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„Identification and validation of new interactors from unbiased Turbo ID screen“

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Abstract

In recent years, there has been a continuing surge of interest in finding and describing lysosomal roles in cell signaling. Studies from the past decade revealed that the lysosome is a coordination center for different signaling events, including autophagy, translation, gene expression, organelle movement, and many other processes. In order to execute these multiple functions the lysosome's surface is decorated with protein and protein complexes. The aim of this project is to map the lysosomal surface in order to get an understanding of the spatial distribution of lysosomal membrane proteins as well as to see which factors are recruited to the specific complex sites. We decided to use a proximity-dependent labeling approach coupled to mass spectrometry. To this purpose seven late endosomal and lysosomal proteins (LAMTOR1, LAMTOR3, Rab7, TMEM192, LAMP1, LAMP2A, and LAMP2B) were fused to the biotin ligase TurboID and expressed in HeLa cells. Moreover, we wanted to investigate the protein interaction dynamics at the lysosomal surface upon spatial reorganization within the cell.

Zusammenfassung

In den letzten Jahren hat das Interesse an der Suche und Beschreibung lysosomaler Rollen bei der Zellsignalübertragung einen anhaltenden Anstieg erfahren. Studien aus dem letzten Jahrzehnt haben gezeigt, dass das Lysosom ein Koordinationszentrum für verschiedene Signalereignisse ist, einschließlich Autophagie, Translation, Genexpression, Organellenbewegung und vielem mehr andere Prozesse. Um diese vielfältigen Funktionen auszuüben, ist die Oberfläche des Lysosoms mit Proteinen und Proteinkomplexen dekoriert. Das Ziel dieses Projektes ist die Kartierung der lysosomalen Oberfläche, um ein Verständnis der räumlichen Verteilung lysosomaler Membranproteine zu erhalten und zu sehen. Wir haben uns für eine gängige Markierungstechnik ("proximity-dependent labeling") gekoppelt mit Massenspektrometrie entschieden. Dazu wurden sieben späte endosomale und lysosomale Proteine (LAMTOR1, LAMTOR3, Rab7, TMEM192, LAMP1, LAMP2A und LAMP2B) mit der Biotin-Ligase TurboID fusioniert und in HeLa-Zellen exprimiert. Außerdem wollten wir die Protein-Interaktionsdynamik an der lysosomaler Oberfläche bezüglich räumlicher Reorganisation innerhalb der Zelle untersuchen.

Contents

List of Abbreviations

AMPK	AMP acivated kinase
APS	Ammonium persulfat
BCA	Bicinchonic assay
IF	Immuno fluorescence
FCS	Fetal bovine serum
FSM	Full Serum Medium
GAF	GTPase activating protein
FLCN	Folliculin
FNIP	FLCN-interacting protein
GEF	Guanine nucleotide Exchange factor
GSK3β	Glycogen synthase kinase 3β
HRP	Horseradish peroxidase
MS	Mass Spectrometry
mTORC1	Mammalian target of rapamycin complex1
LAMTOR	Late endosomal and lysosomal adaptor and MAPK and MiTOR activator
LAMP	Lysosome-associated membrane proteins
LB	Lysogeny broth
NPCs	Neural progenitor cells
ECM	Extracellular matrix
ERK	Ras-dependent extracellular signal-regulated kinase
ER	Endoplasmatic reticulum
LSD	Lysosomes storage dissease
ORP1L	Oxysterol-binding protein-related protein 1
PAGE	Polyacril gel electrophoresis
PBS	Phosphatebuffered saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenamide
PIP5K	phosphatidylinositol 4-phosphate 5-kinases
Rab	Ras associated protein
RILP	Rab-interacting lysosomal acivated protein
RIPA	Radioimmunoprecipipitation assay buffer

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SDS	Sodium dodecyl sulfat
SFM	Serum free medium
SEM	Standard error mean
TAE	TRIS Acetate EDTA
TFEB	Transcription factor EB
TEMED	Tetramethylethylendiamine
TMEM	Transmembrane protein 192
TPC	Two-pore channel
TRIM16	Tripartite motif-conteinig protein 16
TRPML	Transient receptor potential cation channels of the mucolipin family
TSC	Tuberous sclerosis complex
ULK1	Unc-51-like kinase
WB	Western blot

Chapter 1

Introduction

1.1 The Lysosome

Lysosomes were first discovered by Christian de Duve in the 1950s and at that time, described as cytoplasmic organelles enclosed by a membrane having the role in the degradation of macromolecules including proteins, lipids, carbohydrates, and nucleic acids [1]. Nowadays, lysosomes are recognized as cell organelles with a pivotal role in cell metabolism and cell signaling. As such, they take part in other cell function as antigen presentation, cell membrane repair, apoptosis, gene regulations, cell adhesions, migration, and even at tumor invasion and metastasis [2]. The functions are illustrated in image by Ballabio and Bonafacino [1]. The most important factor of these roles is lysosomal interactions with other cell compartments like the endoplasmatic reticulum (ER), peroxisomes, and mitochondria. Moreover, interaction with the Golgi apparatus is crucial for the regulation of mammalian target of rapamycin complex in the perinuclear area [3].

1.1.1 Signaling pathways regulated by lysosomes

Cells and cell organelles communicate via direct contact or through sending signals. Although the main function of lysosomes, such as nutrients degradation, typically happens in its lumen, the signaling occurs on its surface. This is the reason why so many proteins are found on lysosomal membranes. Different lysosomal signaling pathways allow the maintenance of cellular homeostasis and cellular functions. Some of the essential pathways and lysosomal proteins are further described in Section 1.1.4 and illustrated in Figure 1.2.

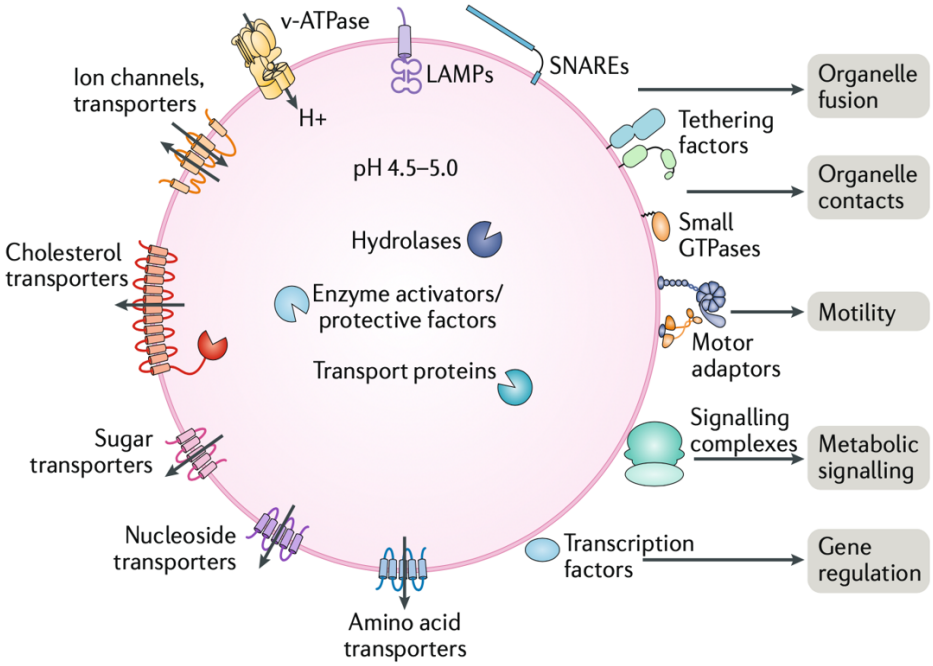

FIGURE 1.1: **Image 1. Lysosomes as signalling hub.** The scheme depicts the diversity of luminal, integral-membrane proteins and peripherally associated proteins. Due to v-ATPase activity the lysosomal lumen has acidic pH. It contains hydrolases and other enzymes for substrate degradations, as well as lipid transport protein NPC2, which transfers cholesterol to NPC1 - another lysosomal membrane protein. Lysosomal membranes contain LAMPs, highly glycolysited proteins, whose role is protecting the lysosomal membrane form acidic pH. Further, on lysosomal membrane are ion channels, ion transporters, lipids transporters and solute carriers for transport of sugers, nucleosids, amino acids and other degradation products. The protein complexes on the cytosolic side of lysosomes are illustrated. Among others they regulate the mTORC pathway and transcription factors such as TFEB and TFE3, that further regulate authophagy, lysosome biogenesis and energy metabolism. Furthermore, small GTPases control lysosomal scaffold complexes and they are responsible for microtubules interactions with lysosomes. Image by Ballabio and Bonifacino[1].

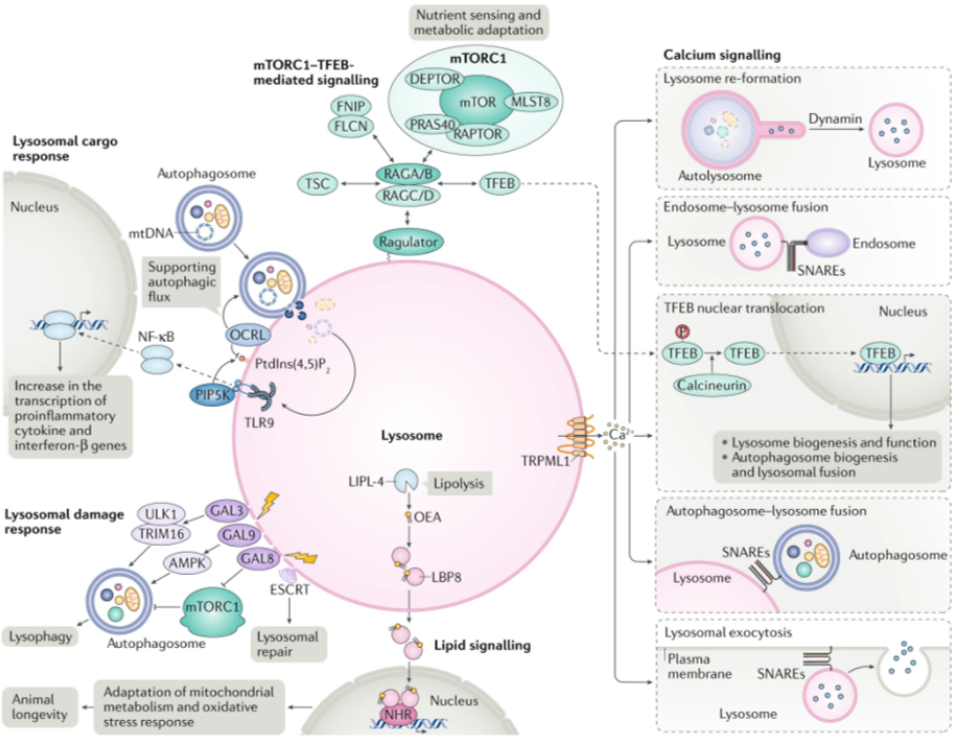

FIGURE 1.2: **Image 2. Cellular processes are modulated by signaling pathways initiated from the lysosomal surface.** mTOR pathway and translocation of TFEB are regulated by several RAG GTPases, among others, such as tuberous sclerosis complex (TSC), folliculin (FLCN), and FLCN-interacting protein (FNIP). Ca^{2+} release initiates the processes such as lysosomal re-formation from hybrid organelles, TFEB nuclear translocation, autophagosome-lysosome fusion, endosome-lysosome, and lysosomal exocytosis. Lysophagy is modulated by mTORC1, 5-AMP activated protein kinase (AMPK), and Unc-51-like kinase (ULK1)-tripartite motif-containing protein 16 (TRIM16). The process is activated by the recruitment of galectins (GAL3, GAL8, and GAL9). Through TLR9 activation, the lysosome senses mitochondrial DNA and starts lysosomal cargo response. Consequently, phosphatidylinositol 4-phosphate 5-kinases (PIP5K) is recruited and phosphatidylinositol 4,5-bisphosphate generated - mediator in SNAREs recycling after autophagosome-lysosome fusion. Image by Ballabio and Bonifacino[1].

1.1.2 The positioning of lysosomes

It could be said that lysosomes in the cell are divided into two groups. The main one is perinuclear, and comprises most of the lysosomes, and the second one is peripheral. The lysosomes interact with other cell structures and can travel fast via microtubules (MT) or by low-speed diffusion [4]. Several MT-associated kinesin motor proteins, such as the Arf GTPase Arl8b and its effectors SKIP and KIF1 - kinesin 1 are responsable for lysosomal motility towards the periphery. Arl8b is recruited by BORC to the lysosomal membrane and leads lysosomes to the cell periphery. BORC inhibition causes Arl8b dissociation from lysosomes, preventing lysosomal trafficking to the cell periphery. It is suspected that an increased number of the translocated lysosomes has impact on adhesion formation and metastatic processes in migratory cells. However, the degradative "housekeeping" functions of lysosomes in the perinuclear area is not affected in this case [5]. Important factors in anterograde lysosomal trafficing are RhoA, PI3Ks and the protein complex FYCO [6]. This sort of motility is also KIF1 dependent [5]. Retrograde movement - from the periphery to the perinuclear area - is directed by the dynein-dynactin motor complex. Recruitment of dynein motors is further explained in Section 1.1.4, as one of the main interactors and regulators of the dynein-dynactin complex is Rab7.

1.1.3 Ca^{2+} release

Ca^{2+} release is important for many signaling events including endocytic membrane trafficking, autophagy and cell membrane repair [1]. There are three types of Ca^{2+} channels: transient receptor potential cation channels of the mucolipin family (TRPML), trimeric Ca^{2+} two-transmembrane channel (P2X4), and two-pore channel (TPC) [1]. Lysosomal Ca^{2+} channels are regulated by different stimuli, such as pH and nutrients, but also by ATP, nicotinic acid adenine dinucleotide phosphate, sphingosine and phospholipids [1].

1.1.4 Selected lysosomal bait proteins

LAMTOR complex

Lysosomal positioning regulates anabolic and catabolic responses to changes in nutrient availability [7]. Cellular functions of lysosomes are regulated by modulating the activity of the mTORC1, 5´- AMP-activated protein kinase (AMPK), and glycogen synthase kinase- 3β (GSK3β). All of these kinases are activated by complexes on a lysosomal membrane in coordination with nutrient levels [8].

The LAMTOR (late endosomal and lysosomal adaptor and MAPK and mTOR activator) complex, also known as Ragulator, is composed of five subunits. The crystal structure of the Ragulator shows that LAMTOR2/3 and LAMTOR4/5 heterodimers are surrounded and held together by LAMTOR1, which anchors the complex to the lysosomal membrane by its N-terminal myristoyl and palmitoyl groups [9]. It has been shown that the LAMTOR2/3 subunit is responsible for activation of MAPK3/ERK1 through interaction with MAPK kinase 1 [10]. However, the main function of LAMTOR complex is to anchor the Rag guanosine triphosphatases (GTPases) to the lysosome. Rags - Rag GTPases - are critical for proper amino acid sensing by conversion from the inactive GDP-bound state to the active GTP-bound state. This conversion is mediated by guanine nucleotide exchange factors (GEFs). The Ragulator also serves as a guanine nucleotide exchange factor (GEF). Therefore, one of the main mechanisms for mTORC1 activation is through amino acids presence with Rags GTPases and Ragulator complex as mediators. Under low amino acid conditions the Ragulator is found in inhibitory state with bounded Rags in their inactive GDP form. Rags in GTP-bound state enhance the association of mTORC1 and late endosomes by bringing mTORC1 close to Rheb, a potent stimulator of the mTORC1 kinase activity, what further regulates cell growth and division [11]. Rheb, in the absence of growth factors under stimulation of the tuberous sclerosis complex (TSC), promotes GTP hydrolysis [12].

A futher regulator of cellular metabolism is AMPK (AMP-activated protein kinases). AMPK is activated upon glucose deficiency - Axin recruits AMPK through interaction with liver kinase B1 and activates AMPK by forming complexes with V-ATPase and Ragulator [13]. It

is not clear which LAMTOR subunit interacts with Axin. It has been suggested that it is the Ragulator complex as a whole entity. This hypothesis is supported by the fact that knockout of any LAMTOR subunit impaired AMPK activation. Simultaneously, Axin inhibits GEF activity of Ragulator toward Rags, causing inhibition of the mTORC1 pathway [13].

Finally, Ragulator interacts with SLC38A9 and vacuolar H+ ATPase, neutral amino acid transporter. When activated, mTORC1 stimulates anabolism, including protein and lipid synthesis and inhibits catabolic processes such as autophagy. The complex between Ragulator and v-ATPase serves as the docking site for AXIN/LKB1 endosomal translocation, and the forming the v-ATPase-Ragulator-AXIN/LKB1-AMPK complex enables switching between anabolic mTORC1 and catabolic AMPK pathways [14].

Lysosomal positioning has been linked to the lysosomal function. Hence, peripheral lysosomes activate the mammalian target of rapamycin complex 1 (mTORC1) pathway i.e. anabolism, and perinuclear lysosomes autophagy i.e. catabolism [7].

Lysosomal associated membrane proteins (LAMPs)

As a membrane protection, lysosomes have high N-glycosylated proteins, lysosomal associated membrane proteins – LAMPs, and lysosomal integral membrane protein-2/lysosomal membrane glycoprotein. Both proteins have their N-terminus directed towards the lysosomal lumen [15]. Furthermore, all the LAMPs have a single transmembrane region of about 20 amino acids, and 10-12 amino acids long carboxyl-terminus exposed on the cytosolic side of the lysosomal membrane [16]. It is assumed that LAMP1 and LAMP2 differ a lot evolutionary, as they have only around 37% amino sequence homology. Further evidence for this comes from their localization on different chromosomes [17]. Most of the LAMPs are localized on the lysosomal membrane. However, sometimes they can be found at the endosomes and at the plasma membrane [18].

There are three LAMP2 isoforms – A, B, and C, which differ in transmembrane and cytosolic parts,whereas the luminal region is conserved [19]. LAMPs isoforms are differentially expressed in different

tissues and differently distributed between the plasma membrane and lysosomes [20]. LAMP2A acts as a receptor in the lysosomal membrane for substrate of chaperone-mediated autophagy, a process that targets proteins to degradation by recognizing specific motifs on their sequence [20]. Mutations in genes coding LAMP2 can lead to the accumulation of late autophagic vacuoles in the heart and muscles, which can cause Danon disease, fatal cardiomyopathy, and myopathy associated with mental retardation [21].

Rab7

Ras-related protein Rab7 is a small GTPase from the Rab family. Generally, Rab GTPases are responsible for intracellular membrane and protein trafficking, vesicle transport, as well as SNARE complex formation and membrane fusion [22]. For the GTPase function guanine exchange factors (GEF) are necessary. GEFs stimulate the exchange of GDP to GTP, thereby activating Rab7, whereas GAP (GTPase activating protein) triggers hydrolysis from GTP to GDP and by that inactivates Rab7. Rab7's main role is bidirectional trafficking of membranous cargo [23]. The minus-end-directed dynein-dynactin motor complex transports its cargos to the perinuclear region, whereas plus-end-directed kinesin motor proteins transport their cargos towards the cell periphery [24]. Rab7 builds a complex with the adaptors RILP (Rab-interacting lysosomal protein) and a cholesterol sensor ORP1L (oxysterol-binding protein-related protein 1) that binds the p150 $^{\text{Glued}}$ protein of dynein-dynactin motor complex on the cell membrane. [25]. In this case, Rab7 boosts lysosomal retrograde movement - from the cell periphery towards the nucleus [26]. In contrast, FYCO1 can be found on late endosomes and lysosomes where it regulates anterograde movement - from the perinuclear area to the periphery by interaction with LC3A and LC3B. Therefore, Rab7 has a role in several physiological processes such as apoptosis, neurotrophin trafficking and signaling, neurith outgrowth, phagocytosis, as well as autophagy [23].

TMEM192

TMEM192 is a lysosomal and late endosomal transmembrane protein. TMEM192 is a non-glycosylated protein with four transmembrane

domains [27]. It cannot be classified in any group of lysosomal proteins, so its function is not well known. However, it is widespread in tissues like human kidney, liver, lung, and pancreas tissue, which suggests it has an important role for lysosomes [27].

1.2 Lysosomes in disease

As it was mentioned in Section 1.1, lysosomes have a central role in many cell processes, and thereby profoundly impact homeostasis. The most famous disorders due to lysosomal dysfunction are lysosomal storage disorders (LSDs), rare, inherited diseases, in which the cell metabolism is impaired. Lately, it became clear with the discovery of new lysosomal functions and observing the change in size and number of lysosomes, as well as lysosomal interactions with other cellular structures, that the lysosomes are important for developing other diseases, besides LSDs. These include neurodegenerative diseases such as Parkinson´s disease, Alzheimer's disease, and Huntington's disease, metabolic disorders as obesity and cardiovascular diseases, and cancer [1].

1.2.1 Lysosomal storage diseases

Lysosomal storage disorders are rare inherited diseases, in which the lysosomes are not able to degrade their substrates due to the absence or reduced activity of lysosomal proteins [28]. This leads to the accumulation of different undegraded substrates in the lysosomal lumen, and impacts autophagy, vesicle trafficking, fusion with other organelles, and mitochondrial function and signaling pathways [29]. Moreover, these changes can result in altered calcium homeostasis. Depending on the specific lysosomal disorder, calcium storage and release can be decreased, for example in NPC cells [30] or as in Mucolipidosis type IV, calcium release can be increased, which leads to enhanced fusion within the endocytic pathway [31]. Activation of the TFEB pathway is considered responsible for the increased size of lysosomal compartments during LSDs. When TFEB is overexpressed, there is enhanced clearance of stored material. Thus, activation of the TFEB/CLEAR network could recover the cellular defect in many LSDs [32].

1.2.2 Neurodegenerative disorders

Autophagic processes are altered in many neurodegenerative diseases, such as Alzheimer´s disease, Parkinson's disease, Huntington's disease, but autophagy dysfunction can also contribute to autoimmune diseases, such as multiple sclerosis. [33], [34]. Lysosomal swelling and accumulation of lipids were reported near the degenerated astrocytes in multiple sclerosis. Based on this fact, it is supposed that due to swelling and permeabilization, lysosomes release hydrolases in the cytosol, where they affect native proteins [35]. Further, mTOR upregulation was described in multiple sclerosis [36], and LAMTOR is an important factor in mTOR pathway control. Alzheimer's disease is characterized by protein aggregates, composed of microtubules-associated protein tau and amyloid- β peptides. In the first phase of Alzheimer's disease, lysosomal activity is increased in order to counteract the toxic effect of proteins aggregates. However, as the disease progresses, lysosomal activity decreases, leading to the further accumulation of toxic proteins and subsequent cell death [37].

1.2.3 Cancer

Cancer cells suffer a change in composition, subcellular localization, and lysosomal volume during cancer transformation and progression [38]. In metastasis, during the invasion phase, it was noticed that more lysosomes localized at the cell periphery. In the invasion phase, massive degradation of ECM happens and because of that, it is assumed that lysosomes, namely their proteolytic enzymes such as matrix metalloproteins and plasminogen, are needed for the degradation of ECM proteins. [5]. In addition, lysosomes at the periphery contribute to the acidic environment, which is neccessary for the internalization of adhesion components. Therefore, also V-ATPase, a proton pump on lysosomes, is highly involved in cancer cell invasion [39]. Moreover, recent findings suggest that due to the lysosomal membrane permeabilization more cathepsins are released. Cathepsins are lysosomal proteases that can be active outside of lysosomes, at pH 7. Oncogenic properties were identified in many cathepsins and they are associated with the stimulation of cancer progression [40]. Their releasing in the cytosol can activate the intrinsic apoptotic

pathway, while the release of cathepsins into the extracellular space stimulates tumor growth. [40].

1.3 Insight in current draft of the lysosomal proteome

The processes in cells are regulated and executed through macromolecular interactions. Interactions between proteins, proteins - DNA, or proteins - RNA are crucial and their dysregulation can lead to different diseases such as cancers, immune disorders, and neurodegeneration. There are several methods to describe and study these interactions, but the disadvantage of many of these is that they fail to reveal transient, short term interactions. The combination of proximity-based labeling and mass spectrometry-based proteomics investigation of previously unknown interactions, including transient and short term, and a deeper understanding of biological processes and cell signaling are becoming possible [41].

As already mentioned in Section 1.1, lysosomes are emerging as a signaling hub regulating many metabolic and signaling processes. Until now, more than 300 proteins have been identified either on or at lysosomes. Currently, proteins involved in lysosomal functions could be classified as:

i. soluble proteins residing in the lysosomal lumen

ii. membrane proteins (present in the cytosol and associated with the lysosomal surface) [3].

To date, different approaches - a variety of methods for lysosomal enrichment and mass spectrometry (MS) - have been utilized to characterize the lysosomal proteome, investigate processes on lysosomal membrane and changes in the lysosomal environment during disease, and identify biomarkers for disease. Through these efforts, the lysosomal proteome is still continuously expanding and novel lysosomal proteins are identified.

1.3.1 Proximity labeling - Turbo ID

A state-of-the-art method for investigating protein-protein interactions is *in vivo* proximity biotinylation. Proximity labeling uses enzymes such as engineered ascorbic peroxidase 2 (APEX), horseradish peroxidase (HRP), and biotin ligase (BioID, BASU, TurboID, mini-TurboID) genetically coupled to the proteins of the interest ("baits"). Upon addition of biotin phenol and H_2O_2 for APEX and biotin for BioID and TurboID, cellular proteins in the proximity of the bait will be biotinylated. The labeling range is 1-10 nm, experimentally determined. Reactive species are membrane impermeant, therefore the reaction always happens inside of the cell [42]. A very convenient way to isolate biotinylated proteins is to utilize the quasi-covalent interaction of biotin with streptavidin in a pulldown setup and later identify them via mass spectrometry.

The peroxidase-catalyzed proximity labeling system - APEX - requires biotin-phenol as the substrate and it is necessary to add hydrogen peroxide for peroxidase activation. As is well-known, hydrogen peroxidase is toxic to living cells and leads to a strong stress response. By contrast, BioID labeling is simple and non-toxic, as it requires only biotin as the substrate. However, a big disadvantage is very slow kinetics that misses the processes that happen in the timescale of minutes or a couple of hours [43]. Important improvement to the proximity-based labeling in the TurboID system (Fig. 1.3) is very fast kinetics.

1.4 Aim of the research

The aim of the project was to conduct a TurboID proximity labeling screen with prominent lysosomal proteins (LAMTOR1, LAMTOR3, Rab7, TMEM192, LAMP1, LAMP2A, and LAMP2B), in order to get a precise and more accurate lysosomes´ interactome. Based on previous screens (unpublished data from Rainer Ettelt), it is assumed that lysosomal proteins´ groups are spatially organized. This means they form so-called "interaction islands". The goal of the project is the identification of novel lysosomal proteins, especially transient interactors, which could not be identified via other methods.

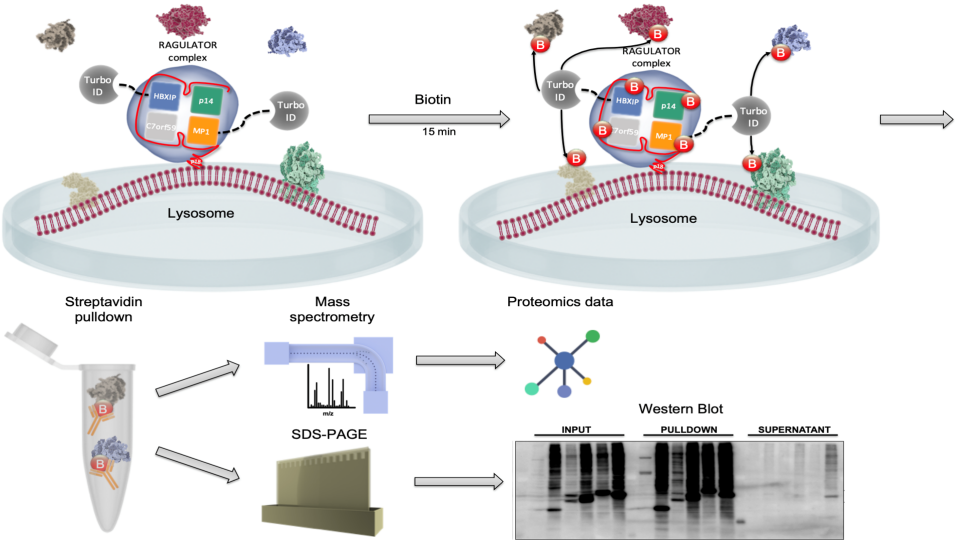

FIGURE 1.3: **Image 3. Workflow of TurboID proximity labeling.** Inducing constructs in stable HeLa cells with doxycycline is followed by the addition of biotin for 15 minutes to the cells. During this time proteins within a radius of 10-20 nm are labeled, and pulldown of biotinylated proteins is possible after cell lysis. Pulled down proteins are analysed via western blot or mass spectrometry. The whole LAMTOR complex was used as an illustration for the work-flow. The constructs which we used are described in Section 1.1.4.

Chapter 2

Results

2.1 Doxycyclin titration

To conduct a proximity-dependent labeling screen coupled to MS analysis, we started by cloning bait fusion proteins (proteins of interest coupled to TurboID plus a V5 tag for simpler detection on WB and IF samples). We confirmed that the identity of the desired constructs were correct by sequencing (complete sequences are shown in Appendix A.1). Next, we verified that the constructs were expressed and active in the cells. As all constructs are doxycyclin dependent, we conducted doxycyclin titration in order to investigate under which doxycyclin concentration our constructs were expressed. Cells without doxycyclin induction were used as controls.

Western blot analyses (WB) (see Figs. 2.1, 2.2 and 2.3) showed that all of our constructs were expressed after doxycyclin treatment. Additionally, we could compare expression levels of the endogenous proteins with those of the exogenous fusion proteins to choose the optimal doxycycline concentrations. This is important to maintain next-to-endogenous stoichiometry in the cell and thus faithful complex formation.

TurboID converts biotin into biotin-adenosine monophosphate, a intermediate that covalently labels lysin residues of proteins in the range of 1-10 nm in living cells. Streptavidin is a sensitive and stable biotin interactor. Therefore, streptavidin staining indicates that the TurboID subunits are active and able to biotinylate proteins in their proximity. This means that streptavidin bands are similar in size to interactors, but their intensity depends on the interactors' molecular weight - number of lysin residues as biotynilation sites - in addition

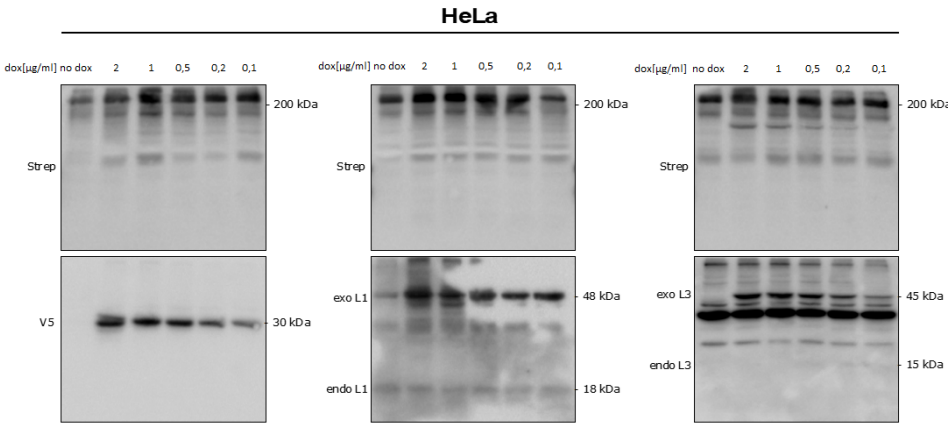

FIGURE 2.1: **Doxycycline titration.** Stable HeLa cells lines were treated with 2 µg/ml, 1 µg/ml, 0,5 µg/ml, 0,2 µg/ml or 0,1 µg/ml of doxycyclin. Top panels: Whole cell lysates obtained from HeLa cells expressing TurboID alone (cyto*, left panel) or as a fusion protein with LAMTOR1 (middle panel) or LAMTOR3 (right panel) were stained with streptavidin in order to visualize the enzymatic activity of the biotin ligase. Bottom panels: The first panel (from left to right) depicts the V5 staining of cyto bait. The other two panels are LAMTOR1 and LAMTOR3 baits, respectively stained for the corresponding proteins - they show endogenous LAMTOR1/LAMTOR3 and exogenous LAMTOR1/LAMTOR3.
*Cyto is a nuclear export sequence (NES) fused to the TurboID. NES is a short peptide, which is exported from the cell nucleus to the cytoplasm through nuclear pore complex, therefore it can be found in the whole cytoplasm.

to the stability and intensity of interaction (e.g. LAMTOR1 and LAMTOR3 are strong interactors, but due to the small molecular weight of LAMTOR3, the streptavidin band at the size of LAMTOR3 on LAMTOR1 panel is not so strong).

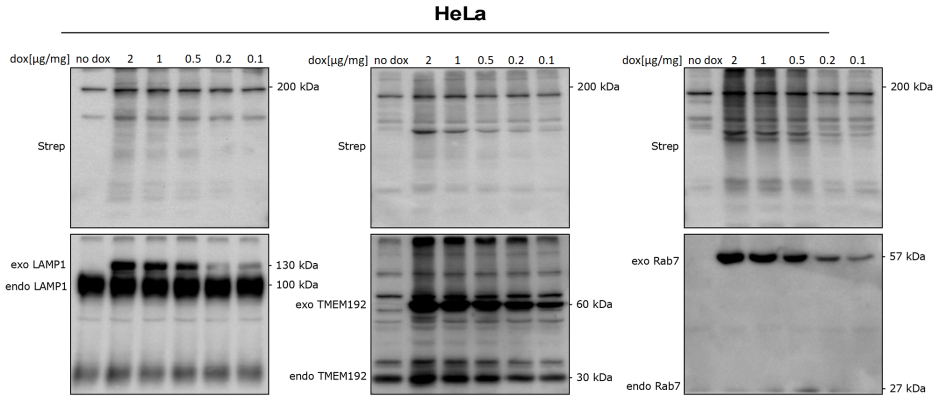

FIGURE 2.2: **Doxycyclin titration.** Stable HeLa cells lines were treated with 2 µg/ml, 1 µg/ml, 0,5 µg/ml, 0,2 µg/ml or 0,1 µg/ml of doxycyclin. Top panels: Whole cell lysates obtained from HeLa cells expressing a fusion protein with LAMP1 (left panel), TMEM192 (middle panel) or Rab7 (right panel) were stained with streptavidin in order to visualize the enzymatic activity of the biotin ligase. Bottom panel: panels are LAMP1, TMEM192 and Rab7, respectively, stained for the corresponding proteins - they show endogenous LAMP1/TMEM192 /Rab7 and exogenous LAMP1/TMEM192 /Rab7.

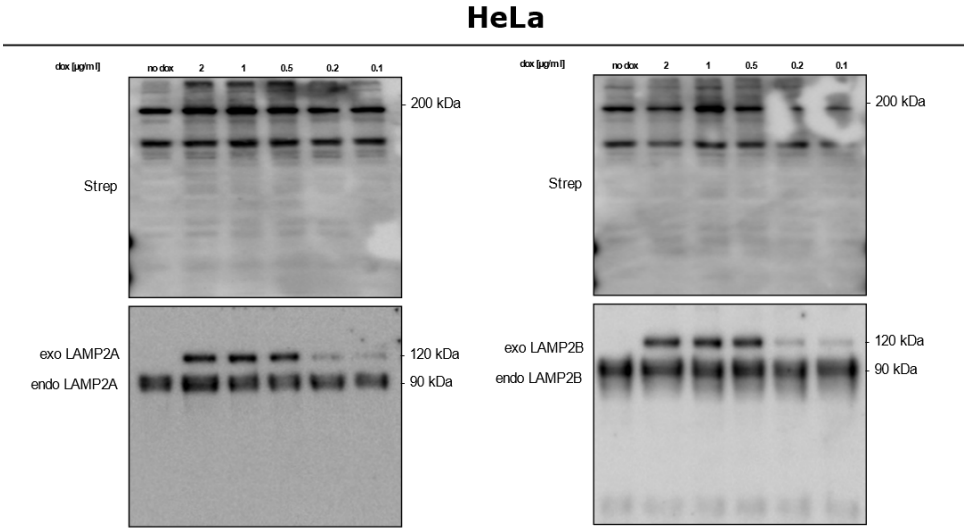

FIGURE 2.3: **Doxycyclin titration.** Stable HeLa cells lines were treated with 2 µg/ml, 1 µg/ml, 0,5 µg/ml, 0,2 µg/ml or 0,1 µg/ml of doxycyclin. Top panels: Whole cell lysates obtained from HeLa cells expressing a fusion protein with LAMP2A (left panel) or LAMP2B (right panel) were stained with streptavidin in order to visualize the enzymatic activity of the biotin ligase. Bottom panel: panels are LAMP2A and LAMP2B, respectively, stained for the corresponding proteins - they show endogenous LAMP2A and LAMP2B and exogenous LAMP2A and LAMP2B.

2.2 Baits expression and localisation

We investigated the expression of the constructs and their localization by Immuno-flourencence (IF). Expression of the constructs was induced with doxycycline at the concentration chosen from the previous experiment, that is the concentration that the best matches the endogenous levels of proteins.

All cells were stained with DAPI, V5, Streptavidin, and LAMP1 antibodies, (Figs. 2.4 and 2.5). The purpose of the LAMP1 staining is to mark the lysosomes, while V5 antibodies mark the baits. Streptavidin staining illustrates the biotinylation cloud generated by the ligase. At the bottom of the figures, there are also merged images, where the colocalization of the baits with lysosomes can be observed. There is no colocalization for the cyto bait because it represents cytoplasmic control.

To further analyze the images, profiling plots were created as follows. A line that connects the cell nucleus with the cell membrane was drawn, highlighted in the merged images in Figs. 2.6 and 2.7. Distribution of staining along this line is shown in Figs. 2.8 and 2.9. The profiling plots clearly illustrated colocalization of V5, streptavidin and LAMP1 signals in all cases except for the cyto bait.

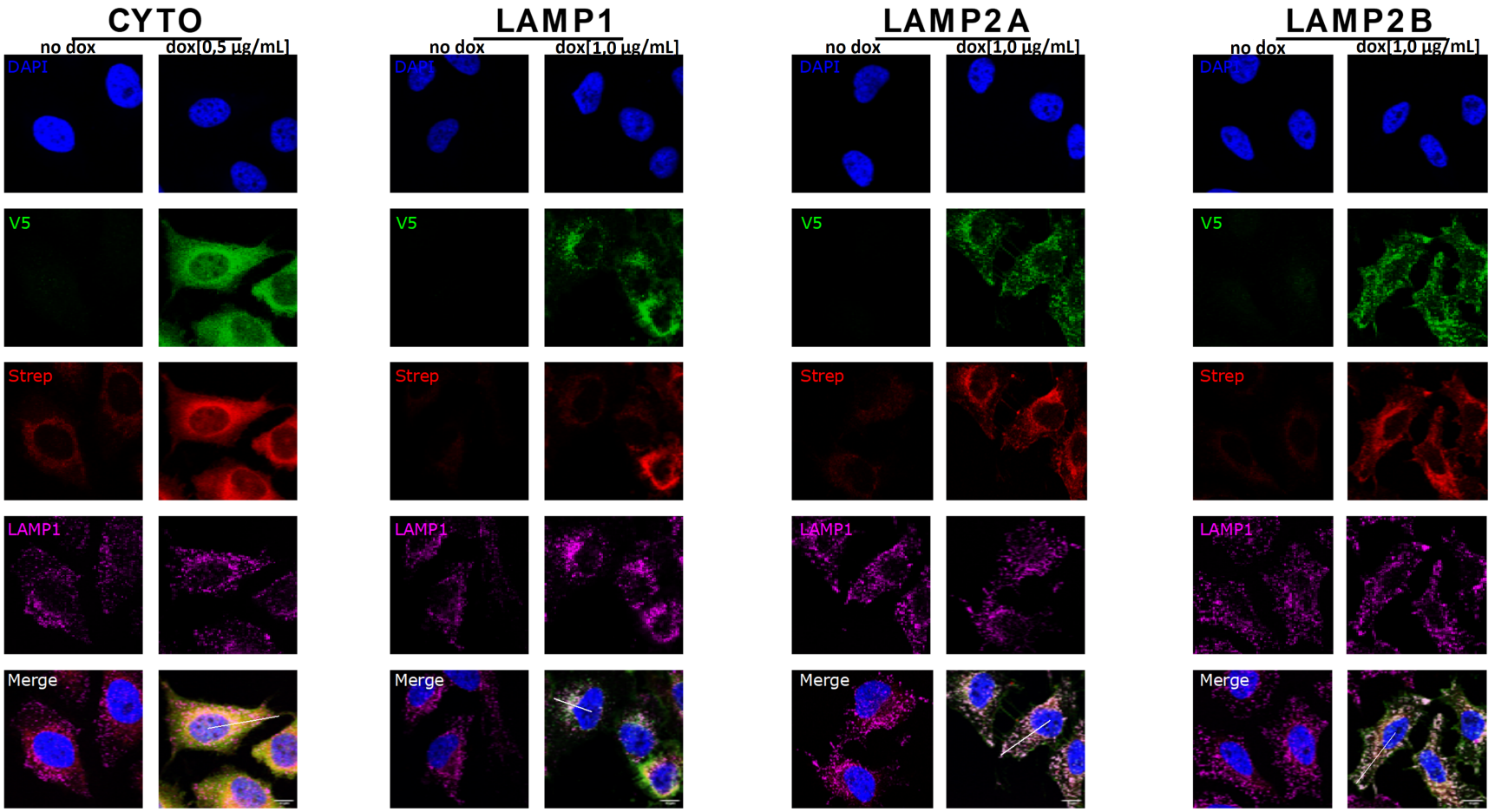

FIGURE 2.4: Baits localization. The figure shows images of the cells after the constructs' expression was induced with doxycyclin (Cyto, LAMP1, LAMP2A and LAMP2B - from left to right). The first column of images for each bait is control (cells without doxycyclin induction). DAPI staining is shown in blue, V5 tag staining in green, streptavidin staining in red. LAMP1 staining (purple) was used as a marker for the lysosomes. The bottom images represent a merged image of the upper four images.

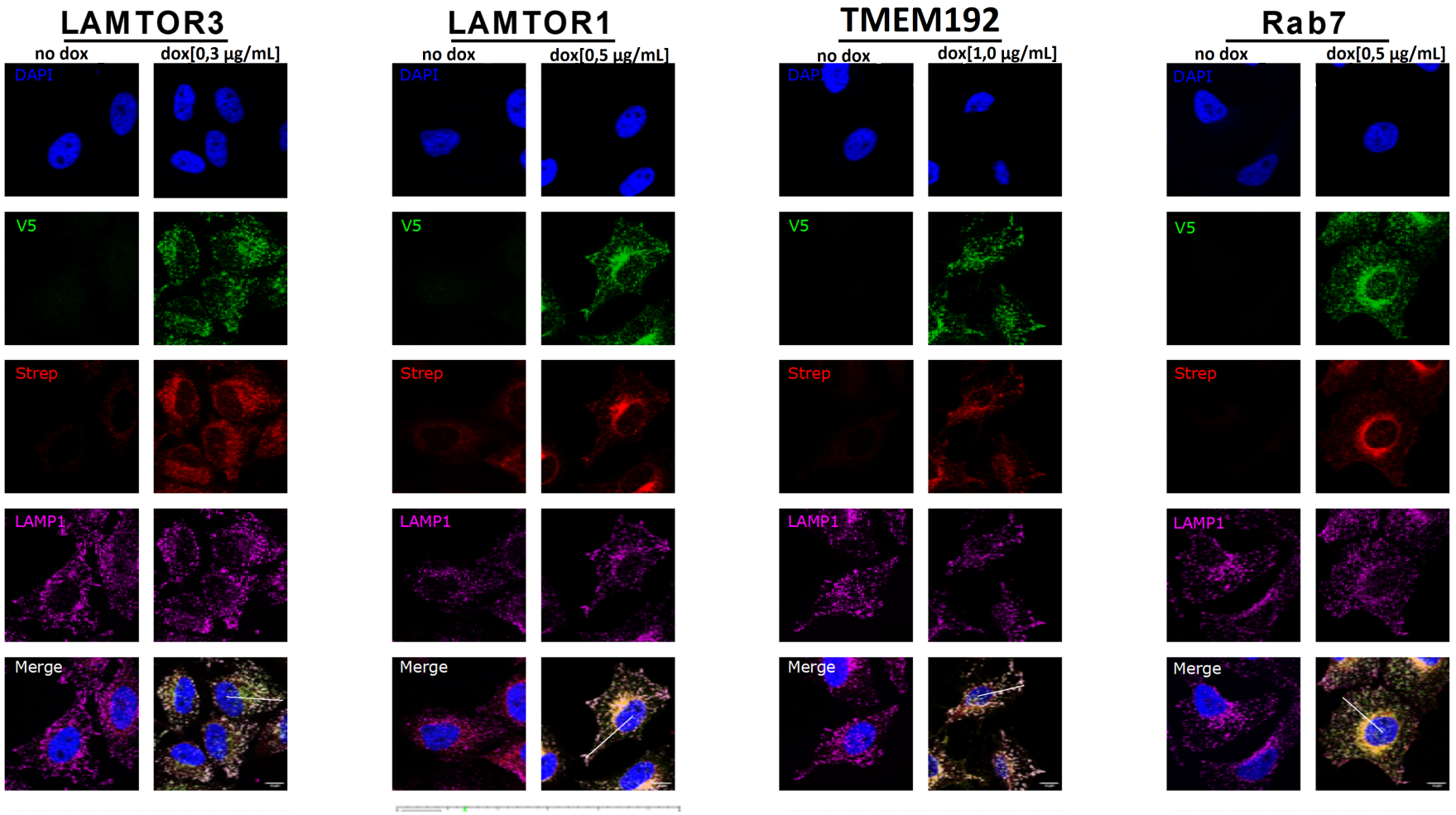

FIGURE 2.5: **Baits localization.** The figure shows images of the cells after the constructs' expression was induced with doxycyclin (LAMTOR3, LAMTOR1, TMEM192 and Rab7 - from left to right). The first column of images for each bait is control (cells without doxycyclin induction). DAPI staining is shown in blue, V5 tag staining in green, streptavidin staining in red. LAMP1 staining (purple) was used as a marker for the lysosomes. The bottom images represent a merged image of the upper four images.

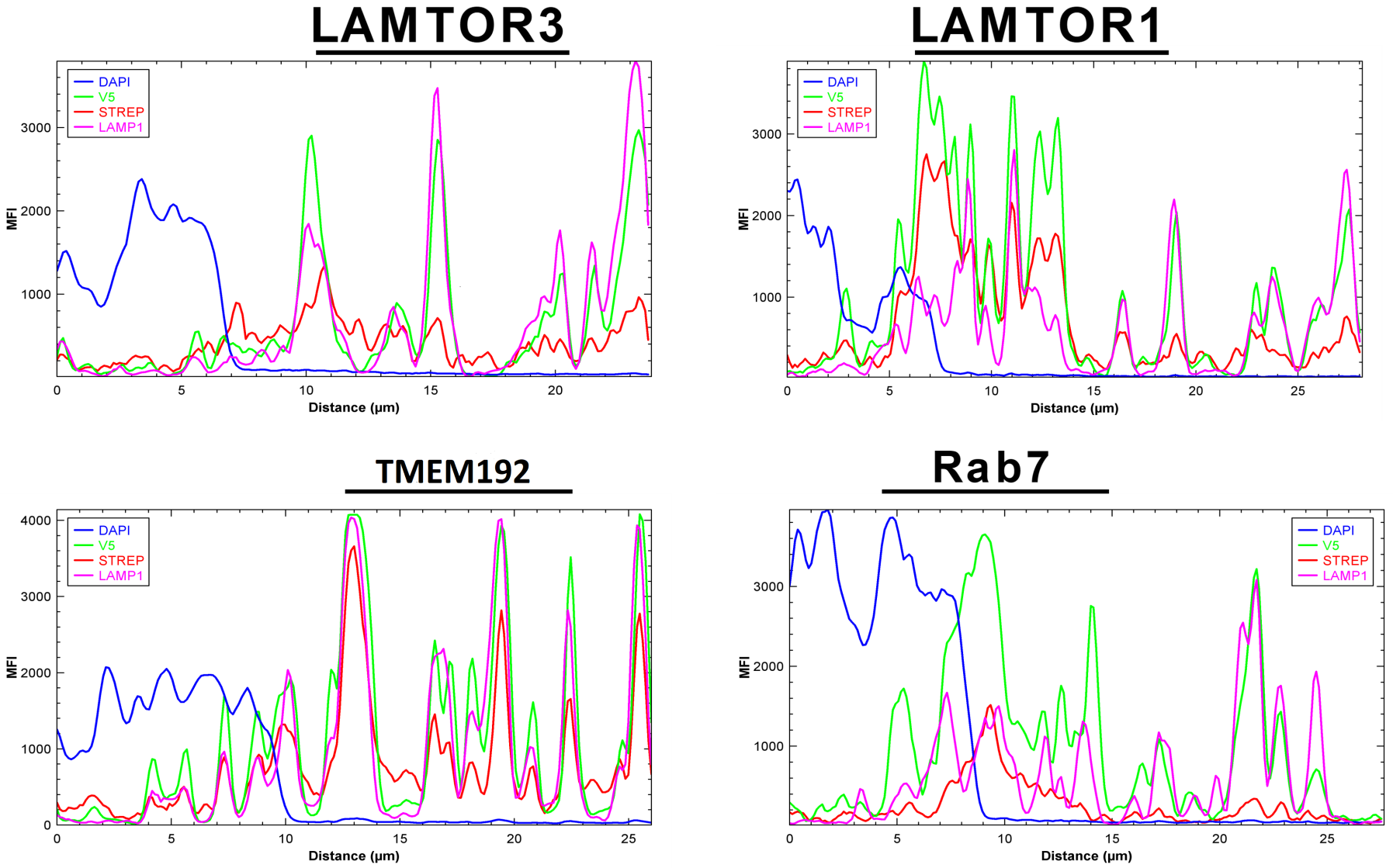

FIGURE 2.6: **Baits localization.** Representative profiling plots of the cells in Figs. 2.4 and 2.5. Shown is the distribution of the markers along the line highlighted in merged images in Figs. 2.4 and 2.4. DAPI staining is shown in blue, V5 tag staining in green, streptavidin staining in red. LAMP1 staining (purple) was used as a marker for the lysosomes.

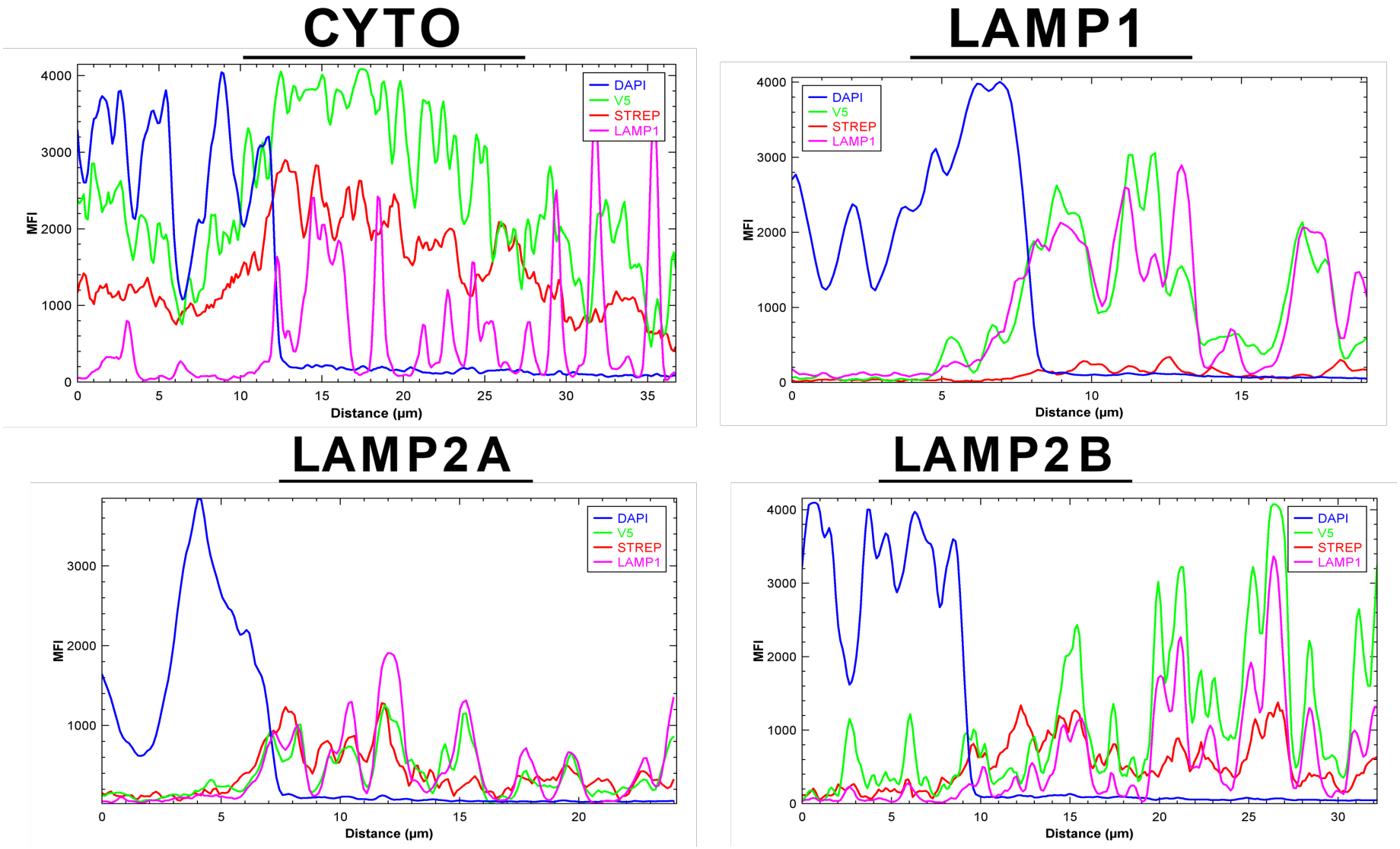

FIGURE 2.7: **Baits localization.** Representative profiling plots of the cells in Figs. 2.4 and 2.5. Shown is the distribution of the markers along the line highlighted in merged images in Figs. 2.4 and 2.4. DAPI staining is shown in blue, V5 tag staining in green, streptavidin staining in red. LAMP1 staining (purple) was used as a marker for the lysosomes.

Parallel with the IF, we performed a western blot to confirm the expression levels and activity of the constructs. The cells for IF and western blot were grown and treated in the same dish, and after biotinylation split into two parts, one of which was further prepared for the IF and the other lysed for western blotting. Figs. 2.8 and 2.9 show streptavidin staining, which reads out TurboID enzymatic activity, and V5 staining showing the expression levels of the fusion proteins. The membranes were also stained with antibodies recognizing the baits (LAMTOR1, LAMTOR3, LAMP1, LAMP2A, LAMP2B, Rab7 and TMEM192) to ensure that the levels of expression of the fusion proteins did not vastly exceed that of the endogenous proteins, which could lead to mislocalization.

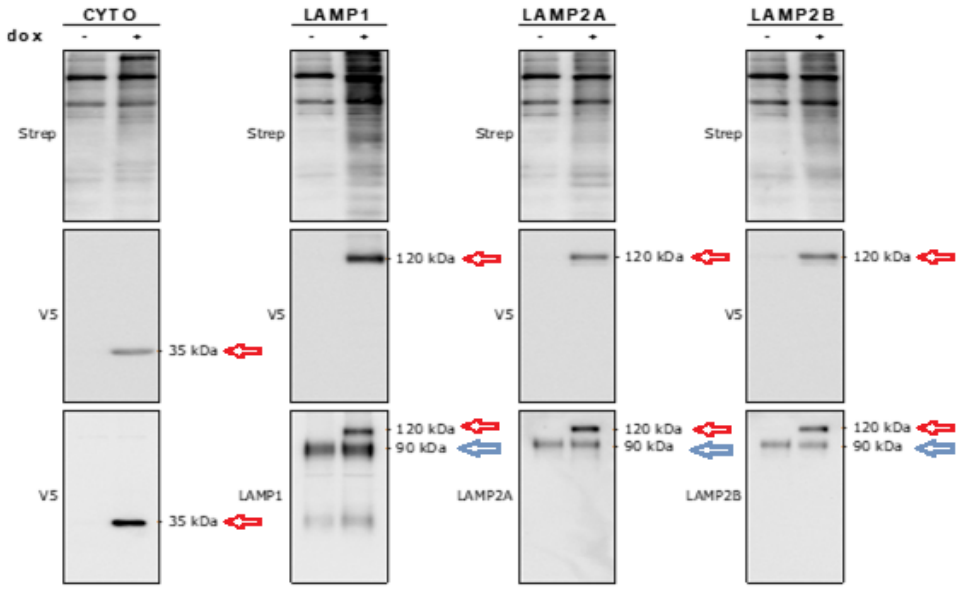

FIGURE 2.8: **Baits expression and activity performed in parallel with the IF.** Top panels: streptavidin staining; Middle panels: V5 tag staining; Bottom panels: stained for Cyto, LAMP1, LAMP2A and LAMP2B (from left to right); for cyto V5 antibodies were used. Red arrows point at the fusions proteins and blue ones at the endogenous proteins.

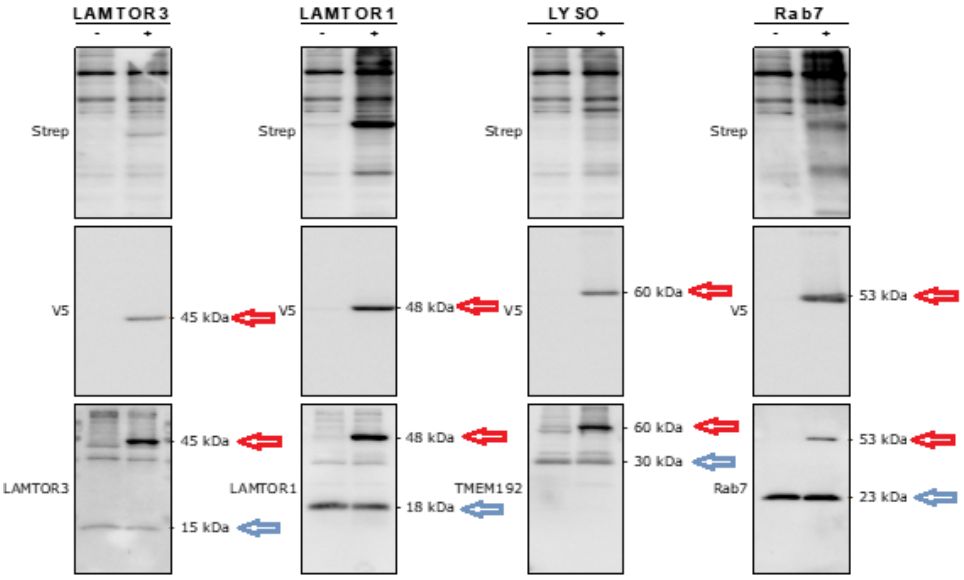

FIGURE 2.9: **Baits expression and activity, done parallel with the IF.** Top panels: streptavidin staining; Middle panels:V5 tag staining; Bottom panels: stained for LAMTOR3, LAMTOR1, Lyso and Rab7 (from left to right). Red arrows point at the fusions proteins and blue ones at the endogenous proteins.

2.3 Lysosomal positioning

According to the literature (see Refs. [7], [44]), lysosomal functions change upon relocalization of the organelles. As already described in Section 1, depending on where lysosomes localize in the cell, they can have different interactors. Thus, to address the question of the positioning of the lysosomes as potential conditions for the MS screen, we starved cells for 18 h with serum free medium (SFM) and then restimulated them with full serum medium (FSM) for 2 h. Figure 2.10 shows that under starvation conditions the lysosomes were concentrated in the perinuclear area compared to the cells that were not starved. The images were analyzed with Image J and the diffrence is illustrated in Fig. 2.11. Hand selected cells in images such as 2.10 were segmented and the density of the lysosomes is indicated as the value raw integrated density (RawIntDen). Normalized RawIntDen represents the sum of all pixel intensity (LAMP1 staining) in the area of interest, divided by the number of pixels in the same area. The LAMP1 pixel intensity density is represented as a function of the distance from the cell nuclei to the cell membrane.

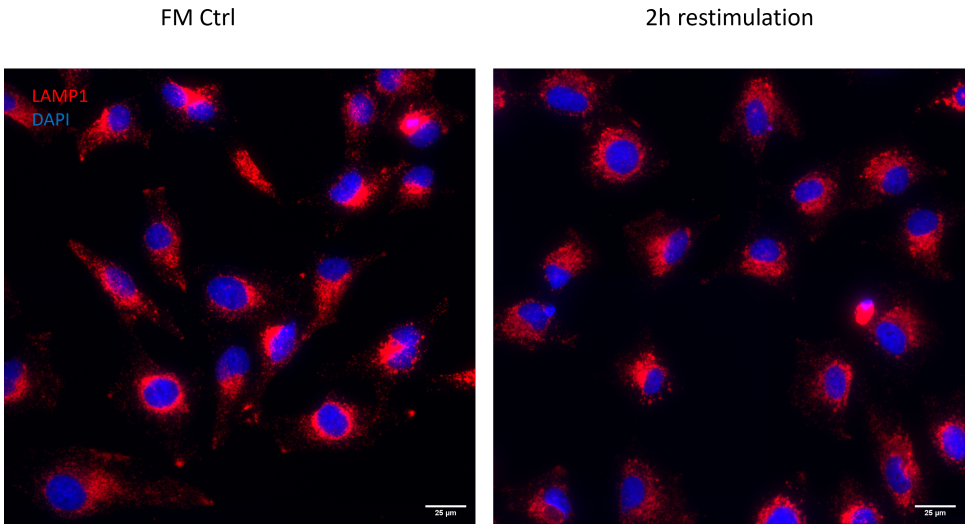

FIGURE 2.10: Left: Lysosomal distribution in cells in full medium. Right: Lysosomal distributions in starved cells in serum free medium for 18 h and restimulated with full medium for 2 h. LAMP1 was used as a marker for the lysosomes (red). Blue color indicates DAPI staining.

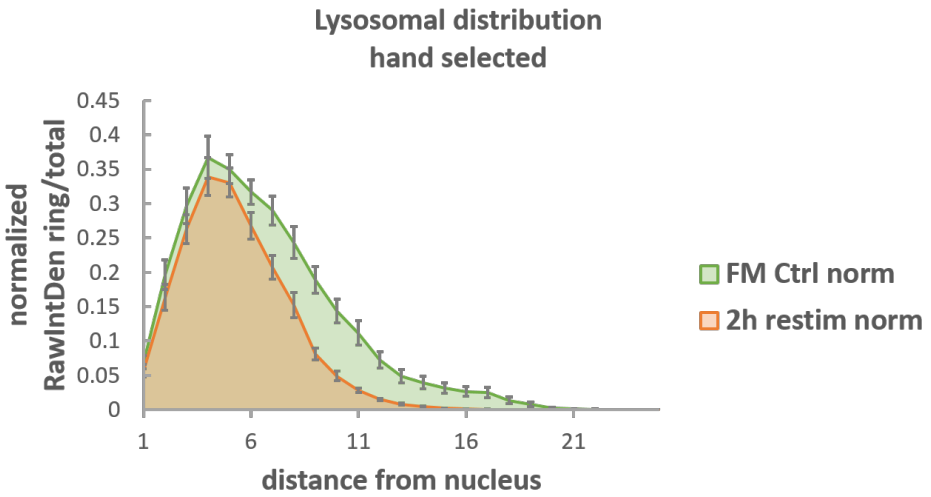

FIGURE 2.11: This graph shows normalized raw integrated density (RawIntDen) as a function of distance to the cell nucleus along a spatial path (the lysosomal density is measured in areas from cell nuclei to cell membranes, distances are measured in µm). The orange curve represents lysosomal distribution in starved cells (18 h serum free medium + 2 h full medium), and the green curve represents untreated cells (grown in full medium). More than 1000 cells pro condition were counted in 3 independent experiments. Vertical bars indicate standard error of the mean.

Chapter 3

Discussion and conclusion

3.1 Discussion

We confirmed that all baits (cyto, LAMP1, LAMP2A, LAMP2B, LAMTOR1, LAMTOR3, Rab7, and TMEM192) are expressed and we titrated them to match endogenous expression levels. The localization of the baits was checked by Immunofluorescence and the colocalization for all constructed lysosomal baits was confirmed by costaining of the lysosomal marker LAMP1 with the bait marker V5. As expected, the cytosolic control didn't show any overlap with the lysosomal marker, but all other baits did (see Figs. 2.4, 2.5, 2.6 and 2.7). At the same time, with the western blot in Figs. 2.8 and 2.9 we confirmed that all constructs are expressed at levels similar to the endogenous proteins and enzymatically active. With that, the tools for a MS screen are set up.

Moreover, with cell starvation we tasted conditions that impact lysosomal positioning. This is important since the literature (see Refs.[7], [44]) suggests that lysosomal positioning coordinates different cell functions, and might therefore impact the proteins/structures the lysosomes interact with. Under steady state conditions - full medium - the lysosomes show full dispersal throughout the cytoplasm. Under starvation and then 2 hours of restimulation, the shift in lysosomal positioning could be observed (shown in Figs. 2.10 and 2.11). However, not all the lysosomes were affected by the treatments but rather a smaller mobile population. A shift of this magnitude would probably not suffice to observe any difference in the interactome. Restimulation was used instead of starvation only, because the cells under starvation only experienced more of a stress phenotype.

The idea for this project came from another screen done by Rainer Ettelt (unpublished data). For his MS screen a smaller number of baits were used - LAMTOR3, TMEM192, and the cyto as control. However, some results were surprising. For example, LAMPs were not biotinylated by a LAMTOR3 bait, although the LAMPs are the most abundant lysosomal proteins. A possible explanation for this could be that proteins cluster on the lysosomal surface, building proteins clusters. By using more baits we expect to define lysosomal proteins belonging to specific clusters, and ideally how the clusters are distributed on the lysosomal surface. In support of this strategy, it has been recently reported that three of the baits we used, namely LAMP1, LAMP2A, and LAMP2B, which have very similar structure and function, have distinct interactomes as determined by a BioID screen [45]. Such difference might be even more pronounced among baits involved in different lysosomal functions, such as mTOR pathway regulation and/or trafficking (LAMTOR1, LAMTOR3, Rab7). It is also possible that some proteins will participate in several clusters or switch between them depending on the signaling events happening.

As the authors of [45] conducted a screen with BioID system, we expect that with TurboID improvements more relevant and preciser data can be collected. BioID is a well-established method for identifying protein-protein interactions, but labeling period requires 15-18 hours for biotinylation which is not ideal for short-term, transient interations. The short biotinylation times typical of TurboID (10-15 minutes) will enable the detection of such short-term, transient interations.

3.2 Conclusion

We generated TurboID fusion constructs with a range of lysosomal proteins and cloned them in doxycyclin-inducible lentiviral backbones. Stable HeLa cell lines were generated by viral infection. The expression and correct localization of our constructs was confirmed by western blotting and immunofluorescence, respectively, and the activity of the biotin ligase moiety could be verified by streptavidin western blotting. The expression levels were titrated close to endogenous levels and therefore the cells are ready for submission to mass spectrometry analysis.

Further plans include the study of different metabolic states (starved and fed) which should result in lysosomal relocalization and therefore possibly in a change in the proxisome of the lysosomal bait proteins.

Chapter 4

Materials and Methods

4.1 Materials

TABLE 4.1: Facilities that were used for the project

Aparatures	Company	Method
ProFlex PCR System	Thermo Fischer	PCR
BioRad Power Supply	BioRad	Electrophoresis
NanoDrop ™2000 spectrophotometer	Thermo Fischer	DNA, RNA concentration
Confocal microscope Zeiss LSM 700	Zeiss	IF
Olympus slide scanner	Olympus life science	IF

4.1.1 Buffers and solutions

TABLE 4.2: 1% agarose gel

Compound	Quantity	Comment
Agarose	1g	
TAE buffer	100 ml	self prepared ((Tris-base, acidic acid and EDTA)
Ethidium bromide	8 µL	8% lab stock solution

TABLE 4.3: Composition for 1 L agar for agar plates. After preparation of agar, kanamycin was added in ratio 1:1000.

Compound	Quantity
Tryptone	10 g
NaCl	10 g
Yeast	5 g
Agar	15 g

TABLE 4.4: Composition for 1 L Lysogeny broth (LB) medium

Compound	Quantity
Tryptone	10 g
NaCl	10 g
Yeast	5 g

TABLE 4.5: RIPA Lysis Buffer pH 7.4 for Western blot

Compound	Final concentration
Tris-HCl (pH 8)	50 mM
NaCl	150 mM
SDS	0,1%
ddH20	

TABLE 4.6: 10x Tris-Glycine SDS-PAGE Running Buffer

Compound	Final concentration
Tris-ultrapure	500 mM
Glycine	1.92 M
SDS	0,1%
ddH20	

TABLE 4.7: 1.5 M TRIS pH 8.8 Buffer for PAGE

Compound	Quantity	Comment
Tris-base	181,71 g	
ddH2O	900 mL	adjust pH with base to 8.8
ddH20	fill up to 1L	

TABLE 4.8: 0.5 M TRIS pH 6.8 Buffer for PAGE

Compound	Quantity	Comment
Tris-base	60 g	
ddH2O	900 mL	adjust pH with acid to 8.8
ddH20	fill up to 1L	

TABLE 4.9: 8%, 10% and 15% gel for Western blot

Compound	Separating gel 8% (mL)	Separating gel 10% (mL)	Separating gel 15% (mL)	Stacking gel (mL)
ddH2O	9.3	7.9	5.9	8.4
30 % Acrylamide	5.3	6.7	8.7	2.5
1.5 M Tris pH 8.8	5	5	5	-
0.5 M Tris pH 6.8	-	-	-	3.7
10 % SDS	0.2	0.2	0.2	0.15
10 % APS	0.2	0.2	0.2	0.15
10 % TEMED	0.02	0.02	0.02	0.015

TABLE 4.10: 10 x Transfer Buffer (Towbin buffer)

Compound	End concentration
Tris	25 mM
glycine	192 mM
SDS	0,1%
ddH20	

TABLE 4.11: Transfer Buffer for Western blot, 3 L

Compound	Volume (mL)
96% Ethanol	300
10x Transfer Buffer	300
ddH20	2400

4.1.2 Antibodies used for Immunoblotting

TABLE 4.12: Primary antibodies for Immunoblotting. All antibodies were disolved in 3% BSA TBST.

Antibody	Company	Species	Cat.No.	Dilution (WB)	dilution (IF)
V5	CST	13202	rabbit	1:2000	1:500
LAMTOR1	CST	8975	rabbit	1:1000	
LAMTOR3	CST	8168	rabbit	1:1000	
TMEM192	abcam	ab185545	rabbit	1:1000	
LAMP1	Santa Cruz	sc20011	mouse	1:1000	1:500
Rab7	CST	9367	rabbit	1:1000	
LAMP2	Santa Cruz	sc18822	mouse	1:1000	
Streptavidin HRP				1:2000	1:200

TABLE 4.13: Reagent for Immunoblotting. The reagent was disolved in 3% BSA TBST.

Reagent	Dilution (WB)	dilution (IF)
Streptavidin HRP	1:2000	1:200

TABLE 4.14: Secondary antibodies for Immunoblotting. All antibodies were disolved in 5% milk TBST.

Antibody	Company	Species	Cat.No.	Dilution
anti-rabbit HRP	Jackson	111-035-003	donkey-anti-mouse	1:1000
anti-mouse HRP	Jackson	115-035-003	goat-anti rabbit	1:1000

TABLE 4.15: Secondary antibodies for IF. All antibodies were disolved in 3% BSA in TBST + 0,01% NaN3 (Sodium azide).

Antibody	Company	Species	Cat.No.	Dilution
Alexa Fluor 647	Invitrogen	A31571	donkey-anti-mouse	1:1000
Alexa Fluor 488	Invitrogen	A11008	goat-anti rabbit	1:1000

TABLE 4.16: Reagent used for IF

Reagent	company	Cat.No.	dilution
DAPI	Sigma	D8417	1:2500

4.2 Methods

In order to create TurboID tagged proteins of interest, we generated lentiviral constructs to create stable cell lines. Some of our proteins of interest could be isolated from existing plasmids while others had to be amplified from cDNA.

4.2.1 RNA isolation and cDNA production

RNA was isolated from Human Embryonic Kidney (HEK293) and HeLa cells with the NucleopSpin RNA isolation kit (Marcherey-Nagel NucleoSpin ®). cDNA was yielded from isolated RNA by RT-PCR using the LunaScript SuperMix kit from NEB.

PCR

Proteins of interest (LAMTOR1, LAMTOR3, Rab 7, TMEM192, LAMP1, LAMP2A, LAMP2B, NES) as well as the corresponding TurboID part were amplified by PCR and later on combined via Gibson assembly into the pCW57.1 backbone (Plasmid #41393). Primers that were used are given in Tables 4.17 and 4.18. An example for the PCR reation mixture that we used, as well as conditions for each reaction step, are given in Tables 4.19 and 4.20, respectively.

TABLE 4.17: The primers used for cloning of proteins of interest into pCW57.1 backbone with TurboID-V5 tag

Plasmid	TurboID-V5 forward primer	TurboId-V5 reverse primer	Ta (°C)
pCW57.1-LAMTOR1-TURBOID-V5	tgggatcccaGCTAGCAAAGACAATACTGTGC	gtggtggtggtggtggtggaccggtTTAGGTGCTGTCCAGGCC	64,7
pCW57.1-V5-TURBOID-Rab7	cagatcgcctggagaattggctagcgc cacATGGGCAAGCCCATCCCC	tagaggtcatCTGCAG CTTTTCGGCAGAC	68,1
pCW57.1-LAMP1-1x-TURBOID-V5	ccagactatcggtggcggtggat ccGCTAGCAAAGACAATACTGTGC	gtggtggtggtggtggtggaccggtTAGGTGCTGTCCAGGCC	64,7
pCW57.1-LAMP2A-1x-TURBOID-V5	tgagcaatttggtggcggtggatccGCTAGCAAAGACAATACTGTGC	gtggtggtggtggtggtggaccggtTAGGTGCTGTCCAGGCC	64,7
pCW57.1-LAMP2B-1x-TURBOID-V5	tcagactctgggtggcggtggatccGCTAGCAAAGACAATACTGTGC	gtggtggtggtggtggtggaccggtTAGGTGCTGTCCAGGCC	64,7

TABLE 4.18: **The primers used for cloning of proteins of interest into pCW57.1 backbone with TurboID-V5 tag.** Plasmid cDNA–V5-TURBOID-TMEM192 was used as template, so the whole sequence was subcloned into pCW57.1 backbone. For the three plasmids that contain LAMPs (pCW57.1-LAMP1-1x-TURBOID-V5, pCW57.1-LAMP2A-1x-TURBOID-V5, pCW57.1-LAMP2B-1x-TURBOID-V5) a GSG linker. pCW57.1-V5-TURBOID-NES and pCW57.1-V5-TURBOID-LAMTOR3 were already used in the lab and not specially prepared for this project

Insert	tagged terminus	Insert forward primer	Insert reverse primer	Ta (°C)
LAMTOR1	C	cagatcgcctggagaattggct agcgccac-cATGGGGTGCTGCTATAGC	ctttgctagcTGGGATCCCAA ACTGTAC	62
Rab7	N	aaagctgcagATGACCTCT AG-GAAGAAAGTG	gtggtggtggtggtggtggaccggtTCA GC AACTGCAGCTTTC	61,6
LAMP1	C	cagatcgcctggagaattggctagcGCC ACCATGGCGGCCCCC	ctttgctagcggatccaccgccaccGATAG TCTG GTAGCCTGCGTGACTC-CTCTTCC	72
LAMP2A	C	cagatcgcctggagaattgg ctagcGC-CACCATGGTGTGCTTC	ctttgctagcggatccaccgccaccAAA TTGCTCATATCCAGCAT-GATG	64
LAMP2B	C	cagatcgcctggagaattggc tagcGC-CACCATGGTGTGCTTC	ctttgctagcggatccaccgcca ccCA-GAGTCTG ATATCCAGCA TAACTTTTTC	65,8
TMEM192*	N	cagatcgcctggagaattggctagcgccacc ATGGGCAAGCCCATCCCC	gtggtggtggtggtggtggaccggtTTA CG TTCTACTTGGCTGACAGC	67,7

TABLE 4.19: PCR reaction mixture

Reagent	Final concentration	Quantity, for 50 µL of reaction mixture	company
dNTPs	10 mM	10	NEB
Forward primer	10 µM	1 µL	Sigma
Reverse primer	10 µM	1 µL	Sigma
Tamplate DNA	diverse	1,000 ng	see section 4.2.1
Q5 High-Fidelity DNA Polymerase	5X	0,5 µL	NEB
Q5 High GC Enhancer	5X	10 µL	NEB
Nuclease - Free Water		up to 50 µL	NEB

TABLE 4.20: PCR reaction steps. * Ta - given for every primers pair Tabels 4.18 and 4.17. ** 30 s pro kb amplified DNA

Step	Temperature	Time (pro cycle)
Initial Denaturation	98 °C	10 s
Annealing	Aligning temperature (Ta)*	30 s
Extension	72 °C	x**
Final extension	72 °C	120 s

Electrophoresis and gel extraction

After PCR amplification the fragments were resolved on a 1% agarose gel for 40 minutes at 80V. The gel was imaged in the Bio-Rad Chemi Dok ™XRS+ with Image Lab TM Software. 10 µL of 6X loading dye (NEB) and gene ruler 1kb+ gene ruler (NEB) DNA were used. Fragments with the correct size were cut out of the gel and isolated with QIAquick Gel Extraction Kit from QIAGEN following their instructions. Finally, the concentrations were measured with a NanoDrop ™2000 spectrophotometer from Thermo Fischer.

Gibson assembly and transformation

pCW57.1 lentivirus backbone was digested with AgeI-HF and NheI-HF for one hour. According to the protocol from NEB Builder for Gibson assembly, 1:2 or 1:3 ratio of backbone and inserts were used accordingly, depending on the number of fragments that should be combined. The following formula were used for calculating the amounts and volumes of DNA fragments:

$$\text{pmol} = \text{ng} * 1000/(\text{Bp} * 650).$$

For the backbone typically 80 ng were used. 10 µl of NEB Assembly Buffer were applied and the total volume was filled up to 20 µl with nucleus free water. The mixture was incubated for 1-2 hours at 50 °C and after incubation time used for transformation. Table 4.21 is given as one example for Gibson assably.

TABLE 4.21: An example for Gibson assamble mixture

compound	volume (µL)
DNA (insert 1)	3,4
DNA (insert 2)	2,3
DNA (backbone)	1,4
Buffer	10
ddH20	2,9
total	20

On 50 µL of dH10B competent E.coli cells we added 5 µL of a Gibson assembly and flicked the vial. Thereafter, the vial was left for 20 minutes on ice and then heat-shocked at 42 °C for 60-80 seconds and after that chilled on ice for at least 2 minutes. LB Medium 4.4 (250

µL) was added and the bacteria was let to grow at 37 °C for one hour. Then, the bacteria were spun down (300 rfc, 5 minutes), the supernatant decanted and the pellet resuspended in remnant LB. The resuspended pellet was distributed on agar plates, previously made (see 4.2). Finally, the plates were stored at 28 °C for 48 h.

4.2.2 Plasmid isolation

The clones were picked and let to grow in 5 mL of LB medium with kanamycin (1:1000) for another 48 h at 28 °C. Minipreps were made from this bacterial culture with GeneJet Miniprep Kit from Thermo Fisher following the manufacture's instructions. Later, minipreps were sent for the sequencing (around 500 µg DNA). The sequencing was performed by Microsynth AG company. Clones with the correct sequence were expanded to the maxiprep. Maxipreps were made with Qiagen Plasmid Maxi Kit following the manufacture's instructions.

4.2.3 Cell culture

Human HeLa cervical cells were maintained in DMEM (D6429, Sigma Aldrich) to which we added 10 % fetal bovine serum (FCS) and Penicillin-Streptomycin (100U/mL) kept at 37 °C, with 5% CO2. The cells were split every two to three days. Before splitting, the cells were washed once with PBS (self-made) and treated with trypsin/EDTA.

Stable cell lines

For virus production, HiEx cells were transfected with the virus packaging system VSVG and GAG-POL as well as the fusion proteins of interest in 10cm dishes. An example is given in Table 4.22. PEI was added to 500 µL of SFM and left for 10 minutes at room temperature, then mixed with the mixture of the other three DNA components and left for 30 minutes at room temperature. After the mixture was added dropwise to the HiEx cells, they were transported into the S2 lab and left to grow for 48 hours. The supernatant was aspirated with a syringe and filtered through 0,45 µm filters and polybrene was added (1:2000). Prepared virus was added on the plates with parental Hela cells (confluency around 50-60 percent), ratio 1 (virus):10 (medium)

for 72 hours. Cells were selected with puromycin with a concentration of 1 µg/mL.

In order to check whether the constructs are expressed, doxycyclin titration was performed. Concentrations of doxycycline that were used are: 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.3 µg/mL and 0,1 µg/mL. Biotinylation was conducted pre immunoblotting, in the way that biotin was added in ratio 1:200 for 15 minutes.

TABLE 4.22: Transfection mixture for virus production. For a 10 cm dish PEI was set to 24 000 ng

compound	ratio	amount (ng)	volume (µL)
DNA (plasmid)	5	3636	5.5
DNA (VSVG)	1	727	1.6
DNA (GAG-POL)	5	3636	6,2
PEI	3	24000	24

4.2.4 Immunoblotting

Cells at a confluency of around 90% were lysed in RIPA buffer (composition given in 4.5) and the concentration of the proteins was measured using Pierce™ BCA Protein assay from Thermo Fisher following their instructions. Equal amounts of protein were subjected (10-20 µg) to SDS-PAGE and immunoblotted. Depending on the size of the protein, the proteins were separated and identified with 8%, 10% or 15 % acrylamide gel (4.9). The primary antibody used are listed in the Table 4.13. Secondary antibodies coupled to horseradish peroxidase (listed in 4.14) were used at 1:5000 for 1 hour at room temperature. SuperSignal™ West Pico chemiluminescence Substrate and SuperSignal™ West Femto Maximum Sensitivity Substrate from Thermo Fischer were used for visualizations of chemiluminescence. Quantification of blots was performed using ImageJ.

4.2.5 Immunofluorescence

First of all, cells were fixed in 4% formaldehyde in PBS for 10 min, washed three times with PBS (Sigma), and blocked for 20 min in 3% BSA solution in PBS with 0,02% saponin. Primary antibodies were left on cells overnight at 4°C. Secondary antibodies were incubated for 1 hour at room temperature. DAPI was used 1:2500 and slides were

mounted with Invitrogen ProLongTM Gold Antifade reagent. The slides were left overnight in the dark to dry out and the following day they were sealed with nail polish. Primary and secondary antibodies used are listed in Tables 4.13 and 4.15. Slides were imaged on the confocal microscope Zeiss LSM 700.

4.3 MS screen conditions

In order to find the right conditions for the MS screen, we conducted an experiment questioning different starvation methods and duration. Positioning of lysosomes was observed, i.e. in which conditions most of the lysosomes are in the perinuclear area and in which mainly at the periphery. First, cells were starved with SFM (serum-free medium) for 18 hours, and re-stimulated with FM (full medium) for 2 hours. Two slides - control and starved cells were treated as described in Section 4.2.5. Furthermore, images were taken with an Olympus slide scanner and analyzed with both macro and excel (more than 1000 cells pro condition. Shown is average +/- standard error of the mean (SEM).

.1 Plasmids maps

The following figures show plasmid maps of the used constructs.

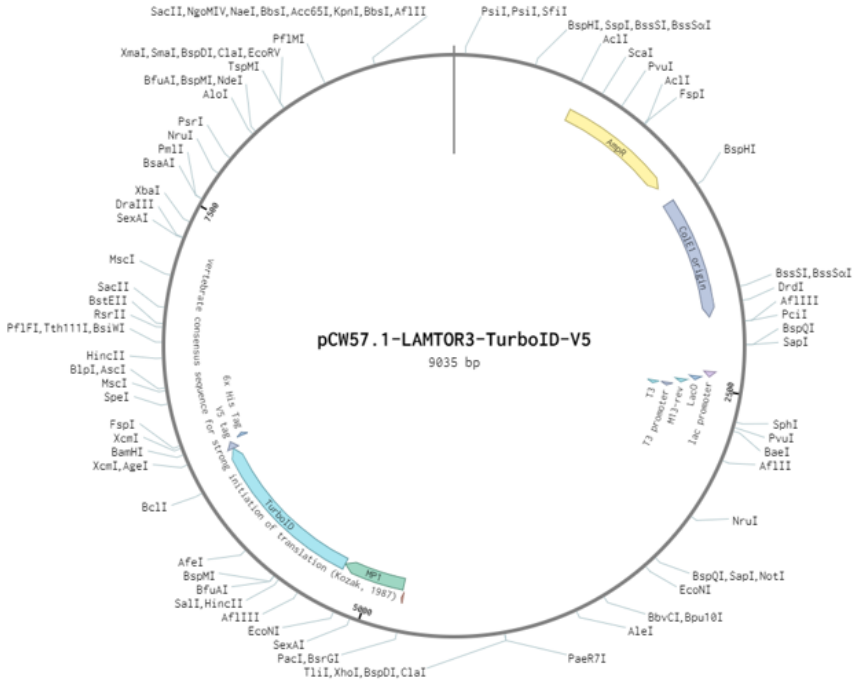

FIGURE 1: pCW57.1-LAMTOR3-TurboID-V5

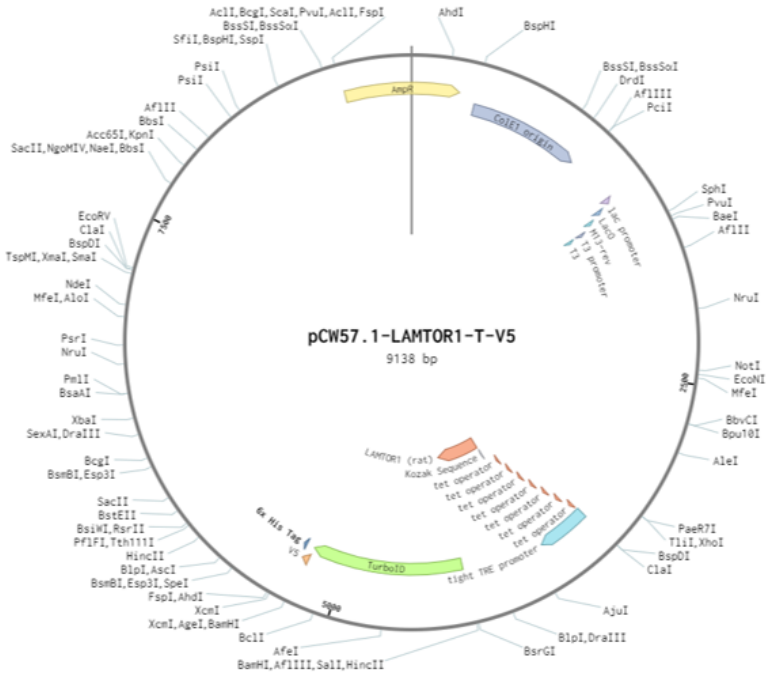

FIGURE 2: pCW57.1-LAMTOR1-TurboID-V5

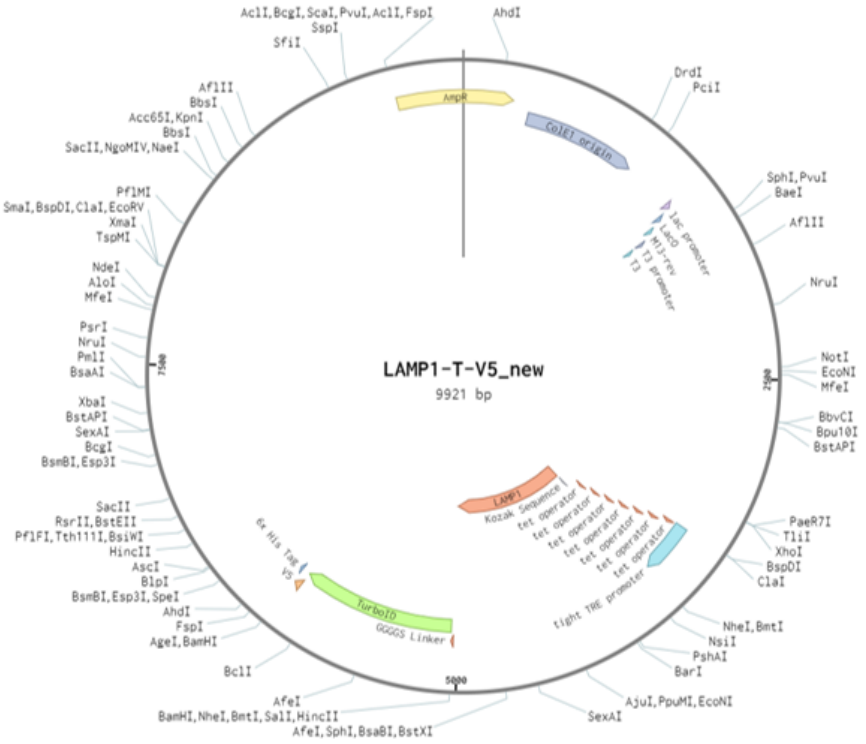

FIGURE 3: pCW57.1-LAMP1-TurboID-V5

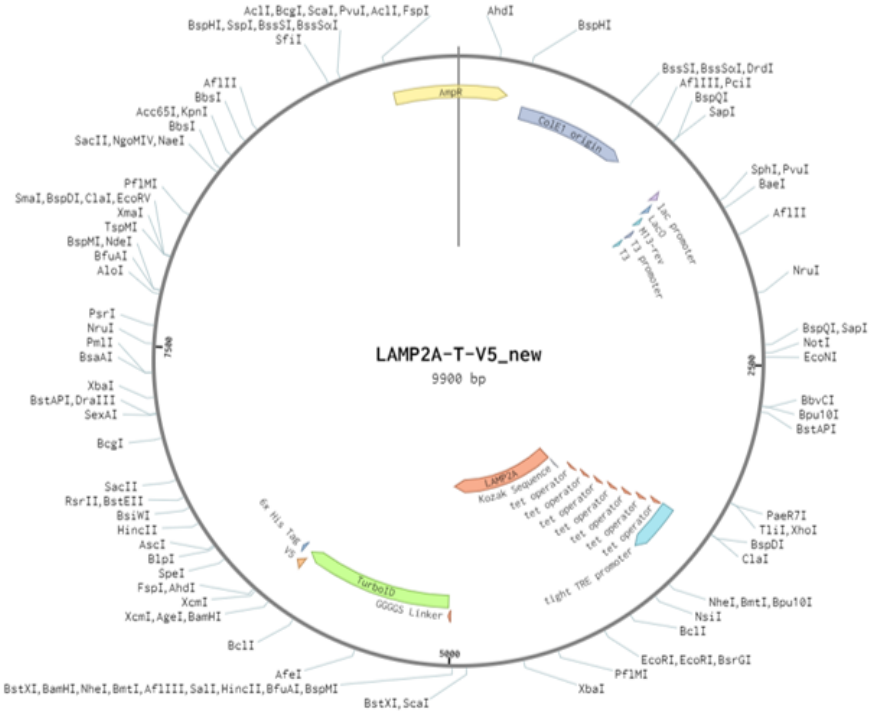

FIGURE 4: pCW57.1-LAMP2A-TurboID-V5

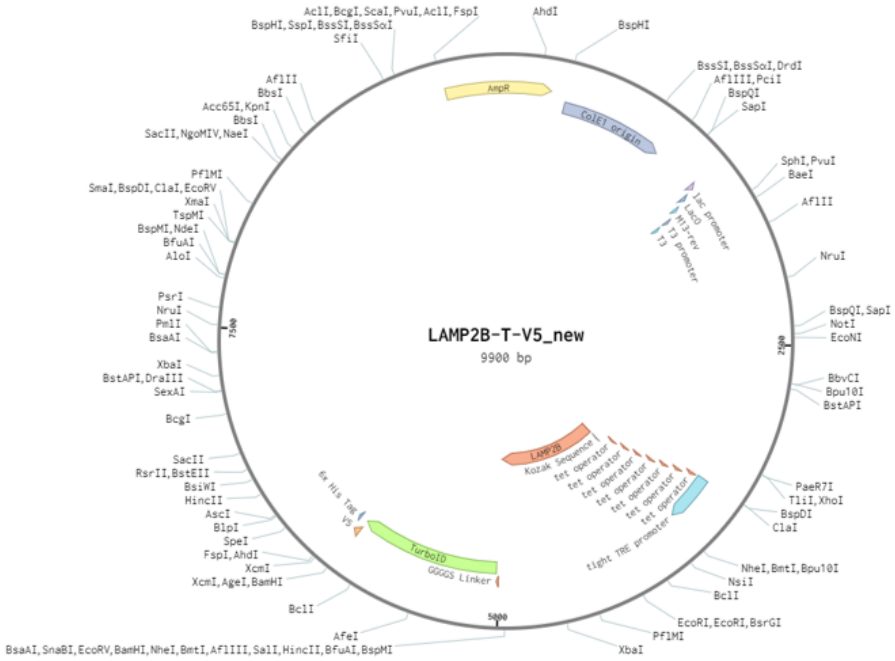

FIGURE 5: pCW57.1-LAMP2B-TurboID-V5

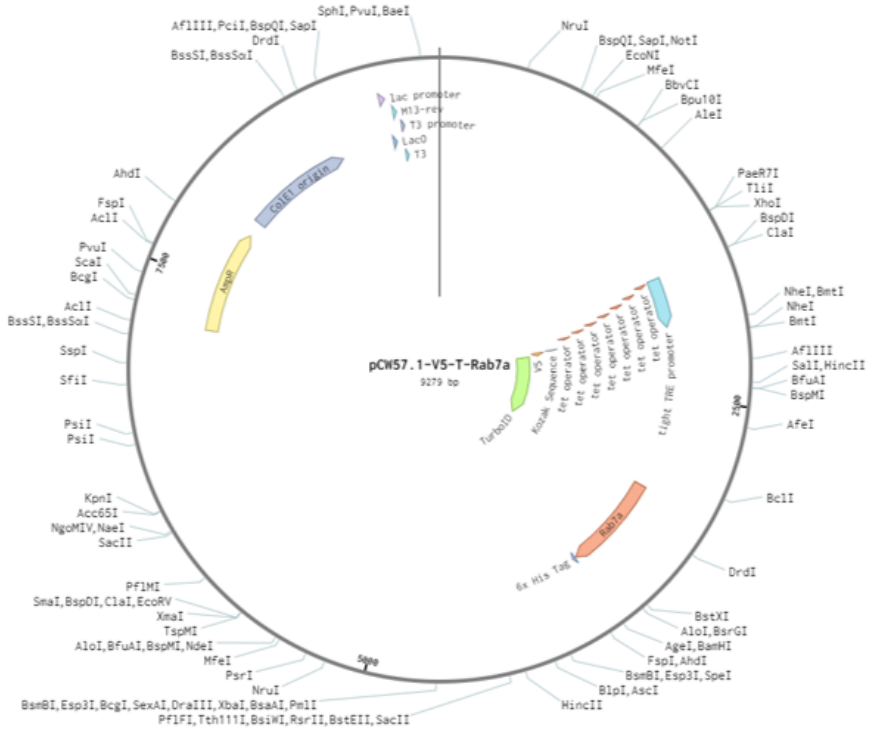

FIGURE 6: pCW57.1-V5-TurboID-Rab7

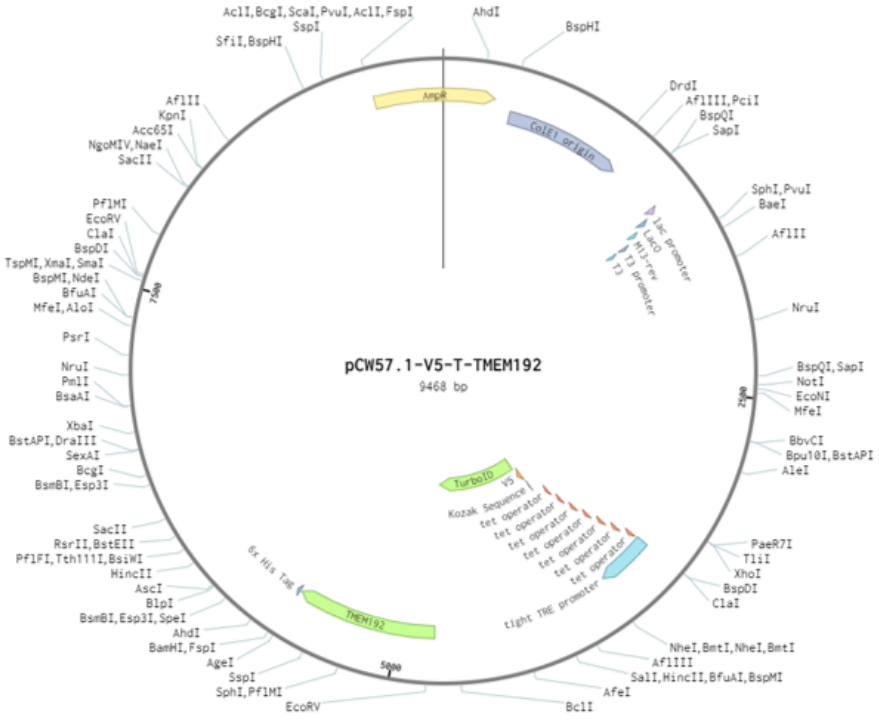

FIGURE 7: pCW57.1-V5-TurboID-TMEM

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