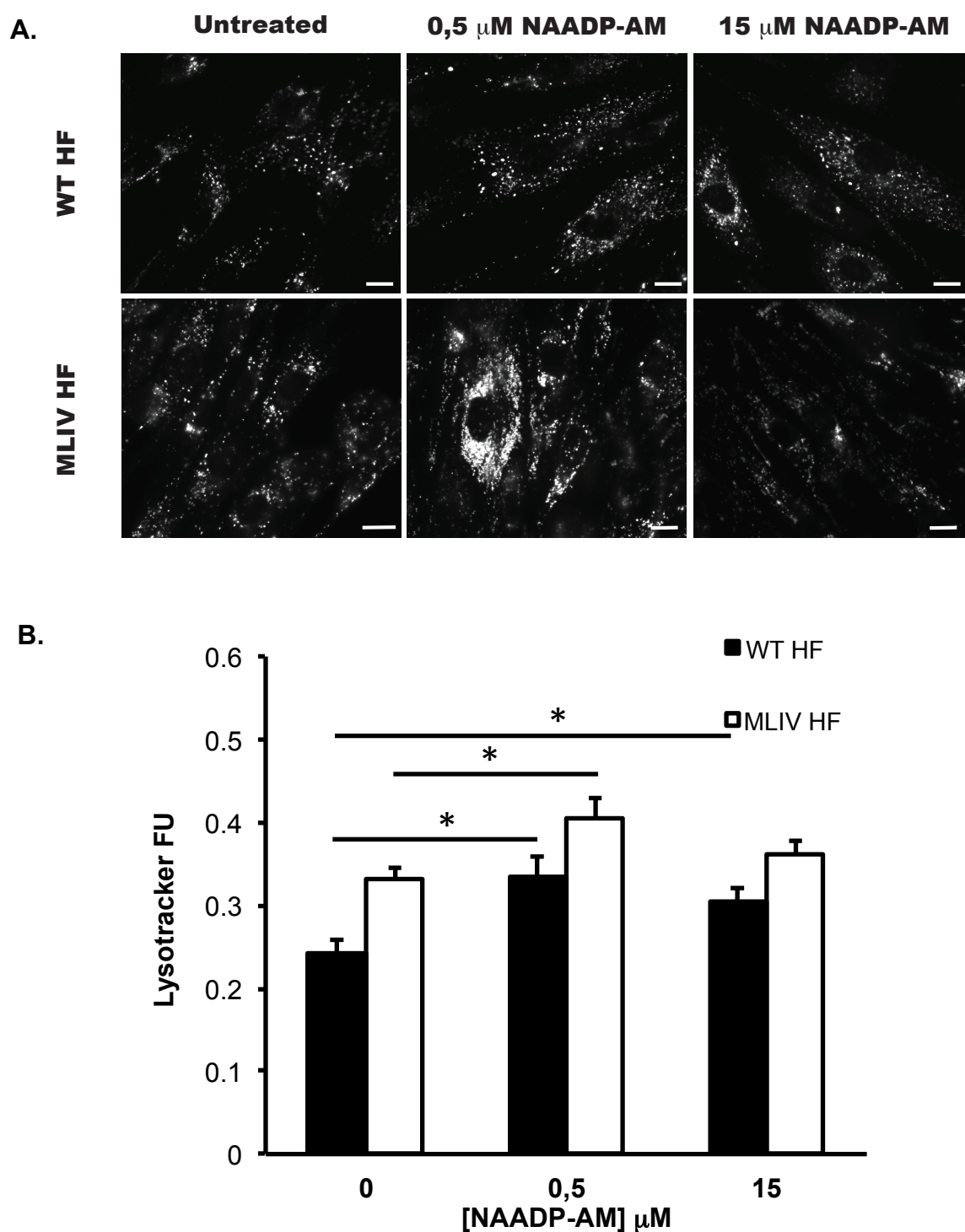


Figure 4.11 NAADP-AM treatment exacerbates the lipid storage phenotype in MLIV fibroblasts. **A.** HFs were pre-treated using 0,5 μ M and 15 μ M NAADP-AM for 24 h then stained with Lysotracker as described in section 2.2.9 and visualized live. Scale bar represents 5 μ m. **B.** Grey scale intensity of Lysotracker fluorescence was quantified using ImageJ software. Error bars represent mean $\pm$ S.E.M.

4.3.8 Treatment of MLIV fibroblasts with the NAADP antagonist Ned19

NAADP signaling was partially inhibited using Ned19 and the effects on MLIV phenotypes determined.

4.3.8.1 Ned19 localisation

As mentioned previously Ned19 is fluorescent in the blue spectrum of visible light. Ned19 localisation was assessed by treating WT fibroblasts with 100 μ M Ned19* (Table 4.1) for 1 h then staining pre-treated cells with LysoTracker Red DND-99 as described in section 2.2.8. We found that Ned19 blue punctate staining completely co-localised with red punctate staining of LysoTracker Red (Fig. 4.12), showing Ned19 is localised to late endosomal / lysosomal compartments.

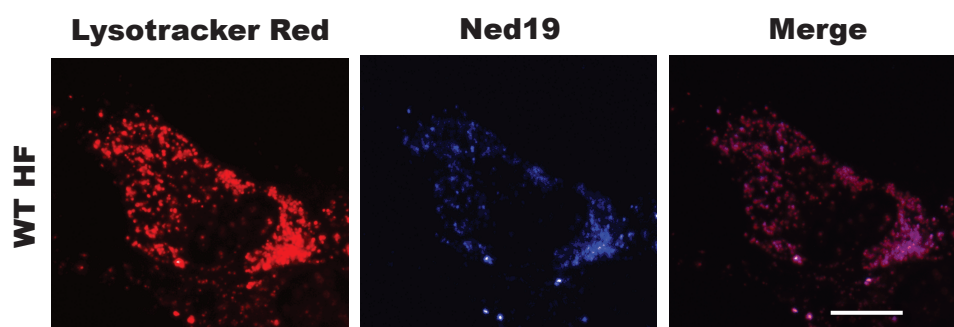


Figure 4.12 Ned19 staining completely co-localises with LysoTracker staining. WT HFs were pre-treated with 100 μ M Ned19 for 1 h before loading with LysoTracker as described in section 4.2.5. Scale bar represents 5 μ m.

4.3.8.2 Treatment of MLIV fibroblasts with the NAADP antagonist Ned19 ameliorates some of the MLIV phenotypes

Treatment of MLIV fibroblasts with either 10-20 μ M *cis/trans* Ned19* (Table 4.1) or 50 μ M PPADS for 12-24 h, reduces lysotracker fluorescence (Fig. 4.14 b and c respectively) indicating that lysosomal storage is reduced. Interestingly, the same concentrations of Ned19 and PPADS used in WT fibroblasts increase lysotracker fluorescence, indicating an increase in lipid storage.

We have found no reduction in lysotracker staining and therefore no improvement in lipid storage when using *trans* Ned19[#] (Table 4.1) (data not shown).

Improvements in trafficking and phospholipid storage phenotypes in MLIV after treatment with Ned19 were followed using Alexa Fluor 594-CtxB or LipidTox respectively. WT and MLIV patient fibroblasts were treated for 18 h using 20 μ M *trans* Ned19*.

Preliminary experiments show that in MLIV patient fibroblasts CtxB localisation changes following Ned19 treatment. As discussed previously, CtxB-GM1 complex is trafficked down the endocytic system to the lysosome (in healthy cells it exits the early endosome and goes to the Golgi, in LSD cells it is mistrafficked with GM1 to lysosomes) and then recycled back to the Golgi. In the untreated MLIV fibroblasts has punctate distribution indicating that CtxB is localized to late endosomes/lysosomes. However *trans* Ned19 treated MLIV fibroblasts show Golgi localization (Fig. 4.15 A) indicating that the delay in trafficking is corrected (any counts?). However in WT fibroblasts 24h treatment with Ned19 causes CtxB to mislocalise to endocytic compartments (Fig. 4.15 B); this phenotype is again similar to that seen in NPC1 where lysosomal Ca²⁺ content is reduced and a delay in trafficking out of the lysosome occurs.

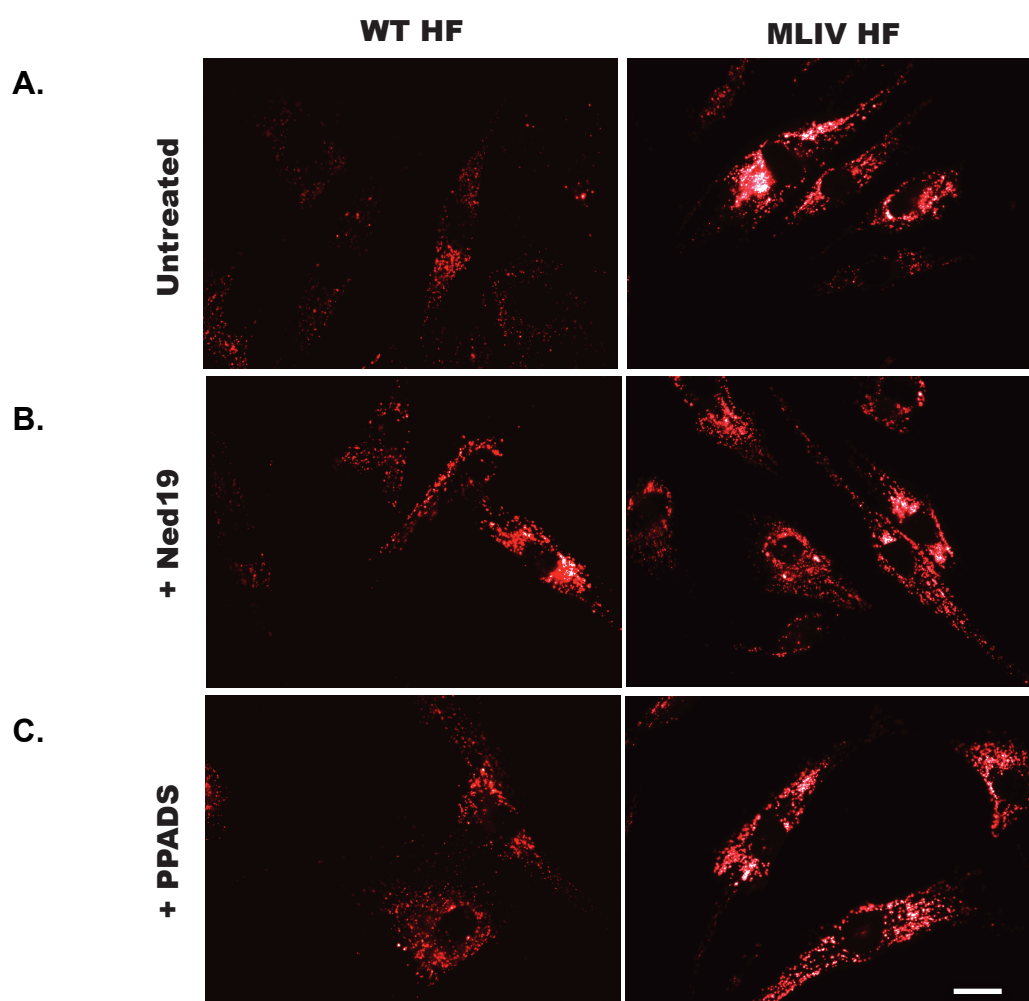


Figure 4.13 Partially inhibiting NAADP receptor using Ned19 corrects lipid storage phenotype in MLIV fibroblasts. a. HF's were untreated (A), pre-treated using 20 μ M Ned19 (B) or 50 μ M PPADS (C) for 18 h then stained with LysoTracker Red DND-99 as described in section 2.2.9 and visualized live. Scale bar represents 5 μ m.

Changes in phospholipid content following *trans* Ned19* (Table 4.1) treatment were assessed using LipidTox Red as described in section 2.2.9 (Fig. 4.16). LipidTox staining in MLIV fibroblasts was not significantly reduced following Ned19 treatment ($p>0.5$), indicating that phospholipid storage is not cleared. This could be explained by Ned19 having the chemical properties of a CAD, more specifically a secondary amine group. However Ned19 predicted pKa = 2.17; predicted pKa of 'ionizable' or lipophilic amine groups is estimated to be > 6 [197] therefore Ned19 would not bind phospholipids and become trapped in the lysosome. Ned19 did not cause an increase in LipidTox staining in WT fibroblasts ($p>0.1$) in the treatment time frame indicating that

it does not interact with phospholipids or the enzymes that affect phospholipid metabolism. Perhaps a longer treatment time frame is required to clear phospholipid build up in the lysosomes of MLIV fibroblasts.

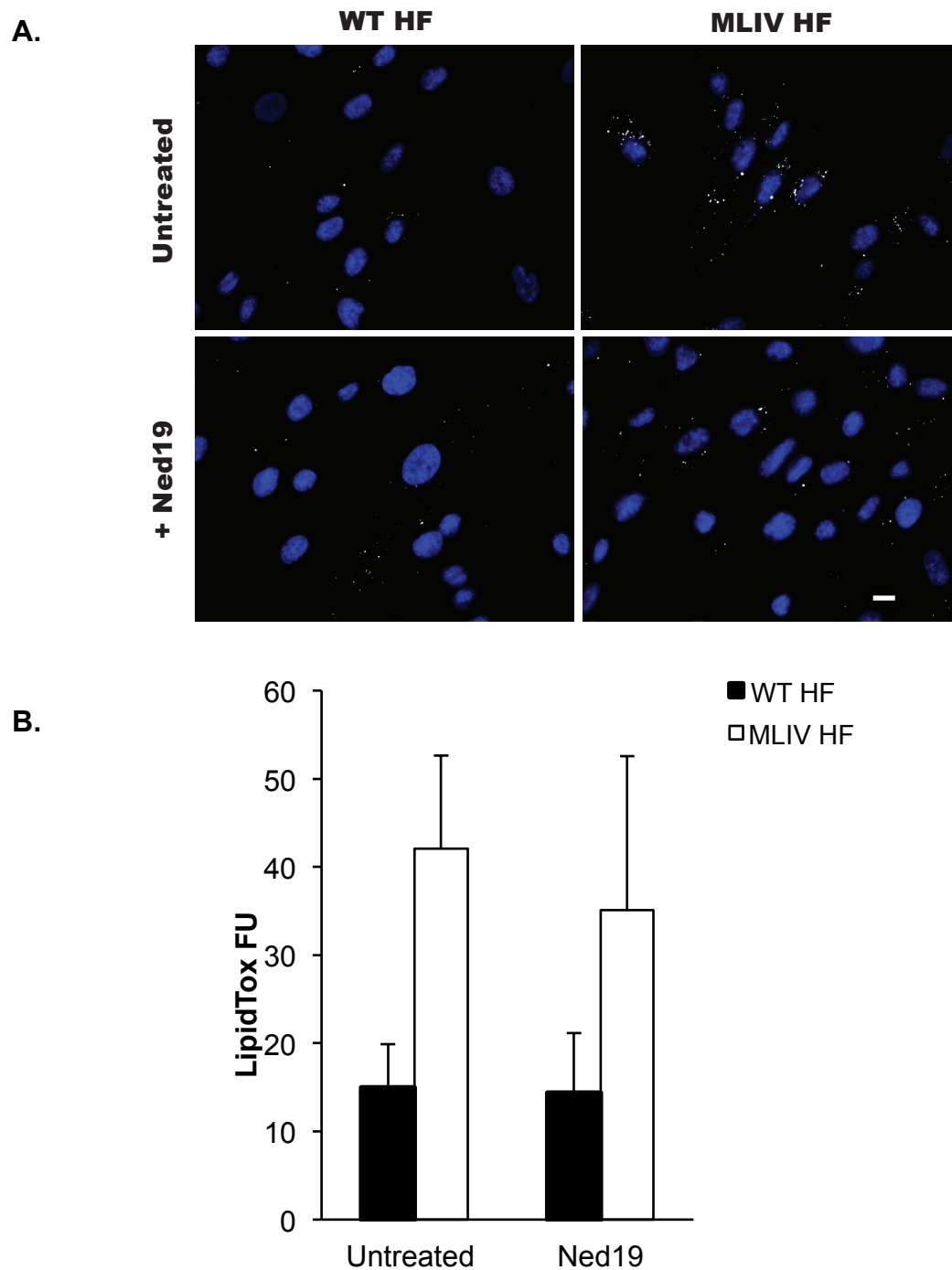


Figure 4.14 Partially inhibiting NAADP receptor using Ned19 does not correct phospholipid storage phenotype in MLIV fibroblasts. **A.** HF s were pre-treated using 20 μ M Ned19 then stained with LipidTox as described in section 2.2.9 and visualized live. Scale bar represents 5 μ m. **B.** Grey scale intensity of LipidTox fluorescence was quantified using ImageJ software. Error bars represent the mean $\pm$ S.D.

Similarly to phospholipid storage, autofluorescence in MLIV fibroblasts, was not reduced following 12-24 h treatment with *trans* Ned19* (Table 4.1) (Fig. 4.15, lower right panel), indicating that lipofuscin was not cleared by Ned19 within the time frame of treatment. Although, preliminary experiments suggest that delay in trafficking was corrected with Ned19 (data not shown), lipofuscin fluorescence remains, indicating that although trafficking out of the lysosome is corrected lipofuscin cannot be effluxed out of the lysosome.

Ned19 treatment did not induce autofluorescence in WT fibroblasts at 470 nm excitation, therefore partially inhibiting Ca^{2+} release via NAADP receptor does not induce lipofuscin storage. Only green ($\lambda_{\text{excitation}} = 470 \text{ nm}$) autofluorescence is shown here since there is no autofluorescence in red ($\lambda_{\text{excitation}} = 520 \text{ nm}$) before or after Ned19 treatment in MLIV or WT fibroblasts. Changes in autofluorescence in blue could not be assessed since Ned19 itself is autofluorescent in blue ($\lambda_{\text{excitation}} = 368 \text{ nm}$).

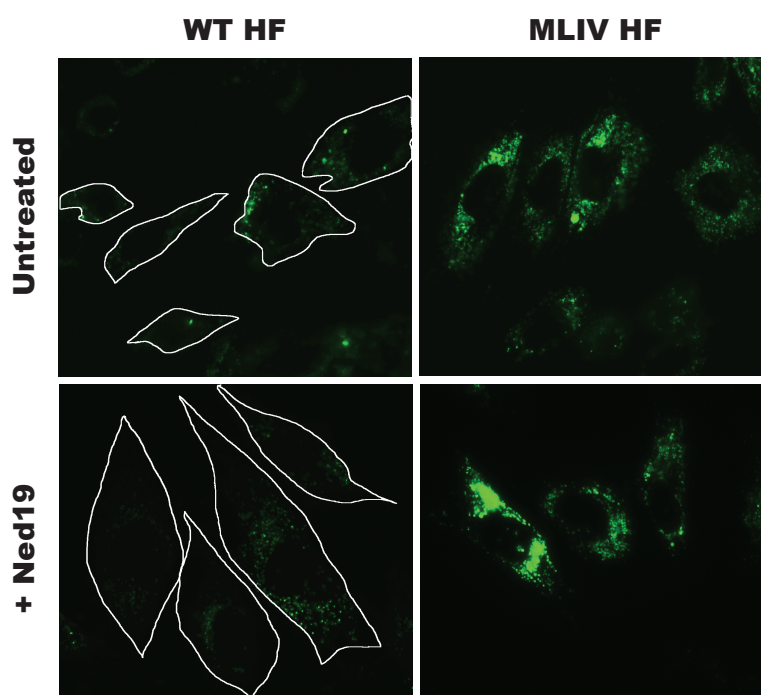


Figure 4.15 Ned19 staining completely co-localises with Lysotracker staining. WT HFs were pre-treated with 100 μM Ned19 for 1h before loading with Lysotracker as described in section 4.2.13. Scale bar represents 5 μm .

4.4 DISCUSSION

The present study shows that in absence of functional TRPML1, NAADP is capable of inducing Ca^{2+} release from the lysosomal compartment, which leads to the conclusion that TRPML1 cannot be the NAADP sensitive lysosomal Ca^{2+} release channel. These data therefore disagree with the findings of Dr. H. Xu and colleagues who have suggested that TRPML1 may be necessary for NAADP induced lysosomal Ca^{2+} signaling [155]. These data do support the findings of Dr. Sandip Patel (UCL, London, UK) and Prof. Shmuel Muallem (NIH, MD, USA) who have suggested that TRPML1 is not an NAADP receptor/channel [104], as there is a detectable Ca^{2+} release in response to NAADP and ET1 in TRPML1 deficient cells. Taken together, our findings clearly indicate that TRPML1 is not an NAADP receptor.

Interestingly, here we showed an enhanced lysosomal Ca^{2+} release in presence of normal levels of NAADP signal and candidate NAADP receptors, TPCs, as well as overall normal lysosomal Ca^{2+} levels. These data suggest a possibility that TRPML1 could be in some way involved in regulating the function of the NAADP receptor, perhaps as a negative regulator and potentially in some way one that modulates endocytosis. Spatial proximity of TRPML1 and TPCs as well as their previously reported association make the model of TRPML1 regulating TPCs plausible [198].

We also showed that inhibiting enhanced lysosomal Ca^{2+} release by a specific NAADP receptor antagonist, Ned19, ameliorates most of MLIV phenotypes. This suggests that enhanced lysosomal Ca^{2+} release is an upstream event that subsequently causes trafficking defects and lipid accumulation confirming that we see in *Mcoln1* silenced cells in Chapter 3 and indicating that the NAADP signaling pathway is a novel therapeutic target for MLIV.

4.4.1 Potential functions of TRPML1 protein

4.4.1.1 TRPML1 is not the principal lysosomal Ca^{2+} release channel

Our results show that lysosomal Ca^{2+} release induced by the endogenous NAADP signal is perturbed in TRPML1 deficient cells. However, the lysosomal Ca^{2+} release is not reduced as Shen *et al.* [96] suggest, but is enhanced in absence of a functional TRPML1 channel. This directly contradicts the suggestion that TRPML1 is the NAADP sensitive Ca^{2+} release channel as, if this were the case, there would be no lysosomal response to NAADP in TRPML1 null cells.

Subsequently, Shen *et al.* [96] went further to suggest that TRPML1 is directly involved in NPC disease pathology, suggesting that inhibition of the functional TRPML1 protein in NPC patient fibroblasts by accumulated sphingomyelin is what causes the reduced lysosomal Ca^{2+} release in response to NAADP in NPC1 null cells. Our results again contradict this finding since if NAADP signalling was mediated by TRPML1 then in the absence of a functional protein there would be no Ca^{2+} release in response to NAADP. We have previously shown that lysosomal Ca^{2+} release in pharmacologically induced NPC disease, patient cells and mouse cells null for NPC1 was in fact reduced, however this was due to a reduced lysosomal Ca^{2+} content, a defect that lies upstream of defects in endocytosis that ultimately cause the sphingomyelin storage. It is therefore unlikely, based on our current findings, that sphingomyelin inhibits TRPML1 in NPC1 null cells as we would expect to see an elevation in NAADP induced lysosomal Ca^{2+} release (as we have now shown in TRPML1 null cells) as opposed to the reduction that is seen in NPC disease. Lysosomal Ca^{2+} levels in NPC measured by Lloyd Evans *et al* were found to be lower compared to lysosomal Ca^{2+} levels in WT controls cells. Hence the reduced NAADP induced Ca^{2+} release in NPC cells is attributed to a reduced lysosomal Ca^{2+} pool as opposed to an inhibition of TRPML1 by sphingomyelin. Moreover lipid storage was reported to occur downstream of the

lysosomal Ca^{2+} defect in a pharmacologically induced model of NPC1, meaning that the Ca^{2+} defect cannot be a result of sphingomyelin storage. In combination with our earlier findings indicating very little similarity between NPC1 null and TRPML1 null patient fibroblasts, almost no similarity between patient phenotypes and our evidence indicating that Ned19 induces an NPC phenotype in wild-type cells by inhibiting NAADP mediated Ca^{2+} release yet correcting phenotypes in TRPML1 null cells, it would appear that there has been an error in the interpretation of Shen *et al.* [96]. Furthermore, if the aforementioned findings were correct, then we would also expect Niemann-Pick A and B patients to present with similar phenotypes to MLIV considering the primary accumulation of sphingomyelin observed in these diseases, we would also expect that overexpression of TRPML1 should rescue all the phenotypes in NPA, NPB and NPC cells, interestingly this was not attempted in Shen *et al.* [96].

The increased Ca^{2+} release in MLIV fibroblasts cannot be attributed to endogenous NAADP levels or the expression level of TPC1 and TPC2 receptors, since both the signal and the receptor levels are not elevated in these cells. The increased Ca^{2+} release in response to NAADP instead suggests that TRPML1 acts a negative regulator of NAADP mediated Ca^{2+} signalling. The lack of TPC and NAADP overexpression in MLIV cells also suggests a loss of regulation rather than an increase in signal.

Enhanced lysosomal Ca^{2+} release can be directly attributed to the absence of TRPML1 receptor, as Ca^{2+} signaling can be normalized by expression of TRPML1 in TRPML1-null cells. This lends itself to speculation that TRPML1 negatively regulates TPC receptors and inhibits release from the TPC receptors once the fusion between late endosome and the lysosome has occurred, and the hybrid organelle has been formed. Inhibiting further Ca^{2+} release would allow the Ca^{2+} to be re-sequestered into the

lysosome and the two organelles to segregate. This may explain the presence of enlarged lysosomes with increased Ca^{2+} levels; these are discussed in more detail in the section below. This hypothesis is supported by evidence from Dr. Sandip Patel group who show that TPC2 receptor, and to a lesser extent TPC1 co-immunoprecipitates with TRPML1 [104]. TRPML1 could be inactive at the lower lysosomal pH and only become activated at the higher pH of the hybrid organelle owing to the fusion of the larger less acidic late endosome with the smaller more acidic lysosome.

4.4.1.2 TRPML1 could function as a lysosomal Ca^{2+} leak channel

Lysosomal Ca^{2+} levels are not significantly different between MLIV and WT patient fibroblasts. There are, however, a few expanded lysosomes in each TRPML1 deficient cell with elevated Ca^{2+} content. We hypothesize that these enlarged organelles are the late endosome-lysosome hybrid organelle. Separation of the hybrid organelle into lysosomes and late endosomes requires a localized re-sequestration of Ca^{2+} [198]. This probably does happen in MLIV cells, however in absence of TRPML1 on the lysosomal membrane, this re-sequestration has to compete with excessive Ca^{2+} release through the NAADP receptor meaning that the hybrid organelle cannot separate into lysosome and late endosomal compartments as quickly as the fusion occurs. Enlarged hybrid organelles form as a result of excessive fusion, subsequently the Ca^{2+} content in these organelles is elevated, it is most likely that they contribute to the enhanced Ca^{2+} release.

It was previously suggested that TRPML1 is regulated by changes in pH [199]. Late endosome fusing with the lysosome causes a slight elevation in lysosomal pH; this may switch off TPCs so that Ca^{2+} can be re-sequestered as the pH drops again.

The hybrid organelle may overfill with Ca^{2+} during re-acidification such that a Ca^{2+} leak channel is required to remove the excess Ca^{2+} without triggering a massive Ca^{2+}

release that would induce fusion again whilst the organelles are separating. In the absence of TRPML1 the excess Ca^{2+} does not leak out so that once the pH has recovered TPCs may become activated and fire again meaning that the hybrid organelle does not separate.

4.4.2 Deregulation in lysosomal Ca^{2+} release contributes to MLIV cellular phenotypes

Partially inhibiting the NAADP receptor using NAADP and a synthetic analogue of NAADP, Ned-19, reduces storage levels in MLIV fibroblasts. This occurs through reducing the Ca^{2+} release in response to NAADP, meaning that Ca^{2+} re-sequestration does not have to compete with enhanced Ca^{2+} release allowing the hybrid organelle to separate. As a result lipid trafficking out of the lysosome can be corrected, and lipid storage levels reduced. This is supported by our preliminary results of correcting GSL trafficking using Ned19.

The lysosomal Ca^{2+} defect is therefore most likely to lie upstream of development of MLIV pathology since correcting this defect can correct both mis-trafficking and lysosomal storage. This supports our findings in Chapter 3 that deregulation in Ca^{2+} signalling is likely an upstream event, and normal Ca^{2+} signalling is required for maintenance of lipid flux out of the lysosome.

Autofluorescence of MLIV fibroblasts was not corrected by inhibiting the NAADP receptor indicating that lipofuscin storage was not reduced. This is most likely because it is a large undegradable material that cannot be digested by lysosomal enzymes or effluxed out of the lysosome even when the hybrid organelle is able to segregate into separate lysosomal and late endosomal compartments.

4.4.2.1 Ned19 as a potential therapy for MLIV

Our results suggest that deregulation of NAADP signalling not only is an early event in pathology of MLIV but is also central to MLIV pathology, and that reducing the

excessive lysosomal Ca^{2+} release ameliorates the trafficking defects and lysosomal storage in MLIV patient fibroblasts. This suggests that decreasing NAADP mediated lysosomal Ca^{2+} release in patient cells could be a promising therapeutic strategy. However, using Ned19 may not be a viable therapy since inhibiting NAADP signalling in healthy control cells leads to trafficking defects and lipid accumulation similar to that seen in NPC disease (Lloyd-Evans *et al.*, unpublished observations), suggesting that the effective dose-response range for Ned19 is very narrow, and overdose of Ned19 could have severe side-effects. There is however a possibility of using a weaker inhibitor of TPC receptors that could be beneficial in terms of providing therapy for MLIV patients without having the adverse effect of Ned19.

A more viable therapeutic avenue would be partial inhibition of CD38, the enzyme that catalyses conversion of NADP into NAADP. Reducing the level of the NAADP signal could ameliorate the excessive Ca^{2+} release and partially correct the phenotypes observed in MLIV, similarly to partially inhibiting the NAADP receptor using Ned19. Several natural products have been shown to reduce the activity of human CD38 [200]. Testing the inhibitors of CD38 in MLIV patient fibroblasts, if successful, could provide an over the counter therapy for MLIV patients. Testing the effect of CD38 inhibitors in normal cells would also be necessary since completely inhibiting NAADP signalling would mimic the reduced lysosomal Ca^{2+} release observed in NPC disease and could lead to development of NPC-like symptoms.

5 Lysotracker as a novel screening tool for LSDs

5.1 INTRODUCTION

LSDs are classed as rare disorders with incidence ranging from 1 in 50,000 to 1 in 4,000,000 live births [201]. Although, individually rare, the combined incidence of LSDs has been estimated to be more than 1 in 5,000 live births in an Australian population [202]. Clinical manifestation of disease may vary depending on the residual activity of the mutated protein. There is no single assay that can be used as a general screen for all LSDs at present. In the absence of a family history of disease, an early diagnosis can only be made if a neonatal screening programme is in place.

5.1.1 Current methods used for LSDs screening

5.1.1.1 Biochemical enzyme assays in dried blood spots

Some LSDs can be diagnosed by measuring enzyme activity in blood or urine. This has been adapted to enzyme assays in a dried blood spot [203], which can be used to measure lysosomal enzyme activity. This method can only be used successfully to diagnose LSDs caused by a reduction in activity or complete deficiency of lysosomal enzymes. LSDs caused by a mutation in a transmembrane protein, such as NPC1 and MLIV, or a deficiency in accessory proteins would not be picked up by this assay.

The cost of running a single enzyme assay is high, and it is usually necessary to test more than one enzyme to make the diagnosis. Enzyme assays are normally performed at specialized clinics where a patient would be referred by a physician suspecting an LSD.

5.1.1.2 Mass spectroscopy of enzymatic products

This method was tested to detect β galactosidase (GALC) an enzyme deficient in Krabbe disease [204]. Enzyme activity was quantified by determining the abundance of

a synthetic substrate of the GALC enzyme, β -Gal-C₈-Ceramide, in a dried blood spot sample using a tandem mass spectrometer. The GALC assay was shown to be precise and the enzyme derived from dried blood sample was stable for transportation to specialized center where mass spectroscopy can be performed.

A pilot screening programme for Krabbe disease using the GALC enzyme assay was conducted in New York in 2006, and the cut off point for GALC enzyme activity established [205]. This value has allowed the identification of individuals with lower than normal enzyme activity. These individuals then undergo further confirmatory testing.

A screening program for Pompe disease was conducted in Austria, where 10,000 newborn blood spot samples were tested against those of 29 known Pompe disease patients and 229 healthy individuals [206]. Mass spectrometry analysis of the acid α glucosidase substrate identified 100% of affected individuals.

Mass spectroscopy testing of a single lysosomal enzyme activity was developed to allow assaying of multiple enzymes derived from a single blood spot. Multiplex mass spectrometry analysis allows the activity of up to 6 lysosomal enzymes to be tested from a single Guthrie card sample.

Although the mass spectrometry based enzyme assays are very effective at identifying individuals with enzyme deficiency, results might be complicated by the presence of residual enzyme activity, and the assay is not able to identify patients with LSDs where there is no defect in lysosomal enzyme activity. In addition, the cost of tandem mass spectrometer maintenance is high, and a trained specialist is required to run the tests and interpret the results.

5.1.1.3 BODIPY-Lactosyl ceramide transport assay

The fluorescent analogue of lactosylceramide (LacCer), BODIPY-LacCer is taken up by cells by clathrin-independent endocytosis and transported through the endocytic

system to the Golgi. In many LSDs sphingolipid trafficking is impaired and the probe cannot exit the lysosome, and so accumulates. Similarly to LacCer, BODIPY-LacCer also accumulates in lysosomes in LSD cells. When BODIPY-LacCer accumulates in the membranes, it is amenable to FRET analysis, whereby excitation of the probe with light with a wavelength of 488nm will excite the probe to fluoresce in green. At high enough concentrations, excited BODIPY-LacCer also fluoresces in red. Thus the emission spectrum of the probe changes from green to red with increasing concentration of sphingolipid. This method successfully identified LSDs with a delay of sphingolipid trafficking and subsequent accumulation in late endosomal / lysosomal compartments [141].

This assay requires the use of tissue culture facilities, a fluorescence microscope and a trained specialist to perform and analyse the images.

5.1.1.4 Electron microscopic examination of skin biopsies

Skin biopsies are used to diagnose two LSDs, neuronal ceroid lipofuscinosis and MLIV. In one study, the ultrastructure of 100 skin biopsy samples representing 35 different LSDs was examined [207]. The authors claim that the ultrastructural appearance of lysosomal storage material may predict the specific lysosomal storage disorder: oligosaccharides stored in glycoproteinoses appear as empty vacuoles, whereas accumulated glycolipids present as fine lamellated membrane structures within the lysosome.

Although examination of skin biopsy samples have been used extensively in the past to confirm LSD diagnosis, it cannot be used a stand-alone method for identification of a specific LSD.

Punch biopsy, used to obtain samples for EM, is an invasive procedure whereby a 4mm circular blade is inserted into a pre-anaesthetised area of the forearm. In addition, the cost of obtaining and processing a skin biopsy has been estimated to be more than

that of a single enzyme assay [28], and requires a trained histologist to examine the samples and make a definitive diagnosis.

5.1.1.5 Use of LAMP1 and LAMP2 as a diagnostic marker

As mentioned previously, lysosome associated membrane proteins 1 and 2 (LAMP1 and LAMP2) are the two most abundant lysosomal transmembrane proteins. Since both proteins are found on the lysosomal membrane, it was expected that both LAMP1 and LAMP2 increase with increased lysosomal volume typical of LSDs. One group has investigated whether LAMP1 [208] and LAMP2 [28] can be used as general clinical markers for LSDs. Plasma concentration of LAMP1 and LAMP2 in 312 affected individuals representing 25 different LSDs was tested against that of 202 control individuals. Interestingly, LAMP1 showed an increase in 17 of 25 disorders tested; LAMP2 was increased in 14 of 25 disorders. Therefore LAMP screening in patients plasma would only identify 70% of affected individuals. Moreover, elevated white cell number in the plasma due to infection may increase the number of lysosomes present and subsequently LAMP concentration, giving a false positive result in an unaffected patient.

In addition, the authors tested the use of LAMP1 and LAMP2 as biomarkers in a neonatal screen. LAMP1 and LAMP2 concentrations in blood spots taken from newborns were measured over a period of 4 weeks. Results showed that the newborn population was skewed towards higher concentrations of both LAMP1 and LAMP2, which if used, could lead to a false positives diagnosis.

The use of a two-tier strategy diagnosis [202] was later proposed for patients identified as high risk by having elevated plasma LAMP1 / LAMP2 concentration, these patients would then be tested for the presence of additional LSD markers.

5.1.2 Use of lysotracker assay in LSD screening

We have developed a microplate assay based on the use of lysotracker probe that specifically label lysosomal compartment. We propose for this assay to be used as an initial screening method to detect patients with LSD. The specific diagnosis can then be established using any of the methods listed above.

Lysotracker assay has already been successful in clinical monitoring of LSD [209]. Use of lysotracker assay to follow substrate reduction therapy using miglustat in B lymphoblasts of an NPC patient has been published [210]. In that instance however lysotracker fluorescence was measured using FACS analysis.

5.1.3 Aims of this chapter

The aim of this chapter is to investigate whether the lysotracker probe can be used to detect storage in patient fibroblasts from a broad range of LSDs where there is an increase in lysosomal volume or a change in acidity. We then adapted the lysotracker assay to measure storage in human lymphoblasts and in the white cell fraction isolated from patients' whole blood using a fluorescence plate reader.

5.2 MATERIALS AND METHODS

5.2.1 *Cell lines and cell culture*

The human patient fibroblasts and human patient lymphoblasts used in this study were obtained from the cell repository at the Coriell Institute for Medical Research (New Jersey, USA).

The following fibroblast cell lines were used in this study: We have used two cell lines of fibroblasts from healthy control individuals (GM05399 and GM05659). Fibroblasts from patients with severe (GM02752) and mild (GM02315) forms of Farber disease and Farber HET (GM02317) were used. We have also used the fibroblasts from patients with MPSII (GM02268) and MLD (GM0243). MLIV cell line used here is the same one as we have used throughout the rest of this thesis (GM02527). Fibroblasts were cultured as described in section 2.2.1.

Lymphoblasts from patients with Krabbe and NPC1 disease as well as WT control were used in this study. Lymphoblasts were cultured in Roswell Park Memorial Institute, RPMI medium (Sigma Aldrich, Poole, UK) supplemented with 10% FCS / 1% Gln. Lymphoblasts were grown in suspension in 5% CO₂ atmosphere humidified incubator at 37°C.

5.2.2 *Lysotracker probe loading*

Lysotracker (Molecular probes, Paisley, UK) is loaded into cells in 1X HBSS (Invitrogen, Paisley, UK) containing 1 mM CaCl₂ (Sigma Aldrich, Poole, UK), 1 mM MgCl₂ (Sigma Aldrich, Poole, UK) and 20 mM HEPES pH 7.4 (Invitrogen, Paisley, UK) pre-warmed to 37°C at a concentration of 200 nM Lysotracker Green DND-26 or 300 nM LysoTracker Red DND-99, for 15 min at 37°C.

5.2.3 Labelling endocytic compartments using Rhodamine Dextran

1 mg / ml Rhodamine Dextran (Sigma Aldrich, Poole, UK) in complete medium was loaded at 37°C for varying amounts of time to label endocytic compartment (Table 5.1).

	Pulse	Chase
Early endosome	5 min	-
Late endosome	15 min	15 min
Lysosome	24 h	24 h

Table 5.1 Varying pulse / chase time of Rhodamine Dextran loading allows labelling of different endocytic compartments.

5.2.4 LysoTracker plate assay for adherent cells

Human patient fibroblasts were counted using hemocytometer counting chambers and seeded into F bottom 96 well microplate at a concentration of 20,000 cells per well, and left for 8-10 h in a humidified 37°C incubator to allow the cells to adhere to the well surface. The medium was removed and decanted.

Fibroblasts were incubated with 200 nM LysoTracker Green DND-26 in 1X Hank's Balanced Salt Solution, HBSS (Invitrogen, Paisley, UK) buffer for 15 min at 37°C. LysoTracker HBSS solution was removed and cells were washed once with 1% HBSS buffer. Fluorescence intensity was measured at λ excitation 470nm / λ emission 520nm for LysoTracker Green DND-26 and λ excitation 570nm / λ emission 590nm for LysoTracker Red DND-99 using a NOVOSTAR fluorescence plate reader (BMG Labtech, Aylesbury, UK) and standardized to the number of plated cells. Background fluorescence was estimated using unloaded cells and subtracted from final LysoTracker fluorescence values.

5.2.5 *B lymphocyte capture assay*

Avidin-biotin interaction was used to capture non-adherent B lymphocytes onto the surface of the well. Streptavidin coated Greiner F bottom 96 well microplate (Pierce) were incubated with mouse IgG1 anti human CD19 antibody (eBioscience) linked to biotin at RT for 1 h. Following the incubation wells were washed with 200 μ l PBS three times to remove any unbound CD19 Ab.

B lymphoblasts were counted using hemocytometer, harvested into 1,5ml eppendorf tubes and centrifuged at 1500 rpm for 5 min to pellet down the cells. 200,000 cells per well were used to cover the surface of the well for the capture assay. Cell pellet was re-suspended in 200 μ l 1X HBSS 1mM CaCl₂, 1mM MgCl₂, HEPES (pH ~ 7.4) buffer and added to the well. Lymphoblasts were incubated on the plate at 4°C for 30 min; the well was then gently washed using 200 μ l PBS three times to remove any unbound cells, being careful not to scrape the cells off with the pipette tip. Cell coverage was checked by light microscopy prior to lysotracker loading.

Lysotracker loading was carried out as described in section 5.2.4. Background fluorescence of each individual well was measured prior to Lysotracker loading at the same λ excitation / λ emission as used for Lysotracker probe using NOVOSTAR fluorescence plate reader.

5.2.6 *Adapting Lysotracker plate assay for use in whole blood*

5.2.6.1 *Collecting, storing and shipping blood*

Patients blood samples were collected into an EDTA sprayed vacutaner tube at a clinic and shipped overnight on ice to the laboratory for analysis. Similarly, blood samples donated by healthy volunteers were collected into an EDTA sprayed vacutaner tubes.

5.2.6.2 Isolating white cell fraction

Sterile non-filled 12 ml Leukosep tubes (Greiner BioOne, Stonehouse, UK) were used to separate the white cell fraction from whole blood. Tubes were filled with 3 ml Histopaque-1077 Hybri-Max separation medium (Sigma Aldrich, Poole, UK) pre-warmed to room temperature. Tubes were closed with a screw cap and centrifuged for 30 s at 1000 g.

3 ml of whole blood samples were put into pre-prepared Leucosep tube and centrifuged at 4°C for 30 min at 4000 g. Following centrifugation the top plasma layer was drawn up carefully without disturbing the white cell fraction underneath using filtered pipette tip (Starlab, Milton Keynes, UK); this prevents contamination of enriched white cells with platelets. Plasma cells were decanted. The white cell fraction, consisting of B cells and T lymphocytes was harvested using a filtered pipette tip into a 15 ml Falcon tube, washed with 10 ml PBS (Sigma Aldrich, Poole, UK), and centrifuged for 10 min at 250 g. The wash step was repeated once.

White cell pellet was resuspended in 1X HBSS 1 mM CaCl_2 , 1 mM MgCl_2 , HEPES (pH ~ 7.4) buffer (3 ml whole blood in 100 μl buffer). Cells in antibody buffer were transferred into an antibody coated well of 96 well plate (plate was prepared as described in section 5.2.5) and incubated at 4°C for 30 min, the plate was shaken using orbital shaking function of the microplate reader for 60 s at 10 min intervals. Following incubation wells were washed with 200 μl PBS three times to wash off white cells unbound to the plate surface. Fluorescence at λ excitation 470nm / λ emission 520nm, was read using NOVOSTAR fluorescence plate reader to determine background fluorescence. Cells bound to the plate surface were incubated with 200 μl of 200 nM LysoTracker Green DND-26 at 37°C for 10 min. Wells were washed twice using 200 μl PBS. LysoTracker fluorescence at λ excitation 470nm / λ emission 520nm was read immediately using NOVOSTAR fluorescence plate reader.

5.3 RESULTS

5.3.1 Lysotracker probe loading is dependent on the pH of the compartment

In order to determine the endocytic compartment labeled by the Lysotracker probe we have loaded WT fibroblasts with Rhodamine Dextran and adjusted the pulse chase time to label different compartments of the endocytic system as described in section 5.2.3. Red punctate staining attributed to Rhodamine Dextran indicates endolysosomal localization.

Rhodamine Dextran loaded fibroblasts were then loaded with Lysotracker Green DND-26 as described in section 5.2.2. Lysotracker was loaded for the same amount of time so that the same compartment is labeled. Yellow staining indicates overlap between red staining of Rhodamine dextran and green staining of Lysotracker Green. There is no co-localisation between Lysotracker staining and staining of the early endosomes; red punctae labeled by Rhodamine Dextran are completely separate from green punctae of Lysotracker Green (data not shown). Some degree of overlap (yellow staining beginning to emerge) starts to occur when Rhodamine Dextran labels late endosome (Fig. 5.1). There is complete co-localisation between Lysotracker staining and Rhodamine Dextran staining when Rhodamine Dextran is pulse/chased to the lysosomes (Fig. 5.1). These results indicate that Lysotracker labels lysosomal and to some extent late endosomal compartment.

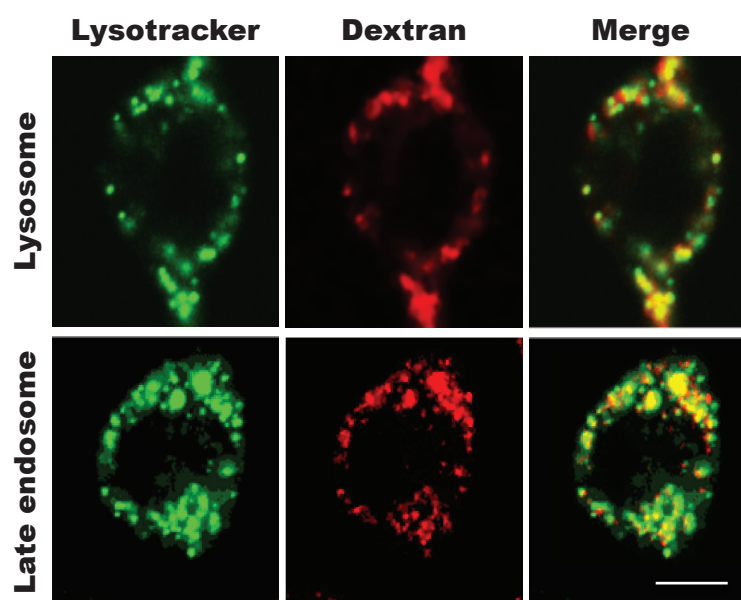


Figure 5.1 Lysotracker is loaded into the acidic endosomal compartment. Rhodamine Dextran is pulsed / chased either to the late endosome or lysosome (Table 5.1) of WT HFs cultured on glass coverslips. Cells are then loaded with Lysotracker as described in section 5.2.2 and visualized live. Scale bar represents 5 μm .

5.3.2 Lysotracker loading is dependent on the size of the compartment

To assess how the Lysotracker probe loading changes with the size of the lysosomal compartment we have treated WT fibroblasts with an amphipathic steroid 3- β -[2-(diethylamino)ethoxy]androst-5-en-17-one, also known as U18666A, which binds to NPC1 protein and interferes with its function [211] thereby inducing an NPC phenotype [45].

Overnight treatment of fibroblasts using 2 μ g / ml U18666A showed an increase in Lysotracker fluorescence compared to that in untreated fibroblasts (Fig. 5.2) indicating that expansion in lysosomal size and number causes an increase in Lysotracker fluorescence due to more probe being loaded into the compartment.

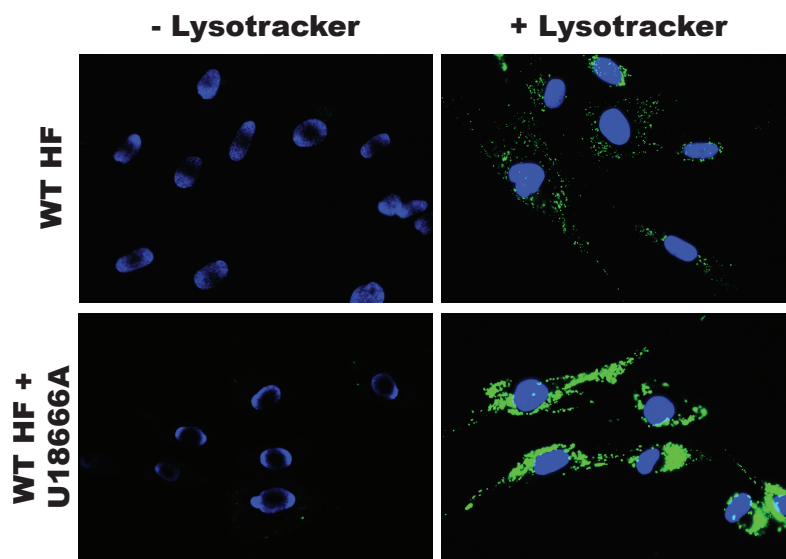


Figure 5.2 Lysotracker loading increases with increasing lysosomal volume. HFs were either untreated or treated using 2 μ g / ml U18666A for 18 h then stained with Lysotracker Green as described in section 5.2.2 and visualized live. Cells treated with U18666A are more fluorescent compared to untreated cells. Scale bar represents 5 μ m.

5.3.3 Lysotracker fluorescence is dissipated following fixation

According to manufacturer's instruction Lysotracker stained cells can be visualized following fixation. However we have found that Lysotracker loaded cells fixed with 4% PFA for 15 min have diminished Lysotracker fluorescence (Fig. 5.3).

We observe the same effect when Lysotracker loaded cells are fixed with cold methanol (data not shown).

Similarly, treating Lysotracker loaded cells with lysomotrophic drugs such as GPN or NH_4 , diminishes Lysotracker fluorescence (data not shown).

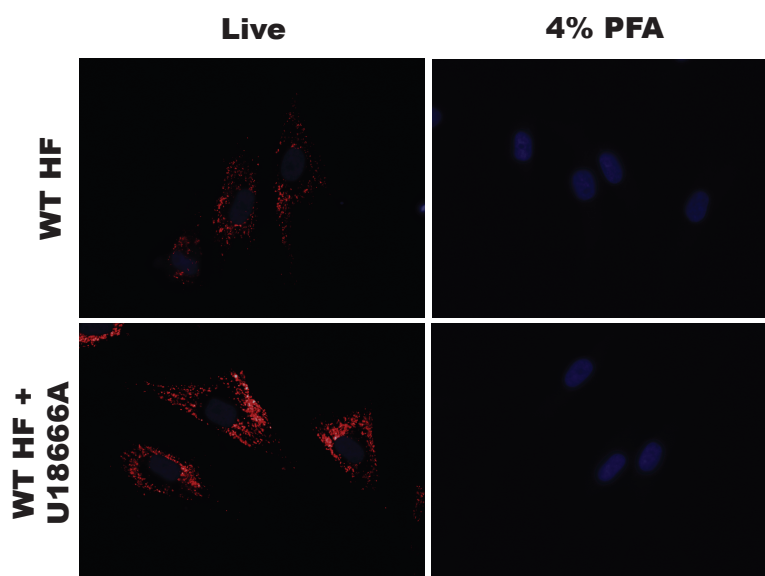


Figure 5.3 Lysotracker loading increases with increasing lysosomal volume. HF cells were either untreated or treated using $2\mu\text{g} / \text{ml}$ U18666A for 18 h then stained with Lysotracker Red for 45 min then Hoesht nuclear stain as described in section 5.2.2 and visualized either live or fixed using 4% PFA. Scale bar represents $5\mu\text{m}$.

5.3.4 Lysotracker fluorescence is standardized to cell number in 96 well plate assay

In the past we have standardised the Lysotracker fluorescence to Hoesht, which labels cell nuclei. However we have since found that some LSD cells are multinucleated meaning that Lysotracker fluorescent does not always correspond to Hoesht signal. We have therefore decided to standardise the Lysotracker fluorescence to cell number instead.

WT fibroblasts were plated into wells of a 96 well plate as described in section 5.3.4. We have plated different number of cells to see how cell number affects Lysotracker signal. We have also treated WT fibroblasts with U18666A to see whether Lysotracker fluorescence to cell number ratio is affected when the cells have expanded lysosomal compartment.

Lysotracker fluorescence of untreated WT fibroblasts follows a linear relationship, with fluorescence increasing with increasing number of cells, when the cell number falls between 10,000 and 30,000 cells (Fig. 5.4, black trace). Fluorescence at or below 5,000 cells per well does not show the linear relationship since this number of cells does not cover the entire surface of the well, with some of the area being devoid of cells. The signal becomes refracted of the surface of the well, generating noise and no longer correlates with actual amount of the probe loaded into cells. Fluorescence at or above 50,000 cells per well also falls outside of the linear relationship since there are too many cells on the surface of the well, such that the cells can no longer form a monolayer but instead start growing on top of each other such that Lysotracker probe loading is no longer uniform.

When cells are treated with U18666A (Fig. 5.4, red trace) the Lysotracker signal to cell number relationship is slightly different with the linear part of the curve being between 10,000 and 20,000 cell per well, with the curve forming a plateau above 25,000 cells.

Based on these findings we have chosen to use 20,000 cells per well of a 96 well plate for a plate based Lysotracker assay.

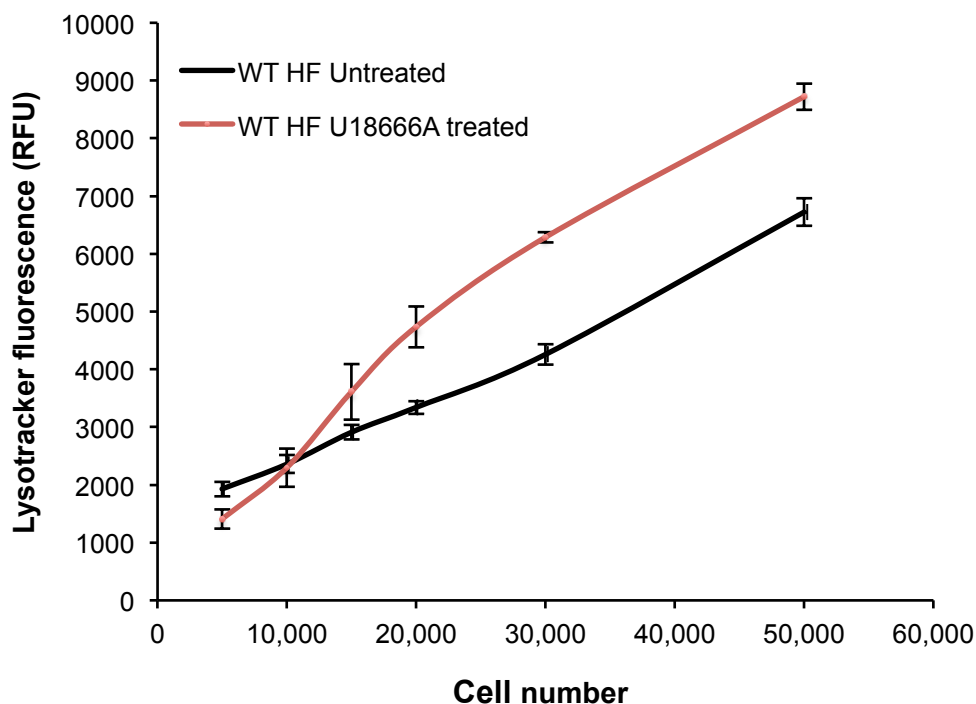


Figure 5.4 Lysotracker fluorescence signal can be standardised to cell number. WT HFs were counted, plated into wells of a 96 well plate and allowed to adhere for 8-10 h. Cells were then loaded with Lysotracker probe as described in section 5.2.2. Mean Lysotracker fluorescence for plated cell number is shown above. Error bars represent S.D., n = 3.

5.3.5 Lysotracker assay can detect lysosomal storage and expansion in patient fibroblasts

The Lysotracker plate assay was previously performed on a panel of LSD patient fibroblasts by Jin Zang who has kindly allowed for the data to be shown here (Fig. 5.5 A). Patient fibroblasts were plated into wells of a 96 well plate and Lysotracker fluorescence intensity in Lysotracker loaded fibroblasts was measured by microplate reader. Please note that here Lysotracker fluorescence was normalised to Hoechst nuclear staining. Lysotracker fluorescence was found to be elevated more than 3 fold in GM2 gangliosidosis, Sandhoff and Tay-Sachs patient fibroblasts, and 4 fold in GM1 gangliosidosis patient fibroblasts compared to WT control fibroblasts. MPS1 patient fibroblasts showed a 5 fold elevation in Lysotracker fluorescence. Farber and α -mannosidosis patient fibroblasts have 2 and 1.5 fold increase in Lysotracker fluorescence respectively. Lysotracker assay picks up lysosomal expansion and storage in fibroblasts with absent or reduced lysosomal hydrolase activity. As you can see with the exception of Gaucher and Krabbe patient fibroblasts, Lysotracker fluorescence of all the other patient fibroblasts is significantly above WT control. In addition, Lysotracker assay detects lysosomal storage in LSDs caused by a loss in lysosomal transmembrane or accessory proteins, NPC1 and MLIV, and NPC2 respectively. Fluorescence in MLIV, NPC1, NPC2 fibroblasts is over 4 fold elevated above that of WT fibroblasts. This has been confirmed by fluorescence microscopy of lysotracker-loaded patient fibroblasts (Fig. 5.5 B).

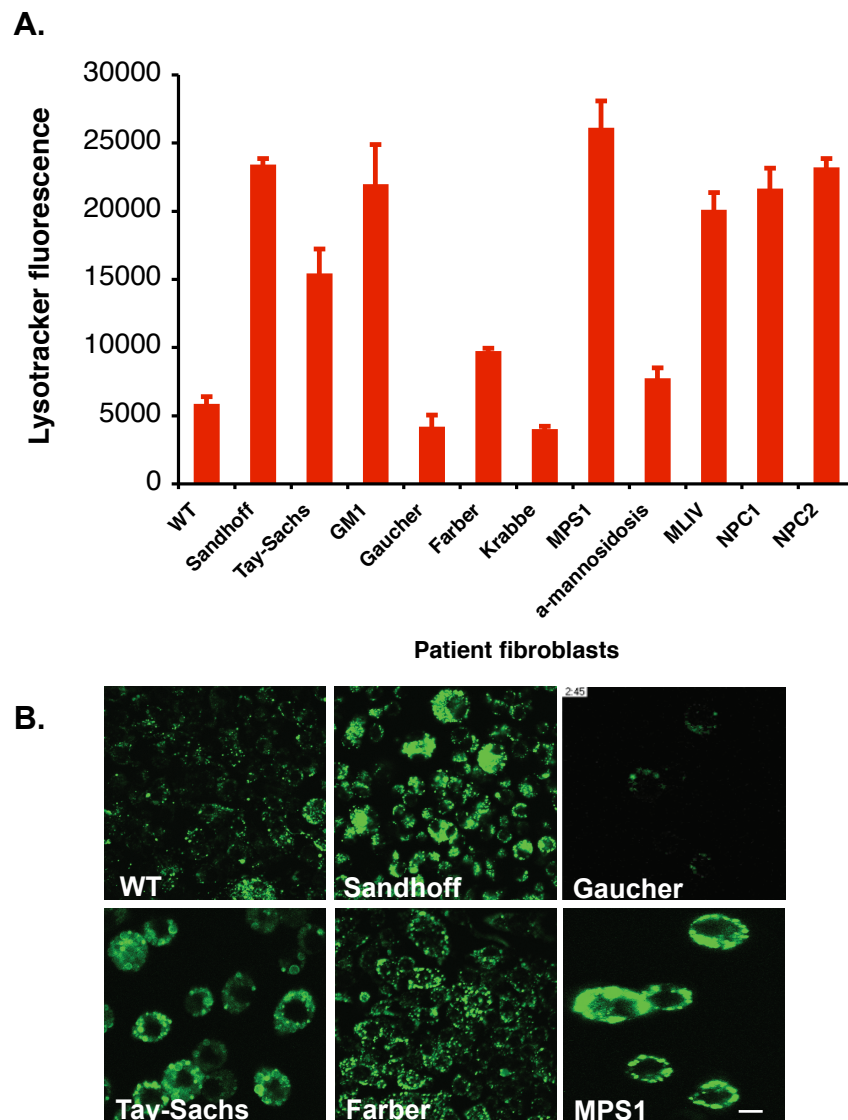


Figure 5.5 Lysotracker plate assay can detect lysosomal expansion and storage in LSD patients. **A.** HF_s were plated into the wells of a 96 well plate and allowed to adhere overnight. Lysotracker probe loading was carried out as described in section 5.2.2. Lysotracker fluorescence was measured using a NOVOSTAR fluorescence plate reader; Lysotracker signal was adjusted to Hoesht nuclear stain. Bars represent mean fluorescence of 3 independent repeats. Error bars represent mean fluorescence $\pm$ S.D. **B.** Fluorescence microscopy images of Lysotracker loaded patient fibroblasts. Scale bar represents 5 μ m.

In this study we validated use of Lysotracker plate assay in several additional lines of LSDs. However, as mentioned in section 5.2.4, Lysotracker fluorescence is normalised to the number of cells plated into wells. Cells were counted using haemocytometer and plated at 20,000 cells per well for 8-10 h to allow the cells to adhere to the surface of the well prior to performing the assay; within this time frame the cells would not divide and double in number.

Lysotracker fluorescence intensity was measured by microplate reader (Fig. 5.6 A). The elevation in Lysotracker signal was confirmed by fluorescence microscopy of Lysotracker loaded patient fibroblasts (Fig. 5.6 B) as before.

We found that Lysotracker fluorescence of MLIV and MPS2 fibroblasts is 1.6 fold elevated above that of WT fibroblasts. The difference in Lysotracker fluorescence is statistically significant ($p < 0.05$) although the difference in Lysotracker signal between WT and MLIV fibroblasts was not as great as a difference detected in the previous study (Fig. 5.5 A). Please note that for this study we have used first generation fluorescence plate reader whereas in previous study we have used a more advanced fluorescence plate reader, which may explain the difference in sensitivity.

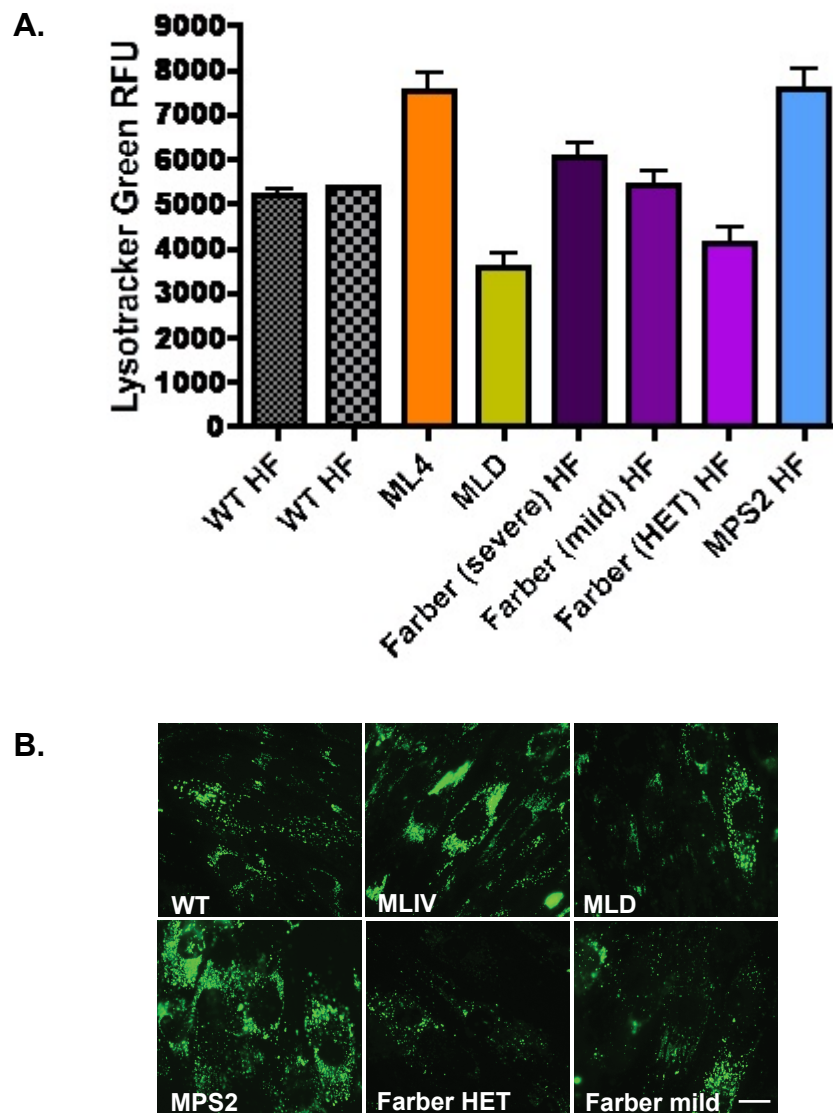


Figure 5.6 Lysotracker plate assay can detect lysosomal expansion and storage in LSD patients. **A.** HFs were plated into the wells of a 96 well plate and allowed to adhere overnight. Lysotracker probe loading was carried out as described in section 5.2.2. Lysotracker fluorescence was measured using a NOVOSTAR fluorescence plate reader; Lysotracker signal was adjusted to Hoesht nuclear stain. Bars represent mean fluorescence of 3 independent repeats. Error bars represent S.D. **B.** Fluorescence microscopy images of Lysotracker loaded patient fibroblasts. Scale bar represents 5 μm .

5.3.6 LysoTracker plate assay can also detect lysosomal storage and expansion in patient lymphoblasts

Human fibroblasts are adherent cells that deposit cell matrix onto the surface on which they are plated and so do not require the surface to be pre-coated. In contrast, patient lymphoblasts are grown in suspension, 96 well plate based assay used for adherent patient fibroblast needed to be modified in order to measure lysosomal storage in patient lymphoblasts using lysotracker probe as shown in Fig. 5.7.

Interaction between streptavidin coating of the well surface and biotin linked to Ab was utilized to capture lymphoblasts onto the plate. Biotin streptavidin interaction is the strongest known non-covalent biological interaction with $K_d \sim 4 \times 10^{-14}$ M [212]. We have used a mouse antibody against human CD19 that specifically recognizes CD19, a surface glycoprotein that is present at all staged of B cell maturation. Therefore all B lymphoblasts would be captured.

Streptavidin coated plates were incubated for 1 h at 4°C to allow for the biotin to interact with streptavidin, we found that extending time of incubation for up to 24 h did not make a difference on cell capture (data not shown). Following the plate preparation, B lymphoblasts were counted and seeded into the wells of the 96 wells plate for 30 min; cell binding was confirmed by light microscopy (Fig. 5.8 (Insert)). LysoTracker loading was carried out using the same conditions as for fibroblasts. We find that LysoTracker signal from NPC and NPB disease cells was higher compared to WT lymphoblasts (Fig. 5.8). In contrast, Krabbe disease lymphoblasts showed reduced lysotracker signal (Fig. 5.8); similarly Krabbe disease fibroblasts show a reduced LysoTracker signal compared to WT fibroblasts (Fig. 5.5 A).

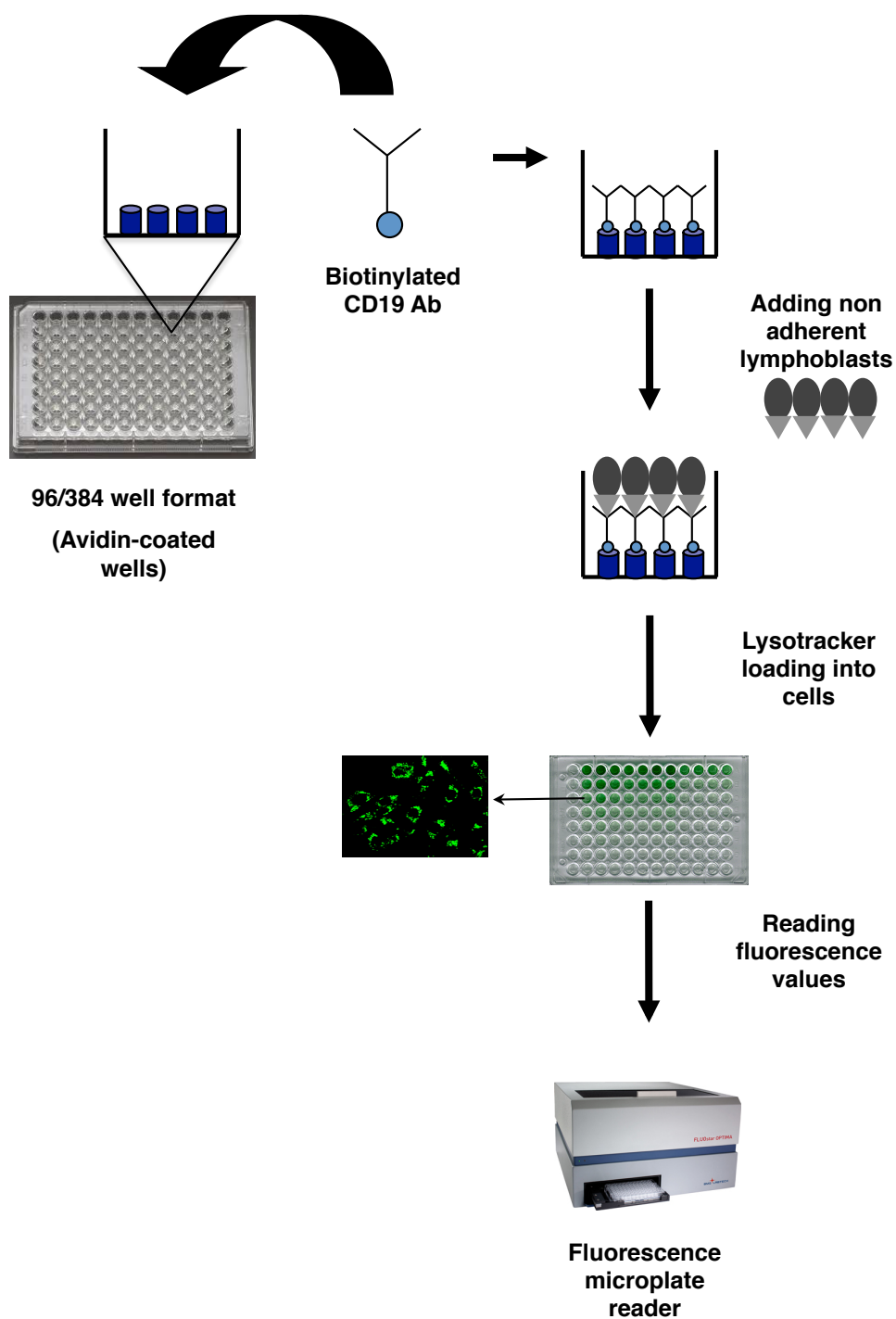


Figure 5.7 Schematic representation of Lysotracker plate assay adjusted for non-adherent cells. B lymphoblasts were captured onto the surface of the plate as described in section 5.2.5. Lysotracker probe loading was performed as described in sections and 5.2.2.

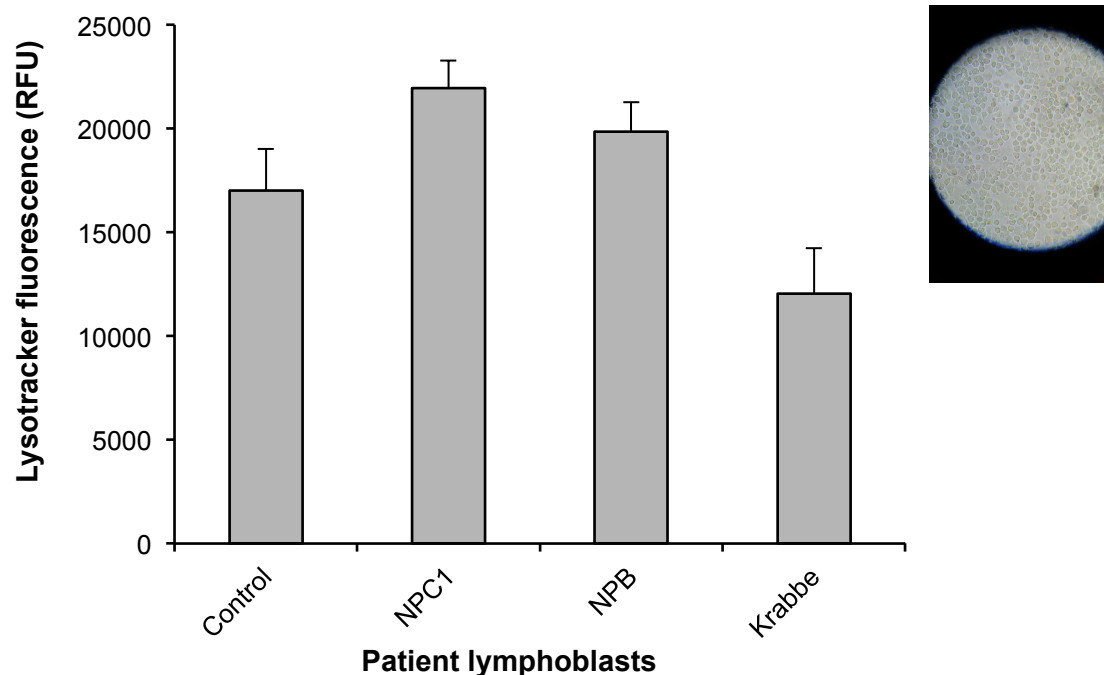


Figure 5.8 Lysotracker plate assay can detect lysosomal expansion and storage in LSD patient lymphoblasts. Lymphoblasts were captured onto the surface of the wells of a 96 well plate as described in section 5.2. Lysotracker probe loading was carried out as described in section 5.2.2. Lysotracker fluorescence was measured using a NOVOSTAR fluorescence plate reader; Lysotracker signal was adjusted to Hoesht nuclear stain. Bars represent mean Lysotracker fluorescence of 3 independent repeats. Error bars represent S.D. Insert is showing a light microscopy image (20x magnification) of a surface of a well of a 96 well plate with lymphoblasts captured on the surface.

5.3.7 Lysotracker assay can be adapted for use in whole blood

Both fibroblasts and lymphoblasts need to be cultured prior to performing the Lysotracker plate assay therefore requiring cell culture facilities. We have adapted the Lysotracker plate assay for use in whole blood so that it can be performed immediately after collecting the blood sample and does not require cell culture. White cell fraction containing B lymphoblasts was isolated from whole blood as described in section 5.2.6.2. After isolating the white cell fraction we have captured the B lymphoblasts onto the surface of a well of a 96 well plate as illustrated in Fig. 5.7. Lysotracker probe loading was carried out as described in section 5.2.2.

Preliminary data of Lysotracker assay performed on the blood of NPC1 patients and healthy controls showed that Lysotracker fluorescence of NPC1 patients is elevated above that of WT control (Fig. 5.9).

We compared the efficacy of a 96 well plate based Lysotracker assay (Fig. 5.9, grey bars) against Lysotracker fluorescence measured by FACS (Fig. 5.9, black bars); FACS assay and data analysis were performed by Dr. Annie Speak (University of Oxford) as described in *te Vruchte et al.* [210]. Measuring Lysotracker fluorescence by FACS gives a more discernible difference in Lysotracker signal of B lymphoblasts isolated from NPC1 patients compared to that of B lymphoblasts isolated from a healthy control; there is also a clearer difference in signal between B lymphoblasts of NPC1 patients. However Lysotracker plate based assay is still able to pick up an elevation in Lysotracker signal between NPC1 patients' lymphoblasts and lymphoblasts from a healthy control. We found that both assay detect an elevation in Lysotracker signal in storage disease lymphoblasts isolated from whole blood.

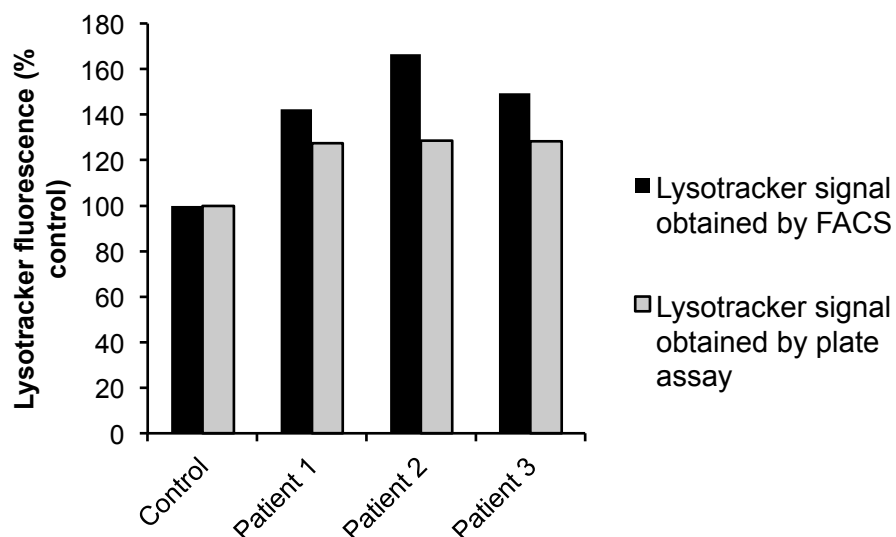


Figure 5.9 Lysotracker assay can detect lysosomal expansion and storage in LSD patient lymphoblasts isolated from whole blood. Lysotracker assay performed as a plate-based assay (grey bars) and as FACS analysis (black bars). Comparison of these two methods has only been performed once.

5.4 DISCUSSION

5.4.1 LysoTracker probe fluorescence is pH dependent

LysoTracker probe fluoresces in subcellular compartments where the pH is below 5.5 – the late endosomes and lysosome (Fig. 5.1). LysoTracker fluorescence does not change with increasing acidity below pH 5.5. Therefore lysoTracker does not act as a pH sensor as there is no linear relationship between fluorescence and pH. However, lysoTracker can be used to indicate the presence of acidic compartments, as it is not fluorescent above pH 5.5. Therefore LSDs with increased lysosomal pH will have a reduced lysoTracker signal if the pH of lysosomes is 5.5 or above as is the case with Krabbe (Fig. 5.5 A) and MLD (Fig. 5.6 A) patient fibroblasts.

5.4.2 LysoTracker probe loading is dependent on volume

Treating WT fibroblasts with U18666A previously shown to cause an increase in lysosomal storage thereby increasing lysosomal volume, also showed an increase in the amount of LysoTracker loaded into a compartment. Therefore LysoTracker probe loading is based on the volume of that compartment. This is a linear relationship: LysoTracker fluorescence intensity increases with increasing compartment volume. This makes LysoTracker a good marker of enlarged lysosomes or an increase in lysosome number, as both will increase the total volume of lysosomes within a cell.

5.4.3 The lysoTracker assay has to be performed in live cells

Contrary to the manufacturer's specifications ([LysoTracker and LysoSensor Probes manual, Molecular probes](#)), LysoTracker fluorescence must be measured in live cells, not post-fixation, if it is to provide an accurate reading of lysosomal volume. Fixing cells with either 4% PFA or methanol reduces LysoTracker signal dramatically. As mentioned in section 5.3.1, LysoTracker is only fluorescent at pH 5.5 or lower and is therefore not fluorescent in the lysosomes of fixed cells. Similarly, lysomotropic drugs such as GPN

permeabilizes the lysosomal compartment, also releasing protons from the lysosome, leading to an increase in pH and a reduction in LysoTracker signal.

In order to overcome the reduction in signal, some groups use increased amounts of LysoTracker and/ or extend the loading time to up to 1 h. However in doing so, LysoTracker probe binds to H^+ ions in the lysosomal lumen, thus increasing the pH of the compartment.

5.4.4 *LysoTracker assay development*

The LysoTracker plate assay was initially used to detect storage in adherent cultured patient fibroblasts from a panel of different LSDs. We have initially normalised LysoTracker signal to Hoechst nuclei staining (section 5.3.5). However upon further investigation we found that in some LSDs the cells become multinucleated therefore normalising the LysoTracker fluorescence to Hoechst signal would yield inaccurate readings. LysoTracker signal is instead normalised to the number of plated cells (section 5.3.5). We have also determined the number of cell that need to be plated per well to give a monolayer on a well surface translating into a uniform loading of LysoTracker probe (section 5.3.4).

We then successfully adapted the assay for use in non-adherent patient lymphoblasts by using streptavidin-biotin interaction to bind an antibody onto the surface of the plate, the antibody can then recognize an antigen on the surface of the lymphoblasts linking the cell to the plate surface (section 5.3.6). This technique is frequently used in Elisa assays. Results obtained from patient lymphoblasts were similar to those obtained for patient fibroblasts.

Finally, we have further developed the assay so that it can be used to detect storage in the white cell fraction isolated from patient whole blood. This would allow for a patients blood to be tested at the hospital where it is collected and avoids the necessity of sending it to a specialized clinic for analysis.

5.4.5 Lysotracker assay works to detect all LSDs

The Lysotracker assay can be used as a broad-spectrum initial screen, capable of detecting almost all LSDs tested. An increase in Lysotracker signal indicates both an increase in volume of individual lysosomes due to lipid storage, and an increase in lysosomal number. The assay can indicate the presence of an LSD, however it does not tell which LSD a patient has. Clinical phenotypes would have to be used to narrow down the type of LSD, and subsequent enzyme assays would have to be performed to confirm the diagnosis.

LSDs with a disruption in lysosomal pH such as Krabbe and MLD can still be picked up by the assay since there is a significant reduction in Lysotracker signal in patient cells compared to healthy controls (Fig. 5.5 A. for Krabbe fibroblasts, Fig. 5.6 A. MLD fibroblasts).

Gaucher disease however cannot be detected by the assay as there is no significant storage in tested patient fibroblasts. This could be due to the presence of Glucocerebrosidase, normally deficient in Gaucher disease, in FCS which is added to the cell growth medium. The Glucocerebrosidase in the medium could therefore act to replace the deficient enzyme and clear stored material, so that it can no longer be detected by the assay. However, if patient blood is assayed, we would expect to see differences in lysotracker signal in Gaucher compared with controls.

The lysotracker assay has been used to monitor NPC1 patients receiving treatment [210]. The assay has been used successfully to monitor a cohort of NPC patients in Europe and the UK on miglustat treatment. As previously mentioned, cholesterol storage is a hallmark of NPC disease [32]. Miglustat has been previously been reported to reduce lysosomal storage in NPC cells and significantly extend the lifespan of the NPC mouse model [45]. Although there is a reduction in lysosomal storage in NPC patients on miglustat treatment compared to pre-treatment levels as detected by

the lysotracker assay, there is no noticeable reduction in filipin staining, indicating that cholesterol is still being stored. Therefore if filipin staining alone would have been used to monitor the efficacy of the treatment, it would not have been able to detect any improvement.

The Lysotracker assay has several advantages over previously proposed assays for a broad screen. The assay is quick to perform; the assay for cultured fibroblasts can be performed within 12 h to allow for cells to adhere. The assay for cultured patient lymphoblasts, together with plate preparation takes only 2 h. It is a very simple assay that only requires a fluorescence plate reader, equipment already present for Elisa assays in most hospitals. The assay is cheap and straightforward to use and so does not require a specialist to perform or analyze.

For many of the lysosomal disorders an assay that would allow pre-symptomatic or as early as possible detection after the symptom onset would be crucial for clinical intervention, and treatment efficacy is often strongly dependent on that.

6 Discussion

6.1 MAIN FINDINGS OF THIS WORK

We have characterized cellular phenotypes in NPC and MLIV patient fibroblasts in Chapter 2 in order to address whether or not these two diseases present with the same cellular phenotypes. Unsurprisingly both diseases have lysosomal storage shown by an increase in LysoTracker fluorescence shown in Chapters 2 and 5, indicating lysosomal storage and expansion of lysosomal volume. However the identity of stored lipids is not the same. We found that unlike in NPC where cholesterol (Fig. 2.1) and sphingomyelin (Fig. 2.2) present are the main storage material, MLIV cells have reduced cholesterol (Fig. 2.1) and sphingomyelin (Fig. 2.2) levels.

In contrast to NPC cells which do not have phospholipid storage, MLIV fibroblasts instead store phospholipids, which has been previously shown biochemically [97]; we confirmed storage of phospholipids in MLIV using a novel fluorescent phospholipid probe LipidTox (Fig. 2.5).

Furthermore, preliminary GSL analysis (Fig. 2.3) revealed that NPC fibroblasts have an elevation in GSL levels, whereas MLIV fibroblasts have an overall reduction in GSLs. This may indicate a defect in GSL biosynthesis in MLIV and certainly warrant further investigation.

We found MLIV fibroblasts to be autofluorescent (Fig. 2.6-7) which limited the use of Green fluorescent probes in this study. In contrast we did not observe autofluorescence in NPC1 fibroblast cell line used in our study.

What we found that defects in endocytosis are present in both NPC and MLIV as demonstrated by trafficking of GM1 ganglioside (Fig. 2.8). As has been previously shown by our group a defect in trafficking in NPC cells is due to a reduction in lysosomal Ca^{2+} levels [45]. When looking at lysosomal Ca^{2+} levels in MLIV fibroblasts

we found that they varied depending on the size of the compartment, with larger lysosomes containing more Ca^{2+} and giving an enhanced Ca^{2+} release in response to stimuli. Enhancing lysosomal Ca^{2+} release in MLIV fibroblasts exacerbated lipid storage phenotype of MLIV fibroblasts (Fig. 4.11). In contrast, partially inhibiting lysosomal Ca^{2+} release ameliorated storage phenotype in MLIV (Fig. 4.13). These findings suggested that lysosomal Ca^{2+} dyshomeostasis is central to the pathology of MLIV disease.

In addition we have both knocked out TRPML1 from healthy cells and reconstituted TRPML1 into MLIV fibroblasts in order to look more closely into the pathogenic cascade of MLIV disease.

6.2 SIMILARITIES BETWEEN MLIV AND NPC

Previous work from our group indicated that there was a reduction in lysosomal Ca^{2+} in NPC disease that leads to reduced NAADP-mediated Ca^{2+} release [45]. The importance of NAADP mediated Ca^{2+} signalling in controlling endocytosis has been demonstrated by our lab [45, 180] and Galione and co-workers [156]. As has been previously suggested [155], if TRPML1 is the lysosomal Ca^{2+} channel that responds to NAADP both NPC and MLIV diseases would be similar in terms of their cellular phenotypes caused by a loss of NAADP mediated Ca^{2+} signaling. Further evidence has recently come to light from the group of Dr. H. Xu [96] indicating that in NPC disease cells the activity of TRPML1 is inhibited by the accumulation of sphingomyelin within the lysosomal compartment. This defect in Ca^{2+} release caused by loss of function of TRPML1 may explain why there are similarities in endocytosis defects in both NPC and MLIV diseases (Fig. 2.8). As such, it would appear on the surface that NPC1 and MLIV cells are mechanistically similarly affected by the absence of lysosomal Ca^{2+} release and that therapies that work for one disease might work for another.

6.3 WHY WAS TRPML1 CONSIDERED TO BE NAADP RECEPTOR?

Consistent with speculation by other groups, we discovered that the lysosomal Ca^{2+} content in MLIV cells is enlarged (Fig. 4.1). This potentially could have been caused by an absence of NAADP mediated signaling within MLIV cells as a direct result of the absence of TRPML1. This multivalent ion channel has been postulated to be the NAADP receptor for a number of years, however no group has ever utilized NAADP in TRPML1 null cells in order to determine whether this is truly the case. Based on our findings in Chapter 4, this was an important experiment to perform to determine whether it was the cause of the elevated Ca^{2+} levels. Interestingly, we did not find the expected reduction in lysosomal Ca^{2+} release but instead found an elevation in NAADP mediated Ca^{2+} release (Fig. 4.4), the opposite of NPC disease cells. This defect could be corrected by reconstituting TRPML1 into MLIV human patient fibroblasts (Fig. 4.8 D) indicating that it is directly caused by the absence of functional TRPML1 rather than any other potential change in gene function (a possibility in a human cell line). As such, we can say with conviction that TRPML1 is not the NAADP receptor, as it remains active, in fact it is more active, in these TRPML1 null cells.

It is plausible that TRPML1 may be regulating the activity of TPC receptors such that in absence of TRPML1, TPC mediated Ca^{2+} release is no longer controlled. It would be interesting to investigate whether TPC overexpressing cells have cellular phenotype similarities to TRPML1 null cell as both have lost regulation of NAADP signalling. It could also act as a pH regulated Ca^{2+} leak channel, which may explain the elevated Ca^{2+} content in TRPML1 null lysosome [40].

Enhanced Ca^{2+} release causes continuous fusion between late endosomal and lysosomal compartments overfilling the hybrid organelle with Ca^{2+} that may explain the presence of expanded lysosomes with an elevated Ca^{2+} content. Continuous Ca^{2+} release causes fusion, preventing resequestration of Ca^{2+} hence inhibiting the

separation of two organelles. The Ca^{2+} defect and mistrafficking are the first phenotypes to emerge following acute knockdown of TRPML1 protein function indicating that alterations in lysosomal Ca^{2+} signalling is central to MLIV disease pathology.

Based on our findings in this thesis, we have conducted the most thorough analysis and comparison of lysosomal phenotypes in NPC1 and MLIV patient fibroblasts in Chapter 2 as well as TRPML1 silenced RAW macrophages in Chapter 3. It is clear from our work that these two diseases are fundamentally opposite to one another. TRPML1 null fibroblasts present with elevated lysosomal Ca^{2+} levels, accelerated defects in endocytosis and fusion, accumulation of lipofuscin and phospholipids. The acceleration in endocytosis appears to be directly caused by the elevated lysosomal Ca^{2+} release triggered by NAADP, indicating that TRPML1 is not the NAADP receptor and in fact lysosomal Ca^{2+} release (not tested using NAADP-AM in [94]) triggered by NAADP is elevated in MLIV cells. NPC cells in comparison are the direct opposite as previously published [45]. Furthermore, MLIV cells do not present with any of the lysosomal lipid storage phenotypes observed in NPC (cholesterol, sphingomyelin, LBPA, gangliosides GM1 and GM2). Fundamentally, the most important difference is the Ca^{2+} homeostasis defects between both diseases. However, the primary example used in [94] to indicate an MLIV phenotype in NPC cells was the presence of autofluorescence. We have analysed different types of NPC cells (fibroblasts, glia, CHO cells) using fluorescence microscopy and histochemistry approaches to identify lipofuscin and do not find any in NPC, which is perhaps why it had never been reported prior to Shen *et al.* [96]. As described in the discussion in Chapter 2, it is likely that this result is an artifact of the experimental conditions used by Shen *et al.* [96]. In addition both diseases have very different clinical presentations. MLIV patients present with

mild neurological symptoms, poor muscle tone, retinopathies and seizures [83, 150]. Patients typically live well into adulthood. In contrast, NPC1 disease (particularly infantile and juvenile forms) present with more aggressive neurological deterioration and visceral organ involvement [125]. Typically patients do not survive beyond their 20s.

In conclusion, it is evident that at the cellular and clinical levels, there is no similarity between these diseases and it is vitally important that care is taken when undertaking studies into the lysosomal Ca^{2+} and endocytic properties of these diseases.

6.4 WHAT CAN WE CONCLUDE FROM THIS STUDY?

Deregulation in NAADP mediated Ca^{2+} signalling has provided us with a potential therapeutic agent, Ned19. While shown to be effective in MLIV at lowering lysosomal storage and correcting lipid trafficking out of the lysosome, it would probably never become a viable therapy due to strong inhibition of NAADP receptor and adverse effect in terms of lowering/completely inhibiting Ca^{2+} release and causing NPC-like phenotypes. A less specific inhibitor of TPC channels, PPADS, may not be a suitable therapy either, as it adversely affects purinergic P2X receptors. However, nifedipine, an L-type Ca^{2+} channel inhibitor was shown to be a weaker inhibitor of TPC receptor (Gruzka and Lloyd Evans unpublished) and may provide an alternative to Ned19. Nifedipine is currently approved for treatment of hypertension and cluster headaches; moreover it has been shown to cross the blood-brain barrier. Another possibility of inhibiting excessive Ca^{2+} release in MLIV would be to reduce the amount of NAADP synthesis and consequently reduce the amount of Ca^{2+} released, lowering the rate of late endosomal lysosomal fusion and allowing the two compartments to separate. Flavonoids have been demonstrated to act as inhibitors of CD38, an enzyme implicated in NAADP, synthesis. If successful in ameliorating MLIV phenotypes they

would provide an over-the-counter therapy, one that drug companies likely would not undertake trials in such a rare disease as MLIV.

If a successful therapy for MLIV was to be discovered, the treatment efficacy could be followed using LipidTox, a new biomarker we have identified for MLIV. However the use of this biomarker is complicated by the necessity of culturing patient cells and having the facilities to do so. We have also developed a plate-based assay, which is a routine method for monitoring lysosomal storage levels. It had already been shown to be useful for monitoring improvement in NPC patients in a flow cytometry based assay ([159, 210]). We have adapted the method to be based on plate assay for use in fibroblasts in B lymphoblasts purified from whole blood, which would avoid the need for cell culturing facilities.

In conclusion, in common with NPC, MLIV is a disease of dysregulated lysosomal Ca^{2+} homeostasis. However, the mechanism and consequences of Ca^{2+} dysregulation in these two lysosomal diseases are opposite to one another. The studies conducted here therefore shed light on fundamental cellular aspects of pathogenesis and will aid the development of more effective therapies for treating these devastating diseases in the future.

7 References

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