Inhibition of TRPML1 by Lysosomal Adenosine Accumulation Involved in ADA Diseases

by

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Submitted in partial fulfilment of requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
March 2015

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Abstract

Adenosine plays important roles in the cell, and impaired adenosine homeostasis has been associated with numerous human diseases. Lysosomes are referred as the cellular recycling centers which generate adenosine by breaking down lysosomal ATP. Recent studies have suggested that lysosomal adenosine accumulation causes phenotypes resemble Mucolipidosis type IV disease that is caused by mutations in TRPML1, a lysosomal ion channel. In this study, we have demonstrated that lysosomal adenosine is elevated by inhibiting or deleting adenosine deaminase (ADA) which degenerates adenosine. Lysosomal adenosine accumulation inhibits TRPML1 activity, which is rescued by overexpressing ENT3, the lysosomal membrane-bound adenosine transporter. Moreover, either ADA inhibition or ADA deficiency results in lysosomal dysfunction, and this is rescued by TRPML1 or ENT3 expression. Our data suggest that lysosomal adenosine accumulation inhibits TRPML1, and subsequently impairs lysosome function. This finding could lead to a new therapeutic strategy for human disorders caused by mutations in ADA and ENT3.

List of Abbreviations Used

5'-NT	5’-nucleotidase
$A_1/A_{2A}/A_{2B}/A_3$	adenosine $A_1/A_{2A}/A_{2B}/A_3$ receptor
ADA	adenosine deaminase
ADP	adenosine 5’-diphosphate
AK	adenosine kinase
amidoPRT	amidophosphoribosyltransferase
AMP	adenosine 5’-monophosphate
ATP	adenosine 5’-triphospate
Baf-A1	bafilomicyn A1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphates
CNS	central nervous system
CNT	concentrative nucleoside transporter
dADP	deoxyadenosine 5’-diphosphate
dATP	deoxyadenosine 5’-triphosphate
DMEM	dulbecco's modified eagle's medium
EGTA	ethylene glycol tetra-acetic acid
EHNA	erythro-9-(2-hydroxy-3-nonyl)-adenine

ENT	equilibrative nucleoside transporter
Epac1	exchange protein activated by cAMP 1
ER	endoplasmic reticulum
FHC	familial histiocytosis syndrome
G437R	mutation on ENT3 changing glycine 437 to arginine
GECO-ML1	The genetically encoded Ca^{2+} indicators for optical imaging fused with ML1 channel
GPN	glycyl-L-phenylalanyl-2-naphthylamide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
LMP	lysosomal membrane protein
LSD	lysosomal storage disease
ML1	transient receptor potential cation channel, mucolipin subfamily, member 1
ML1-4A	mutation on ML1, L15L/AA-L577L/AA
ML1-DDKK	mutation on ML1, D471K/D472K
ML-IV	type IV mucolipidosis
ML-SA1	mucolipin synthetic agonist 1
ML-SI1-3	mucolipin synthetic inhibitor 1-3
NK	Nature Killer
NPA/B/C	Niemann-Pick disease type A/B/C

PHID	pigmented hypertrichotic dermatosis with insulin-dependent diabetes
$PI_{(3,5)}P_2$	phosphatidylinositol 3, 5- bisphosphate
$PI_{(4,5)}P_2$	phosphatidylinositol 4, 5- bisphosphate
PKA	protein kinases A
PM	plasma membrane
SAHH	S-adenosylhomocysteine hydrolase
SCID	severe combined immune disease
SMPD1	sphingomyelin phosphodiesterase 1
SHML	sinus histiocytosis with massive lymphadenopathy
SLC	solute carrier
TG	thapsigargin
TM	transmembrane-spanning proteins
TPC	two pore channel
TRP	transient receptor potential cation
TRPM2	transient receptor potential cation channel, melastatin subfamily, member 2
WT	wild type

Acknowledgements

I would like to thank my supervisor, Dr. Xianping Dong for his constant guidance, encouragement and support during my graduate study. My research projects would not have been possible without his tremendous help and valued input.

I would also like to thank my supervisory committee members, Dr. Robert Rose, Dr. Stefan Krueger and Dr. Alexander Quinn for their assistance and suggestions throughout my study.

Thanks to all members of the Dong lab, especially, Qi Cao, Xi Zoë Zhong, Kevin Zhao, Gaofeng Dong and Haichuan Yu for their valued input.

Thanks to Department of Physiology & Biophysics at Dalhousie University for providing an enjoyable place to stay.

Thanks to my family and friends for the comfort they provided during my hard time.

Chapter 1 Introduction

1.1 Overview

Lysosomes are important organelles that digest and dispose of unwanted protein, DNA, RNA, carbohydrates, and lipids in the cell. Dysfunction of lysosomes has been associated in numerous human diseases called lysosomal storage diseases (LSDs) which are characterized by an accumulation of waste products in the lysosomes, resulting in the formation of large intracellular vacuoles or lysosomes and defect in the clearance of apoptotic cells due to dysfunction of lysosomes (Tardy, Andrieu-Abadie et al. 2004, Saftig and SpringerLink (Online service) 2005). However, the molecular mechanisms underlying these diseases remain largely unknown. Mucolipidosis type IV (ML-IV) is a type of LSD that is caused by the loss of function of transient receptor potential cation channel, mucolipin subfamily member 1 (ML1). Enlarged vacuoles or lysosomes and defective apoptotic cell clearance are observed in ML-IV cells (Venkatachalam, Long et al. 2008).

Adenosine is an important molecule in the cell. Impaired adenosine homeostasis results in numerous human diseases (Giblett, Anderson et al. 1972, Apasov, Blackburn et al. 2001, Blackburn 2003, Blackburn and Kellems 2005, Mohsenin and Blackburn 2006, Aiuti, Cattaneo et al. 2009, Boison, Chen et al. 2010). Particularly, lysosomal adenosine accumulation causes enlarged vacuoles or lysosomes and defective apoptotic cell clearance (Hsu, Lin et al. 2012). These phenotypes resemble those of patients with mutations in ML1, suggesting that lysosomal adenosine accumulation may impair ML1 and then lead to subsequent lysosomal dysfunction. In this study, we have demonstrated that lysosomal

adenosine is elevated by inhibiting or deleting ADA, the enzyme to degenerate adenosine. We have also shown lysosomal adenosine accumulation inhibits ML1 activity, which is rescued by overexpressing ENT3, the adenosine transporter situated in lysosome membrane. Moreover, either ADA inhibition or ADA deficiency results in lysosomal dysfunction, which is rescued by ML1 or ENT3 expression. Our data suggest that lysosomal adenosine accumulation inhibits ML1, and subsequently impairs lysosome function. This study provides a molecular mechanism to explain the cellular phenotypes of cells with ADA and ENT3 deficiency. This finding could lead to a new therapeutic strategy for a number of human disorders caused by mutations in ADA and ENT3.

1.2 An Overview of the Lysosome

Fifty years ago, a tissue fraction study was conducted in order to characterize hepatic glucose 6-phospate by Dr. Christian de Duve. In addition to his original aim, unexpected discovery of the abnormal activity and distribution feature of an acid phosphatase led to the discovery of a new membrane-bound organelle (Appelmans, Wattiaux et al. 1955). Since then, more enzymes related to this organelle, mostly hydrolytic enzymes with acidic pH optima for activity, were identified. This strongly indicated the digestive role of the new organelle. As a result, the organelle were referred as ‘lysosome’, which was derived from the Greek word ‘digestive body’ (de Duve 2005). This was originally a biochemical concept, but was quickly examined by morphologists. Using electron microscopy, morphologists distinguished peroxisomes from lysosomes (Baudhuin, Beaufay et al. 1965), and confirmed that lysosomes consisted of up to 5% of the total intracellular volume, with heterogeneous

size and morphology (Novikoff, Beaufay et al. 1956).

Generally, lysosomes are membrane-bound acidic organelles which are widely distributed in almost all types of cells. They serve as the cell's recycling centers in which unwanted materials are broken down into building bricks by acidic hydrolases. They are differentiated from other organelles by many specific enzymes in the lumen and transmembrane proteins (Luzio, Pryor et al. 2007). In the lysosome lumen, more than fifty acid hydrolases actively break the chemical bonds to catalyze the hydrosis of different substrates. Most of these substrates are obsolete molecules, engulf viruses, and bacteria (Settembre, Fraldi et al. 2013). The majority of these lysosomal enzymes worked with acidic pH optima (Mellman, Fuchs et al. 1986).

The acidic lysosomal pH was maintained by a number of lysosome membrane proteins (LMPs), including V-type ATPase, ion channels, and transporters. They build up an acidic environment to ensure lysosomal enzymes to function properly. However, the identity of some players in this pH building processes still remains uncertain (Mindell 2012). More than 25 types of LMPs have been discovered and new members are still being identified (Eskelinen, Tanaka et al. 2003, Lubke, Lobel et al. 2009, Saftig and Klumperman 2009). Apart from the roles in maintaining a proton gradient, they also participate in regulating other ions' gradients across lysosomal membrane. For example, lysosomes are demonstrated to be an important Ca^{2+} pool in cells, and the LMPs play a critical role in maintaining lysosomal Ca^{2+} homeostasis (Lloyd-Evans and Platt 2011). Moreover, due to lysosome's roles in molecule digestion and recycling, substrates or metabolites have to be transported

across the lysosomal membrane efficiently. LMPs are responsible for the delivery and transport of proteins and other substances into and out of lysosomes.

Defects in lysosomal enzymes and lysosomal transmembrane proteins results in a group of disorders called lysosome storage diseases (LSDs). They are characterized by the accumulation of waste products in lysosomes, so called lysosomal storage, which ultimately result in cell dysfunction and clinical abnormalities. Although enzyme replacement is a promising therapeutic approach for enzymatic LSDs, promoting lysosomal membrane trafficking could be another treatment for both enzymatic LSDs and LSDs associated with dysfunctional lysosome membrane proteins (Medina, Fraldi et al. 2011, Shen, Wang et al. 2012, Chen, Keller et al. 2014, Samie and Xu 2014).

1.2.1 Lysosomes in Molecular Recycling

Almost all kinds of unwanted materials can be digested in lysosomes. For example, extracellular soluble molecules, waste proteins, cell debris and obsolete cytoplasmic organelles are delivered to lysosomes where they are turned into their building bricks to reparticipate in biological processes (Appelqvist, Waster et al. 2013). In the immune system, engulfed pathogens, such as viruses and bacteria, are digested by lysosomal enzymes and the products are provided for major histocompatibility complexes (MHCs) for antigen processing (Geuze 1998). After macromolecules are degraded in lysosomes, basic building bricks are released to cytoplasm by transporters situated on lysosomal membranes or to extracellular environment through lysosomal exocytosis.

Nucleic acid is one of the important molecules which are recycled in lysosomes, and

this salvage pathway serves as the major resource of nucleoside in cells. In this pathway, nucleic acids from wasted materials are degraded into nucleotides first, and then dephosphorylated into basic nucleosides through 5'-nucleotidases (5'-NTs) in lysosomes (Arsenis, Gordon et al. 1970, Zimmermann 1992). Although a portion of the salvaged nucleosides stay in lysosomes, either entering further degeneration reactions or building up nucleoside storage for future mobilization, most nucleosides generated from this salvage pathway are released to cytosol via nucleoside transporters on the lysosomal membrane to return to biological processes (King, Ackley et al. 2006).

1.2.2 Lysosomal Membrane Trafficking

Worn out organelles or substances are actively delivered to lysosomes for recycling through various pathways. Extracellular macromolecules and pathogens, they are engulfed and delivered to lysosomes through endocytosis and phagocytosis, respectively, and then broken down into basic molecules. During these processes, extracellular materials are internalized into endosomes and phagosomes (Mellman 1996), which then fuse with lysosomes and transfer their contents into lysosomes for further degradation (Bright, Gratian et al. 2005). To recycle intracellular worn out organelles, a double-layer membrane-bound structure called autophagosome is formed (Mizushima, Ohsumi et al. 2002) to process the obsolete components. The autophagosome is then directly fused with lysosomes. During the fusion event, only its outer membrane fuses with lysosomes. Its inner membrane and contents are both degenerated (Luzio, Pryor et al. 2007). Therefore, lysosomes are the critical organelles involved in the endocytic, phagocytic, and autophagic membrane-trafficking pathways.

Recently, researchers suggest that lysosomes also serve as secretory vesicles to release their contents to extracellular space. This process is called lysosomal exocytosis, which is widely observed in immune synapse and during bone formation (Andrews 2000, Blott and Griffiths 2002). Lysosome fusion with the plasma membrane (PM) also provides membrane patch to help repair the PM after cell damage (Reddy, Caler et al. 2001, McNeil and Kirchhausen 2005, Luzio, Pryor et al. 2007).

1.2.3 Lysosomal Ca^{2+} Homeostasis

Mounting evidence suggests that lysosome itself is an important Ca^{2+} reservoir in the cell, containing 0.5~0.6 mM Ca^{2+} which is about 5000 folder higher than that in the cytoplasm (Christensen, Myers et al. 2002). Currently, the molecular mechanism for lysosomal Ca^{2+} accumulation is largely unknown. Earlier studies suggest that the building of lysosomal Ca^{2+} content partially relies on the endocytosis, but it only contributes 10% of the total Ca^{2+} content in lysosomes (Gerasimenko, Tepikin et al. 1998). Recent studies suggest that lysosomal Ca^{2+} homeostasis is regulated by a series of LMPs including membrane transporters and ion channels (Morgan, Platt et al. 2011). Some LMPs are responsible for lysosomal Ca^{2+} uptake, whereas some are important for lysosomal Ca^{2+} release. For instance, a putative Ca^{2+}/H^{+} exchanger has been suggested to take Ca^{2+} up into lysosomes. Some Ca^{2+} transporter candidates for yeast vacuole, the mammalian lysosome counterpart, have also been reported (Peters, Bayer et al. 2001), although such Ca^{2+} transporters have not been identified in mammalian lysosomes yet (Patel and Docampo 2010). On the other hand, several Ca^{2+} channels on the lysosomal membranes, including ML1 (Shen, Wang et al. 2012,

Cheng, Zhang et al. 2014) and P_2X_4 (Huang, Zou et al. 2014), mediate lysosomal Ca^{2+} release. Recently, two pore channels 1 and 2 (TPC1/TPC2) (Zhu, Ma et al. 2010), and transient receptor potential cation channel, melastatin subfamily, member 2 (TRPM2) (Lange, Yamamoto et al. 2009) are also suggested to be Ca^{2+} release channels on lysosomal membranes. However, the exact roles of these lysosomal Ca^{2+} channels remain controversial and have to be further elucidated (Fig. 1).

Lysosomal Ca^{2+} homeostasis is important for lysosome functions, particularly lysosomal membrane trafficking (Peters and Mayer 1998, Pryor, Mullock et al. 2000, Piper and Luzio 2004, Hay 2007, Luzio, Bright et al. 2007, Cheng, Shen et al. 2010, Lloyd-Evans and Platt 2011, Morgan, Platt et al. 2011, Pittman 2011). It has been suggested that the lysosome itself provides the main Ca^{2+} source to initiate both lysosome fusion and fission, although the Ca^{2+} release channels and Ca^{2+} sensor proteins involved in the fusion and fission remain elusive (Pryor, Mullock et al. 2000, Morgan, Platt et al. 2011). For example, the fusion of lysosomes with other organelles including endosomes, phagosomes, and autophagosomes, is critically Ca^{2+} dependent (Pryor, Mullock et al. 2000, Stockinger, Zhang et al. 2006). Consistent with this, deficiency in these Ca^{2+} release channels and Ca^{2+} sensitive proteins has been associated with a number of LSDs (Lloyd-Evans and Platt 2011).

1.2.4 Lysosomal Storage Diseases

The LSDs are a group of approximately 50 disorders that result from defects in lysosomal function, and new types continue to be identified. They are inherited diseases characterized by the accumulation of waste products in lysosomes (so called lysosomal storage), which

ultimately results in cell dysfunction and clinical abnormalities. Although each types of LSDs is rare individually, when taken together they are estimated to affect about 1 in 7,700 births, making them relatively common and significant health problems. LSDs affect most body organs, but severe neurodegeneration and motor deterioration are the prominent pathological hallmark of most LSDs.

In general, LSDs are subdivided into two groups. One subgroup of LSDs is caused by a deficiency of specific enzymes that are normally required for the breakdown of certain complex macromolecules including carbohydrates, lipids, proteins, and nucleic acids. The other subgroup of LSDs result from some defective LMPs which are essential for creating optimal environment for lysosomal enzymes, transporting metabolites, or lysosomal membrane trafficking (Platt, Boland et al. 2012).

1.3 ML1 Channels

1.3.1 An Overview of ML1

Transient receptor potential (TRP) proteins are a large cation channel superfamily, comprising 28 mammalian members which are classified into 7 subfamilies. They have diverse tissue distributions, subcellular localizations, physiological functions and regulatory mechanisms (Venkatachalam and Montell 2007). Among them, mucolipin family proteins, including 3 members (ML1,2,3) that share around 75% amino acid similarities, have been relatively poorly characterized (Puertollano and Kiselyov 2009). They are structurally similar to other TRP channels which contain 6 putative transmembrane spanning segments (S1-S6), a pore forming loop between S5 and S6, and intracellular located -NH2 and -COOH

termini. However, their cytosolic tails are shorter than those in other TRP channels (Cheng, Zhang et al. 2014) (Fig. 2).

ML1 channel is encoded by the MCOLN1 gene localized on chromosome 19 in human beings (Bargal, Avidan et al. 2000, Bassi, Manzoni et al. 2000). It is the earliest identified and best studied member in the mucolipin family. ML1 expressed in all types of mouse tissues with relatively high level of mRNA in brain, kidney, heart, spleen and liver (Falardeau, Kennedy et al. 2002, Samie, Grimm et al. 2009). It is highly abundant on lysosomal membranes, although a minority can also be found at the PM with low activity (Thompson, Schaheen et al. 2007, Dong, Shen et al. 2010). This localization on lysosomes is navigated by the two di-leucine motifs separately located in the N-terminal and C-terminal cytosolic tails (Pryor, Reimann et al. 2006). Unlike other TRP channels, ML1 possesses a large loop with four N-glycosylation sites on the luminal side of lysosomes. On the N-terminal side, ML1 bears two lipid binding sites. Like other TRP channels, ML1 is a non-selective channel permeable to a variety of cations, including Na^+, Ca^{2+}, K^+, Fe^{2+}, Zn^{2+}, and Mg^{2+} (Dong, Cheng et al. 2008, Cheng, Shen et al. 2010).

Evidence suggests that ML1 activity is regulated by the binding of two lipids. Phosphatidylinositol 3,5-bisphosphate [$PI_{(3,5)}P_2$], the major form of phosphoinositide on the lysosomal membrane, can bind to the Arg61 and Lys62 sites to activate ML1. In contrast, phosphatidylinositol 4,5- bisphosphate [$PI_{(4,5)}P_2$], the predominant phosphoionositide on the PM, binds to the Arg42/Arg43/Arg44 sites to inhibit ML1. Since $PI_{(3,5)}P_2$ is enriched in the lysosomes but $PI_{(4,5)}P_2$ is predominant on the PM, compartment-specific phosphoinositides

may provide an economical and efficient means to regulate ML1 channel activity in a compartment-specific manner (Dong, Shen et al. 2010). In addition, a number of small molecules, including SF-22 (renamed MK6-83) and SF-51 (renamed mucolipin synthetic agonist 1 (ML-SA1)), that increase ML1 activity have also been identified by high throughput screening (Grimm, Jors et al. 2010, Shen, Wang et al. 2012). Meanwhile, some other small molecules, such as mucolipin synthetic inhibitor 1-3 (ML-SI1-3) (Cheng, Zhang et al. 2014), have been described to inhibit ML1 activity. These small molecules are powerful tools to study ML1 function, and could provide potential treatment for some lysosomal storage diseases.

Although a role of ML1 in lysosomal Ca^{2+} homeostasis, lysosomal pH regulation, and lysosomal membrane trafficking has been suggested (Thompson, Schaheen et al. 2007, Cheng, Shen et al. 2010, Shen, Wang et al. 2012), the biological function of ML1 remains a matter of debate. Some studies suggest that loss of ML1 results in the elevation of lysosomal Ca^{2+} and lysosomal alkalization (Bach, Chen et al. 1999, Vergarajauregui, Connelly et al. 2008, Wong, Li et al. 2012) but some other studies suggest that lysosomal Ca^{2+} and pH are not altered by ML1 deletion (Venkatachalam, Wong et al. 2014). In spite of the controversy, it is consistently reported that loss of ML1 results in enlarged lysosomes by using cells from a variety of organisms. Current hypothesis is, by releasing Ca^{2+}, ML1 activation triggers lysosomal fusion and/or fission processes. On one hand, loss of ML1 causes defective fusion of autophagic vacuoles with lysosome in either mammalian cells or *Drosophila* cells, leading to autophagic vacuoles accumulation (Vergarajauregui, Connelly et al. 2008, Wong, Li et al.

2012). On the other hand, ML1 mediated lysosomal Ca^{2+} release also regulates fission event in lysosomal reformation from hybrid organelles (Pryor, Mullock et al. 2000). In addition, ML1 has also been suggested to promote lysosomal exocytosis (Medina, Fraldi et al. 2011, Samie and Xu 2014), and thereby plays a significant role in phagocytosis and membrane repair following cell damage (LaPlante, Sun et al. 2006, Sardiello, Palmieri et al. 2009, LaPlante, Falardeau et al. 2011, Cheng, Zhang et al. 2014). As a lysosomal channels involve in membrane trafficking, ML1 is also required for clearance of apoptotic cells by immune cells (Venkatachalam, Long et al. 2008).

1.3.2 Mucolipidosis type IV and Other ML1 Related Human Disorders

Dysfunction of ML1 results in ML-IV, a type of LSD which was first reported in the year of 1974 (Kogot-Levin, Zeigler et al. 2009, Platt, Boland et al. 2012, Parkinson, Baines et al. 2014). Since 2000, more than 20 types of mutations of ML1 leading to ML-IV have been identified (Berman, Livni et al. 1974, Sun, Goldin et al. 2000), and most of them lead to severe loss of channel function (Dong, Cheng et al. 2008). In general, ML-IV is a type of autosomal recessive developmental diseases with disturbance in brain, eye and gastric functions. Patients begin to display severe growth and psychomotor retardation from the first or the second year and remain on this developmental level. Therefore, most patients are not able to speak or walk independently (Riedel, Zwaan et al. 1985, Frei, Patronas et al. 1998, Schiffmann, Dwyer et al. 1998, Sun, Goldin et al. 2000). Currently, no effective cure has been developed for ML-IV. At the cellular level, loss of ML1 activity results in accumulation of the substrates in enlarged vacuoles. Biochemical investigations revealed that the abnormal

storage in ML-IV cells is attributed to the trafficking defects but not the loss of lysosomal enzyme function (Puertollano and Kiselyov 2009). Lysosomes from ML-IV cells also display Ca^{2+} overload and elevated pH, although some other studies do not agree with it (Bach, Chen et al. 1999, Kogot-Levin, Zeigler et al. 2009, Venkatachalam, Wong et al. 2014).

Impaired ML1 has also been associated with certain LSDs with defective activities of specific lysosomal enzymes. For example, ML1-mediated Ca^{2+} flux was greatly impaired in a group of inherited severe metabolic disorders with sphingomyelin accumulation in lysosomes, known as Niemman-Pick (NP) diseases including type A (NPA), type B (NPB) and type C (NPC). NPA and NPB are caused by mutations in the sphingomyelin phosphodiesterase 1 (SMPD1) gene that produces an enzyme called acid sphingomyelinase (da Veiga Pereira, Desnick et al. 1991, Pavlu-Pereira, Asfaw et al. 2005). This enzyme is responsible for the conversion of sphingomyelin into another type of lipid called ceramide. Mutations in SMPD1 lead to a shortage of sphingomyelinase, which results in reduced breakdown of sphingomyelin, causing this fat to accumulate in lysosomes. NPC is caused by mutations in NPC1 or NPC2 gene (Carstea, Morris et al. 1997, Verot, Chikh et al. 2007). The proteins produced from these genes are involved in the movement of lipids (cholesterol and other lipids) across lysosomal membrane. Mutations in these genes lead to lysosomal accumulation of cholesterol, sphingomyelin, and other lipids, eventually causing cell dysfunction and cell death. Sphingomyelin accumulation in the NP diseases inhibits ML1 channel activity, which subsequently leads to membrane trafficking defect and lysosomal

storage (Shen, Wang et al. 2012). Overexpression of ML1 corrects the excessive lysosomal storage in the NPC cells (Parkinson, Baines et al. 2014).

Defects in lysosomal Ca^{2+} release and membrane trafficking have been associated with not only LSDs (Dong, Cheng et al. 2008, Lloyd-Evans, Morgan et al. 2008, Kiselyov, Yamaguchi et al. 2010, Lloyd-Evans and Platt 2011, Shen, Wang et al. 2012), but also classical forms of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (Jeyakumar, Dwek et al. 2005, Pan, Kondo et al. 2008, Zhang, Sheng et al. 2009, Coen, Flannagan et al. 2012, Funk and Kuret 2012, Wang, Chan et al. 2013, Neefjes and van der Kant 2014). Interestingly, ML1 has been implicated in Alzheimer's disease by promoting sphingomyelin and Aβ clearance from lysosomes (Bae, Patel et al. 2014).

Recent evidence also suggests that ML1 mediated Ca^{2+} flux plays an important role in preventing muscular dystrophy (Cheng, Zhang et al. 2014) and large particle phagocytosis (Samie, Wang et al. 2013).

1.4 Adenosine in Lysosomes

1.4.1 An Overview of Adenosine

Nucleosides are critical molecules in human physiology, playing important roles in nucleotides formation, signaling transduction, neurotransmission and energy transfer. The basic structure shared by the nucleosides is a nitrogenous base attached to a pentose sugar. When the nucleosides are phosphorylated on the pentose sugar with one or more phosphate groups, they turn into nucleotides which build the skeleton of nuclei acids. There are

generally two different classes of nucleosides in human body, ribonucleosides and deoxyoribonucleosides. They can be distinguished from each other by the types of sugars (ribose for ribonucleosides and deoxyribose for deoxyribonucleosides) connected to the nitrogenous bases.

Adenosine and deoxyadenosine participate in biochemical metabolisms in a variety of ways. For example, adenosine is the most important nucleosides involved in energy transfer processes. Although all nucleoside phosphates with phosphoanhydride bonds can transfer energy, adenosine phosphates, including 5'-adenosine triphosphate (ATP) and 5'-adenosine diphosphate (ADP), are the most common types that are required in almost all the energy-related biological processes (Knowles 1980). In addition to ATP and ADP, deoxyadenosine 5'-triphosphate/diphosphate (dATP/dADP) also acts as an important energy supplier in the biological processes, such as DNA synthesis in the nucleus.

With regard of signaling transduction, intracellular adenosine is the basic structure element of the widely distributed second messenger, cyclic 5'-adenosine monophosphate (cAMP). Cyclic AMP is synthesized from ATP by cyclic adenylyl cyclase. It plays an important role by activating cAMP-dependent kinases and proteins, such as protein kinases A (PKA) and exchange protein activated by cAMP 1 (Epac1). It can also directly activate some cyclic-nucleoside gated ion channels (Jefferson, Exton et al. 1968, Meinkoth, Alberts et al. 1993, de Rooij, Rehmann et al. 2000). As a second message, cAMP possesses diverse roles in a number of biochemical processes, such as gene expression, hormone regulation and glycogen metabolism (Walsh and Van Patten 1994). Extracellular adenosine itself

directly transduces signal by interacting with adenosine receptors. Four types of adenosine receptors ($A_1/A_{2A}/A_{2B}/A_3$) have been discovered so far and they all belong to G-protein coupled receptors (Fredholm, Abbracchio et al. 1994). In heart and lung, the activation of adenosine receptors can regulate cellular processes by regulating the level of intracellular cAMP. This regulatory mechanism has critical role in maintaining the normal function of these organs (Jacobson and Gao 2006, Mitchell and Lazarenko 2008). In the central nervous system (CNS), adenosine is considered to be an inhibitory neurotransmitter. By interacting with A_{2A} receptor and Gs proteins, adenosine antagonizes against the excitatory neurotransmitters (Latini and Pedata 2001).

1.4.2 Adenosine Metabolisms

Adenosine is produced constitutively by cells under normal conditions and its concentration increases under metabolic stress (e.g. ischemia) or at sites of tissue injury (Obata 2002, Ribeiro, Sebastiao et al. 2002, Blackburn 2003) (Fig. 3). Adenosine can be produced by either *de novo* synthesis or ATP breakdown. In *de novo* synthesis progress, amino acid glutamine and phosphoribosyl pyrophosphate (PRPP) are directly used to synthesis adenosine, catalyzed by amidophosphoribosyltransferase (amidoPRT) (Farrell and Golan 2008). This synthesis pathway does not require the participation of nitrogen bases like adenine and guanine. However, *de novo* generation is not the main source of adenosine production. Adenosine is mainly produced from the ATP breakdown mechanism, especially in ischemic conditions, and to a less extent during strenuous physical exercise. Extracellular adenosine is generated from the dephosphorylation of adenosine monophosphate (AMP) by

ecto-5'-nucleotidase. Intracellular adenosine is generated from the dephosphorylation of AMP by a cytosolic form of nucleotidase, cyto-5'-nucleotidase, or the hydrolysis of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase (SAHH) (Lloyd, Deussen et al. 1988, Deussen, Lloyd et al. 1989, Barsotti and Ipata 2004, Burgos, Gulab et al. 2012).

The removal of adenosine is carried out by two metabolic pathways: deamination into inosine via adenosine deaminase (ADA) and phosphorylation into AMP via adenosine kinase (AK) (Blackburn 2003, Blackburn and Kellems 2005). ADA is an enzyme produced in all cells, but lymphocytes in immune system contains the highest levels of ADA. The function of ADA is to convert adenosine and deoxyadenosine into inosine and deoxyinosine. Among them, deoxyadenosine has been suggested to be cytotoxic but adenosine's role in pathology remained to be explored (Cohen, Hirschhorn et al. 1978).

In addition, transport mechanisms across the membranes are also critical for the homeostasis of adenosine. In mammalian cells, adenosine can be carried across PM by the concentrative nucleoside transporters (CNTs, also known as SLC28 family proteins) and the equilibrative nucleoside transporters (ENTs, also known as SLC29 proteins) (Molina-Arcas, Casado et al. 2009).

1.4.3 Adenosine Metabolisms in Lysosomes

Owing to the central role of lysosomes in molecule recycling and the abundance of ATP in lysosomes, it is not surprising that adenosine and deoxyadenosine are generated in lysosomes (Maguire and Luzio 1985, Wada, Eto et al. 1987, Tanaka, Himeno et al. 1989, Zimmermann 1992). However, adenosine biogenesis pathway in lysosomes has not been

described. Lysosomes contain abundant ATP, which might be generated by recycling nuclei acids that are delivered from autophagy pathway (Arsenis, Gordon et al. 1970, Lardeux and Mortimore 1987, Heydrick, Lardeux et al. 1991) or by the SLC17A9-mediated ATP transport from the cytosol (Cao, Zhao et al. 2014). Lysosomal ATP could be eventually converted into adenosine by lysosomal 5'-NT (Pisoni and Thoene 1989, Zimmermann 1992, Lindley and Pisoni 1993). It has been suggested that ADA is expressed in lysosomes, and the lysosomal ADA accounts for around 10% percent of the total ADA in human fibroblasts. Lysosomal ADA may catalyze the same reaction as its counterpart in the periphery, turning adenosine and deoxyadenosine into inosine and deoxyinosine (Lindley and Pisoni 1993), especially during nutritional deprivation when lysosomes recycle more cell debris and obsolete organelles (Fox and Kelley 1978, Lindley and Pisoni 1993). The identification of ENT3 in lysosomes and lysosomal adenosine accumulation in ENT3 deficient cells suggest that excess adenosine in lysosomes can be transported out through ENT3 (Hsu, Lin et al. 2012) (Fig. 3).

1.4.4 Transport of Adenosine across Lysosomal Membrane

There are four types of known ENTs, designated ENT1, ENT2, ENT3, and ENT4. All four isoforms are widely distributed in mammalian tissues. The best-characterized members of the family, ENT1 and ENT2, appear to function primarily at the cell surface although ENT1 has also been detected in liver mitochondria (Lai, Tse et al. 2004) and in the nuclear envelopes of cultured cells (Mani, Hammond et al. 1998). In contrast, ENT3 (Baldwin, Yao et al. 2005, Kang, Jun et al. 2010, Hsu, Lin et al. 2012) and ENT4 (Barnes, Dobrzynski et al.

2006) are predominantly expressed in intracellular membranes although some ENT3 was found in the PM (Govindarajan, Leung et al. 2009). Intracellular ENT3 was detected in the lysosomes (Baldwin, Yao et al. 2005, Hsu, Lin et al. 2012) as well as the mitochondria (Kang, Jun et al. 2010). Uniquely, both ENT3 and ENT4 are pH sensitive, and are optimally active under acidic conditions (around pH 5.5), which probably reflected the subcellular location of the transporters in acidic compartments (Baldwin, Yao et al. 2005, Barnes, Dobrzynski et al. 2006).

Human ENT3 is a protein with 475 residues 29% identical to hENT1 and 74% to mENT3 (Hyde, Cass et al. 2001, Baldwin, Yao et al. 2005). It possesses a long, hydrophobic, N-terminal structure which contains two dileucine motifs that target the proteins on lysosomal membranes (Sandoval, Martinez-Arca et al. 2000). Because it reaches its maximal activity at low pH (around 5.5), it is an ideal candidate to conduct nucleoside transport on the membranes of acidic intracellular organelles such as endosomes and lysosomes (Baldwin, Yao et al. 2005, Young, Yao et al. 2008). Indeed, ENT3 transports both adenosine and deoxyadenosine out of lysosomes and ENT3 deficient cells are characterized by lysosomal adenosine accumulation (Baldwin, Yao et al. 2005, Hsu, Lin et al. 2012).

1.5 Adenosine Related Human Diseases

Impaired adenosine homeostasis has been associated with numerous human diseases. ADA and ENT3 are two major genes which mutations lead to adenosine accumulation and subsequent disorders. ADA deficiency causes an elevation in the level of adenosine and deoxyadenosine in the plasma. Consequently, individuals with ADA deficiency display

impaired lymphocyte differentiation, cell function and viability, resulting in lymphopenia and severe combined immunodeficiency disease (SCID), a systematic immune problem characterized with the loss of function of T cells, B cells, and Natural Kill (NK) cells. SCID patients with ADA mutations are extremely vulnerable to the infectious from bacteria, fungi and viruses (Giblett, Anderson et al. 1972, Buckley, Schiff et al. 1997, Aloj, Giardino et al. 2012). Patients with ADA deficiency also show neurological abnormalities (Apasov, Blackburn et al. 2001, Blackburn 2003, Blackburn and Kellems 2005, Mohsenin and Blackburn 2006, Aiuti, Cattaneo et al. 2009, Boison, Chen et al. 2010). Loss of ENT3 results in an increase in adenosine in the cytosol as well as lysosomes. Mutant ENT3 has recently been associated with familial Rosai-Dorfman disease, Faisalabad histiocytosis (FHC), and H syndrome (Morgan, Morris et al. 2010, Hsu, Lin et al. 2012).

Adenosine and its receptors also provide a protective mechanism during the injury and stress conditions like ischemia, hypoxia and seizure. When ischemia occurs, the level of extracellular adenosine will increase dramatically by 2 to 4 fold in the tissues. The excessive adenosine can efficiently generate anti-inflammatory effects in order to prevent further damages (Jacobson and Gao 2006).

1.5.1 SCID and Mouse Model with ADA-deficiency

ADA-deficiency accounts for around one fifth of the total SCID cases. In ADA associated SCID, the thymus is absent or small because immune cells, including T, B and NK cells, experience severe apoptosis during development (Aloj, Giardino et al. 2012). Other characteristic symptoms found from the SCID patients include marked lymphopenia, failure

of thrive and recurrent fungal, viral and bacterial infections (Buckley, Schiff et al. 1997, Blackburn and Kellems 2005, Sauer and Aiuti 2009). Without intervention, most of the patients will die from various kinds of infections in the first year of life. A minority of the patients may be able to survive longer with less severe immunodeficiency (Sauer and Aiuti 2009).

Several strategies have been developed to treat ADA associated SCID. The best among them is histo-compatible bone marrow transplantation from a sibling possessing matching human leukocyte antigen (Kohn, Hershfield et al. 1998). However, the chance to receive this type of therapy is obviously low. Various substitutions are developed, such as T cell-depleted haploidentical bone marrow transplantation from a parent and biomedical approach of replacement of ADA by a bovine form with polyethylene glycol-modifications, but the chance of cure is limited (Hershfield, Buckley et al. 1987).

Great efforts have been devoted to develop a mouse model with ADA deficiency, which provides an opportunity for examining the systematic role of purine salvage disturbance *in vivo* (Migchielsen, Breuer et al. 1995, Wakamiya, Blackburn et al. 1995, Blackburn, Datta et al. 1998). However, the ADA deficient mice died prenatally, which are similar to some of the ADA deficiency SCID patients, preventing further studies of the consequences of ADA deficiency in postnatal animals (Migchielsen, Breuer et al. 1995, Wakamiya, Blackburn et al. 1995). To solve this problem, a two-stage genetic engineering strategy is used to generate ADA deficient mice. After ADA knockout mice are generated, ADA expression is restored in the trophoblast which is critical for the natal survival of the

animal. In this condition, mice with complete ADA deficiency are allowed for postnatal survival. This model ideally mimics the phenotypes observed in ADA deficient humans, in particular a severe lymphopenia and combined immunodeficiency. Notably, Adenosine accumulation is detected in a variety of tissues in this model, but the deoxyadenosine accumulation is restricted to lymphoid tissues (Blackburn, Datta et al. 1998).

1.5.2 ENT3 Associated Human Diseases

Mutations of ENT3 have been identified in numerous human diseases. The majority of the mutations compromise ENT3's function in its transport ability of adenosine and other nucleosides although some mutations affect the turnover speed of ENT3 (Molho-Pessach, Lerer et al. 2008). Accumulating evidence reveals that mutations in hENT3 cause a spectrum of human genetic disorders, including H syndrome, FHC, sinus histiocytosis with massive lymphadenopathy (SHML, also known as familial Rosai-Dorfman disease), and pigmented hypertrichosis with insulin-dependent diabetes mellitus (PHID) syndrome (Kang, Jun et al. 2010). They share some common phenotypes including histocytosis which might be due to defective phagocytosis of apoptotic cells. The similar symptoms among those diseases suggest ENT3 associated human diseases might have common genetic basis, which have been verified by the discovery of mutation on ENT3 changing glycine 437 to arginine (G437R) in all of these diseases (Kang, Jun et al. 2010, Morgan, Morris et al. 2010) (Fig. 4). Currently, no therapeutic strategy for the treatment of ENT3 associated diseases is available yet.

ENT3 knockout mouse was successfully generated in 2012. In the absence of ENT3,

mice display defective phagocytosis apoptotic cells and suffer from immune problems due to the overexpansion of histiocytosis, and eventually die 30 weeks after birth (Hsu, Lin et al. 2012). At the cellular level, the loss of ENT3 results in lysosomal adenosine buildup. Notably, lysosomal adenosine accumulation leads to enlarged lysosomes, elevated intralysosomal pH, altered macrophage function, and defective apoptotic cell clearance (Hsu, Lin et al. 2012). These phenotypes resemble the cells with ML1 deletion, suggesting that impaired ML1 and subsequent lysosomal dysfunction might be the reasons causing the aforementioned phenotypes in ENT3 deficient cells.

1.6 Research Objective and Hypothesis

Both ADA and ENT3 have been detected in lysosomes (Pisoni and Thoene 1989, Lindley and Pisoni 1993). Loss of either ADA or ENT3 results in adenosine accumulation and defective apoptotic cells clearance (Apasov, Blackburn et al. 2001, Fredholm 2007, Hsu, Lin et al. 2012). Particularly, ENT3 mutant cells show enlarged lysosomes and defective apoptotic cells clearance (Hsu, Lin et al. 2012), suggesting that adenosine accumulation in lysosome may impair lysosome membrane trafficking and subsequently lead to defective apoptotic cell removal and histiocytosis. Interestingly, ML1 regulates lysosomal membrane trafficking, ML1 mutant cells also exhibit enlarged lysosomes, and defects in the clearance of apoptotic cells and histiocytosis (Nilius, Owsianik et al. 2007, Venkatachalam and Montell 2007, Venkatachalam, Long et al. 2008, Puertollano and Kiselyov 2009, Cheng, Shen et al. 2010, Dong, Wang et al. 2010). We therefore hypothesize that ML1 activity might be compromised by accumulated adenosine and deoxyadenosine in lysosomes, leading to the

phenotypes mentioned in ADA and ENT3 mutant cells.

In this research, my primary purpose is to investigate whether nucleosides accumulation in lysosomes impairs ML1's activity, and whether upregulated ML1 rescues cellular phenotypes associated with lysosomal nucleoside accumulation. To achieve these goals, the effect of adenosine and deoxyadenosine on ML1 activity will be examined first. Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), an ADA blocker, will be used to trigger the nucleoside accumulation. ENT3 transporter will be introduced to facilitate the nucleosides release, which would suggest whether the compromised ML1 is caused by nucleoside accumulation. In addition, cells treated with EHNA and ADA patient human fibroblasts will be examined to test whether nucleoside accumulation causes lysosome dysfunction and whether this can be rescued by ML1. Specifically, we plan to examine the following hypothesis by conducting eletrophysiological recordings, Ca^{2+} imaging, and confocal microscopy.

1. High concentration of nucleosides would inhibit ML1 currents.
2. ML1-mediated Ca^{2+} release from lysosomes would be diminished when intralysosomal nucleosides were elevated by ADA inhibitor EHNA.
3. ENT3 but not its G437R mutant would rescue the impaired ML1-mediated Ca^{2+} release induced by EHNA pretreatment.
4. ADA deficiency would result in compromised ML1-mediated Ca^{2+} release from lysosomes, which can be rescued by ENT3 but not ENT3-G437R.
5. ADA inhibition by EHNA or ADA deficiency would cause lysosome dysfunction, which

would be rescued by ENT3 and ML1 overexpression.

Chapter 2 Methods and Materials

2.1 Cell Culture and Transfection

Cos-1 and HEK293T cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Wild type (WT) human fibroblasts (GM00969) and ADA deficient human fibroblasts (GM02605) were obtained from Coriell Institute (Camden, NJ, USA) and were cultured in Dulbecco's Modified Eagle's Medium supplemented with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37℃ in a 5% CO_2 atmosphere. For some experiments, cells were seeded on 0.01% poly-lysine coated coverslips and cultured 24-48 hrs before further experiments. Cells from passage numbers 5-30 were used for subsequent assays. Cos-1 and HEK293T cells were transfected using Lipofectamine 2000. Human fibroblasts were transfected by electroporation with the Neon® Transfection System (Invitrogen) following manufacturer's optimized protocol (1525V, 25 ms, 1 pulse). About 70% transfection efficiency was regularly achieved.

2.2 Antibodies and Reagents

Antibodies anti-Lamp1 (H4A3, Developmental Studies Hybridoma Bank), and Alexa 488 goat-anti-mouse (Invitrogen) were used for immunofluorescence staining and western blotting. LysoTracker DND-99 Red (Invitrogen, 50 nM) was used to label lysosomes. DQ-BSA (Invitrogen, 10 μg/ml) was used to assess lysosome function. The following reagents were also used: Glycyl-L-phenylalanine 2-naphthylamide (GPN) (Santa Cruz

Biotechnology), adenosine (Sigma-Aldrich), deoxyadenosine (Sigma-Aldrich), ML-SA1 (Princeton Biomolecular Research), ionomycin (Cayman Chemical), A23718 (Tocris), Thapsigargin (TG) (Tocris), Bafilomycin A1 (Baf-A1) (Tocris), DQ-BSA (Invitrogen), Fura-2 AM (Invitrogen), inhibitor EHNA（Cayman Chemical), ATP-Na, ADP-Na and Adenosine standard (Sigma, St. Louis, MO, USA), methanol (High Performance Liquid Chromatography (HPLC) grade) (Sigma-Aldrich, St. Louis, MO, USA), phosphoric acid and absolute ethanol (analytical grade) (Thermo Fisher Scientific, Inc, USA). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA).

2.3 Immunocytochemistry

Cells grown on coverslips were washed with PBS twice and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then blocked with 3% bovine serum albumin (BSA) in PBS for 60 min at room temperature. After washing three times with PBS, cells were incubated with primary antibodies at 4°C overnight. After washing three times with PBS, cells were incubated with fluorescence-conjugated secondary antibodies for 45 min at room temperature in the dark. Images were acquired using a confocal microscope (LSM510, Zeiss) with a 63X oil-immersion objective lens and captured using ZEN2009 software (Zeiss).

2.4 Confocal Microscopy

Confocal fluorescent images were taken using an inverted Zeiss LSM510 confocal microscope with 10X plain or 63X oil-immersion objectives. Sequential excitation

wavelengths at 488 nm and 543 nm were provided by argon and helium-neon gas lasers, respectively. BP500-550 and LP560 emission filters were used to collect green and red images in channels one and two, respectively, and then images of the same cell were saved and analyzed with ZEN software. The term co-localization refers to coincident detection of above-background green and red fluorescent signal. Image size was set at 1,024 × 1,024 pixels.

2.5 Plasmid Constructs

The genetically encoded Ca^{2+} indicators for optical imaging fused with ML1 channel (GECO-ML1) construct was made by inserting the full length GECO sequence (Zhao, Araki et al. 2011) between the HindIII and BamHI sites of a pcDNA6 plasmid that contains the mouse ML1 cDNA at the XhoI site. ML1-L15L/AA-L577L/AA (abbreviated as ML1-4A) and the ML1 non-conducting pore mutant (D471K/D472K; abbreviated ML1-DDKK) were constructed using a site-directed mutagenesis kit (Qiagen). All constructs were confirmed by sequencing, and protein expression was verified by western blot. HA-ENT3 and HA-ENT3-G437R were kindly provided by Neil Morgan and Eamonn Maher (University og Birmingham). Cos-1, HEK293T, and human fibroblasts were transiently transfected with GECO-ML1 for electrophysiology, Ca^{2+} imaging, and confocal imaging.

2.6 Whole-cell Electrophysiology

The pipette solution contained 140 mM K-gluconate, 4 mM NaCl, 1 mM ethylene glycol tetra-acetic acid (EGTA), 2 mM $MgCl_2$, 0.39 mM $CaCl_2$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2; free $[Ca^{2+}]_i$ = 100

nM). The bath solution (modified Tyrode's solution) contained 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose (pH 5.5). Cells were recorded within 0.5-2 hrs after splitting. All bath solutions were applied via a perfusion system to achieve a complete solution exchange within a few seconds. Data were collected using an Axopatch 200B patch clamp amplifier, Digidata 1440 digitiza, and pClamp 10.0 software (Axon Instruments). Whole-cell currents were digitized at 10 kHz and filtered at 2 kHz. ML1 currents were evoked by voltage ramps from -140 mV to 140 mV within 4 s, $V_{holding}$ = 0 mV. All experiments were conducted at room temperature (21-23 ℃), and all recordings were analyzed with pClamp 10.0, and Origin 8.0 (OriginLab, Northampton, MA) (Fig. 5).

2.7 Fura-2 Based Ca^{2+} Imaging

Cells were loaded with 1 μM Fura-2 AM (Invitrogen) in the culture medium at 37℃ for 60 min. Fluorescence was recorded at different excitation wavelengths using an EasyRatioPro system (PTI). Emission fluorescence was obtained in both 340 nm and 380 nm excitation of light (background subtracted). Fura-2 ratios (F340/F380) were used to monitor changes in intracellular $[Ca^{2+}]_i$. Lysosomal Ca^{2+} release was measured under a 'low external Ca^{2+}' solution, which contained 145mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM Glucose, 1 mM EGTA, and 20 mM HEPES (pH7.4). Ca^{2+} concentration in the nominally-free Ca^{2+} solution is estimated to be 1-10 μM. With 1 mM EGTA, the free Ca^{2+} concentration is estimated to be < 10 nM based on the Maxchelator software. GPN (200 μM), a substrate of the lysosomal exopeptidase cathepsin C that induces lysosome osmodialysis , and Baf-A1 (500 nM), an inhibitor of vacuolar type H^+-ATPase, were used to release the Ca^{2+} content in lysosomes.

TG (2 μM), a non-competitive inhibitor of ER Ca^{2+} ATPase, was applied to induce ER Ca^{2+} release. The Ca^{2+} ionophore, Ionomycin (1 μM, in 2 mM Ca^{2+} Tyrode's solution) was added in the end of all experiments to induce a maximal response for comparison. For endogenous lysosomal Ca^{2+} release measurement, Ca^{2+} imaging was carried out within 0.5-2 hrs after plating when cells still exhibited a round morphology.

2.8 GECO Based Ca^{2+} Imaging

Cells were trypsinized and plated onto glass coverslips after 18-24 hrs transfections with GECO-ML1. Most experiments were carried out within 0.5-2 hrs after plating when cells still exhibited round morphology. Lysosomal Ca^{2+} release was measured under a 'low external Ca^{2+}' solution, which contained 145mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM Glucose, 1 mM EGTA, and 20 mM HEPES (pH7.4). Ca^{2+} concentration in the nominally-free Ca^{2+} solution is estimated to be 1-10 μM. With 1 mM EGTA, the free Ca^{2+} concentration is estimated to be < 10 nM based on the Maxchelator software. The fluorescence intensity at 470 nm (F470) was monitored. Lysosomal Ca^{2+} release caused an increase in the fluorescence, which was indicated as a change of fluorescence ΔF over basal fluorescence F0; $\Delta F/F_0$).

2.9 HPLC

2.9.1 Lysosome Isolation by Subcellular Fractionation

Lysosomes of Hela cells and human fibroblast (WT and ADA) cells were isolated as described previously (Cao et al., 2014). Briefly, cell lysates were obtained by Dounce homogenization in a homogenizing buffer (HM buffer; 0.25 M sucrose, 1 mM EDTA,

10 mM HEPES; pH 7.0), and then centrifuged at 1,500 × g (4200 rpm, Fisher, ST-16R, F15 rotor) at 4℃ for 10 min to remove the nuclei and intact cells. Postnuclear supernatants were then subjected to ultracentrifugation through a Percoll density gradient using a Beckman Optima L-90K ultracentrifuge. An ultracentrifuge tube was layered with 2.5 M sucrose, 18% Percoll in HM buffer and supernatant (top). The centrifugation was carried out at 90,000 × g (31300 rpm), 4℃, for 1 hr using a Beckman Coulter 70.1 Ti rotor. Samples were fractionated into light, medium, and heavy membrane fractions. Heavy membrane fractions contained concentrated bands of cellular organelles and were further layered over a discontinuous iodixanol gradient, generated by mixing iodixanol in HM buffer with 2.5 M glucose (in v/v; 27%, 22.5%, 19%, 16%, 12%, and 8%) and with osmolarity maintained at 300 mOsm for all solutions. After centrifugation at 4℃ for 2.5 hr at 180,000 × g (44200 rpm), each sample was divided into twelve fractions (0.5 ml each) for further analyses. Note that the biological and ionic compositions of the lysosomes were largely maintained due to the low rate of transport across the lysosomal membrane at 4℃.

2.9.2 HPLC Analysis

HPLC analysis was carried out using a Prostar HPLC system equipped with a diode array ultraviolet UV-vis detector (Varian Canada Inc., Mississauga, Ontario, Canada). A Cosmosil C18 packed column (250 mm X4.6 ID, Nacalai tesque, Inc. Japan) was used. The column temperature was maintained at 30℃. The standards and samples were separated using a gradient mobile phase consisting of 0.4% phosphoric acid (Solvent A) and methanol (Solvent B). The linear gradient conditions were: 0-25 min, 90% A; 25-35 min, 20% A;

35-40 min, 90% A and 40-65 min, 90% A. The flow rate was set at 0.9 ml/min and the injection volume was 100 μl. The detection wavelength was set at 257 nm. Identification of ATP, ADP, and adenosine was based on the retention time when co-injected with standards.

2.9.3 Sample Extraction Procedure

Isolated lysosomes were re-suspended with 1ml internal buffer and sonicated in 20% power input (6X 20s sonication on ice). After centrifuging at 12, 500 rpm for 10 min at 4℃, the supernatants were saved at -80℃. For further HPLC analysis, 300 μl supernatant was mixed well with 1 ml cooled extraction medium of TE statured phenol/chloroform/deionized water (6:2:2) and centrifuged at 10, 000 g for 5 min at 4℃. The upper aqueous phase was carefully collected and filtered through a filtering cartridge containing a 0.45 μm nylon membrane using a disposable syringe set before HPLC analysis.

2.9.4 Preparation of the Standard Curve

Quantification was based on the external standard method. The stock solutions of ATP-Na, ADP-Na and adenosine standard (1 mg/ml) was prepared by dissolving them in 80% ethanol. The working standard solutions for linear calibration were prepared by diluting the stock solution to produce a concentration sequence of 0.1, 1, 5, 10, 20, 50, 100 and 200 μg/ml. Both the stock and working solutions were kept at 4℃ for 1 month.

2.10 Data Analysis

Data are presented as mean ± SEM. Statistical comparisons were made using analysis of ANOVA or Student's t test. P values of < 0.05 were considered statistically significant. *: $P < 0.05$; **: $P < 0.01$.

Chapter 3 Results

3.1 Adenosine and Deoxyadenosine Inhibit ML1-4A Current in the Plasma Membrane

To test whether adenosine and deoxyadenosine inhibit ML1, HEK293T cells were transfected with ML1 surface-expressed mutant (ML1-L15L/AA-L577L/AA, abbreviated as ML1-4A), which serves as a surrogate of lysosomal ML1 (Vergarajauregui and Puertollano 2006, Grimm, Jors et al. 2010). Because lysosomal pH is elevated to around 5.5 by adenosine accumulation (Hsu, Lin et al. 2012), to mimic the environments of lysosomes with adenosine accumulation, extracellular solutions (analogous to the intralysosomal luminal side) were adjusted to pH 5.5. ML1-4A in the PM was activated by ML-SA1 (Shen, Wang et al. 2012). As shown in Figure 6, adenosine (10 mM) and deoxyadenosine (10 mM) inhibited ML1 current by 68.56 ± 5.60 % (n=5) and 58.63 ± 4.87 % (n=5), respectively. The inhibition of ML1 current by adenosine and deoxyadenosine exhibited a dose-dependent effect (Fig. 7).

3.2 Adenosine and Deoxyadenosine Inhibit ML1-4A Mediated Ca^{2+} Entry in the Plasma Membrane

ML1 acts as a Ca^{2+} permeable channel (Dong, Cheng et al. 2008, Parkinson, Baines et al. 2014). To further evaluate the inhibitory effect of adenosine and deoxyadenosine, ML1-4A activity was measured by Fura-2 based Ca^{2+} imaging assay. In consistent with other reports (Shen, Wang et al. 2012), activation of ML1-4A by ML-SA1 induced a rapid increase in Fura-2 signal followed by a quick rundown.. The mechanism underlying the rundown remains unclear. To avoid the contamination of the rundown, we co-applied ML-SA1 and

adenosine or ML-SA1 and deoxyadenosine followed by ML-SA1. If adenosine and deoxyadenosine inhibit ML1, we expect to see a smaller Ca^{2+} signal followed by a larger one. As shown in Figure 8, the Fura-2 signal in cells expressing ML1-4A was increased by co-application of ML-SA1 (10 μM) and adenosine (10 mM), presumably due to incomplete inhibition of ML1 by adenosine. However, much larger response was observed when ML-SA1 was applied alone. Similarly, co-application of ML-SA1 (10 μM) and deoxyadenonsine (10 mM) also induced a smaller Ca^{2+} response than ML-SA1 alone (Fig. 9). These data suggest that adenosine and deoxyadenonsine inhibit ML1 activity.

3.3 A Lysosome-targeted Genetically Encoded Ca^{2+} Indicator

To measure lysosomal ML1 activity and lysosomal Ca^{2+} release in intact cells, we fused GECO, a single-wavelength genetically encoded Ca^{2+} indicator (Zhao, Araki et al. 2011), to the cytoplasmic amino terminus of ML1 (Fig. 10). When it was transfected into HEK293T or Cos-1 cell line, GECO-ML1 was mainly localized in the lysosomal-associated membrane protein 1 (Lamp1) and LysoTracker-positive compartments, suggesting a lysosomal localization of GECO-ML1 (Fig. 11). Since many Ca^{2+} permeable channels are also present on the PM, low external Ca^{2+} (nominally free Ca^{2+} with 1 mM EGTA; free Ca^{2+} estimated to be < 10 nM) was used for GECO-ML1 fluorescence measurement. To determine the specificity of the GECO-ML1 fluorescence signal, we tested a variety of Ca^{2+} release reagents and found that GECO-ML1 fluorescence (F470) responded preferentially to GPN (200 μM), a Cathepsin C substrate that induces osmotic lysis of lysosomes to release lysosomal Ca^{2+}, but not to TG (2 μM), a reagent releasing the endoplasmic recticulum (ER)

Ca^{2+} storage (Fig. 12). Therefore, GECO-ML1 preferentially detects juxta-lysosomal Ca^{2+} increases and can be used to measure ML1 activity in intact cells. Application of ML-SA1 (10 μM) induced rapid increases in GECO fluorescence ($\Delta F/F_0 = 0.35 \pm 0.04$, n = 70 cells) under low external Ca^{2+} in HEK293T cells expressing GECO-ML1 (Fig. 13A, 13B). Both GPN (200 μM, 1 hr) and Baf-A1 (500 nM, 30 min), a specific and potent inhibitor of V-ATPase that can deplete lysosome Ca^{2+} (Parkinson, Baines et al. 2014), largely abolished ML-SA1-induced increase in GECO fluorescence in cells expressing GECO-ML1 (Fig. 13C, 13D). In contrast to GECO-ML1, no significant increase in GECO fluorescence ($\Delta F/F_0 = 0.020 \pm 0.003$; n = 37 cells) was observed in cells expressing GECO fused with non-conducting pore mutant of ML1 (GECO-ML1-DDKK) (Fig. 13E). Collectively, these results suggested that GECO-ML1 could be used as a probe to measure ML1-mediated lysosomal Ca^{2+} release, i.e. ML1 activity (Fig. 13F).

3.4 Reduced ML1 Activity in Cells Treated with EHNA

ADA activity has been detected in lysosomes isolated from human fibroblasts (Pisoni and Thoene 1989, Lindley and Pisoni 1993), and ADA deficiency causes an elevation in the level of adenosine and deoxyadenosine (Apasov, Blackburn et al. 2001, Blackburn 2003, Blackburn and Kellems 2005, Aiuti, Cattaneo et al. 2009). To test whether adenosine or deoxyadenosine inhibits ML1 in intact cells, lysosomal adenosine and deoxyadenosine were elevated by EHNA, a potent ADA inhibitor (Lum, Sutherland et al. 1977) which was reported to efficiently induce adenosine accumulation. As shown in Figure 14A and 14E, EHNA (3 μM, 30 min) significantly decreased ML-SA1 induced GECO-ML1 signals. The

GECO fluorescence was reduced from 0.38 ± 0.06 (n =72) to 0.04 ± 0.01 (n = 58). However, the GPN-induced GECO response was not decreased by EHNA treatment (0.50 ± 0.01 in control group and 0.56 ± 0.01 in EHNA group, Fig. 14C, 14F), suggesting that EHNA did not reduce lysosomal Ca^{2+} content. In addition, the expression levels of GECO-ML1, as estimated from the maximal fluorescence intensity induced by ionomycin (1 μM, in 2 mM Ca^{2+} external solution) (Shen, Wang et al. 2012, Parkinson, Baines et al. 2014), were comparable between control and EHNA treated cells (Fig. 14G). These data suggest that EHNA treatment dramatically reduce ML1 channel activity.

ENT3 was shown to release adenosine and deoxyadenosine from lysosomes (Baldwin, Yao et al. 2005, Govindarajan, Leung et al. 2009, Hsu, Lin et al. 2012). To further demonstrate that the inhibitory effect of EHNA on ML1 was caused by adenosine accumulation, we tested whether ENT3 rescued EHNA induced inhibition on GECO-ML1 response. ENT3-HA overexpression remarkably increased ML-SA1 induced GECO-ML1 responses in EHNA treated cells (Fig. 14B, 14E). By contrast, ENT3-G437R, a mutant ENT3 with compromised transport activity but normal subcellular localization (Kang, Jun et al. 2010, Morgan, Morris et al. 2010), exhibited smaller ML-SA1 induced GECO-ML1 responses in EHNA treated cells (Fig. 14B, 14E). Again, the GPN-induced GECO-ML1 response and the expression levels of GECO-ML1 were not altered by ENT3 or G437R co-expression with GECO-ML1 (Fig. 14D, 14F).

Ca^{2+} release via ML1 can cause cytosolic Ca^{2+} increase. To further test the inhibitory effect of EHNA on ML1, ML1 channel activity was measured by Fura-2 based Ca^{2+} imaging

assay. In accordance with ML-SA1 induced GECO-ML1 response, ML-SA1 induced a smaller Fura-2 response in EHNA treated cells (Fig. 14H). Consistently, ENT3 but not ENT3-G437R rescued the EHNA inhibition on ML-SA1 induced Fura-2 response (Fig. 14H).

3.5 Reduced ML1 Activity in ADA Cells

ADA deficiency results in an elevation in the level of adenosine and 2'-deoxyadenosine (Apasov, Blackburn et al. 2001, Blackburn 2003, Blackburn and Kellems 2005, Mohsenin and Blackburn 2006, Aiuti, Cattaneo et al. 2009, Boison, Chen et al. 2010). Because ADA activity has been detected in lysosomes (Pisoni and Thoene 1989, Lindley and Pisoni 1993), loss of ADA presumably leads to adenosine and deoxyadenosine accumulation in lysosomes. If adenosine and deoxyadenosine inhibit lysosomal ML1 activity, we expect to see less ML-SA1 induced GECO-ML1 response in ADA cells. Thus, we compared ML-SA1 (10 μM) induced GECO-ML1 response between WT human fibroblasts and fibroblasts derived from ADA patients, which completely lack the activity of ADA. As shown in Figure 15A, ML-SA1 induced GECO fluorescence intensity in ADA cells was significantly smaller, decreased from 0.51± 0.17 (n = 11) in control fibroblasts to 0.24 ± 0.04 (n = 22) in ADA fibroblasts. In contrast, GPN-induced GECO fluorescence increase in ADA cells was similar to that of WT cells (Fig. 15B). The expression levels of GECO-ML1 were also comparable between control and ADA cells (Fig. 15C). Altogether, these data suggest a compromised ML1 activity in lysosomes of ADA cells.

ML1 activity in ADA cells was further measured by Fura-2 based Ca^{2+} imaging.

Consistently, ADA cells exhibited significantly smaller Fura-2 signal change in response to ML-SA1 (20μM), reducing from 0.047 ± 0.0043 (n = 29) in control fibroblasts to 0.031 ± 0.0028 (n= 30) in ADA fibroblasts (Fig. 15D). This reduction in Fura-2 signal in ADA cells was not caused by a decrease in lysosomal Ca^{2+} content because ADA cells showed a higher GPN-induced Fura-2 signal (Fig. 15E). Collectively, ADA lysosomes may contain higher levels of adenosine and deoxyadenosine, which compromised ML1 activity.

3.6 EHNA Treatment and ADA Deficiency Result in Adenosine Accumulation in Lysosomes

EHNA administration and ADA deficiency are predicted to elevate the level of adenosine and deoxyadenosine in lysosome. To co-relate the inhibitory effect of EHNA treatment and ADA deletion on ML1 activity with nucleoside accumulation, we measured the adenosine levels in lysosomes isolated from cells treated with EHNA or ADA human fibroblasts using HPLC (Fig. 16). EHNA treatment significantly increased lysosomal adenosine but not ATP level, suggesting that lysosomal adenosine accumulation is likely resulted from inhibiting adenosine degradation by ADA (Fig. 17). In addition, adenosine accumulation was also observed in ADA deficient cells although lysosomal ATP was comparable between WT and ADA deficient cells (Fig. 18). These data further consolidate our conclusion that adenosine accumulation in lysosomes results in a compromised ML1 activity.

3.7 Lysosome Dysfunction Induced by Adenosine and Deoxyadenosine Accumulation in Lysosomes

Compromised ML1 results in lysosome dysfunction (Cheng, Shen et al. 2010). To test

whether adenosine and deoxyadenosine accumulation impair lysosomal dysfunction, we assessed lysosomal function using DQ-BSA, a dye that indicates active proteolysis in lysosomes (Cao, Zhao et al. 2014). Strong loss of DQ-BSA signals were observed in Cos-1 cells treated with EHNA (1 μM) but not control vehicle (Fig. 19). ENT3-GFP but not ENT3-G437R overexpression rescued attenuated DQ-BSA signals induced by EHNA, suggesting that lysosome dysfunction induced by EHNA results from adenosine accumulation. The loss of DQ-BSA signals in EHNA treated cells was also rescued by the expression of ML1-GFP but not its mutant ML1-DDKK-GFP, suggesting that lysosomal dysfunction induced by EHNA is attributed to impaired ML1 activity. In agreement with this, strong DQ-BSA signals were seen in control human fibroblasts, but not in ADA deficient cells (Fig. 20), suggesting that ADA loss impairs lysosomal function. The loss of DQ-BSA signals in ADA cells was rescued by the expression of ENT3-HA but not its mutant G437R, suggesting that lysosomal dysfunction in ADA cells is caused by nucleoside accumulation in lysosomes. The loss of DQ-BSA signals in ADA cells was also rescued by the expression of ML1-GFP but not its mutant ML1-DDKK-GFP. Taken together, our data suggested that lysosomal adenosine accumulation impair ML1 activity, which might be the molecular mechanism associated with ADA diseases.

Chapter 4 Discussion

Adenosine, a ubiquitous signal molecule that is produced constitutively by the cell under both normal and pathological conditions, is engaged in regulation of numerous physiological systems, such as cardiovascular, nervous, lungs, kidneys, renal and immune systems. The effects of adenosine are largely mediated by activation of adenosine receptors. Adenosine is commonly thought as a beneficial signaling molecule, and adenosine receptors are targets for potential drugs in many pathophysiological situations (Obata 2002, Boison, Chen et al. 2010). Although adenosine is well characterized in the PM, little is known about its roles in intracellular organelles, particularly in the lysosome where adenosine accumulation results in lysosomal dysfunction and defective apoptotic cell clearance (Hsu, Lin et al. 2012). In this study, we found that ML1 activity was directly suppressed by adenosine and deoxyadenosine. The inhibition of ML1 by adenosine and deoxyadenosine was rescued by increasing ENT3. Accumulation of adenosine and deoxyadenosine by ADA inhibitor EHNA or ADA deficiency led to dysfunctional lysosomes, which can be rescued by either ML1 or ENT3 upregulation. Our data suggest that lysosomal accumulation of adenosine and deoxyadenosine inhibits ML1, and subsequently impairs lysosome function. This study provides a modular mechanism to explain cellular phenotypes observed in patients or mice with ADA and ENT3 deficiency. This finding may lead to a new therapeutic strategy for ADA and ENT3 associated human disorders.

4.1 Adenosine Homeostasis and Lysosome Function

4.1.1 Adenosine Homeostasis in the Lysosome

There are several pools of adenosine which is generated by intracellular and extracellular enzyme pathways under specific conditions. Previous studies indicate that some pathological conditions such as cellular stress or tissue damage give rise to an increase in extracellular adenosine level. However, whether adenosine is produced in secretory vesicles (except for some synaptic vesicles) and whether vesicular adenosine contributes to the elevated extracellular adenosine are uncertain.

Lysosomes are a group of secretory vesicles that contain a variety of destructive enzymes capable of breaking down of all types of biological macromolecules including proteins, nucleic acids, carbohydrates, and lipids. The degradation of nucleic acids in lysosomes is one of the major sources of nucleoside recycling (Heydrick, Lardeux et al. 1991). Meanwhile, lysosomes take up ATP from the cytosol via SLC17A9, an ATP transporter localized in lysosomal membrane (Cao, Zhao et al. 2014). Since 5'-NT (Arsenis, Gordon et al. 1970, Zimmermann 1992) has been found in lysosomes, it is likely that ATP breakdown in the lysosomal interior acts as an alternative pathway that generates adenosine. In addition, cytosolic adenosine could also contribute to lysosomal adenosine accumulation by putative transporters. Lysosomal adenosine homeostasis is maintained not only by the biogenesis pathway but also the degradation pathway which may be dominated by lysosomal ADA (Pisoni and Thoene 1989, Lindley and Pisoni 1993), and the release pathway mediated by ENT3 (Hsu, Lin et al. 2012). In supporting this, the loss of either ADA or ENT3 causes lysosomal adenosine accumulation. Therefore, lysosomal adenosine homeostasis is tightly

controlled by generation, degradation, and transport pathways. Disruption of either pathway would result in lysosomal adenosine dys-homeostasis. Under certain abnormal conditions, for example the loss of ADA or ENT3 could cause an increase in lysosomal adenosine level, and subsequently results in human diseases. Although our study, together with other reports, suggests that ADA and ENT3 are involved in lysosomal adenosine regulation, it is still not clear whether other enzymes and proteins involved in cytosolic adenosine metabolisms also contribute in lysosome adenosine homeostasis. Addressing these questions would further our understanding adenosine regulation in lysosomes.

Interestingly, the activity of both ADA and ENT3 is pH dependent, with an increased activity when pH changing from 4.0 to 6.0 (Lindley and Pisoni 1993, Baldwin, Yao et al. 2005). It was reported that adenosine accumulation increases lysosomal pH to 5.5-6.0, which could remarkably increase both ADA and ENT3. Therefore, adenosine accumulation in lysosomes would facilitate ADA and ENT3 activity by increasing lysosomal pH that is favorable for ADA and ENT3. This provides a feedback mechanism to maintain a normal lysosomal adenosine level.

4.1.2 Molecular Mechanisms of Lysosome Dysfunction in Response to Adenosine Accumulation

The normal luminal pH (around 4.5-5.0) are critical for maintaining the activity of lysosomal hydrolases. Disturbance of lysosomal pH regulation, especially elevated lysosomal pH, usually causes severe dysfunction of lysosome by perturbing the enzyme activity. It has been suggested that excessive lysosomal nucleosides compete for free protons within lysosomes

and lead to alkalization, up to pH 5.5-6.0 (Hsu, Lin et al. 2012). In this study, we also observed a pH increase when adenosine is added in the low pH Tyrode's solution that mimic lysosomal environment (data not shown). Therefore, lysosomal pH increase in ADA deficient cells or cells treated with EHNA could be one reason that leads to compromised lysosomal function. In supporting this, the signal of DQ-BSA, a fluorogenic substrate for proteases that requires enzymatic cleavage in acidic lysosomal compartments to generate a highly fluorescent product, is dramatically suppressed in both ADA deficient cells or cells treated with EHNA. Meanwhile, ML1 activity decreases as pH increases (Dong, Cheng et al. 2008). It is also possible that elevated lysosomal pH induced by adenosine accumulation inhibits ML1 activity, and further leads to dysfunctional lysosomes. Furthermore, because ML1 is required for maintaining lysosomal pH (Bach, Chen et al. 1999), compromised ML1 activity in lysosomes with adenosine accumulation may further increases lysosomal pH (Vergarajauregui, Connelly et al. 2008, Wong, Li et al. 2012). In agreement, ML1 overexpression rescued the compromised DQ-BSA signals in ADA deficient cells or cells treated with EHNA, likely through correcting lysosomal pH and membrane trafficking. Overall, lysosomal dysfunction induced by adenosine accumulation might be caused by both defects in hydrolyses and compromised ML1 activity. In addition, some other mechanisms could also contribute to the lysosomal dysfunction induced by adenosine accumulation. For example, P2X4, a lysosomal Ca^{2+} channel that is promoted by increased lysosomal pH, could contribute to the defective lysosome membrane trafficking and lysosomal dysfunction induced by adenosine accumulation. This awaits further exploration.

4.1.3 Physiological Role of Lysosomal Adenosine

Although this study together with previous findings suggests that adenosine accumulation impairs lysosomes, the physiological role of adenosine in lysosomes remains unclear. However, because lysosomes keep recycling nucleic acids and lysosomes contain high level of ATP, the nucleoside concentration in lysosomes is expected to be higher than that in the cytosol. It is very likely that ENT3 mediated adenosine release can replenish cytosolic adenosine, which is important for many intracellular processes under normal physiological condition. We can also speculate that lysosomal adenosine may be released into cytosol to participate in ATP synthesis, especially during nutritional deprivation of lysosomal adenosine (Lindley and Pisoni 1993). Unfortunately, the physiological and pathological roles of lysosomal adenosine have been barely discovered. This could be an interesting topic for future study.

Lysosomes are dynamic organelles that constitutively fuse with the PM to release luminal substances. Lysosomes might replenish extracellular adenosine, in conditions requiring the appearance of large amount of adenosine, for instance the membrane damage. Lysosomal exocytosis has been implicated in membrane repair during cell damage (Reddy, Caler et al. 2001, Huynh, Roth et al. 2004, Cheng, Zhang et al. 2014). Since cell damage induces massive lysosome exocytosis, and since extracellular adenosine is largely thought to serve cytoprotective roles during tissue damage (Obata 2002, Ribeiro, Sebastiao et al. 2002, Blackburn 2003, Fredholm 2007), it is important to study whether lysosomal adenosine has a role in membrane repair upon tissue damage in which adenosine plays a significant role. We

can envisage that adenosine secretion via lysosomal exocytosis induced by cell damage may contribute to membrane repair and protect tissue from damage. Further investigation is needed to test this.

4.2 A Clinical Perspective of Adenosine Accumulation

Both ADA and ENT3 deficiency lead to a number of human diseases (Apasov, Blackburn et al. 2001, Fredholm 2007, Hsu, Lin et al. 2012). However, the mechanisms for these diseases are not fully understood. Our findings provide a cellular and molecular mechanism for the pathogenesis of these diseases, which may develop a potential therapeutic strategy for these diseases by upregulating ML1. For instance, the newly identified ML1 agonist could be applied to patient cells or animal with ADA and ENT3 deficiency to test whether activating ML1 alleviates the pathogenesis of these syndromes. However, because the lysosomal ADA only accounts for around 10% of the total ADA and ENT3 is also expressed in other organelles, adenosine accumulation in the cytosol and other organelles need to be considered when lysosomal function is evaluated. Although the loss of ADA and ENT3 primarily leads to defects in immune system in which ADA and ENT3 are highly expressed, our data using non-immune cells suggest that other tissues other than immune systems might also be affected in patients with ADA and ENT3 deficiency.

4.3 Shortcomings and Future Plan

In this study, we have shown that adenosine accumulation in lysosomes inhibits ML1 channel function, subsequently leading to lysosome dysfunction. We have also shown that ML1 overexpression rescued lysosomal defects in ADA cells that displayed adenosine

accumulation in lysosomes and compromised ML1 activity. Our studies provide a molecular mechanism for the pathogenesis of ADA and ENT3 associated human diseases. However, future experiments are required to provide a better understanding based on this thesis.

4.3.1 Lysosomal Patch-clamp Recording

We have shown that both adenosine and deoxyadenosine inhibited ML1-4A, the lysosomal ML1 surrogate that is located onto the PM, by using whole-cell patch-clamp. However, the molecular components in the PM are different from that of lysosomal membrane. It has been suggested that ML1's activity relies on the lipid, $PI_{(3,5)}P_2$, preferably distributed on the endolysosomal membrane. In contrast, a different lipid, $PI_{(4,5)}P_2$ that predominantly located on the PM, inhibits ML1. Although ML1-4A has been repeatedly used as a lysosomal ML1 surrogate, whole lysosomal recording would provide a better way to examine endogenous ML1 function. Unfortunately, the pipette solution which mimics the luminal content is unable to be changed during lysosomal recording. This prohibited us from measuring the inhibitory effect of adenosine on ML1 using lysosomal patch clamping.

Another strategy to directly measure the inhibitory effect of adenosine on ML1 current is to record ML1 under luminal-side out patch recording mode (Dong, Cheng et al. 2008). However, little ML1 current in a small patch has been limited us from drawing a solid conclusion. Nevertheless, this approach is still worth trying in the future.

4.3.2 Selectivity of EHNA

In this research, EHNA was used to induce adenosine accumulation in lysosomes. However, EHNA acts not only as an ADA inhibitor but also a phosphodiesterase inhibitor of PDE2

which might play an important role in the regulation of intracellular concentrations of cAMP (Michie, Lobban et al. 1996). It is unclear whether cAMP is involved in the effect of EHNA on lysosomal function. Although we cannot exclude the possible involvement of cAMP, our data strongly suggest that EHNA might affect cell function by regulating lysosomal adenosine accumulation and subsequent ML1 activity. This is based on following reasons. First, EHNA treatment results in adenosine accumulation in lysosomes. Second, ENT3 rescues the EHNA inhibition on ML1 activity. Third, EHNA treatment causes the same cellular phenotypes as ADA deficiency.

4.3.3 Rescuing ML1 Defects in ADA Human Fibroblasts by ENT3

In this research, EHNA pretreatment induced defect in ML1 mediated Ca^{2+} release can be rescued by overexpression of ENT3. In contrast, overexpression of ENT3-G437R mutant that has impaired nucleoside transport function cannot rescue the phenotype. Since ML1 activity is compromised in ADA deficient human fibroblasts, overexpressing ENT3 is supposed to rescue ML1 activity. This experiment is expected to be conducted in the near future.

4.3.4 ML-IV Like Phenotypes in ADA and ENT3

ML-IV cells display enlarged lysosomes, lysosomal storage, defects in lipid trafficking, Ca^{2+} accumulation, and lysosomal alkalization (Bach, Chen et al. 1999, Kogot-Levin, Zeigler et al. 2009, Venkatachalam, Wong et al. 2014). Since ML1 is inhibited by adenosine accumulation in ADA and ENT3 deficient cells, it would be interesting to test whether ADA and ENT3 deficient cells develop all these ML-IV like phenotypes.

4.3.5 ADA in Other Cellular Components

ADA and ENT3 are also expressed in other cellular components. Although we have shown that ML1 overexpression rescues the cellular phenotypes of cells with ADA and ENT3 deficiency, the contribution of other cellular components to the cellular defects in ADA and ENT3 deficient cells cannot be excluded.

4.3.6 Measurement of Lysosomal Deoxyadenosine Using HPLC

ADA catalyzes the degradation of both adenosine and deoxyadenosine. Besides adenosine accumulation, EHNA treatment and ADA deletion are supposed to cause deoxyadenosine accumulation. Lysosomal deoxyadenosine levels in control, EHNA treated cells, and ADA deficient cells will be also measured using HPLC.

4.3.7 Study the Involvement of ML1 in Immune Cells with ADA or ENT3 Deficiency

Because the loss of both ADA and ENT3 primarily leads to defects in immune system, immune cells should be adopted. Unfortunately, we current do not have access to both immune cells from patients or animals with ADA and ENT3 deletion. We plan to collaborate with other laboratories that can provide us these cells.

4.3.8 Examination of Animal Model

In this research, we mainly use cell lines including human skin fibroblasts from the patients to test our hypothesis. Recently, ADA (Migchielsen, Breuer et al. 1995, Wakamiya, Blackburn et al. 1995, Blackburn, Datta et al. 1998) and ENT3 (Hsu, Lin et al. 2012) knockout mice have been successfully generated. We speculate that activation of ML1 by

ML-SA1 injection would alleviate the defects in ADA and ENT3 knockout mice. This experiment would dramatically increase the significance of the work.

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Appendix: Figures

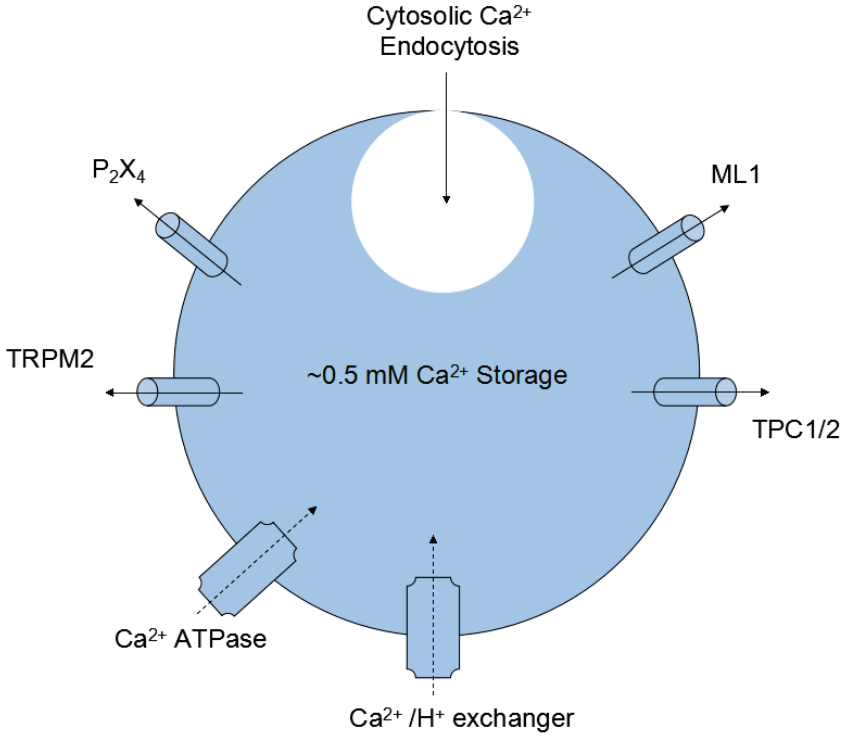

Figure 1 Regulation of Lysosomal Ca^{2+} Homeostasis

Lysosomal Ca^{2+} homeostasis is regulated by a series of channels and transporters. Mechanisms of Ca^{2+} accumulation in lysosomes have not been fully deciphered. Recent evidence suggests that lysosomes obtain Ca^{2+} through endocytosis (10%), Ca^{2+} transporters, and Ca^{2+}/H^+ exchangers (in yeast vacuole). Lysosomes release Ca^{2+} through ML1, P_2X_4, TRPM2, and TPC1/2 channels.

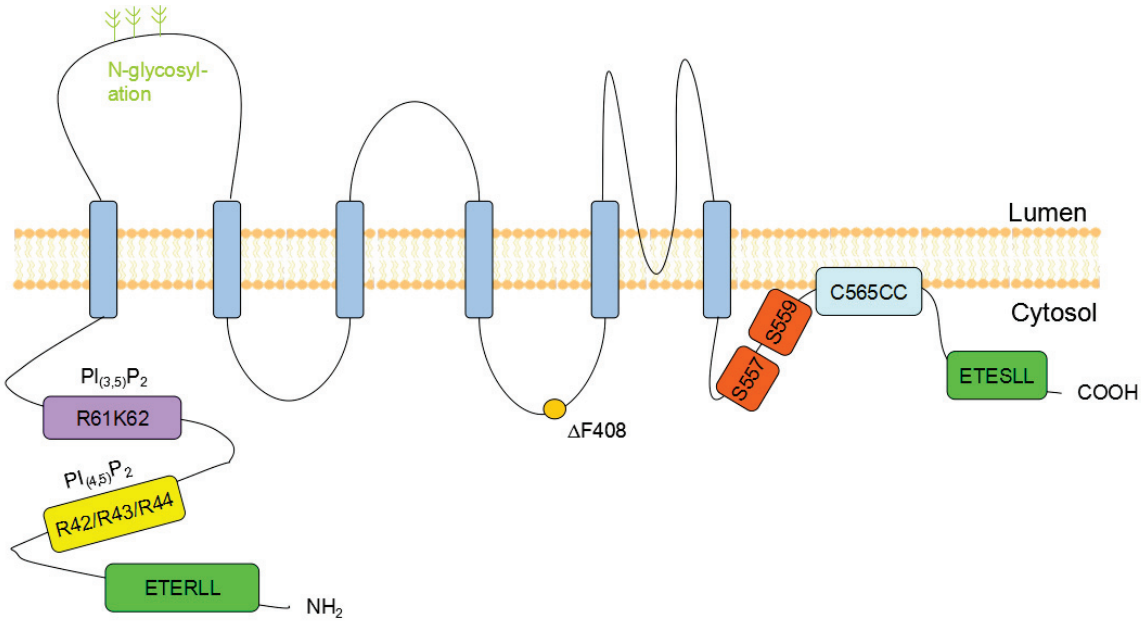

Figure 2 Structure of ML1

ML1 consists of six transmembrane (6TM) domains with the amino-terminal (NH_2) and carboxyl-terminal (COOH) tails facing the cytosol. The first luminal loop is uniquely large and contains four N-glycosylation sites and a cleavage site. Two di-leucine motifs ETERL[15]L and EEHSL[577]L are located separately at each tail to mediate the localization of ML1 to lysosomes. At the N-terminus of ML1, several positively charged amino acid residues are predicted to interact with phosphoinositides (Arg61/ Lys62 and Arg42/Arg43/Arg44 for $PI_{(3,5)}P_2$ and $PI_{(4,5)}P_2$, respectively). The figure is modified from Wang, Zhang et al. 2014

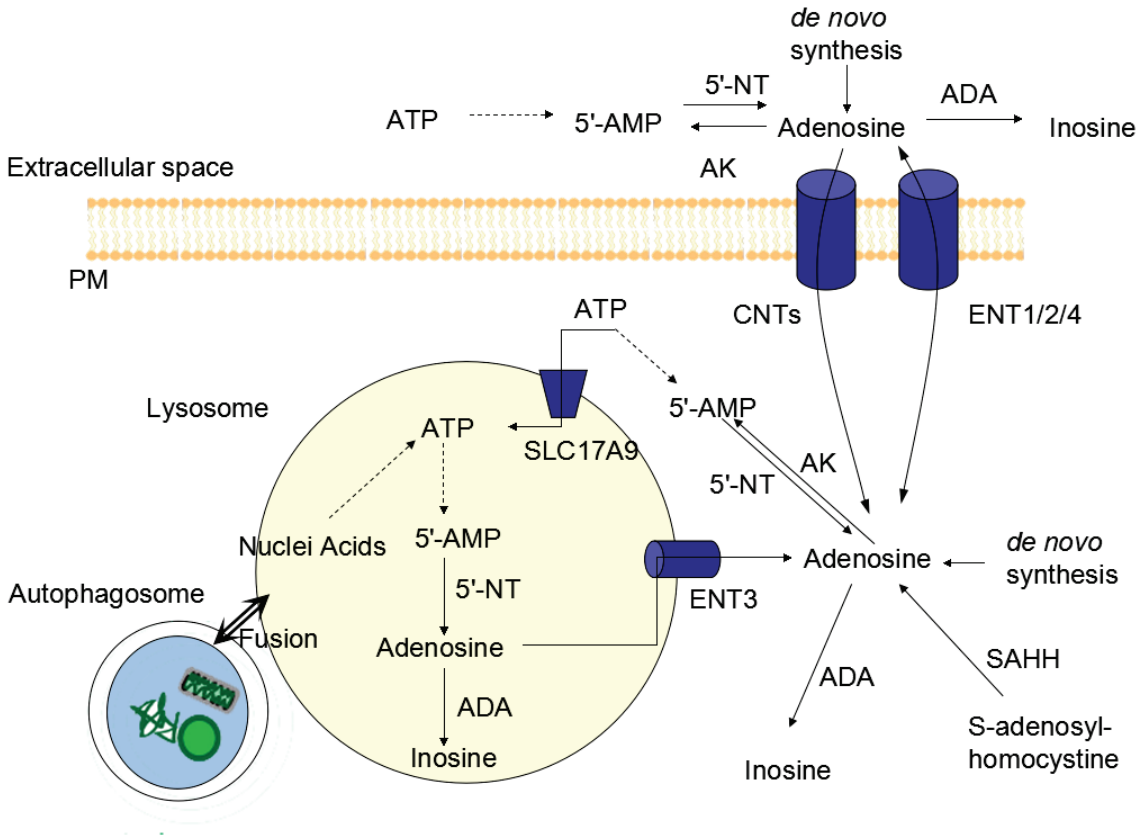

Figure 3 Adenosine Metabolism

Adenosine is generated both extracellularly and intracellularly. A small amount of adenosine can be produced through *de novo* synthesis pathway. The majority of adenosine comes from the breakdown of adenosine phosphates by 5'-NT. In addition, adenosine can be generated from S-adenosylhomocystine by SAHH. Adenosine can be removed by two metabolic pathways: deamination into inosine via ADA and phosphorylation into AMP via AK. Cellular adenosine level is also controlled by CNTs and ENTs (ENT1/2/4) situated in the PM. Interestingly, lysosomes contain high level of ATP, which is generated by either the breakdown of nucleic acids during autophagy, or SLC17A9-mediated ATP transport.

Lysosomal ATP could be converted into adenosine, and lysosomal adenosine homeostasis might be controlled by similar biogenesis pathway and removal pathway that regulate cytosolic adenosine level. In agreement with this notion, 5'-NT, ADA, and ENT3 have been found in lysosomes.

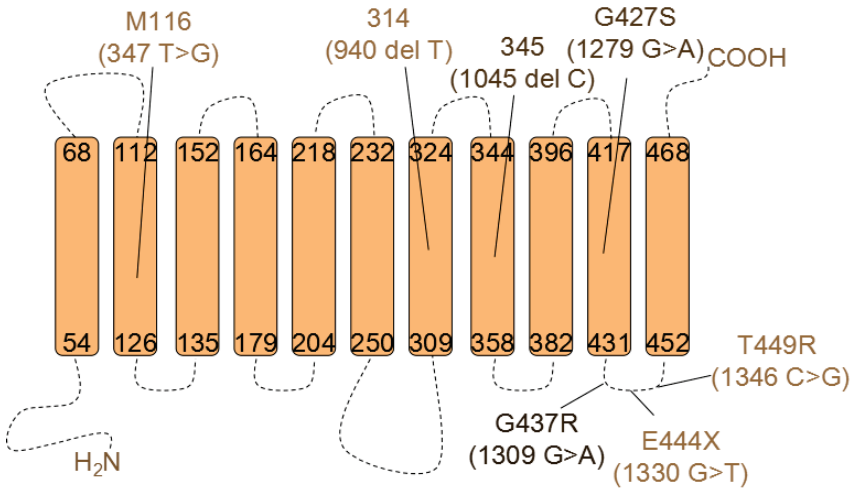

Figure 4 Mutations of ENT3 in Human Syndromes

Diagram showing the topology of ENT3 and common mutations associated with human diseases. Among them, G437R is the most widely discovered mutations. This figure is modified from Kang, Jun et al. 2010.

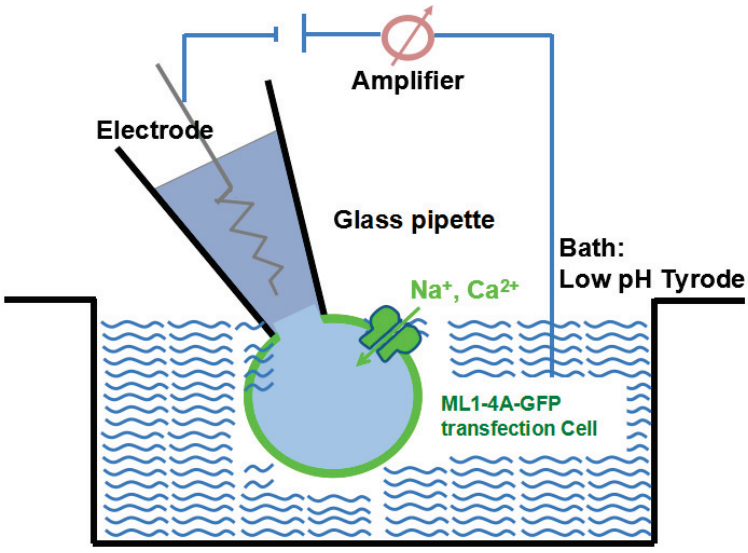

Figure 5 Diagram Showing Whole Cell Electrophysiological Recording

HEK293T cells are transfected with ML1-4A-GFP 24 hrs before recordings. Cells are seeded on coverslips and placed in pH 5.5 Tyrode's solution. Currents are recorded using electrode containing intracellular solution. Activation of ML1-4A channels on the PM induces inward Na^{+}/Ca^{2+} currents.

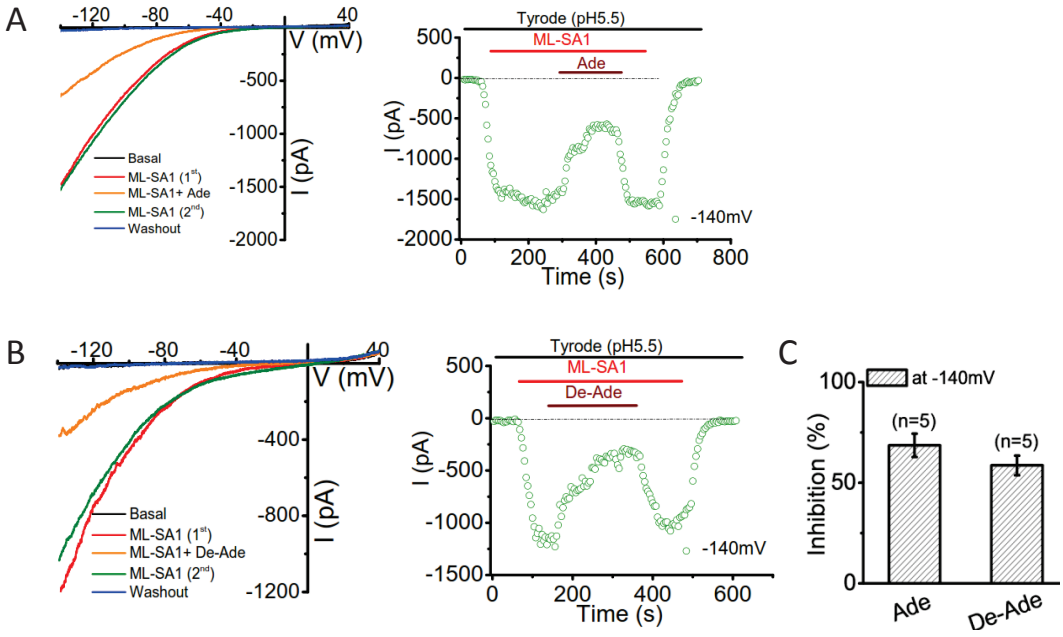

Figure 6 Adenosine and Deoxyadenosine Inhibits ML1-4A Current

(A and B) HEK293T cells transfected with GFP-ML1-4A was recorded using whole-cell recording approach. Administration of ML1 agonist, ML-SA1 (10 μM), induced ML1-4A current that was suppressed by adenosine (10 mM) or deoxyadenosine (10 mM) on top. (Left) representative currents in response to voltage ramps from -140 mV to 40 mV before and after bath application of ML-SA1 ± adenosine or deoxyadenosine. (Right) representative trace of current development at -140 mV responding to indicated treatments. (C) Statistic data showing the inhibitory effect of 10 mM adenosine or 10 mM deoxyadenosine on ML1-4A currents induced by ML-SA1. Currents measured at -140 mV were normalized to the peak currents under ML-SA1.

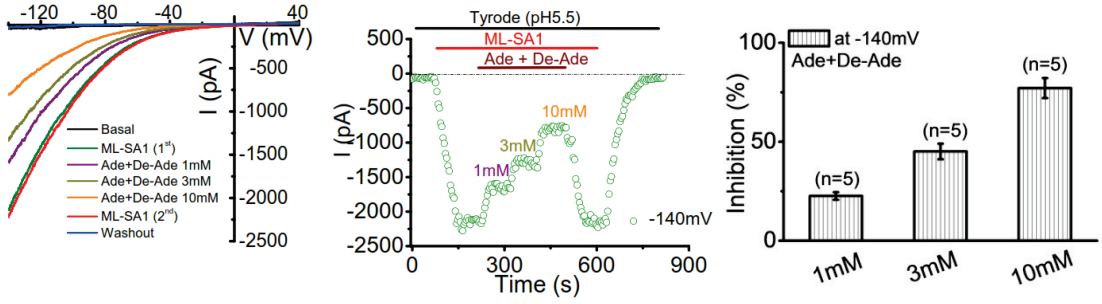

Figure 7 Dose Dependent Inhibition of ML1-4A Current by Adenosine and Deoxyadenosine

Adenosine and deoxyadenosine (1 mM, 3 mM and 10 mM) led to a dose-dependent inhibition on ML-SA1 (10 μM) induced ML1-4A currents. (Left) representative currents in response to voltage ramps from -140 mV to 40 mV before and after bath application of ML-SA1, and 1 mM, 3 mM, 10 mM adenosine + deoxyadenosine on top of ML-SA1. (Middle) representative trace of current development at -140 mV responding to indicated treatments. (Right) Currents measured at -140 mV were normalized to the peak currents under ML-SA1.

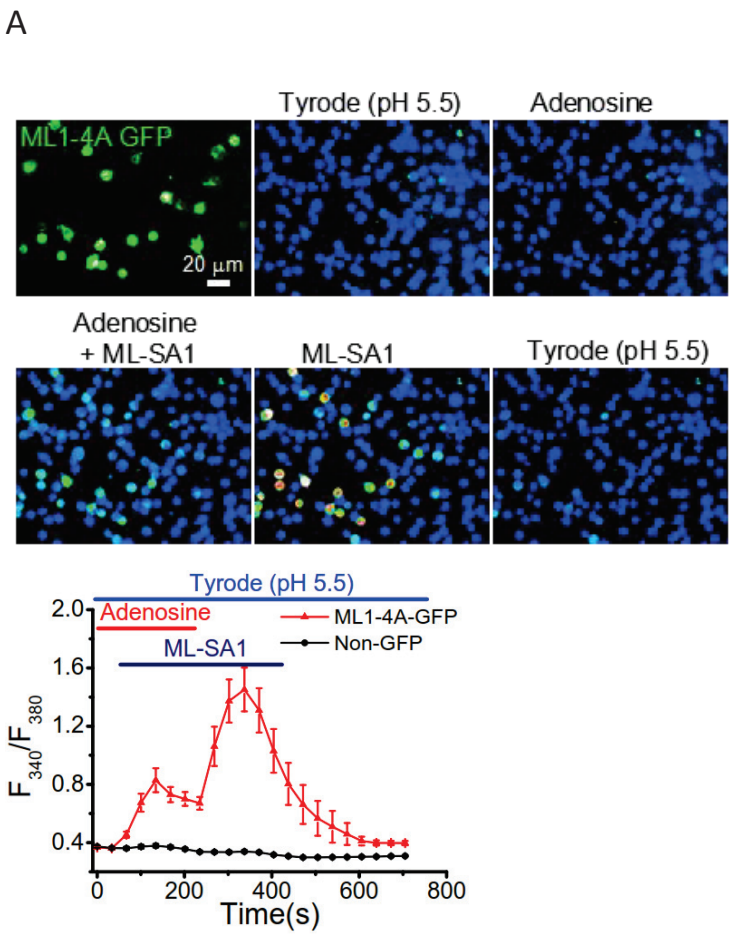

Figure 8 Adenosine Inhibits Ca^{2+} Flux through ML1-4A

(A) Representative images showing the changes of Fura-2 fluorescence upon bath application of adenosine (10 mM), adenosine + ML-SA1 (10 μM), and ML-SA1 in ML1-4A transfected HEK293T cells.

(B) The removal of adenosine (10 mM) significantly increased ML-SA1 (10 μM) induced lysosomal Ca^{2+} release (measured with Fura-2 ratios) in ML1-4A transfected HEK293T cells but not non-transfected cells, suggesting that Ca^{2+} influx through ML1 was suppressed by adenosine.

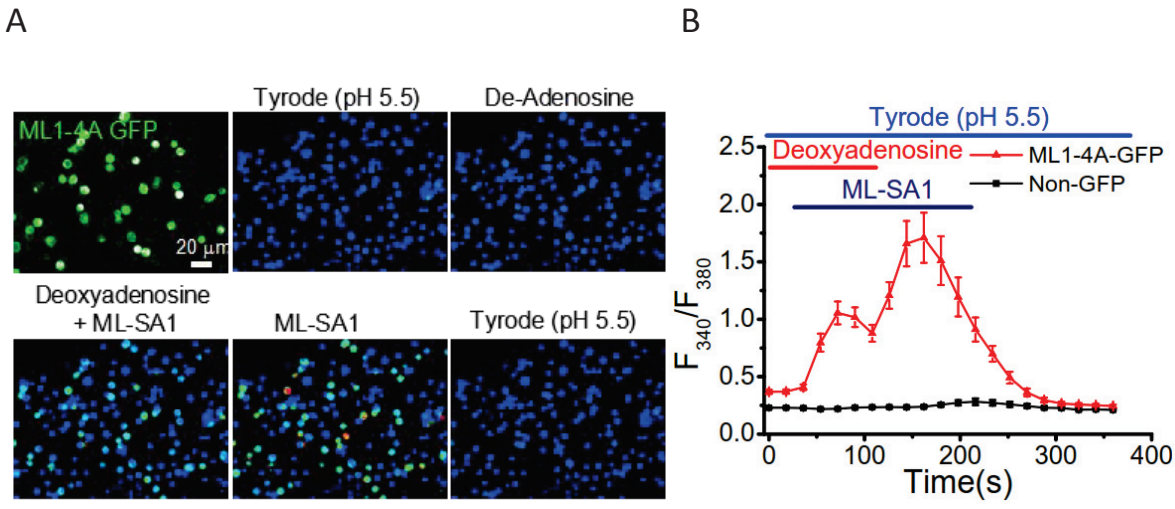

Figure 9 Deoxyadenosine Inhibits Ca^{2+} Flux through ML1-4A

(A) Representative images showing the changes of Fura-2 signals upon bath application of deoxyadenosine (10 mM), deoxyadenosine + ML-SA1 (10 μM) and ML-SA1 in HEK293T cells expressing ML1-4A.

(B) The removal of deoxyadenosine (10 mM) significantly increased ML-SA1 (10 μM) induced lysosomal Ca^{2+} release (measured with Fura-2 ratios) in ML1-4A transfected HEK293T cells but not non-transfected cells, suggesting that Ca^{2+} influx through ML1 was suppressed by deoxyadenosine.

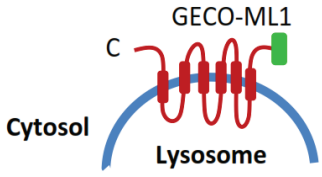

Figure 10 GECO-ML1 Fusion Strategy

The Ca^{2+} indicator GECO was fused on the N-terminal of ML1.

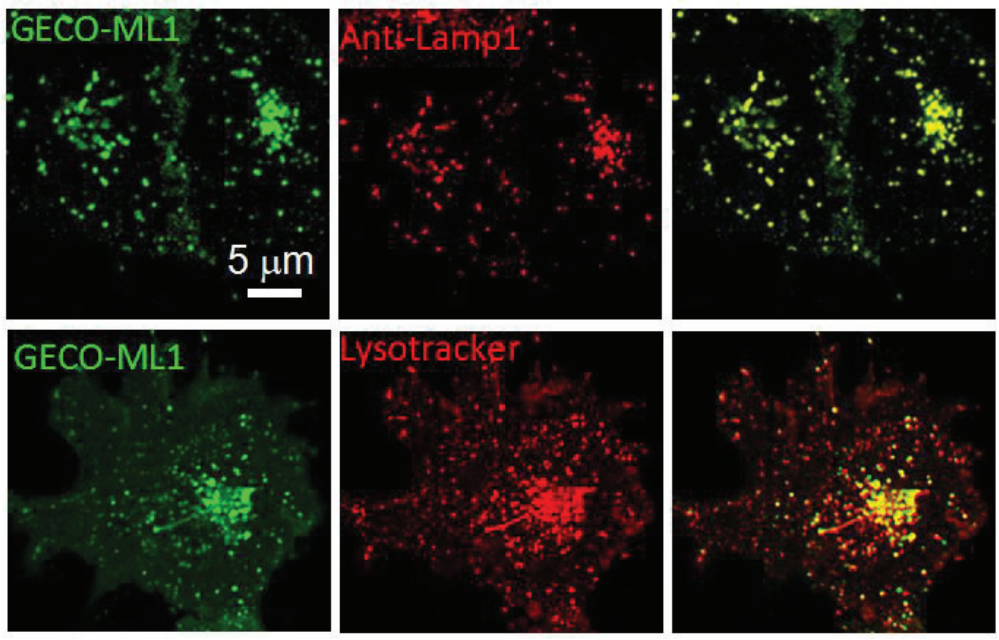

Figure 11 Lysosomal Localization of GECO-ML1

Co-localization of GECO-ML1 with Lamp1 (upper panel) and LysoTracker (bottom panel), two lysosomal markers, in Cos-1 cells overexpressing GECO-ML1.

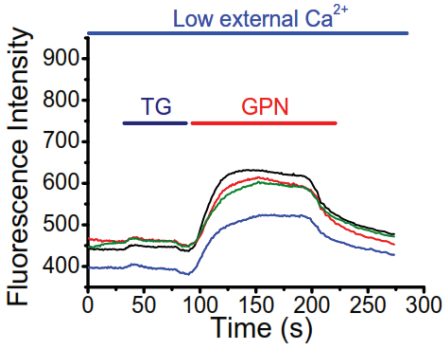

Figure 12 Selective Detection of Ca^{2+} Release from Lysosome Stores by GECO-ML1

ER Ca^{2+} release was triggered by TG (2 μM) and lysosome Ca^{2+} release was trigged by GPN (200 μM) measured by GECO-ML1 fluorescence. GECO-ML1 transfected HEK293T cells only responded to GPN but not TG, suggesting that GECO-ML1 preferentially detects juxta-lysosomal Ca^{2+} increases, and thus could be used as a probe to measure ML1 activity in intact cells.

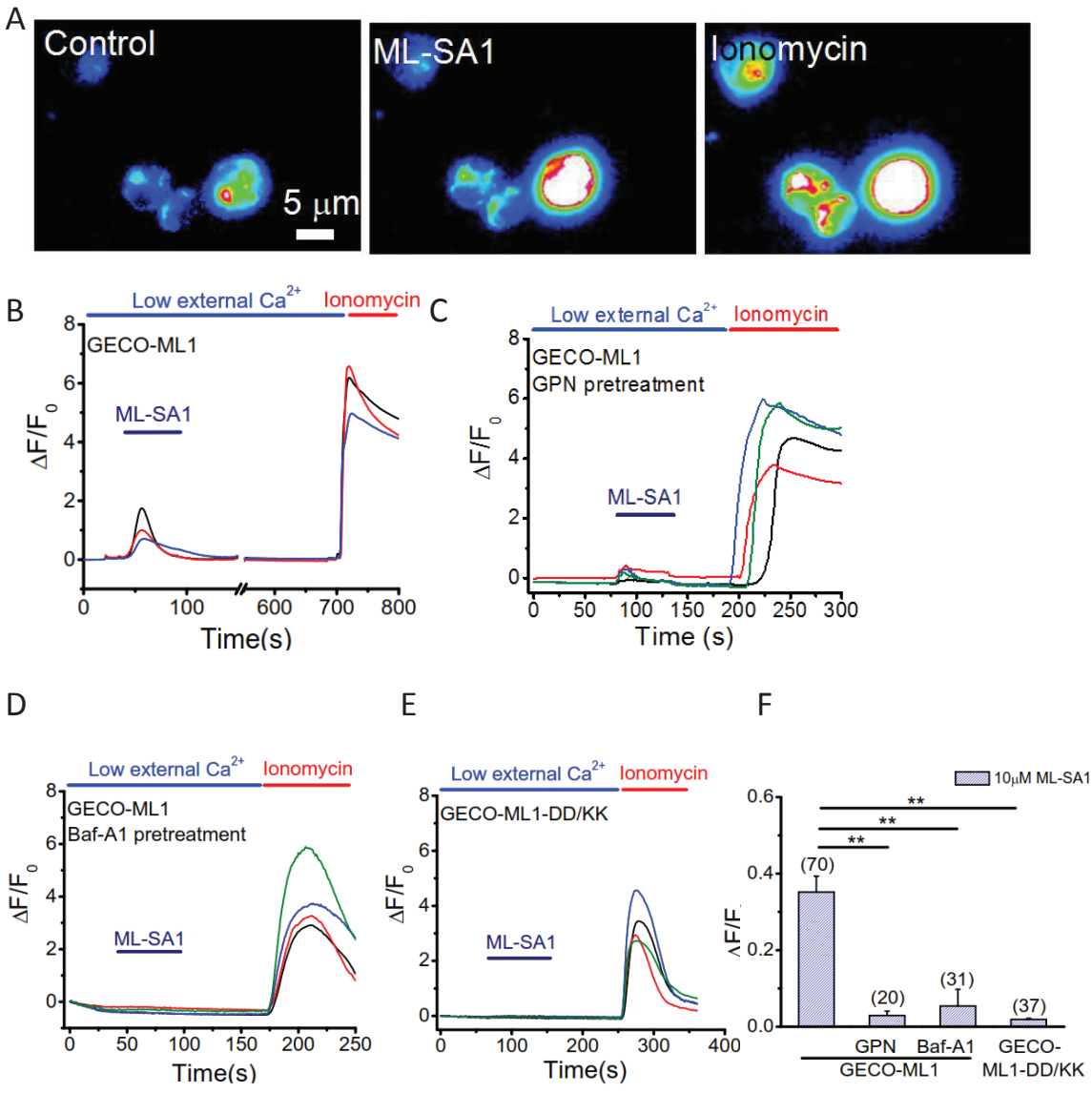

Figure 13 Measuring Lysosomal Ca^{2+} Release through ML1 using GECO-ML1

(A) Representative images showing the changes of GECO fluorescence upon bath application of ML-SA1 (10 μM) and ionomycin (1 μM) in HEK293T cells expressing GECO-ML1.

(B) ML-SA1 (10 μM) induced rapid elevation in GECO fluorescence (measured as change

of GECO fluorescence ΔF over basal fluorescence F0; ΔF/F0) under low (< 10 nM) external Ca^{2+} in HEK293T cells overexpressing GECO-ML1.

(C and D) Pretreatment of GPN (200 μM, 1hr) or Baf-A1 (200 nM, 30 min) suppressed ML-SA1 (10 μM) induced GECO responses, whereas ionomycin (1 μM) induced responses were maintained.

(E) ML-SA1 (10 μM) failed to induce any change in GECO fluorescence in HEK293T cells transfected with GECO-ML1-DDKK (non-conducting pore mutation) whereas ionomycin (1 μM) induced responses were maintained.

(F) Summary of ML-SA1-induced GECO responses in HEK293T cells under conditions indicated. It suggests that GECO-ML1 can be used to measure ML1 mediated Ca^{2+} release in intact cells.

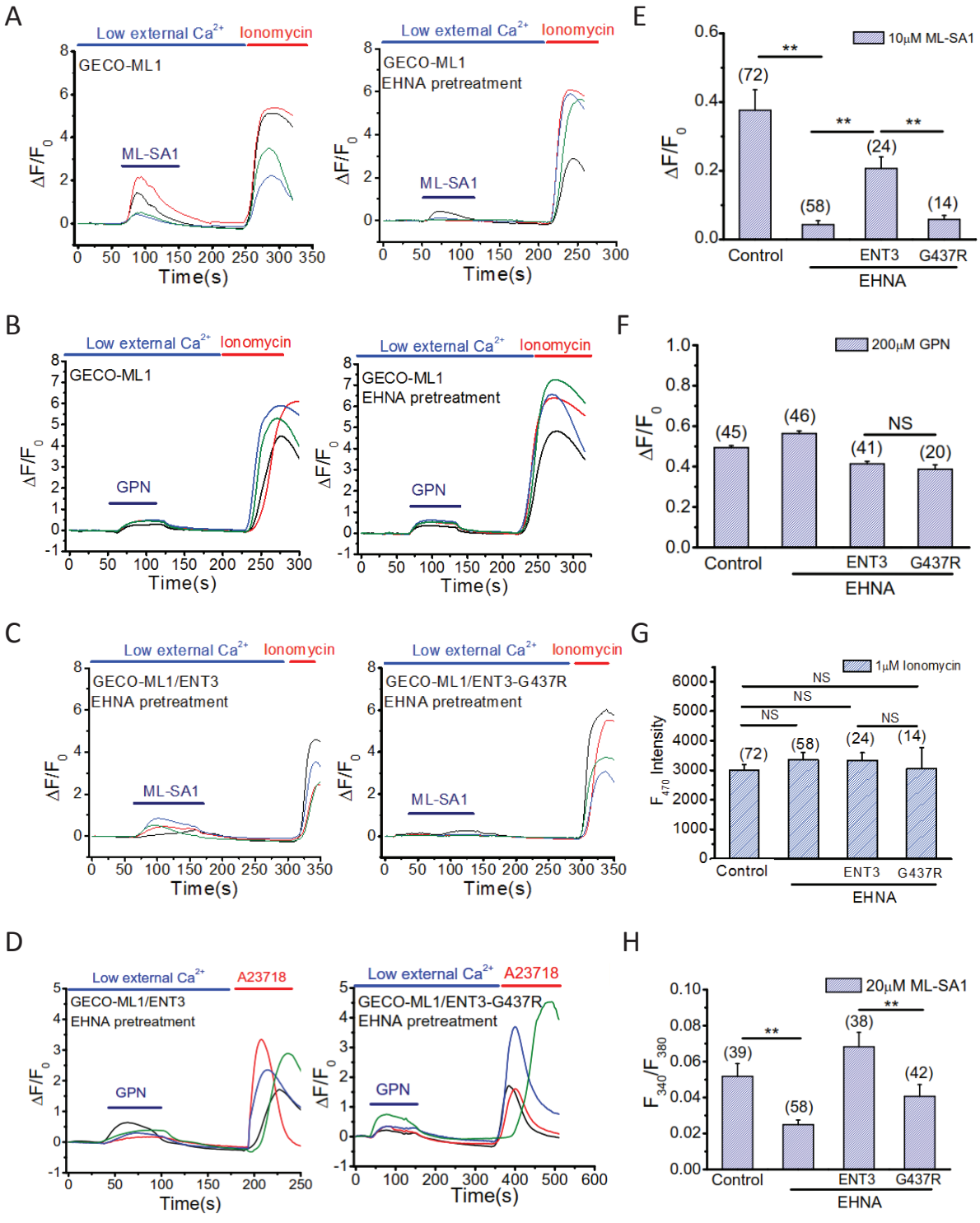

Figure 14 ADA Inhibitor EHNA Compromises Ca^{2+} Release through ML1

(A) EHNA pretreatment (3μM, 30 min) reduced ML-SA1 (10 μM) induced GECO-ML1 responses in HEK293T cells transfected with GECO-ML1, suggesting a smaller lysosomal Ca^{2+} release through ML1.

(B) EHNA-induced reduction in GECO-ML1 responses was partly rescued by overexpressing ENT3 but not ENT3-G437R mutant in HEK293T cells.

(C and D) GPN (200 μM), a substrate of the lysosomal exopeptidase cathepsin C that induces lysosome osmodialysis to release Ca^{2+}, induced comparable GECO-ML1 responses under each condition indicated, suggesting EHNA pretreatment did not lead to lysosomal Ca^{2+} content disruptions. Therefore, the change in SA1-induced GECO-ML1 responses (as shown in A, B and E) is not due to a change in lysosomal Ca^{2+} content.

(E) Summary of ML-SA1 induced GECO-ML1 responses in each group indicated. Pretreatment of EHNA (3 μM, 30 min) suppressed ML1-mediated Ca^{2+} flux, which was rescued by ENT3 but not ENT3-G437R overexpression.

(F) Summary of GPN induced Ca^{2+} release among each group indicated. Note that EHNA pretreatment did not lead to lysosomal Ca^{2+} content disruptions, , suggesting that the decreased GECO-ML1 is not due to a reduction in lysosomal Ca^{2+} content.

(G) Comparable expression levels of GECO-ML1 under conditions indicated. Expression level of GECO-ML1 was estimated by the maximal GECO fluorescence signal (at 470 nM) induced by ionomycin (1 μM).

(H) ML-SA1 (20 μM) induced lysosomal Ca^{2+} release was monitored by measuring Fura-2 ratios. EHNA pretreatment (3μM, 30 min) suppressed ML-SA1 induced lysosomal Ca^{2+}

release in HEK293T cells. The suppression was rescued by expressing ENT3 but not ENT3-G437R mutant. This is consistent with the data obtained by measuring GECO-ML1 responses as shown in A-F.

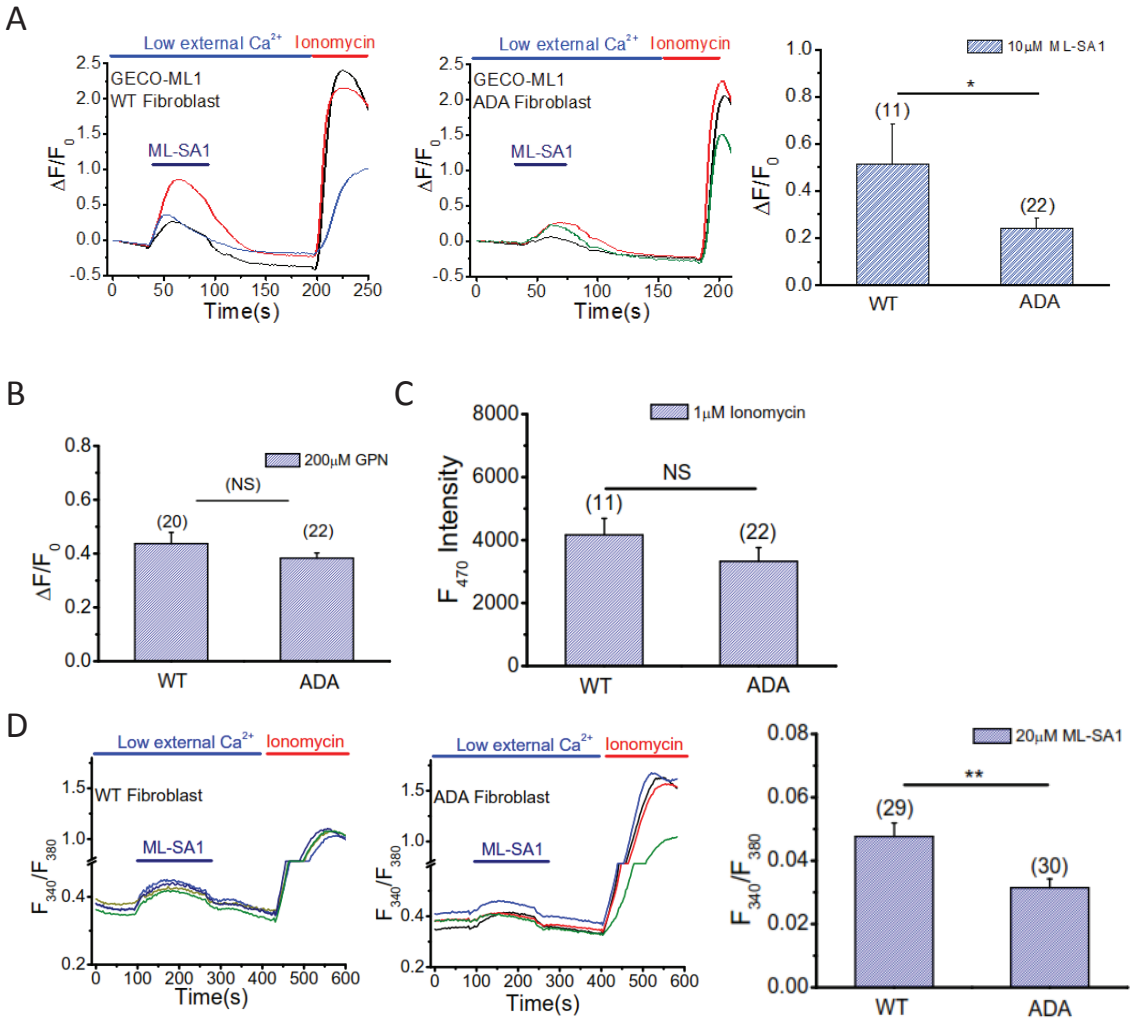

Figure 15 Smaller Ca^{2+} Release through ML1 in ADA Deficient Cells

(A) Cells were transfected with GECO-ML1. ML-SA1 (10 μM) induced GECO-ML1 responses in ADA deficient human fibroblasts was much smaller than that in WT human fibroblasts.

(B) GPN (200 μM)-induced comparable GECO-ML1 responses in WT and ADA deficient human fibroblasts, indicating similar lysosomal Ca^{2+} contents.

(C) Ionomycin (1 μM)-induced comparable GECO-ML1 responses in WT and ADA

deficient human fibroblasts, indicating similar expression level of GECO-ML1 in WT and ADA deficient human fibroblasts.

(D) Lysosomal Ca^{2+} release was monitored by measuring Fura-2 ratios. ADA deficiency caused a decrease in ML-SA1 (20 μM)-indcued lysosomal Ca^{2+} release, suggesting a smaller ML1 activity in ADA human fibroblasts. This is consistent with the data obtained by measuring GECO-ML1 responses as shown in A-C.

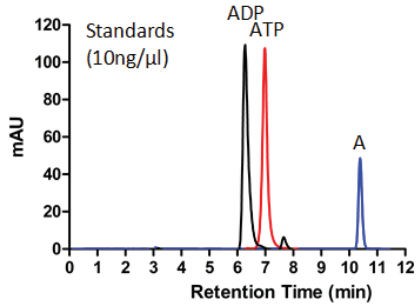

Figure 16 HPLC chromatogram of Adenosine and Adenosine Phosphates Standards

Representative HPLC chromatogram showed the separation of adenosine (A) and adenosine phosphates (ADP and ATP) standards. Standard concentrations were 10 ng/µl. All concentrations of adenosine (A) and adenosine phosphates (ADP and ATP) of lysosome samples were measured based on the standard curves of adenosine, ADP and ATP obtained by HPLC chromatogram.

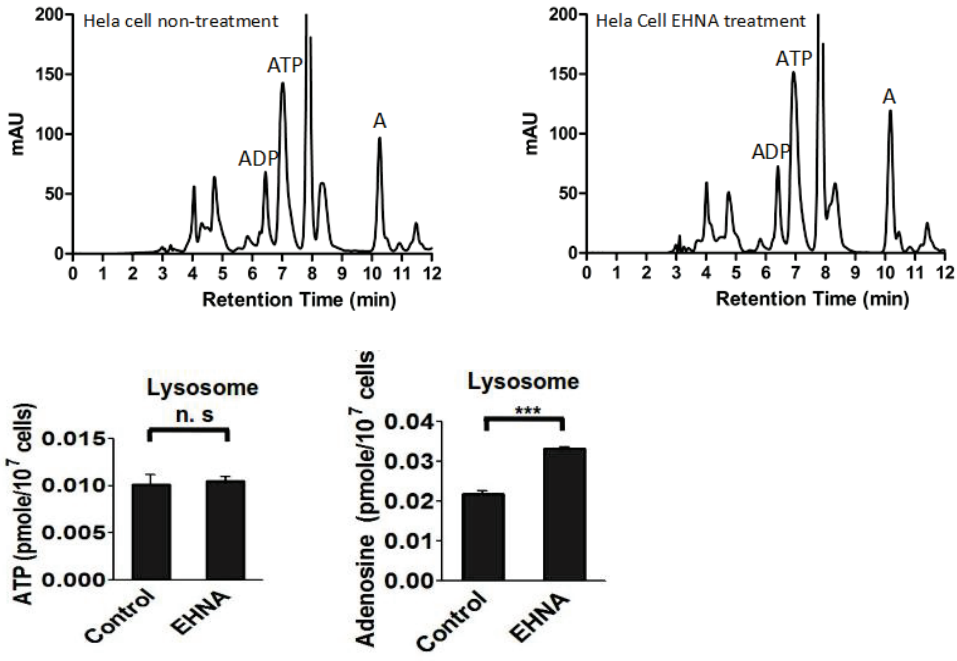

Figure 17 Effect of EHNA on the Level of Adenosine and Adenosine Phosphates in Isolated Lysosomes

ADA activity was inhibited by EHNA. In lysosomes isolated from cells pretreated with EHNA, ATP level did not alter (around 0.010 pmole/10^7 cells) while adenosine level was elevated prominently (from 0.02 to 0.03 pmole/10^7 cells). This result suggests that EHNA treatment effectively induces adenosine accumulation in lysosomes.

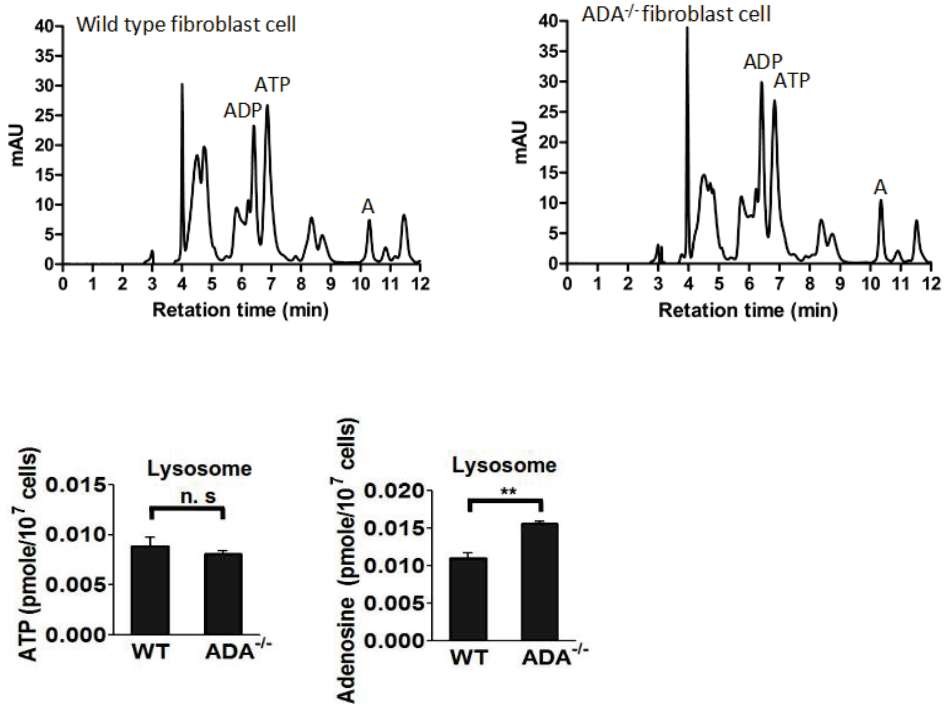

Figure 18 Measurement of Adenosine and Adenosine Phosphates in Lysosomes from the WT and ADA Deficient Fibroblasts

The lysosomes were isolated from WT and ADA deficient fibroblasts. Although lysosomal ATP levels were comparable between WT and ADA deficient fibroblasts (around 0.0085 pmole/10^7 cells), lysosomal adenosine level in ADA deficient fibroblasts was remarkably increased (from 0.01 to 0.15 pmole/10^7 cells).

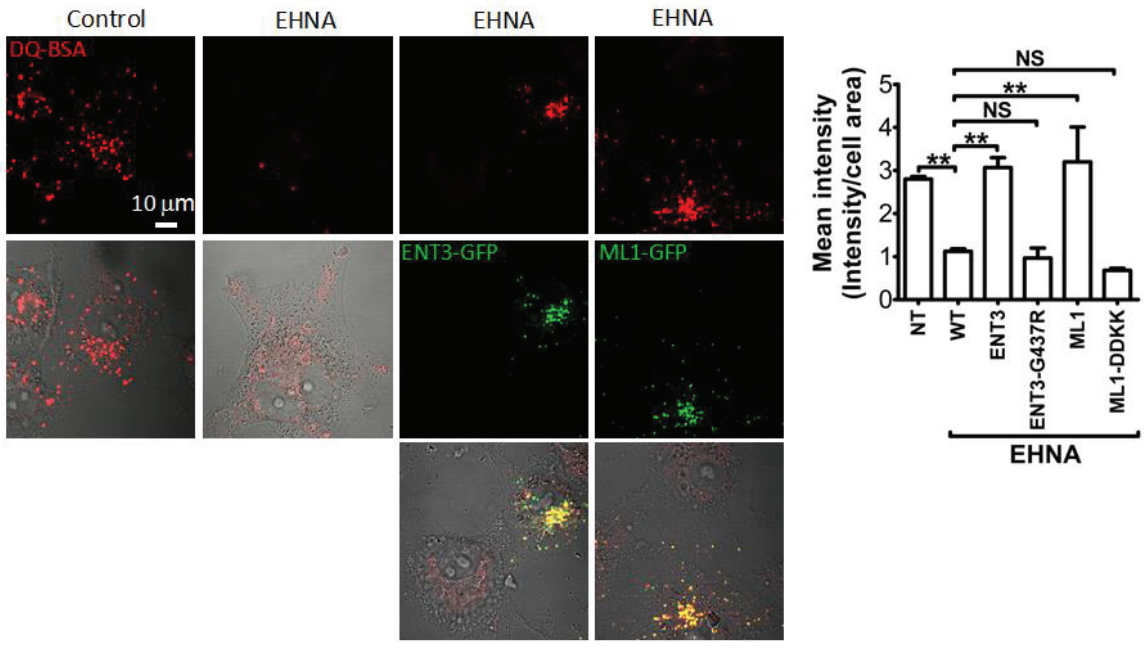

Figure 19 Lysosomal Dysfunction Induced by EHNA.

Lysosomal function was evaluated by DQ-BSA (10 µM) staining. Lysosomal adenosine accumulation was induced by treating EHNA (1 µM, 2 hrs) in Cos-1 cells. Strong DQ-BSA signals were observed in Cos-1 cells treated with control vehicle but not in cells treated with EHNA. This EHNA effect was rescued by ENT3 but not ENT3-G437R overexpression. It was also rescued by overexpressing ML1, but not ML1-DDKK-GFP mutant. These data suggest that EHNA treatment induces lysosomal adenosine accumulation, which inhibits ML1 and subsequently lead to compromise lysosome function.

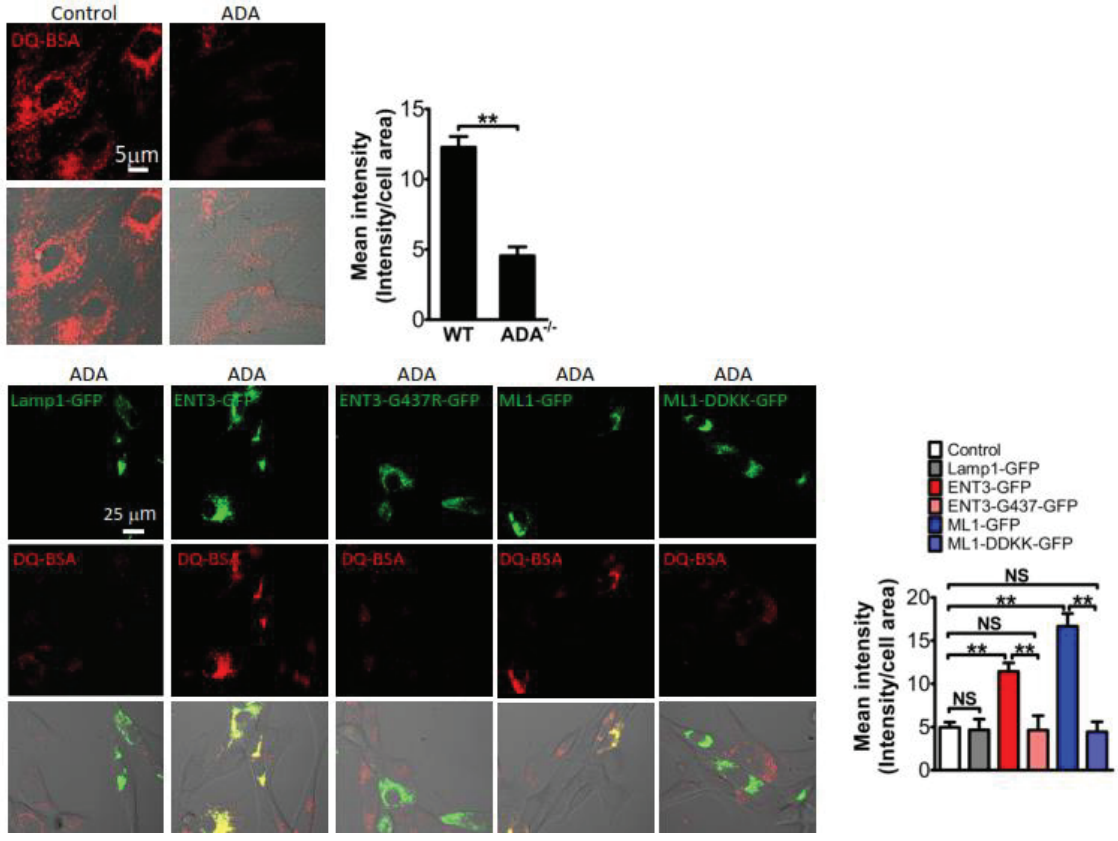

Figure 20 Impaired Lysosomal Function in ADA Deficient Fibroblasts

DQ-BSA signals were greatly reduced in ADA deficient but not WT fibroblasts. ENT3 but not ENT3-G437R overexpression could successfully rescue this inhibitory effect. Attenuated DQ-BSA signal in the ADA deficient fibroblasts was also rescued by ML1-GFP but not ML1-DDKK-GFP. These data suggest that lysosomal dysfunction observed in ADA fibroblasts is due to adenosine accumulation and impaired ML1 activity.