UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

NINA SEKEREŠ

MAGISTRSKA NALOGA

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NINA SEKEREŠ

VLOGA NARDILIZINA 1 IN Z LIMFOCITNO FOSFATAZO POVEZANEGA FOSFOPROTEINA V AVTOFAGIJI RAKAVIH CELIC

THE ROLE OF NARDILYSIN 1 AND LYMPHOCYTE PHOSPHATASE-ASSOCIATED PHOSPHOPROTEIN IN AUTOPHAGY OF CANCER CELLS

Ljubljana, 2016

The research work was carried out at the Institute of Pharmacology at the University of Bern, with collaboration of the Faculty of Pharmacy, University of Ljubljana. I worked under the supervision of Prof. Dr. Dr. Hans-Uwe Simon and home mentorship of Prof. Dr. Irena Mlinarič-Raščan.

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Statement

Hereby, I testify having performed the experiments to the best of my knowledge and having written this thesis independently under guidance of my supervisors: Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Dr. Hans-Uwe Simon.

Ljubljana, September 2016 Nina Sekereš

President of the Thesis defense committee: Prof. Dr. Aleš Obreza

Member of the Thesis defense committee: Asist. Dr. Rok Frlan

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Abstract

Autophagy is a homeostatic degradation process whereby cellular components are engulfed into autophagosomes, digested in autophagolysosomes and recycled. This catabolic process is considered a vital component of normal cell turnover, survival during nutrient deprivation, cell clearance of microbial pathogens, and it has also a role in the pathogenesis of various diseases, including cancer. Therefore, research continues to focus on the elucidation and analysis of the proteins involved in the regulation of this process. A previously performed proteomic analysis of the host laboratory suggested that nardilysin (NRD1) and lymphocyte phosphatase-associated phosphoprotein (LPAP) represent targets of the autophagy pathway. NRD1 knockdown in Jurkat and MEF GFP-LC3 cells resulted in decreased autophagic activity as assessed by both immunoblotting and immunofluorescence techniques. In contrast, knockdown of LPAP was associated with increased autophagy levels in Jurkat and primary T cells. Taken together, the data in this thesis confirm the assumption that NRD1 and LPAP are involved in the regulation of autophagy. Additional work is required to understand their functions in the context of different cell types.

Keywords: Autophagy, NRD1, LPAP, cancer

Razširjeni povzetek

Avtofagija je homeostatski proces v katerem so celične komponente dostavljene do lizosoma in razgrajene. Vloga avtofagije v celicah ni omejena zgolj na odstranitev odvečnih intracelularnih molekul, ampak ima pomembno vlogo tudi v zagotavljanju energije in nujno potrebnih hranil v stanjih, ko celici le-teh primanjkuje. Proces avtofagije je natančno uravnavan in v grobem ga lahko razdelimo na tri dele. V fazi iniciacije se začne oblikovanje dvomembranskih veziklov, ki zajamejo celične sestavine namenjene razgradnji. V naslednji stopnji, stopnji elongacije, se dvojna membrana sklene in tvori se za avtofagijo specifični organel avtofagosom. Avtofagosom se nato zlije z lizosomom v fazi degradacije in v avtofagolizosomu se s pomočjo hidrolizirajočih encimov zajete sestavine razgradijo. Razgradni produkti preidejo v citosol in so na voljo za nadaljnje anabolične procese. Določen nivo avtofagije je v celicah normalno prisoten in tako zagotavlja vzdrževanje homeostaze v stanjih, ko ni pomanjkanja hranil. V stanjih pomanjkanja hranil, kot so hipoksija, oksidativni stres, pomanjkanje rastnih faktorjev in inzulina ali po invaziji patogena ter v raznih bolezenskih stanjih pa je avtofagija povečana.

Avtofagija v zadnjih letih pridobiva na pomenu v razvoju različnih bolezni, tudi raka, čeprav natančen mehanizem ostaja nejasen. Dosedanje študije kažejo dvojno vlogo avtofagije v rakavih celicah. V začetni fazi kancerogeneze se s procesom avtofagije odstranjujejo reaktivne kisikove spojine, odvečni ali poškodovani proteini in organeli. Tako se prepreči njihovo kopičenje in možnost za nadaljnji razvoj raka je zmanjšana. Z napredovanjem tumorja se zaradi pospešene rasti in nezadostne prekrvavitve rakavega tkiva njihove potrebe po hranilih in kisiku povečujejo. Stanje stradanja aktivira avtofagijo, ki deluje kot alternativen vir hranil in kisika ter tako zagotavlja preživetje rakavim celicam.

V magistrski nalogi smo podrobneje raziskovali vlogo dveh proteinov v avtofagiji, in sicer NRD1 (nardilizina) in LPAP (z limfocitno fosfatazo povezanega fosfoproteina), za katera je bilo s proteomsko analizo dokazano, da se njuna ekspresija v tem procesu spreminja. NRD1 je endopeptidaza, ki cepi peptidne substrate na N-koncih argininskih ostankov v dvobaznih parih. Vloga NRD1 ni popolnoma jasna, a naj bi kot endopeptidaza imel vlogo v aktivaciji transmembranskih proteinov kot sta HB-EGF (heparin vezoči rastnemu faktorju podoben epidermalni rastni faktor) in TNF-α (tumor nekrotizirajoči faktor alfa). LPAP je transmembranski fosfoprotein, ki v 75 % tvori kompleks z limfocitno fosfatazo CD45 in naj bi sodeloval pri regulaciji aktivacije limfocitov.

Namen magistrske naloge in eksperimentalni postopki

Naš cilj je bil ugotoviti ali NRD1 in LPAP regulirata proces avtofagije. Torej, ali bo prišlo do spremembe v nivoju avtofagije po utišanju izražanja, bodisi NRD1 bodisi LPAP. Transfekcijo in vnos siRNA smo izvedli z metodo elektroporacije. Izražanje različnih proteinov po transfekciji smo preučevali z western blot analizo in fluorescenčno mikroskopijo. Preverili smo tudi viabilnost transficiranih rakavih celic po aplikaciji protirakave učinkovine, kar smo izvedli z označevanjem mrtvih celic in sledečo pretočno citometrijo.

Raziskovalno delo

Naš prvi cilj je bil ugotoviti ali različna ekspresija NRD1 vpliva na nivo avtofagije. Izvedli smo transfekcijo Jurkat celic z metodo elektroporacije. Nadalje smo z western blot analizo in fluorescenčno mikroskopijo ocenili izraženost proteinov značilnih za avtofagijo in ugotovili, da je nivo avtofagije v Jurkat celicah po utišanju NRD1 znižan. Podoben poskus smo naredili na MEF GFP-LC3 celicah in po analizi s fluorescenčnim mikroskopom v celicah z utišanim NRD1 opazili nižji nivo proteina LC3, torej manj avtofagije. Mehanizem interference NRD1 v proces avtofagije ostaja nepojasnjen. Predpostavljamo, da je direktno vpleten v formacijo avtofagosoma, ali pa avtofagijo uravnava preko signalizacije s TNF-α. Da bi preverili, ali rakave celice z utišanim NRD1, torej manj avtofagije, ki deluje kot proces preživetja, tudi umirajo hitreje, smo naredili test celične smrti. Celice Jurkat smo po transfekciji zdravili s protirakavo učinkovino in po označevanju mrtvih celic izvedli pretočno citometrijo, ki je pričakovano pokazala več mrtvih celic v skupini z utišanim NRD1. LPAP je naslednji protein katerega vlogo v avtofagiji smo raziskovali. V Jurkat celicah in prav tako v primarnih T-celicah smo opazili indukcijo avtofagije po utišanju izražanja LPAP. Dokazano je bilo, da avtofagija inducira izražanje LPAP in obratno, LPAP inhibira avtofagijo verjetno po principu negativne povratne zanke v T –celicah. Zatorej lahko trdimo, da LPAP potencialno preprečuje pretirano aktiviranje avtofagije.

Razumevanje proteinske signalizacije v avtofagiji rakavih celic ima še veliko neodgovorjenih vprašanj. V raziskovalnem delu smo potrdili predpostavko, da NRD1 in LPAP sodelujeta v regulaciji procesa avtofagije. Oba proteina bi lahko v prihodnosti bila tarči za proti-rakava zdravila, a potrebne so dodatne raziskave za podrobnejše razumevanje njune funkcije.

Ključne besede: avtofagija, NRD1, LPAP, rak

List of abbreviations

ADAM	A disintegrin and metalloproteinase
ATG	Autophagy-related gene
Bcl-2	B-cell lymphoma/leukemia-2
CQ	Chloroquine
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FIP200	Focal adhesion kinase family-interacting protein of 200 kD
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescence protein
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HRP	Horseradish peroxidase
Hsc70	Heat shock cognate 70
hVps34	Vacuolar protein sorting 34
LAMP-2A	Lysosome-associated membrane protein 2A
LC3	Microtubule-associated protein 1 light chain 3
LPAP	Lymphocyte phosphatase-associated phosphoprotein
mRNA	Messenger RNA
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NRD1	Nardilysin
PAF	Paraformaldehyde
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PI3K	Class III phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PMSF	Phenylmethylsulfonyl fluoride

RT	Room temperature
siRNA	Small interfering ribonucleic acid
SQSTM1	Sequestosome 1
TACE	Tumour necrosis factor-α-converting enzyme
TNF-α	Tumour necrosis factor-α
ULK	Unc-51-like kinase

1 INTRODUCTION

1.1 Autophagy

1.1.1 Introduction to autophagy

The term autophagy originates from Greek words “auto” and “phagy”; meaning “eating of self” or “self-eating”. With this term a catabolic process is described in which cellular components are delivered to the lysosome for degradation. The purpose of autophagy is not just a simple elimination of materials, but instead, autophagy is a dynamic process which is needed for the homeostasis of the cell. Cellular homeostasis is achieved when the biosynthesis and turnover are balanced. In the turnover, beside the ubiquitin-proteasome system, autophagy has a significant role. In this process, firstly, double-membrane vesicles, called autophagosomes are formed. Their formation begins with an ‘isolation membrane’, also known as phagophore, whose precise origin is still unknown. Autophagosomes engulf damaged organelles, aggregated proteins and even pathogens and upon closure of the membrane, they fuse with lysosomes (forming autolysosomes) where the cargo gets degraded by lysosomal hydrolases. The resulting macromolecules are then reused for providing building blocks and energy for future anabolic processes. A low level of autophagy is present in almost all mammalian cells. This type of autophagy is defined as “basal autophagy” and it is present when in a cell there is an abundance of nutrients. However, when the cell undergoes stress conditions such as lack of nutrients (growth factor or insulin deprivation), hypoxia, oxidative stress, endoplasmic reticulum stress or pathogen invasion, autophagy is up-regulated. In this case we are talking about “induced autophagy” (1, 2).

1.1.2 Types of autophagy

There are three major types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Figure 1). All three types include degradation of the substrate by the lysosome and recycling of degradation products, but differ in selected substrates (2, 3).

In macroautophagy cellular elements are first encapsulated in autophagosomes, which bring the cargo to the lysosome and fuse with it. Cellular contents such as organelles, lipid droplets, proteins and pathogens are, after the autolysosome formation, degraded by lysosomal enzymes. Microautophagy, on the other hand, involves a direct engulfment of cellular components into the lysosome by invagination of its membrane. Macro- and microautophagy can include similar substrates; organelles and large structures through non-selective and

selective mechanisms. Chaperone-mediated autophagy is a complex and specific pathway, which selectively targets soluble long-lived proteins. The proteins with the pentapeptide KFERQ-like sequence get recognized by the Hsc70 chaperone and are taken to the lysosome. The substrate-chaperone complex is associated with the lysosomal trans-membrane receptor, called Lamp-2A, which enables the translocation of the substrate into the lysosome (2, 3, 4). This master thesis focuses on macroautophagy, hereafter referred to as autophagy.

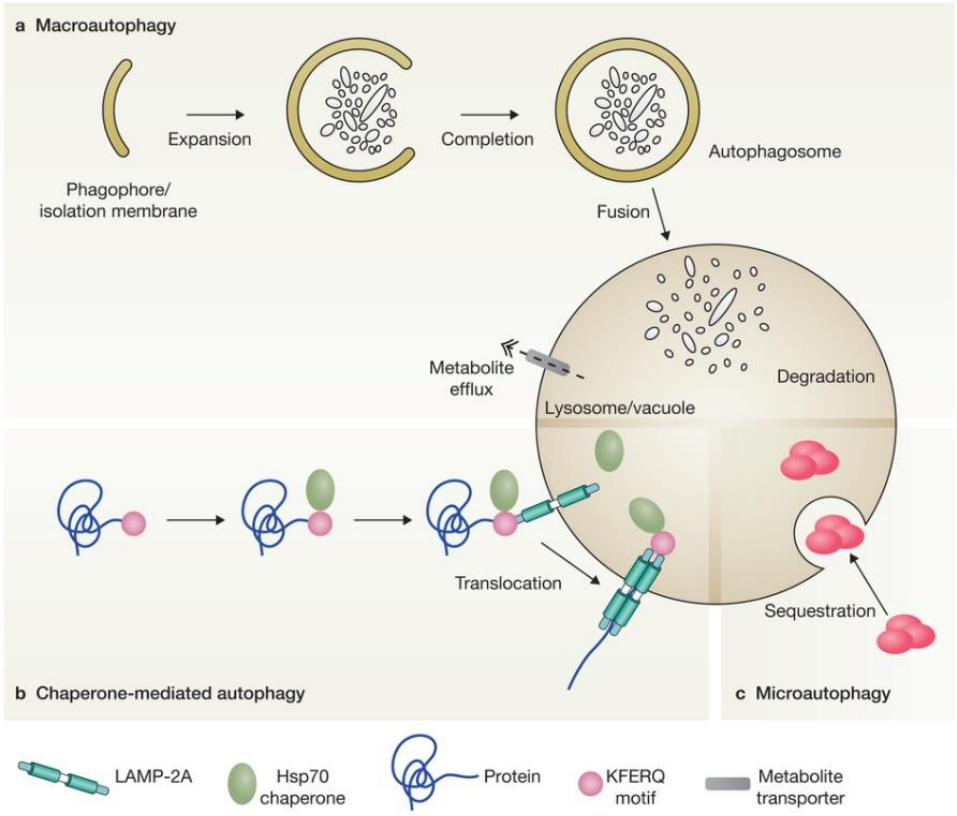


Figure 1: The three different types of autophagy

(A) In macroautophagy the substrates are engulfed by double-membrane autophagosomes which eventually fuse with lysosomes and finally expose their content to the hydrolytic enzymes for degradation. (B) During chaperone-mediated autophagy, Hsc70 and co-chaperones recognize autophagy substrates which carry the pentapeptide KFERQ-like sequence, and these are taken up by the lysosome with the help of receptor Lamp-2A. (C) Microautophagy directly recruits cytosolic components into the lysosome via invagination of its membrane. Common grounds of macroautophagy, chaperone-mediated autophagy, and microautophagy are lysosomal degradation and recycling of the degradation products for anabolic processes (4).

Autophagy can also be divided by its substrate selectivity. There are two main types: bulk autophagy or non-selective autophagy, and selective autophagy. Whereas bulk autophagy randomly targets and degrades cytoplasmic components, selective autophagy specifically chooses its substrates. Regarding the origin of the target, selective autophagy can be further

divided into xenophagy (selective for intracellular pathogens), mitophagy (selective for mitochondria), pexophagy (selective for peroxisomes), ER-phagy (selective for endoplasmic reticulum), ribophagy (selective for ribosomes), and so on (5).

1.1.3 Molecular mechanism of autophagy

Autophagy is a process that is evolutionarily conserved among eukaryotes. It was discovered in yeast, where eventually a network of more than 30 autophagy-related (ATG) genes was worked out. Since then many genetic homologs have been found in mammals (6). Autophagy is a highly regulated process and ATG proteins, autophagy related gene corresponding products, play a significant role. Besides ATG proteins, autophagy is regulated by the mammalian target of rapamycin (mTOR) as well as class I and class III phosphoinoside 3-kinase (PI3K) pathways (3).

Induction. mTOR senses nutrition levels, regulates cell growth and is a well-known repressor of autophagy. Under nutrient-rich conditions mTOR is activated, thus, autophagy as well as protein degradation are inhibited. On the other hand, in nutrient-poor conditions autophagy is induced, since mTOR is deactivated. mTOR regulates the activity of ULK1/2 kinase complex (ULK1, ULK2, FIP200 and ATG13). Sufficient amount of oxygen and nutrients activates mTOR and inhibits autophagy by mTOR-ULK1/2 association. During nutrient deprivation mTOR phosphorylates the complex and dissociates from it leading to its activation. Activated ULK1/2 complex localizes itself at the isolation membrane, whose origin is still a matter of debate (6, 7). Studies have implicated plasma membrane, Golgi apparatus, endoplasmic reticulum, and mitochondria as possible sources for phagophores (8).

Autophagosome nucleation. ULK1/2 complex has also a role in localizing the phosphatidylinositol 3-kinase (PI3K-III) class III complex to the isolation membrane. The catalytic subunit of PI3K-III complex is the lipid-kinase VPS34. It interacts with VPS15, a protein kinase, with Beclin 1 (yeast ATG6) and additional regulatory proteins like UVRAG and AMBRA1 to subsequently generate phosphatidylinositol 3-phosphate (PI3P), which recruits other molecules necessary for the elongation of autophagosomal membrane. On the other hand, Beclin 1 can associate with the oncoproteins Bcl-XL and Bcl-2. This association reduces the PI3-kinase activity and inhibits autophagosome formation (9).

Autophagosome completion. Completion of autophagosome is mediated by two ubiquitin-like systems. The first system yields the ATG5–ATG12 conjugate while the second pathway carries out the conjugation of phosphatidylethanolamine (PE) with LC3 (yeast Atg8) (10).

Atg12 is associated with Atg5 in a process assisted by Atg7 and Atg10 enzymes. Atg12–Atg5 conjugates bind to ATG16 and form larger oligomers. In this manner, the autophagosome is elongated and closed. LC3-I is a form of LC3, which had the C-terminus cleaved by ATG4, a cysteine protease. In order to get anchored into the autophagosomal membrane, ATG7 (E1-like enzyme) and ATG3 (E2-like enzyme) assist the LC3-I lipidation with phosphatidylethanolamine (PE), to form LC3-II. The final role in the LC3-II binding to the autophagosome has the ATG12-ATG5-ATG16L or E3-like enzyme which recruits LC3-II to the autophagosomal membrane (9, 11). LC3-II is specifically anchored in the autophagosome and therefore it is used as a precise marker of autophagy. It is located on both, inner and outer membrane. The intraluminal LC3-II is degraded together with the transported cargo after the fusion with lysosome and the exterior LC3-II gets cleaved by ATG4 and recycled (11).

Autophagosome-lysosome fusion and content recycling. After the autophagosome completion it fuses with the lysosome and the cargo transported in the autophagosome comes to contact with lysosomal enzymes. The outer autophagosomal membrane and the lysosome fuse, while the inner membrane gets degraded along with the autophagosomal content. Lysosomal hydrolases work as endopeptidases at acidic pH. The generated small molecules are then released into the cytosol and are used as building blocks for new cellular components (9, 11).

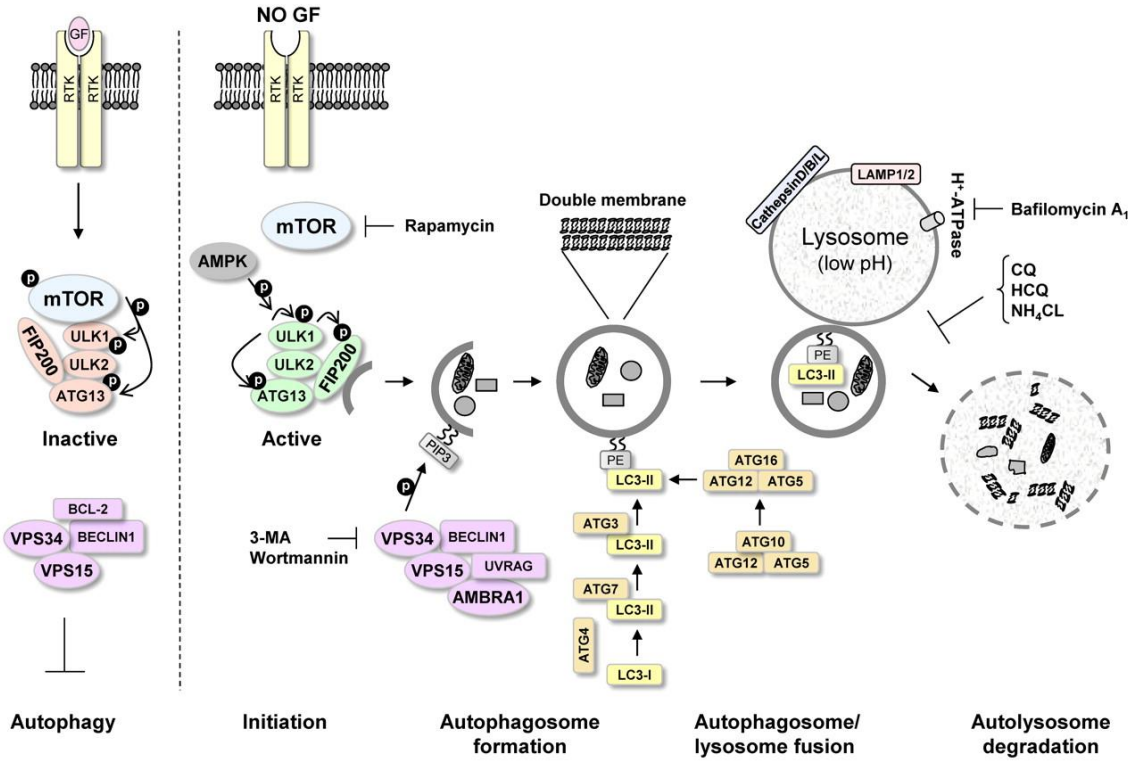


Figure 2: Schematic representation of the autophagic machinery

Under nutrient rich conditions, the mTOR protein kinase is associated with the ULK1/ULK2 complex (ULK1, ULK2, ATG13 and FIP200) and autophagy is negatively regulated. Under nutrient deprivation or with the use of rapamycin, mTOR dissociates from the complex and activates it. The activated ULK1/ULK2 complex localizes itself to the phagophore membrane, and initiates the autophagosome formation. Active ULK1/ ULK2 complex interacts with class III phosphatidylinositol 3-kinase (PI3K-III) complex formed by Vps34, Vps15, UVRAG, AMBRA1, and Beclin1. This complex stimulates the nucleation of the autofagosomal membrane. Autophagosome completion is mediated by two systems: the ATG8 (LC3)-PE and ATG12-ATG5 conjugation systems. They perform the lipid modification of LC3-I, which leads to the binding of LC3-II to the autophagosomal membrane. This conversion to LC3-II is commonly used to monitor autophagy. Completed autophagosomes contain proteins and organelles that are digested after the fusion with lysosomes. Lysosomes have low pH and an abundance of endopeptidases that can break down waste materials and cellular components. The autolysosome degradation can therefore provide new building blocks for cellular maintenance and health (6, 12, adapted from 12).

1.2 Methods to study autophagy

1.2.1 Pharmacological regulation of autophagy

There are several pharmacological mediators, which are commonly used to study autophagy. 3-Methyladenine (3-MA), PI3-kinase inhibitor, can be used as an inhibitor of the early stages of autophagy by blocking the formation of autophagosomes. Chloroquine (CQ) and bafilomycin A1 inhibit endosomal or lysosomal acidification in later stages of the process.

Bafilomycin A1 is often used to study autophagy, since it inhibits the fusion of autophagosomes with lysosomes via V-ATPase inhibition. As a week base CQ accumulates inside lysosomes, and the resulting increased lysosomal pH reduces the hydrolytic enzyme activity and eventually also blocks the autophagosome-lysosome fusion (13). CQ is frequently used in the treatment of malaria. In cancer treatment, CQ has been combined with chemotherapeutic and immunotherapeutic drugs. These anticancer treatments can induce autophagy in tumor cells, which then helps the tumor cells to survive. Therefore, blocking the autophagy with CQ produces a synergistic effect and enhances drug cytotoxicity. However, studies suggest that the effect of CQ on the efficacy of tumor cell killing is likely tissue- or tumor-type-dependent (14). On the other hand, autophagy can be induced by starvation or rapamycin. Nutrient starvation is one of the main stress factors that induce autophagy by inactivation of mTORC1. Lack of amino acids for 1 hour can induce autophagy in most cell lines (15). Autophagy is negatively regulated by mTOR. The activity of mTOR can be inhibited by rapamycin, a macrocyclic immunosuppressive drug, which is also a well-established inducer of autophagy. Studies have shown that it has some powerful antifungal properties, however later on it was discovered to possess anti-T and anti-B cell activity and has been used as immunosuppressant in solid organ transplantations. Furthermore, it was reported that rapamycin derivatives act as anti-tumor agents; since they block the proliferation of tumor cells similarly as they inhibit B and T cell proliferation. This was shown to be the case in treatments of rapamycin alone or combined with other modes of therapy (16).

1.2.2 Monitoring the process of autophagy

One way to monitor the process of autophagy is the detection of autophagosome formation.
LC3 is the most commonly monitored autophagy marker. The amount of LC3-II is tightly associated with autophagosomes, since it is present on both, the inner and outer autophagosomal membrane. Western blot analysis can be used to detect the conversion of endogenous LC3-I to LC3-II when there is induced autophagosome formation. Following SDS-PAGE and incubating with antibodies, LC3 can be observed as two bands; cytosolic LC3-I as a band at 18 kDa and the lipidated LC3-II at 16 kDa. When autophagy is induced either by starvation or rapamycin, there will be a more intensive band for LC3-II and a weaker band for LC3-I. Although LC3- II accumulation is a marker for induction of autophagosome formation, this accumulation can also be seen if the progress of autophagy is inhibited, for example, if the degradation of LC3-II is blocked. With the use of CQ, the autophagy is inhibited by blocking the lysosomal degradation, which leads to an increase in LC3-II

accumulation; in this case, the increase in LC3-II expression does not prove increased autophagy. Another way to monitor autophagosome formation is GFP-LC3 puncta formation assay. A fusion protein of LC3 and a fluorescent protein GFP (GFP-LC3) can be used to observe the formation of autophagosomes by fluorescence microscopy, to determine the increase in puncta formation and the accumulation of LC3 (11, 15).

To monitor the autophagic flux, the degradation of SQSTM1/p62, one of the best-studied autophagy substrates, can be observed. The p62 protein has both LC3 and ubiquitin binding domains. It can directly interact with LC3, and incorporate subsequently into the autophagosome, and get degraded. The p62 cellular expression level is down- regulated by autophagic activity and can serve as a marker of autophagic degradation. The GFP-LC3 can be used as an autophagy indicator as described above, and it can be also used to monitor the autophagic flux by immunoblotting. The idea of the GFP-LC3 cleavage assay is that free GFP, which is cleaved from GFP-LC3 in the autolysosome, is relatively resistant to degradation by lysosomal enzymes. Therefore, the free GFP detected by immunoblotting with an anti-GFP antibody can be used for determination of autophagic flux (15).

1.3 Autophagy in cancer

1.3.1 The role of autophagy in cancer

Autophagy has emerged as an important pathway in tumor development and cancer therapy, although the exact mechanism is still unclear. In normal cells, autophagy acts as a surveillance mechanism which removes damaged organelles and misfolded proteins. In cancer cells, studies suggest, autophagy has a dual role. In the beginning of tumor formation, autophagy functions as a tumor-suppressing mechanism. On the other hand, in already established tumors, autophagy acts as protective mechanism favoring tumor cell survival (3, 14).

1.3.2 Autophagy as a tumor-suppressing mechanism

Autophagy was in the beginning considered a tumor-suppressing mechanism. This idea aroused from early studies of Beclin1, a protein which is an important regulator of autophagy and cell death. Studies found a monoallelic deletion of ATG6/BECN1 (gene encoding Beclin 1) in many human breast, ovarian, and prostate malignancies (17, 18). Further research of malignant breast tissue showed a decreased amount of Beclin1 when compared with healthy breast tissue (19). Following these studies, two groups showed that monoallelic deletion of Beclin1 in mice results in higher frequency of tumorigenesis. The lack of one copy of beclin1

resulted in development of spontaneous tumors, including lung, liver and B cell malignancies (20, 21). This data provided the first evidence that beclin1, as a significant autophagy regulator, functions as tumor suppressor. Furthermore, genetic alterations of other autophagy-related genes were shown to be associated with different cancers. Deletion of ATG5, a gen involved in autophagosome formation, was found in NK-cell malignancies (22). Further, nonsense mutations in UVRAG, a Beclin1 binding protein, and a positive regulator of autophagy, were found in colon cancer cells (23) and gastric carcinomas (24). Also down-regulation of Bax-binding protein-1 (Bif-1), another positive regulator of autophagy, in colorectal adenocarcinomas was reported (25). Collectively, these data supports the idea that different components of autophagy pathway play a tumor-suppressing role in cancer.

How exactly the inhibition of autophagy leads to tumorigenesis remains elusive. It is suggested that defects in autophagy result in elevation of reactive oxygen species, since the damaged molecules and organelles, particularly mitochondria cannot be removed. Therefore, deficiencies in autophagy are finally associated with the accumulation of oncogenic mutations and higher frequency of tumorigenesis (26).

1.3.3 Autophagy as a tumor-progressing mechanism

Tumor cell proliferation requires a great amount of oxygen and nutrients in order to meet its high energy demands. Cancer tissues, especially poorly vascularized solid tumors, often contain areas with significantly lower oxygenation. Consequently, the cells inside of a tumor mass have more autophagy than the marginal ones because they are looking for an alternative source of metabolites and energy. Because the cancer cells have the nutrients provided by autophagy, they are protected from apoptosis and necrosis, and therefore the cancer can progress (19). Additionally, autophagy can play a role in cancer metastasis. When the cells detach from extracellular matrix, they undergo a special kind of cell death, commonly termed anoikis. At the same time, in these cells autophagy is induced, which interferes with anoikis and protects them from dying. The resistance of cancer cells to anoikis is crucial for metastatic colonization and tumor survival (19, 27). Many studies have reported that autophagy helps cancer cells to overcome stress conditions and promotes tumor progression; Altman et al. found that autophagy is imperative to permit BCR-Abl leukemogenesis. They showed that inhibition of autophagy after the deletion of Atg3 prevents BCR-Abl-mediated leukemogenesis in a cell transfer model (28). Furthermore, increased levels of autophagy were monitored in human pancreatic cancer cells, and they were shown to support the tumor cell growth by maintaining energy production. Moreover, inhibited autophagy in these cells

was shown to cause tumor regression (29). Additionally, in residual human ovarian cancer cells after anticancer treatment, autophagy induction was shown to support tumor cell dormancy, which may subsequently lead to tumor recurrence and progression (30).

Similarly to its ability to promote cell survival or induce cell death, autophagy has a contradictory role following anticancer therapy either boosting or reducing its cytotoxic activity. Autophagy is frequently up-regulated in both normal and tumor cells exposed to anticancer therapy in order to protect them from stress and to promote cell survival.

This is assumed to contribute to chemotherapy resistance in certain tumors. Therefore, with autophagy inhibition, the cells might be sensitized to antitumor treatments and may overcome chemotherapy resistance (31). Accumulating data indicates that CQ sensitizes cancer cells to anticancer drugs. On the basis of the evidence from a number of clinical trials, it can be implied that this drug may alter cancer treatment strategies (13).

Nonetheless, autophagy may also induce cell death, so it can actually enhance the effect of chemotherapy. If after applied chemotherapy cells fail to undergo apoptosis, it was shown that, autophagic cell death can be chosen as an alternative pathway of dying. In fact, activating autophagic cell death could be another way of killing cancer cells without resistance to anticancer treatment (31).

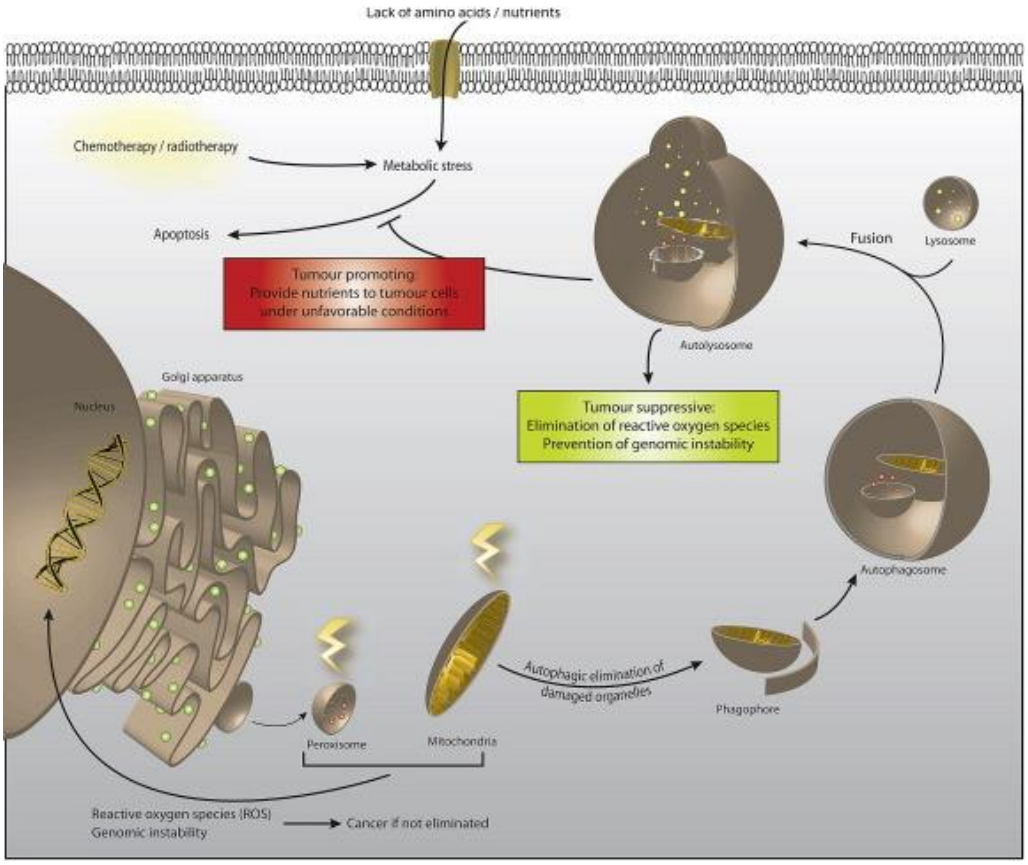


Figure 3: Autophagy in tumor suppression and promotion.

In the lower part of the picture autophagy as a tumor-suppressive mechanism is presented; if damaged peroxisomes and mitochondria are not eliminated by autophagy, reactive oxygen species (ROS) will get accumulated. This may cause mutagenesis and genomic instability. The upper part of the picture illustrates how autophagy can actually help tumor cells to survive under stress conditions like nutrient deprivation and cytotoxic therapy by providing them nutrients and energy (32).

1.4 Identification of autophagy related proteins and regulators

Autophagy network components represent promising novel cancer drug targets, since autophagy was shown to have a role in cancer development and also contributes to treatment resistance. Additionally, various anticancer agents aberrantly activate autophagy as part of cellular stress and cell survival response (31). Therefore, selectively targeting autophagy network components may offer new opportunities to overcome drug resistance of cancer (30). Proteins are the principal targets of drug discovery. One approach to study different properties of proteins that are involved in a process like autophagy is the use of proteomics. The goal of proteomic analysis, a large-scale systematic study, is the understanding of proteins including their expression, function, structure, and interactions with an endpoint of discovering protein biomarkers. Specific biomarkers can be used for more precise interpretation of diseases, easier and faster recognition of pathological conditions and improving the development of new, specific drugs (33).

The genome of an organism normally does not differ between cells; this is, however, not the case for the proteome. In just one cell, the protein expression will differ following different triggers, thus a proteomic analysis has a complex body of information to collect. Current proteomics technologies allow large-scale, high-throughput, mainly MS- and micro array-based identification and quantification approaches (33, 34).

Mass spectrometry (MS) represents an analytical tool that sorts the ions from the sample regarding their mass-to-charge (*m/z*) ratio. Protein samples are usually very complex. For this reason, two-dimensional gel electrophoresis and high performance liquid chromatography are commonly used techniques to separate the proteins in the mixture prior to MS analysis. MS-measurements are then used to estimate the exact molecular weight of proteins in question. Mass spectrometers consist of three components: an ion source that converts a portion of the sample into gas phase ions, a mass analyzer which separates ionized molecules, and a detector that measures the quantity of different ions based on their *m/z* ratio (34). Mass spectrometry is considered as an indispensable tool for molecular and cellular biology.

Protein microarrays are powerful, robust and reproducible tools for capturing and measuring proteins from biological samples. They are used for identifying, for instance, posttranslational modifications or new interaction partners, and also for relative protein quantification. A protein microarray typically consists of thousands of capture molecules immobilized on modified glass slides. Fluorescently labeled probe molecules are added to the array. The emitted fluorescent signal following the reaction between the immobilized protein and the probe is then read by the scanner. Microarrays provide the option of miniaturization and parallelization. They contain thousands of capture features aligned in a grid, each specific for a particular protein; therefore, they can test for multiple biomarkers simultaneously (35).

The validation of information obtained in proteomic studies is essential. Global approaches such as microarray techniques or MS can lead to false discovery and over-interpretation. The usual purpose of large-scale techniques is to identify a subset of cases that are interesting for further investigation (36, 37). The targets discovered by proteomic screening should be confirmed and validated using a second method, e.g., immunocytochemistry or western blotting. After the identification of target protein is confirmed, additional detailed studies can ensue. These may include gene expression manipulation using antisense RNA, RNA interference, specific pharmacological inhibitors, or gene knockout experiments. Such analysis of proteomic pathway reveals a deeper understanding of cellular signaling or may even identify novel drug targets (37).

1.4.1 Nardilysin (NRD1)

Nardilysin or N-arginine dibasic convertase is an endopeptidase that cleaves peptide substrates at the N-terminus of arginine residues in dibasic pairs. NRD1 is a member of the M16, a zinc peptidases family. It was first discovered in rat brain cortex, and later purified from testis, where it is particularly abundant. Two isoforms of NRD were found, NRD1 and NRD2, located both in the cytosol and at the cell surface or in the extracellular milieu (38, 39). In vitro, this metalloendopeptidase cleaves various peptides such as somatostatin-28 (S-28), dynorphin A, α-neoendorphin and glucagon (40). Comparisons of the rat and human full-length cDNAs show a similarity of about 94%. In humans, NRD1 convertase is mostly expressed in heart, skeletal muscle, and testis. At lower levels, it is expressed in other tissues like thymus, prostate, ovary, small intestine, and leukocytes (41). It has also been found in adult and in developing human brain where it possibly plays a role in brain development (40).
It has been identified that NRD1 is a protein which specifically binds heparin-binding epidermal growth factor-like growth factor (HB-EGF), a chemotactic and mitogen factor and therefore has a role in cell migration and proliferation (42). HB-EGF can be, as a transmembrane protein, shed enzymatically to release a soluble growth factor in the extracellular space. It was shown that NRD1 enhances ectodomain shedding, a post-translational modification which releases the extracellular domain of transmembrane proteins, such as of HB-EGF. The effect of NRD1 appears not to be direct, but mediated by tumor necrosis factor-α-converting enzyme (TACE) leading to enhanced cell migration and proliferation (43). Similarly, through the interaction with TACE, NRD1 enhances the cleavage of tumor necrosis factor alpha (TNF-α) anchored in the cell membrane by which it detaches from the cell surface. The interaction between NRD1 and TACE increases TACE-induced TNF-α shedding (44). It is reported that NRD1 interacts further with other functionally important molecules such as malate dehydrogenase, beta-secretase 1, neuregulin 1, or the histone H3 dimethyl Lys4 (41).

1.4.2 Lymphocyte phosphatase-associated phosphoprotein (LPAP)

CD45, a leukocyte-specific phosphatase, has previously been shown to associate with a 32-kDa phosphoprotein in human T lymphocytes and T-cell lymphoma cell lines. Since the expression of the protein was found to be restricted to T and B lymphocytes it was named lymphocyte phosphatase-associated phosphoprotein. LPAP is a transmembrane protein, containing a potential transmembrane domain and a short, 10-amino acid-long extracellular domain. In resting human T lymphocytes, there are two differently phosphorylated forms of

LPAP (29kDa and 32kDa), which both undergo alterations during the activation of a lymphocyte. About 75% of total CD45 and LPAP exist in a form of an intermolecular complex (45). CD45 has protein tyrosine phosphatase (PTP) activity which is important for the signaling in T and B cell activation. Various interacting molecules participate in regulating CD45 activity like Src family protein tyrosine kinases such as Lck, however, the exact mechanism remains unexplained (46).

It has been shown, that the expression of LPAP depends on the presence of CD45. CD45-deficent Jurkat T cell line expresses LPAP mRNA, and similar amounts of LPAP protein are synthesized, however, the protein's half-life is shorter than in the wild-type cells. Therefore, it seems that LPAP protein is degraded in the absence of its binding partner, CD45 (47). On the other side, Matsuda et al. investigated the importance of LPAP in CD45 expression and showed reduced CD45 expression in T and B lymphocytes of LPAP–null mice. Further, the phosphatase activity was reduced proportionally to its decreased expression, indicating that the absence of LPAP does not directly change the CD45 activity. They also showed that "CD45/Lck-complex in T lymphocytes of LPAP-null mice is disturbed and that the responses towards a variety of stimuli of LPAP-null lymphocytes are impaired" (46). However, there are studies that confirm the decreased CD45 expression and no change in enzyme activity, but claim, that LPAP is not crucial for the regulation of Src-family kinase activity by CD45 (48) and that there is no major alteration in the association between Lck and CD45 in LPAP-null mice (49). Therefore, further studies should ensue to resolve the question of the role and importance of LPAP in CD45 expression.

Although, the physiological role of LPAP is not yet clarified, it is suggested that LPAP acts as an adaptor protein which enables the interactions of CD45 with other molecules and may serve an important role in regulation of lymphocyte activation.

2 OBJECTIVES

In the present thesis the role of two proteins, nardilysin and lymphocyte phosphatase-associated phosphoprotein, in autophagy will be investigated. Both proteins have been identified as potential targets of autophagy via proteomic approach. Our aim will be to figure out whether NRD1 and LPAP also take part in the regulation of autophagy. Therefore, we will look for a potential change in autophagy level, after knocking down either NRD1 or LPAP expression in cancer T cells or other cells. The siRNA transfection will be carried out by an electroporation method. The expression of various proteins after transfection will be analyzed by western blotting and immunofluorescence staining followed by confocal microscopy. Furthermore, we will also check the viability of transfected cells after the application of an anticancer drug by flow cytometry.

3 MATERIALS AND METHODS

3.1 Materials

Table 1: Devices

Devices	Type	Company
Aspirating pipette	2 mL	Greiner Bio-One
Autoradiography film	Amersham Hyperfilm ECL	GE Healthcare
Cell culture flasks	TC Flasks with filter cap (50 ml, 250 ml, 550ml)	Greiner Bio-One
Cell culture plates	6-, 24- and 96-well plates	Greiner Bio-One
Centrifuge	Heraeus Multifuge 3SR	Thermo Fisher Scientific
Centrifuge	Heraeus Multifuge 40R	Thermo Fisher Scientific
Confocal microscope	LSM 700	Carl Zeiss
Counting chamber	Neubauer chamber	Marienfeld
Cover glasses	Φ12mm	Thermo Scientific
Cryogenic vial	Nunc CryoTubes; 1.8 mL	Sigma
Cytocentrifuge	Shandon CytoSpin 3	Therno Scientific
Cytoslides	Shandon Single Cytoslides	Thermo Scientific
Electronic analytical balance	XP205	Mettler Toledo
Electrophoresis machine	Powerpac 3000	BioRad
Electrophoresis system	XCell SureLoc Mini-Cell	Life Technologies
FACS Verse Flow cytometer	BD FACSVerse 6 color Flow Cytometer	BD Biosciences
Falcon tubes	15mL, 50mL	Greiner Bio-One
Filters	Filter paper	GE Healthcare
Filter cards for cytospin	For use with 0.5mL samples	Thermo Scientific
Freezer (-20°C)	G 3513	Liebherr
Freezer (-80°C)	MDF-5386SC	Sanyo
Imaging System	X-Omat 2000 processor	Kodak
Incubator	Heraeus HERAcell 150i	Thermo Fisher Scientific
Light microscope (inverted fluorescent)	Zeiss Axiovert 35	Carl Zeiss
Micro Test Tube	3810X; 1.5 ml	Eppendorf
Microcentrifuge	Centrifuge 5415 D	Eppendorf
Microcentrifuge	Centrifuge 5417 R	Eppendorf
Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units	0.2µm pore size, 75mm diameter, 500mL	Thermo Scientific
Neon® Transfection System	Neon® Transfection System Starter Pack	Invitrogen
Pipette boy	Pipetboy acu	INTEGRA Biosciences
Pipette boy	Accu-jet® pro Pipette Controller	BrandTech Scientific
Pipettes	10µL, 100µl, 1000µL	Eppendorf
PVDF Transfer membrane	Immobilon - P	EMD Millipore
Serological pipettes	2ml, 5mL, 10mL, 25mL	Eppendorf
Spectrometer	SpectraMax M2	Molecular Devices
Stripping owen	Hybridiser HB-1D	Techne
Sysmex	Sysmex KX—21	Sysmex Digitana AG
Vortex mixer	Vortex-Genie 2	Scientific Industries

Table 2: Chemicals and media

Chemical/medium	Cat. number	Company
Amersham ECL Prime Western Blotting Detection Reagent	RPN2232	GE Healthcare
Amersham Hyperfilm™ ECL	KNO90029	GE Healthcare
Bovine Serum Albumin (BSA) Fraction V Solution 7.5%	A-8412	SIGMA
Chloroquine diphosphate salt	C6628	SIGMA
DPBS	17-512F	Satorius
DMEM	31966-021	Life Technologies
DMSO	D2650	SIGMA
DTT(Dithiothreitol)	197 777	Roche
Fetal Calf Serum	A15-104	GE Healthcare
Gibco® EBSS	14155-048	Life Technologies
Glycine	50049	Fluka
Goat serum blocking solution	S-1000	Vector
Human T cell enrichment kit	19051	Stem cell technologies
Methanol	1.06009.2511	Merck
Novex® Sharp Pre-Stained Protein Standard	LC5800	Invitrogen
NuPAGE LDS Sample Buffer (4x)	NP0007	Invitrogen
Pancoll human	P04-60500	Pan Biotech
Paraformaldehyde extra pure	16005	Riedel-de-Häen
Phenylmethylsulfonylfluoride (PMSF)	837 091	Roche
Phosphate buffered saline (PBS) pH 7.4	P3813	Sigma
Pierce BCA Protein Assay Kit	23255	Thermo Scientific
Pierce™ ECL Plus Western Blotting Substrate	32132	Thermo Scientific
ProLong® Diamond Antifade Mountant with DAPI	P36962	Life Technologies
Proteases inhibitor cocktail (PIC)	P-8340	SIGMA
Rapamycin (sirolimus)	BML-A275-0005	Enzo
Restore™ Western Blot Stripping Buffer	21059	Thermo Scientific
RPMI Medium 1640 + GlutaMAX	61870	Life Technologies
RunBlue 12% SDS PAGE Precast Gel	NXG01212K	Expedeon
RunBlue SDS Running Buffer (20x)	NXB50525	Expedeon
Saponin 10% (1:200 in water)	47036-50G-F	Sigma
Streptomycin/Penicillin	15140-122	Invitrogen
Trizma Base (TRIS)	T-6066	SIGMA
TWEEN® 20	P2287	SIGMA
Zombie Violet™ dye	423113	BioLegend

Table 3: Solutions

Solution	Composition
Lysis buffer	50 mM Tris [pH 7.4], 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 2 mM EDTA, 10 mM NaPyrophosphate, 50mM NaF, 200µM Na_3VO_4
Blocking buffer for western blot	5% milk powder in TBST
Blocking buffer for immunofluorescence staining	3% goat serum and 1:200 of 10% saponin in PBS
Blocking buffer for FACS	2/3 of FACS Buffer + 1/3 of 2,4 G2 batch 3
PBS Plus buffer/FACS buffer	2% FCS +1mM EDTA in PBS (stock EDTA 0,5M)
TBS (10x)	0.20 M Tris, 1.50M NaCl [pH 7.6]
TBST	0.1% Tween 20 in TBS
Transfer buffer 10x	0.25 M Tris, 1.87M Glycine
Transfer buffer 1x	20% MeOH +10% Transfer buffer (10x) + ddH_20

Table 4: Antibodies

Target protein	Cat. Number	Company	Source
CD3 epsilon, PerCP	345766	BD Biosciences	Mouse
Goat Anti-Rabbit IgG, HRP-linked Ab	NA934V	GE Healthcare	Goat
Sheep Anti-Mouse IgG, HRP-linked Ab	NXA931	GE Healthcare	Sheep
GAPDH	MAB374	Millipore	Mouse
Goat anti-Mouse IgG, Alexa Fluor® 555	A-21422	Molecular Probes	Goat
Goat anti- Rabbit IgG, Alexa Fluor® 488, 568	A-11034 A-11011	Molecular Probes	Goat
LC3B	NB600-13	novusBiologicals	Rabbit
LC3 (clone 5F10)	0231-100	nanoTools	Mouse
LPAP	sc59290	Santa Cruz Biotechnology	Mouse
Nardilysin	NBP2-19473	NovusBiologicals	Rabbit
p62 (SQSTM1)	P0067	Sigma	Rabbit

3.2 Methods

3.2.1 Cell lines

Two types of human cell lines are cultured. Human T-cell lymphoma cell line (Jurkat) as suspension cells and mouse embryonic fibroblasts with stable expression of green fluorescent protein GFP-LC3 (MEF GFP-LC3) as adherent cells.

Jurkat cells are cultured in RPMI medium with 10% fetal calf serum (FCS) and 1% solution of antibiotics penicillin and streptomycin in the incubator at 37°C with 5% CO_2. For changing the medium cell suspension is centrifuged, the supernatant is removed and the pellet is resuspended in new medium.

MEF GFP-LC3 cells are cultured in DMEM medium with 10% FCS and 1% penicillin and streptomycin. For medium change, the medium is aspirated without touching the cells. Cells are washed with DPBS and new medium is added.

Counting cells

Cell concentration is evaluated by cell counting with Neubauer chamber. A diluted suspension with a suitable concentration is prepared and the chamber is loaded with 10 µL diluted sample. The Neubauer chamber is placed on the microscope stage and cells in 4 counting grid squares are counted. The starting cell concentration can be calculated with the following equation:

cell concentration [cells/mL]=1/4 x number of counted cells x dilution factor x 10^4 cells/mL

Jurkat cells as blood cells can also be count by Sysmex hematology analyzer. 80-100 µL cell suspension is transferred to microcentrifuge tube and analyzed by Sysmex.

Freezing and thawing cells

To freeze the cells, cell pellet is resuspended in 900 µL filtered FCS and transferred into a cryotube. 100 µL DMSO is added and the suspension is put in a Nalgene® Cryo freezing container, which provides a slow cooling rate. It is placed in the freezer at -80°C and 24 hours later the cells are put to liquid nitrogen at -196°C.

When we want to thaw new cells, first, the cryotubes with cells are transferred from liquid nitrogen to a 37˚C water bath, in order to thaw the cells more rapidly. The cell suspension is then transferred to a Falcon tube with 9 mL of culture media and centrifuged at 1400 rpm for 5 min at room temperature (RT). After centrifugation, the cell pellet is resuspended in 1 mL culture media and the suspension is transferred to a flask with new medium.

Cell passaging

Cell passaging involves splitting cells and transferring them into new flasks. Splitting should be done before cells reach confluency.

Suspension cells are transferred into a Falcon tube and centrifuged at 1400 rpm for 5 min at RT. After the supernatant is removed, the cell pellet is resuspended in new medium and a suspension with a concentration of 200.000-500.000 cells/mL is made in a new flask. If there is no significant change in culturing media color, appropriate amount of cells can be directly transferred to a new flask with new media. These can be left in the flask and fed every 2 to 3 days by adding fresh culture media until they reach confluency.

Adherent cells are washed with DPBS without Mg2+ and Ca2+, and then trypsin 0, 25% is let to act for 1 min at 37°C to detach the cells from surface. After 1 minute new medium is added to the flask and the suspension is mixed by pipetting up and down. The cell suspension is then centrifuged and the pellet is resuspended in new medium. The volume of 500.000 cells is calculated and added to new middle flask with 10 mL medium in total.

3.2.2 Cell treatment

To induce autophagy, MEF GFP-LC3 cells are treated with rapamycin for 5 hours. Chloroquine (CQ) is used as a lysosomal degradation inhibitor. For the experiment, rapamycin 10 mM and CQ 100 mM stock solutions are used.

250.000 cells/ condition are after transfection seeded onto cover slips in a 24-well plate. For the treatment both rapamycin and CQ are used at a 10 µM concentration. New mediums are prepared for each condition;
Condition I as control; 500 µL DMEM
Condition II, stock rapamycin is diluted 1:1000 in 500 µL DMEM
Condition III, stock CQ is diluted 1:10000 in 500 µL DMEM and
Condition IV both rapamycin (1:1000) and CQ (1:10000) are added to DMEM with final volume of 500 µL.
After the mediums are changed the cells are incubated at 37°C for 5 hours.

To inhibit the lysosomal degradation of NRD1, Jurkat cells are treated with CQ.
400.000 cells/ condition are spinned down and the pellet is resuspended either in 200 µL RPMI as control condition or in 200 µL 10 µM CQ in RPMI. The cells are incubated at 37°C for 24 hours.

To estimate cell death, Jurkat cells are treated with cisplatin. 500.000 cells/ condition are centrifuged at 1400 rpm for 5 min at room temperature. The supernatant is removed and the cell pellet is resuspended in 500 µL RPMI for control condition or 500 µL of 3 µM, 10 µM or 25 µM cisplatin. They are treated for 20 hours at 37°C.

3.2.3 Cell lysis

By cell lysis we break down the membrane of a cell in order to further study its contents. Firstly, cells are centrifuged at 1400 rpm for 5 min at RT. Supernatant is removed and the pellet is washed with 1 mL cold DPBS (1400 rpm, 5 min, RT). Meanwhile, total cell lysis buffer is prepared; 0,5 µL of protease inhibitor cocktail and 0,5 µL of PMSF are added to 500 µL of lysis buffer. Sample, as well as lysis buffer, should always be kept on ice. Cell pellet is resuspended in 20 -50 µL lysis buffer, depending on the size of the pellet, and incubated for 20 min on ice. After 20 min cells are spinned down at 13.300 rpm, 10 min, 4°C and the supernatant is transferred to pre-labeled microcentrifuge tubes. Samples are stored at -20°C.

3.2.4 Protein concentration measurement

Samples are taken from -20°C and put on ice for 10 min. Protein concentration measurement is performed in 96–well plate. From A-H are standards with defined decreasing concentrations of albumin prepared from 2 mg/mL albumin stock solution. 25 µL of standards are pipetted to parallel wells in first two columns. In the two following wells 25 µL of double-

distillated water (blank sample) are pipetted. The supernatants of samples are diluted 1:10 with double-distillated water (54 µL water+ 6 µL sample) and 25 µL are added to parallel wells following the standards and the blank sample.

To each well 200 µL of reagents A+B from the Thermo Scientific Pierce BCA Protein Assay Kit are added. A mixture of A and B reagents is prepared in ratio 50:1. The 96-well plate is then incubated at 37°C for 5 min and the absorbance is measured with a spectrophotometer (λ=562 nm) from which the protein concentration is determined by a protein standard curve. The measurement bases on the colorimetric detection of Cu1+ ions by bicinchoninic acid. The cuprous cations are caused by the reduction of Cu2+ by proteins in an alkaline medium.

3.2.5 Western blot

Western Blot is a common method to detect and analyze proteins. In the first step, we separate the proteins by their molecular weight (kDa) using gel electrophoresis. After the separation, the proteins are transferred or blotted onto a polyvinylidene difluoride (PVDF) membrane and the membrane is blocked to avoid any nonspecific binding. The target proteins are detected with specific antibodies by visualizing them on X-ray films by chemiluminiscent reaction, where the intensity of the signal correlates with the abundance of the protein on the membrane.

Sample preparation. After the protein concentration is measured, exact volumes of double-distilled water, DTT (1:10), protein lysates, and loading buffer (1:4) are calculated and mixed in microcentrifuge tubes. The amount of protein varies between 20-50 µg. The tubes are heated on a water bath for 5 min at 90°C to denature the proteins, then cooled and centrifuged for 3 min at 13000 rpm.

Electrophoresis. The 12% SDS-PAGE gel is put into the electrophoresis chamber and the running buffer (20x) is added. The wells are washed carefully with running buffer by pipetting up and down. Sample dilutions with the same amount of proteins are pipetted slowly into the wells and 7 µL of pre-stained molecular weight markers are pipetted to the two wells enclosing the samples. An 80 V electric field is applied over the gel for 25 min and then changed to 135 V for another 1 hour and 20 min.

Electrotransfer. While the electrophoresis is running, the transfer buffer is prepared.

700 mL double-distilled water, 200 mL methanol and 100 mL transfer buffer (10x) are added to a measuring cylinder and poured to a plastic box with other equipment for the electrotransfer. For the transfer, six sponges, two filters, and one membrane are stacked in the transfer chamber in the following order (bottom-up): lower part of the transfer chamber, three

sponges, one filter, the membrane, one filter, three sponges and the upper-part of the transfer chamber.

Before the membrane is put in the transfer stack, it should be activated in methanol for 1 minute, then rinsed in tap-water and labeled. Until the electrophoresis is finished the transfer buffer is put to the fridge to cool down, together with the cassette holder, sponges, filters and the membrane. After electrophoresis, the gel is taken out and placed between the lower filter and the membrane. Bubbles are removed from the sponges by pressing them with a glass bar. The stack is then put to the electrophoresis chamber, the transfer buffer is added, and the transfer is run for 1 h at 30 V and 4°C. In case of two gels, both can be transferred in the same electrophoresis chamber at 60V for 1 h at 4°C.

Blocking and incubation. When the transfer is finished the molecular weight markers are labeled with a pen. Afterwards, the membrane is first washed in TBST for 5 minutes, shaking and then incubated in blocking buffer for 1 h, shaking. After blocking, the membrane is incubated overnight in diluted primary antibody at 4°C on the rotor. Primary antibodies are diluted 1:500 – 1:10.000 in blocking buffer, depending on their strength of binding. Usually they are diluted 1:1000.

After incubation with primary antibody, the membrane is washed 3 times for 5 min in TBST, shaking at RT. Horseradish peroxidase (HRP) conjugated secondary antibody is diluted 1:10000 in blocking buffer, added to the membrane, and incubated for 1 h at RT.

Detection. After the incubation with secondary antibody, the unbound secondary antibodies are removed by washing 3 times for 5 min in TBST. Next, the ECL Western blotting substrate is prepared. Two different substrates are used, depending on the expected signal strength;

ECL: the substrate working solution is prepared by mixing Substrate 1 and Substrate 2 at a ratio of 1:1; ECL Plus: the substrate working solution is prepared by mixing Substrate A and Substrate B at a ratio of 40:1. The protein side of the membrane is incubated for 2 minutes in ECL solution on a balanced base. The membrane is then removed from the working solution and placed in the developing cassette between two plastic sheets with the protein side facing up. The bubbles between the plastic sheets are gently removed with a tissue. The film is developed in the darkroom. Amersham Hyperfilms ECL which detect chemiluminescent reactions are placed on the top of the membrane and exposed for a certain time. The horseradish peroxidase conjugated to the secondary antibody catalyzes a light generating reaction using the ECL detection reagent as a substrate. The exposure time depends on the signal intensity, which differs with individual antibody. The films are then developed by X-Omat 2000 processor. If other proteins are being checked, the membrane is shortly washed in

TBST and incubated with stripping buffer at 50 ˚C for 35 min, shaking. Then, after washing with TBST and blocking, the next primary antibody can be applied.

3.2.6 Immunofluorescence staining

Immunofluorescence staining is based on a reaction between antibodies labeled with a fluorescent dye and their specific antigen, followed by the observation of the reaction product under fluorescence microscope. 100 µL of cell suspension with 200.000-250.000 cells is pipetted into cuvettes and cytospinned onto pre-labled slides (2000 rpm, 1 min). Alternatively, the adherent cells are seeded onto cover slips and are, after washing with DPBS, directly fixed with 4% PAF.

After cytospin cells are being fixed onto slides with 4% PAF for 10 min. The slides are washed with PBS pH 7.4 twice for 3 minutes. To permeabilize the cells, first, they are incubated in 80 µL 0,005% saponin in water for 5 min. Secondly, after 3 minutes washing, they are transferred to the slide chamber with ice-cold aceton and incubated for 10 min at -20°C. The slides are washed twice for 3 min with PBS pH 7.4 and incubated in 80 µL/slide blocking solution (3% goat serum + 0,005% saponin in PBS pH 7.4) for 1 h in moist chamber at RT. Meanwhile primary antibodies are diluted 1:100-1:200 in blocking solution and 70 µL/ slide is applied after the blocking buffer is removed. The slides are incubated overnight in moist chamber at 4°C.

Next day, the unbound antibodies are washed away three times for 5 min with PBS pH 7.4. Secondary antibodies are diluted 1:100 in 3,5% BSA in PBS and 70 µL are applied to each slide and incubated for 1 hour on RT. After the incubation with secondary antibodies, the slides are washed three times for 5 minutes. 7 µL of mounting medium with DAPI are pipetted to coverslips and put to microscope slides. The slides are put in dark place for 30 min and then to 4°C until microscopy.

3.2.7 Neon transfection

The Neon® transfection system is a device using an electroporation technique for siRNA transfection. Plates or small flasks with media without antibiotics, RPMI or DMEM with 10% FCS, are prepared and put to 37°C to warm up. Cells are count and a certain amount is centrifuged at 1400 rpm, 5 min at RT. The cell pellet is resuspended in T Buffer (Jurkat and T cell) to make the final concentration of $2x10^7$cells/mL. MEF cells are resuspended in R Buffer and a suspension with the concentration of $5x10^6$cells/mL is made. The suspension is then transferred to a sterile microcentrifuge tube. From 10 µM stock siRNA the appropriate amount is pipetted to the microcentrifuge tube with cells, to make the working solution 10-

200 nM, mostly 200 nM. Either control siRNA and NRD1 siRNA or control siRNA and LPAP siRNA are used, depending on the gene we want to knockdown. The siRNA is added shortly before the transfection is performed.

The power supplier and the pipet station are properly attached to the transfection machine and the desired transfection program is entered. 3 mL of Electrolytic Buffer are added to the Neon tube and inserted into the pipet station until a click is heard. For the 10 µl Neon Tip Buffer E is used and for the 100 µl Neon Tip Buffer E2 is used. A Neon Tip is inserted into the Neon Pipette and the cell-DNA mixture is aspirated into the tip avoiding air bubbles. The Neon Pipette is then inserted into the Neon tube with Electrolytic Buffer. Jurkat cell line and T cells are pulsed three times with a voltage of 1,350 and width 10 ms. MEF cells are pulsed once with 1,350 V and 30 ms width. After the pulses, cells are quickly transferred into the culture plate with antibiotic-free medium and placed at 37˚C in a 5% CO_2-humidified atmosphere for 6-, 24-, or 48 -hours. Transfected cells are then used for further experiments.

3.2.8 Flow cytometry (FACS)

Jurkat cell death measurement

For the cell death measurement, two fluorescent dyes are used: ethidium bromide and Zombie Violet™ dye.

When ethidium bromide is used as a dead cell marker no previous sample preparation is needed. Right before the FACS measurement, 100 µL of ethidium bromide is added to 100 µL cell suspension and shortly vortexed. An unstained sample should be measured to make sure that the staining of the cells is appropriate.

When Zombie Violet™ dye is used, 500.000 cell/ condition are spinned down and resuspended in 100 µL PBS with 1 µL of Zombie added. After Zombie is added, the work should be performed protected from light. The cell-dye mixture is incubated on RT 15-30 minutes. After incubation the cells are washed with 500 µL PBS with 1% FCS and then resuspended in 200 µL PBS+ 1% FCS. Lastly, the amount of dead cells is measured by FACS.

Isolated T cell purity analysis

FACS Buffer and FACS Blocking buffer are put on ice. 500.000 Jurkat cells are spinned down and washed with cold FACS buffer (1500 rpm, 5 min, 4°C). The supernatant is removed and the pellet is resuspended in 100 µL of FACS blocking buffer. The cells are incubated with blocking buffer on ice for 5–10 minutes. Next, 0,5 µL of anti-CD3 antibody is

added, the cell suspension is briefly vortexed, and incubated on ice for 45 minutes in the dark. After incubation the cells are centrifuged at 1500 rpm for 5 min at 4°C. The supernatant is removed and the cell pellet is resuspended in 500 µL FACS Buffer. The FACS analysis is conducted.

3.2.9 T cell isolation

50 ml blood is collected from donor's vein. Into three 50 mL Falcon tubes 15 mL of Pancoll, a density gradient media, is added. Blood is diluted 1:1 in PBS and collected in a middle flask. Diluted blood is then carefully pipetted onto the Pancoll, without mixing the two fluids. Falcon tubes are put to the centrifuge and spin down at 800xg for 20 min at RT. After the centrifugation multiple layers are visible. The upper layer is aspirated, leaving the mononuclear cell layer (lymphocytes, monocytes, and thrombocytes) undisturbed at the interphase. The mononuclear layer is carefully transferred to two new 50 mL Falcon tubes. These are then filled up with PBS Plus buffer, mixed and centrifuged at 1400 rpm, 7 min at RT. The supernatant is removed completely. For removal of platelets, the cells are washed again and the pellets are resuspended in 1 mL PBS Plus. Further, the cells are count by Sysmex and a cell suspension at a concentration of 5 x 10^7 cells/mL is prepared. Cells are placed in a 5 mL polystyrene tube and EasySep Human T cell Enrichment cocktail is added at 50 µL/mL cells. The suspension is mixed by pipetting and incubated at room temperature for 10 minutes. Meanwhile the EasySep D Magnetic Particles are vortexed for 30 seconds to make a uniform suspension with no aggregates. After 10 minutes incubation, EasySep D Magnetic Particles are added at 50 µL/mL cells, mixed, and incubated for 5 minutes at RT. Next, the cell suspension is brought up to a total volume of 2,5 mL by adding PBS Plus Buffer. After pipetting up and down 2–3 times the tube is placed into the magnet and set aside for 5 minutes. The EasySep Magnet is then in one continuous motion picked up and inverted, pouring off the desired fraction into a new 5 mL polystyrene tube. The magnet with the tube is left in inverted position for 2–3 seconds then returned to upright position. The magnet should not be shaken and any off the drops that may remain hanging from the mouth of the tube should not be blotted off. For better efficiency, the tube is taken out of the magnet, filled up to 2,5 mL, and the separation is repeated. For better purity, the cell suspension is poured back to the first tube and the steps are repeated. The fractions are then centrifuged and collected in one tube. The purity of isolated T cells is checked by staining the CD3+ cells (T lymphocytes) followed by FACS Verse analysis.

4 RESULTS

To study the proteins involved in autophagy network of cancer cells, a global proteomic analysis was conducted. Upon induction of autophagy, up- and down-regulated proteins were identified by a quantitative mass-spectrometry (MS) in Jurkat cells. NRD1 and LPAP expressions were both found up-regulated. The data was confirmed also using second methods such as western blotting and immunocytochemistry (Zhaoyue He). Based on this data, my goal was to further characterize NRD1 and LPAP in autophagy. With conducted gene knockdown experiments, we were manipulating the expressions of NRD1 and LPAP and analyzed the impact of the silencing of these two proteins on autophagy.

4.1 Down-regulation of autophagy level upon NRD1 knockdown in Jurkat cells

4.1.1 Western blotting

Our first goal was to study the effects of NRD1 on autophagy. Control siRNA (non-targeting siRNA) and siRNA for human NRD1 were transfected into Jurkat cells by Neon® Transfection System, to silence the NRD1 expression. Firstly, the most efficient, 200 nM NRD1 siRNA dose was found by transfecting the cells with different concentrations of NRD1 siRNA, from 10- to 200 nM. The cells were then transfected with 200 nM control siRNA and 200 nM NRD1 siRNA and cultivated for 24 hours. After 24 hours, cell lysates were collected and expressions of NRD1, p62, LC3-I/LC3-II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping protein involved in glycolysis, were investigated by western blotting (Figure 4A). Further, we wanted to see how long the NRD1 expression is silenced, thus we cultivated the cells for 24 h and 48 h after transfection with 200 nM siRNA. We lysed the cells 24 h and 48 h post-transfection and checked the protein expression by western blot (Figure 4B). Upon NRD1 knockdown, a decrease in NRD1 protein expression was observed which confirms a successful transfection. LC3-II and p62/SQSTM1 can both be used as markers to study autophagy. As compared with control, the NRD1 knockdown cells show a slightly decreased expression of LC3-II and accumulation of p62 both indicating less autophagy. Furthermore, the siRNA induced potent NRD1 knockdown which was observed 24 hours post-transfection and still evident after 48 hours.

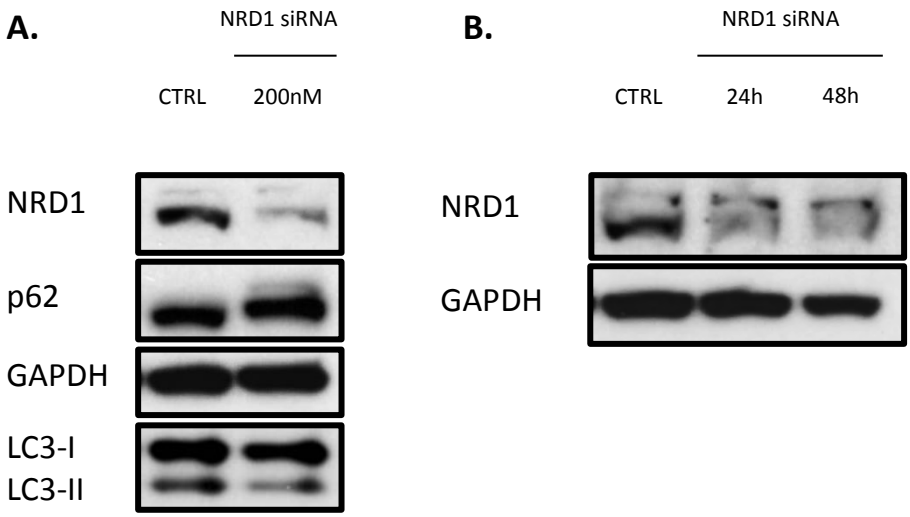


Figure 4: Down-regulation of autophagy level upon NRD1 knockdown in Jurkat cells and time course of NRD1 silencing.

NRD1 was knocked down in Jurkat cells by RNA-mediated interference. For the knockdown, a dose of 200 nM NDR1 siRNA was used. As negative control, cells were infected with control siRNA, a non-targeting 20-25 nucleotide siRNA that cannot lead to the specific degradation of any cellular message. Cells were lysed and western blotting was performed, incubating with rabbit-NRD1- (1:1000), rabbit-p62-(1:1000), rabbit-LC3- (1:1000) and mouse-GAPDH-antibodies (1:1000). (A) Lower level of NRD1 in NRD1 knockdown cells confirms a successful transfection. Decreased level of LC3-II, a marker of autophagosomes, indicates less autophagy in knockdown cells. Accumulation of p62, an autophagy substrate, also suggests a lower autophagy level. The house-keeping protein GAPDH serves as a loading control. (B) A successful knockdown of NRD1 is observed 24 hours after transfection and lasts up to 48 hours.

4.1.2 Immunofluorescence staining

The level of autophagy in NRD1 knockdown Jurkat cells was further investigated by immunofluorescence staining and visualized by confocal microscopy. Jurkat cells were transfected with 200 nM siRNA and after 24 h treated with 10 mM CQ, a lysosomal degradation inhibitor. As a control sample, the cells were transfected with control siRNA and treated with 10 mM CQ. Microscopic preparations of Jurkat cells were stained with rabbit-NRD1-antibodies (1:100), mouse-LC3-antibodies (1:100) and DAPI (Figure 5).

In control cells CQ increased the level of LC3-II and slightly the level of NRD1, suggesting that both proteins undergo lysosomal degradation. The difference in NRD1 expression is not so obvious, since the control cells already have a quite high expression of NRD1. In NDR1-silenced cells we observed similarly increased NRD1 signals comparable to increasing LC3-II

when treated with CQ. Interestingly, there were some cells which had a lower level of NRD1 (cells in white circles), and these cells also had less LC3-II, therefore less autophagy. These are most probably the cells that underwent a successful transfection and the NRD1 expression was potently silenced.

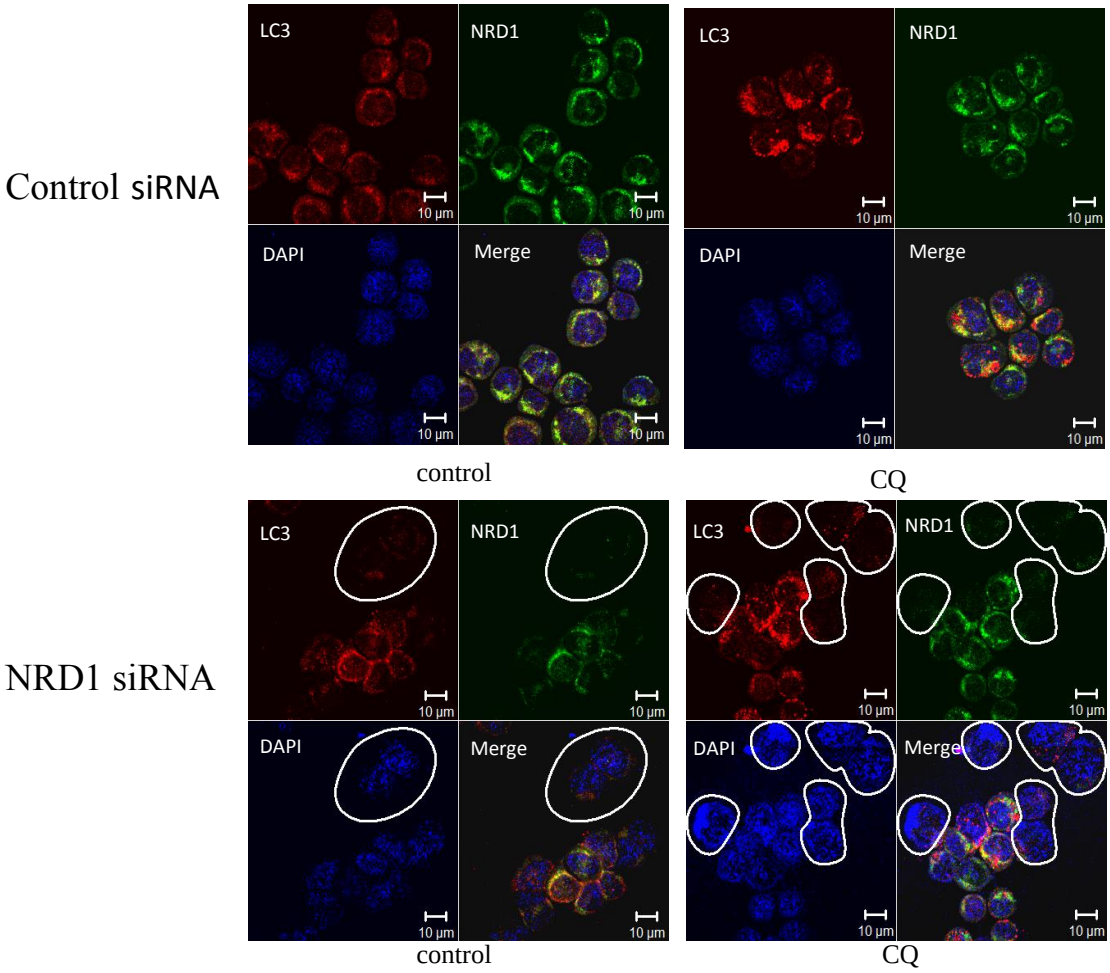


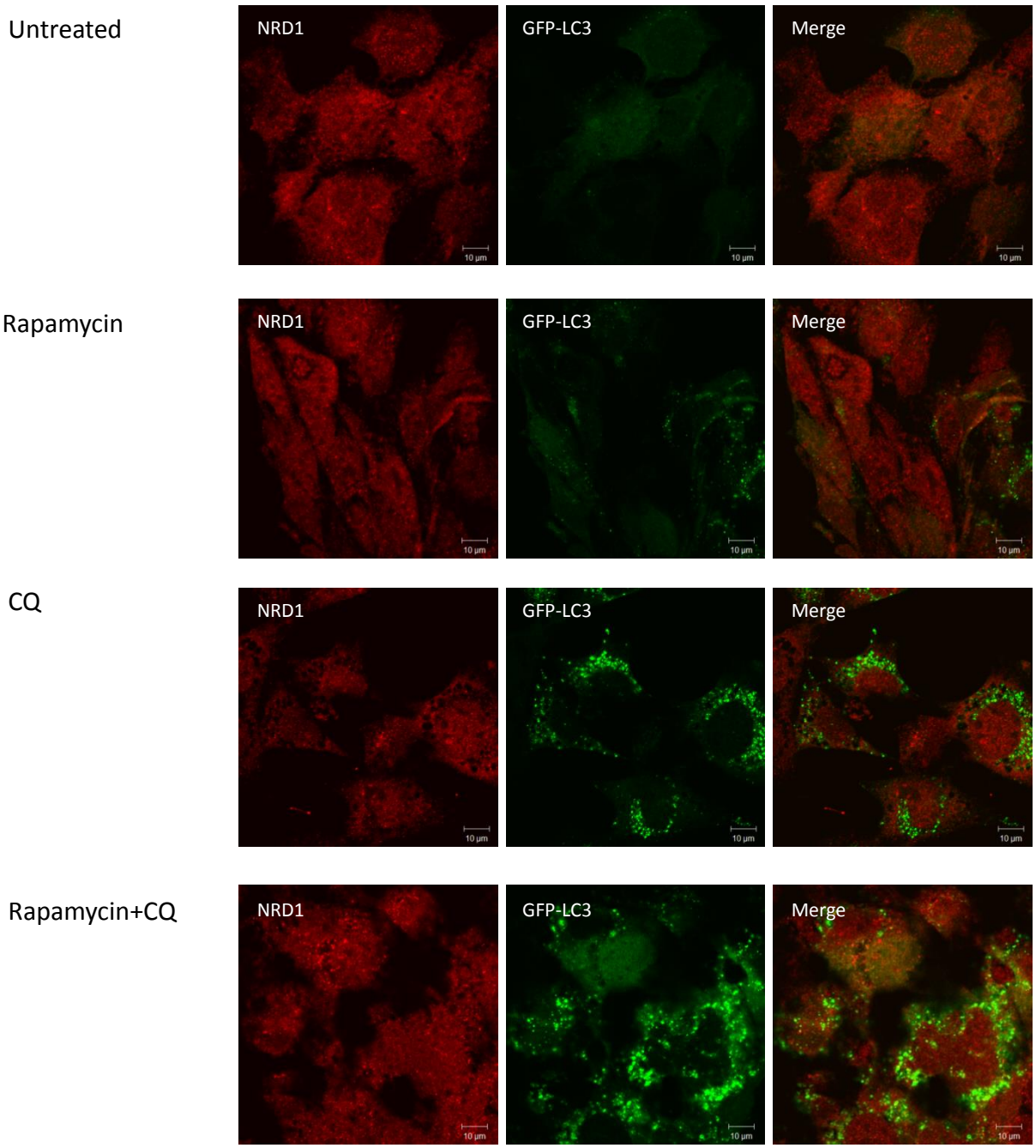
Figure 5: Lower level of autophagy in successfully transfected Jurkat cells with NRD1 siRNA.

Jurkat cells were transfected with 200 nM control siRNA or 200 nM NRD1 siRNA. Both control and transfected cells were then either left untreated or were treated with lysosomal degradation inhibitor, CQ, for 24 h. After immunofluorescence staining of Jurkat cells with rabbit-NRD1 (1:100), mouse-LC3 antibodies (1:100) and DAPI, cells were observed under the confocal microscope. In control cells an accumulation of LC3-II occurred after CQ treatment. The level of NRD1 was quite high in untreated cells and it didn't increase much more with CQ. Interestingly, in NRD1 silenced sample, not all the cells had a lower expression of NRD1. But, the ones that had are most probably the cells that were transfected successfully and these had in parallel lower levels of LC3, indicating less autophagy. With CQ the lysosomal degradation of LC3 and NRD1 was inhibited thus we see a slight accumulation of both proteins.

4.2 Down-regulation of autophagy level upon NRD1 knockdown in MEF GFP-LC3 cell line

Additionally, we wanted to check the effect of NRD1 knockdown on autophagy in another type of cells. MEF GFP-LC3 cells were transfected either with control siRNA, a non-targeting siRNA, or with NRD1 siRNA. Cells were grown onto cover slips and after 20 h, treated with rapamycin, an autophagy inducer or/and CQ. After 5h treatment the immunofluorescence staining was performed. The cells were stained with rabbit-NRD1-antibodies (1:100), and analyzed by confocal microscopy (Figure 6). In cells transfected with control siRNA, rapamycin nicely induced the GFP-LC3 puncta formation which is further increased in combination with CQ. As expected, the expression of NRD1 profoundly decreased when cells were transfected with NRD1 siRNA, indicating inhibition of NRD1 synthesis. Cells with less NRD1 showed also a decrease in GFP-LC3 puncta formation thus also less autophagy. In CQ-treated cells we observed higher levels of GFP-LC3, but when compared to the control group, the level of GFP-LC3, and thus autophagy, was still clearly lower.

Control siRNA



NRD1 siRNA

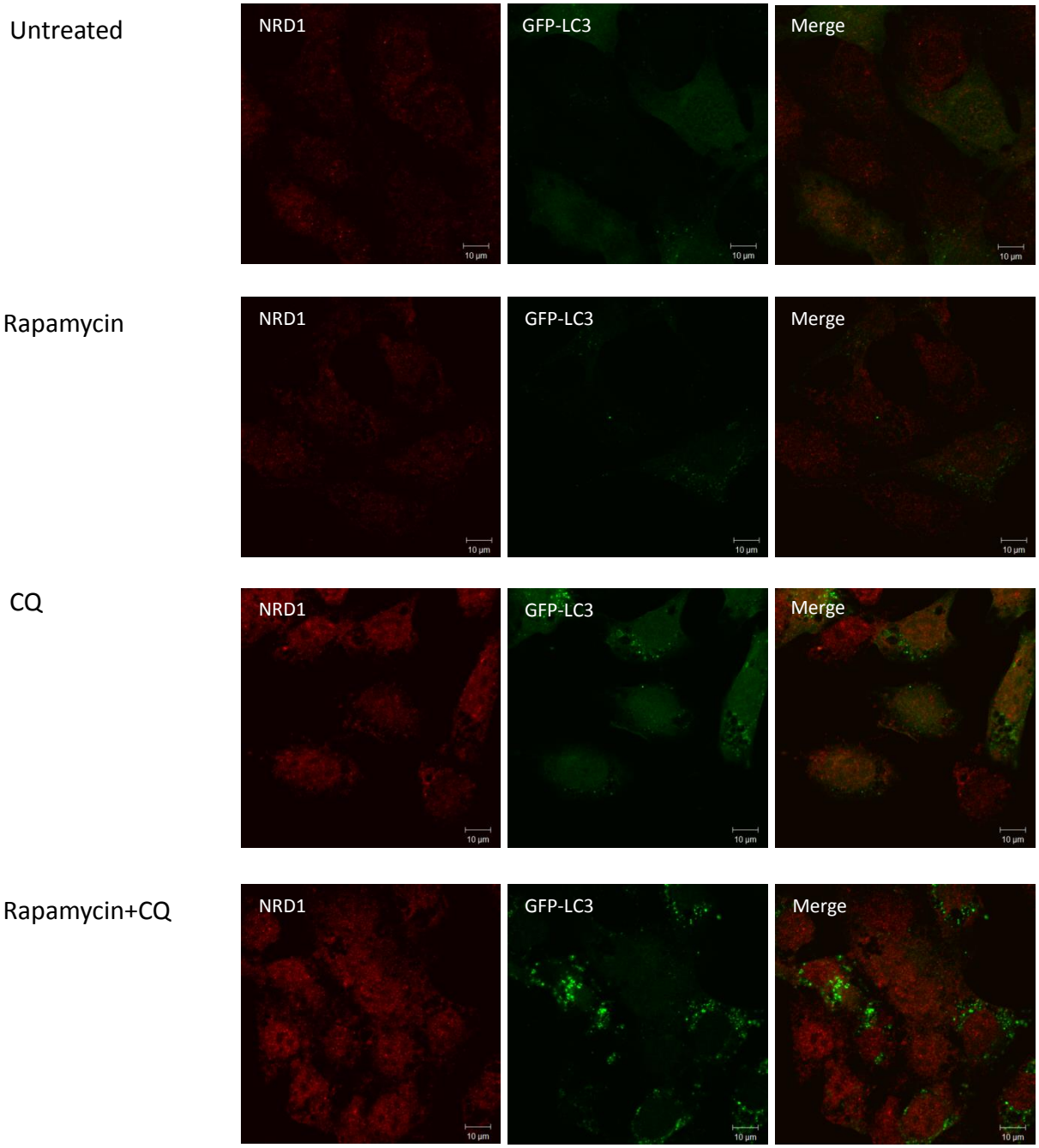


Figure 6: Down-regulation of autophagy level upon NRD1 knockdown in MEF GFP-LC3 cell line.

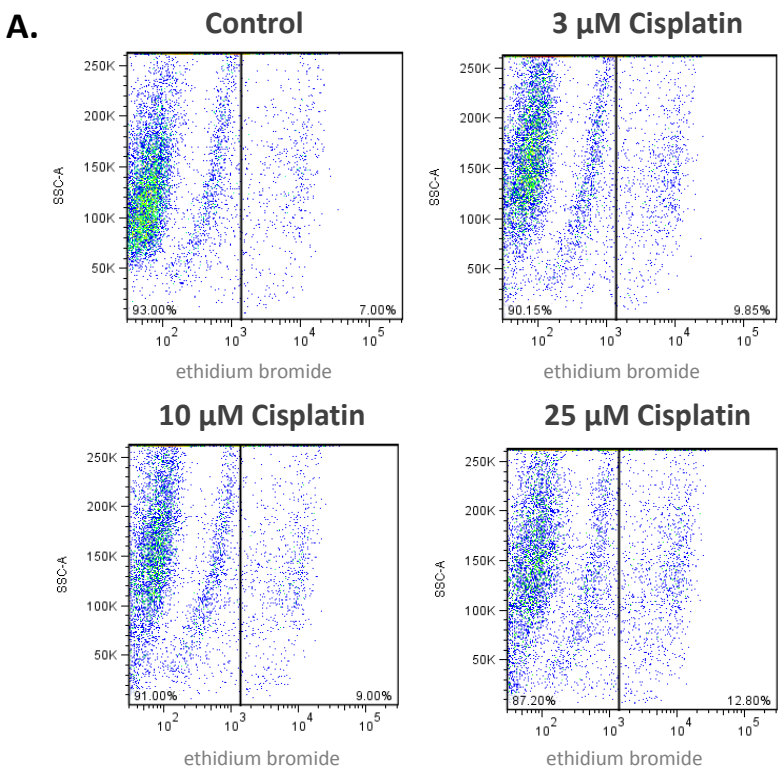
Control siRNA or NRD1 siRNA were transfected into MEF GFP-LC3 cells. Both control and knockdown cells were then either left untreated or treated with rapamycin, CQ or both together. After treatment the cells were stained with rabbit-NRD1 antibodies (1:100) and analyzed under confocal microscope. In control samples an increase of GFP-LC3 puncta formation was observed with rapamycin treatment, which was even more distinctive when combined with CQ. In knockdown cells, lower expression of NRD1 confirmed the inhibition of synthesis by knockdown. Cells with less NRD1 showed also a decrease in GFP-LC3 puncta formation, indicating less autophagy. With CQ, NRD1 expression and also GFP-LC3 were slightly increased but compared to the control samples clearly lower.

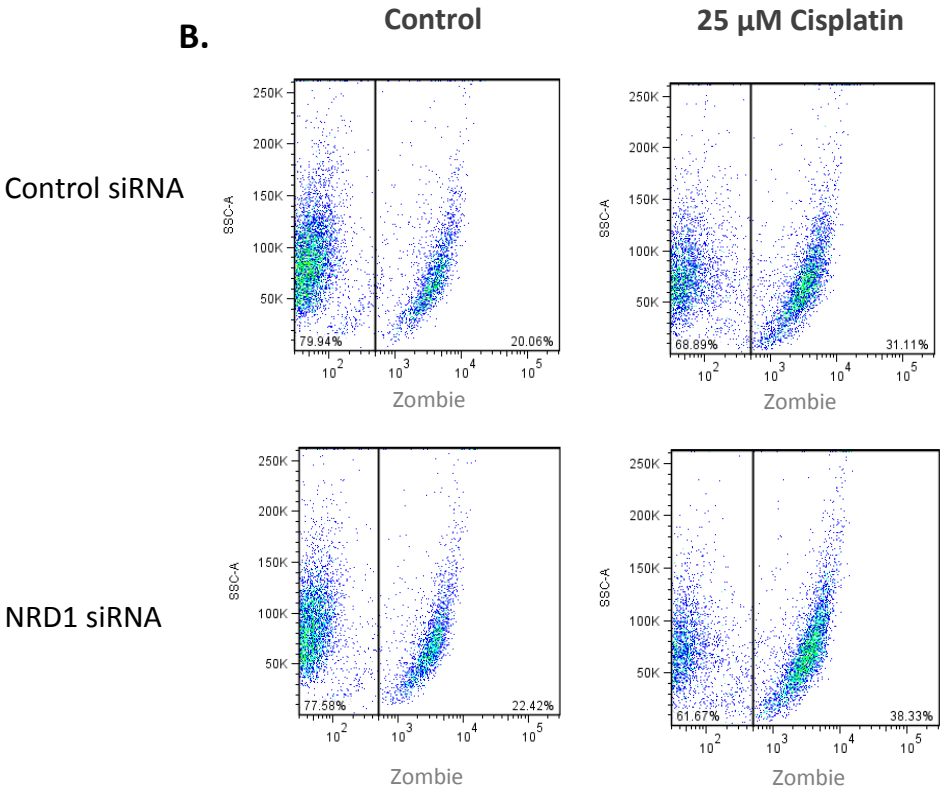
4.3 Higher cell death in NRD1 knockdown cells after anticancer drug treatment

Autophagy has different roles in cancer. One explanation says that autophagy is a protective mechanism which helps cancer cells to survive. As we showed, cells with silenced expression of NRD1 have lower levels of autophagy, therefore less protective mechanism and they are expected to die faster. We decided to investigate, if this is the case with Jurkat cells after we knockdown the NRD1 expression. First, we wanted to determine the concentration needed to kill a sufficient amount of Jurkat cells. We treated the cells with different concentrations of cisplatin in RPMI (3 µM, 10 µM and 25 µM) and assessed their viability using flow cytometer. Ethidium bromide, a dead cell marker, was added directly to the sample just before the FACS measurement. A very small population of cells underwent cell death with 3 µM and 10 µM cisplatin, while 25 µM cisplatin killed nearly 13% of cells compared to 7% in the control sample (Figure 7A). In following experiments 25 µM concentration of the anticancer drug was used. Furthermore, cells were transfected either with control siRNA or with NRD1 siRNA and incubated for 24 hours. After 24 hours, 25 µM cisplatin was added to the control and NRD1 knockdown cells and incubated for 20 hours. Zombie violet^{TM} dye was used to assess live vs. dead status of the cells (Figure 7B). NRD1 knockdown cells showed increased cell death comparing with those of control cells. The data was analyzed by FlowJo software.

Figure 7: Analysis of cell death in Jurkat cells by FACS.

(A) Cells were treated with different concentrations of cisplatin to determine the amount of the drug needed to see a relevant change in cell death. Cells were treated with 3 µM, 10 µM and 25 µM cisplatin for 20 hours and before the FACS analysis, ethidium bromide was added as a dead cell marker. 25 µM showed nearly 13% dead cells compared to 7% in the control sample and was chosen for further experiments. (B) Cells were transfected with control siRNA or NRD1 siRNA and treated with 25µM cisplatin for 20 hours. Zombie violet™ dye was used to distinguish live from dead cells. NRD1 knockdown cells showed increased cell death comparing to those of control cells.





4.4 Up-regulation of autophagy upon LPAP knockdown in Jurkat cells and T cells

LPAP is another protein we were investigating. Firstly, we knocked down LPAP expression in Jurkat cell line. 200 nM concentrations of siRNAs were used. After 24 hours incubation, lysates were collected and expressions of LPAP, NRD1, p62, LC3-I/LC3-II and GAPDH were examined by western blot (Figure 8A). LPAP knockdown cells showed lower level of LPAP which demonstrates an efficient transfection. Upon LPAP knockdown, an increase in LC3-II was observed and at the same time decrease in p62 expression, indicating up-regulation of autophagy.

Furthermore, we isolated T lymphocytes from blood and showed 96% purity of the isolate by FACS. In isolated cells, we checked the autophagy level after transfection with either NRD1 siRNA or LPAP siRNA. NRD1 knockdown was not efficient in T cells; actually, the level of NRD1 in primary cells was already very low. However, we did see a slight decrease in LPAP after LPAP siRNA transfection, meaning the transfection was more successful. Similarly as in Jurkat cells, in T cell there was an up-regulation of LC3-II after LPAP knockdown. In p62 expression there was no significant change, but interestingly we observed an up-regulation of NRD1.

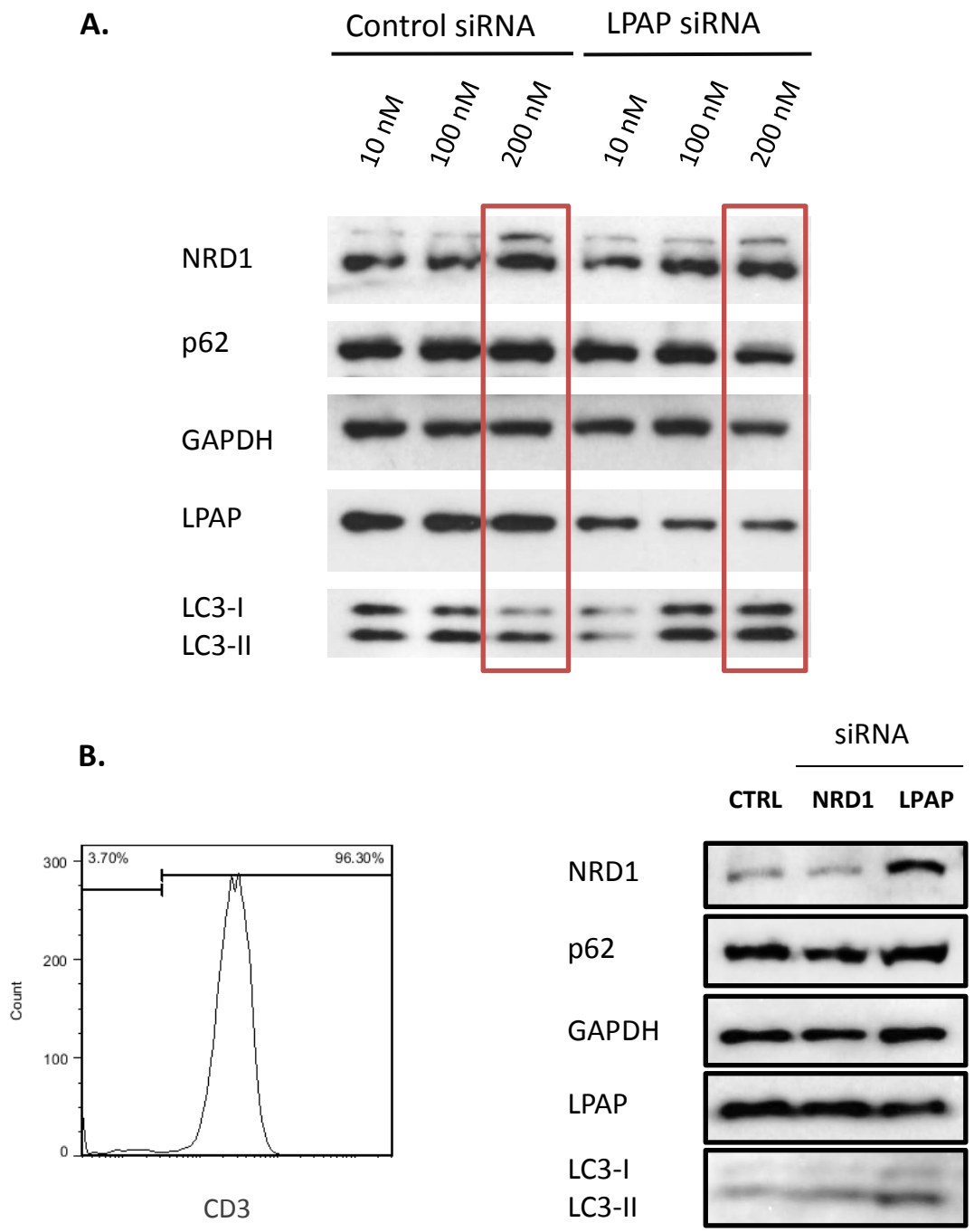


Figure 8: Up-regulation of autophagy upon LPAP knockdown in Jurkat cells and T cells.

(A) Jurkat cells were transfected with control siRNA or with LPAP siRNA. After 24 hours incubation, the cells were lysed and western blotting was performed incubating with rabbit-NRD1, rabbit-p62, mouse-LPAP, rabbit-LC3, and mouse-GAPDH antibodies, all diluted 1:1000 in blocking buffer. Down-regulation of LPAP defines an efficient knockdown. Upon the knockdown of LPAP, with a concentration of 200 nM, we observed an increase in LC3-II and, at the same time, degradation of p62, both suggesting an induction of autophagy. (B) T lymphocytes were isolated from blood and the purity of the isolated cells was checked by FACS. Approximately 96% of the isolated cells were CD3+, representing T lymphocytes. The cells were then used for the NRD1 or LPAP silencing. The NRD1 knockdown was less efficient in T cells. After more efficient LPAP transfection, we observed an increase in LC3-II, similarly as in Jurkat cells. In p62 expression there was no significant change, but interestingly there was an up-regulation of NRD1.

5 DISCUSSION

In the past decade there has been an immense step forward in understanding of the molecular signaling of mammalian autophagy. In spite of all the outcomes, many questions remain unanswered. As proteins have an important role in mediating cellular processes, proteomic analysis gives us an insight into how autophagy impacts these processes and might provide new targets for drug development. In our research, we showed that both NRD1 and LPAP may regulate autophagy. The knockdown of NRD1 resulted in less autophagy which we observed with western blot analysis and immunofluorescence staining in Jurkat and MEF GFP-LC3 cells. We also demonstrated that NRD1 knockdown cells have lower viability after treatment with an anticancer drug. Inversely, the cells with silenced expression of LPAP showed a higher autophagy level, both in Jurkat and in healthy T cells.

Recently it has been found that NRD1 is a protein regulated by autophagy, since its expression was changing when autophagy was being manipulated. Our first goal was to check if different expressions of NRD1 have an impact on the process of autophagy. We knocked down the NRD1 expression in various cell lines with Neon transfection system. This system uses an electroporation RNA-mediated interference technology. With short-duration, high-amplitude, pulsed electric fields applied to the sample, the permeability of the cell membrane is increased and small RNA molecules can be taken up from the environment into the cell. The siRNAs target their complementary site on mRNA and after base pairing the mRNA is cleaved and degraded. In this manner, the translation is inhibited and the NRD1 expression is silenced (50, 51). Firstly, Jurkat cells were transfected and to quantify autophagy, we made use of LC3 and p62 protein expression. LC3-II is covalently bound to autophagosomal membranes and after the fusion with lysosomes the intraluminal LC3 gets degraded and the outer LC3-II is delipidated and recycled. The more autophagosomes are formed, the more LC3 is degraded. Therefore, lysosomal turnover of LC3 is a fine way to estimate autophagic activity (52). Another protein to monitor autophagic flux is p62, a substrate of autophagy. p62 is localized at the autophagosome formation site where it directly interacts with LC3. It is incorporated into the completed autophagosome and is degraded in autolysosomes (15, 53).

To analyze the expression of LC3 and p62 in Jurkat cells we used western blotting and immunofluorescence staining followed by confocal microscopy. With western blotting we observed a decrease in LC3-II expression and at the same time p62 accumulation in NRD1 transfected cells, both indicating a lower autophagy level. We further decided to check the same phenomenon by immunofluorescence staining. As for this experiment a 48- hour

incubation is needed (24-hour for knockdown, 24-hour for treatment), before conducting the experiment we used western blotting to prove that the knockdown of NRD1 is still evident after 48 hours. With immunofluorescence staining we observed a similar phenomenon, since we found NRD1 silenced cells which had knocked down expression of NRD1 and at the same time lower levels of LC3-II. However, not all the cells had a decrease in this two protein expression. Most probably the cells with lower level of NRD1 are the ones that were successfully transfected and had the NRD1 silenced. From this data we concluded, that upon a successful NRD1 knockdown there is a down-regulation of autophagy in Jurkat cells. Therefore, NRD1 is not only regulated by autophagy, but it potentially regulates the process of autophagy. Furthermore, we knocked down the NRD1 expression in another type of cells, MEF GFP-LC3, to see if we can find similar observations in different cells. After immunofluorescence staining we observed the expression of NRD1 and LC3 under confocal microscope. Similarly to Jurkat cells, MEF GFP-LC3 cells which had successfully silenced NRD1 expression, had in parallel lower level of GFP-LC3. With this data we showed that also in MEF cells, NRD1 has a role in autophagy regulation.

Mechanism of the interference of NRD1 in autophagy remains unclear. It is possible that NRD1 is directly involved in the autophagosome formation. On the other hand, the protein has been described to indirectly enhance the ectodomain shedding of tumor necrosis factor-α (TNF-α), a member of epidermal growth factor (EGF) family (54). TNF-α is released from cells by cleavage of a membrane-anchored precursor. ADAM proteases have the major role in ectodomain shedding of TNF-α and NRD1 enhances the activity of these proteases. Among the diverse range of roles TNF-α has within organism from regulation of immune cells and induction of inflammation to signaling events leading to necrosis or apoptosis, it has also been shown to induce autophagy (52, 55). Recently, it has also been reported that NRD1 plays a potential role in gastric tumor growth via enhanced ectodomain shedding of TNF-α (56). Following this data, the potential mechanism of NRD1 interference is through TNF-α signaling pathway. Since NRD1 increases the activation of TNF-α and subsequently induces autophagy, knocking down the expression of NRD1 might lead to less TNF-α shedding and a decreased level of autophagy. Furthermore, it was reported that TNF-α acts as tumor-promoting factor and has a role in all steps of tumorigenesis including transformation, proliferation, angiogenesis, invasion and metastasis (57). Therefore, we decided to analyze whether the NRD1 knockdown cells with less tumor-promoting factors and less autophagy, have reduced cell viability. We analyzed Jurkat cells treated with cisplatin, an anticancer drug, after we silenced the NRD1 expression. Cisplatin is a member of a class of platinum-

containing anti-cancer drugs. Its cytotoxic effect is based on the interaction with DNA and formation of crosslink adducts which activate several signal transduction pathways and finally leading to cell death (58). We observed 11% dead cells in wild type Jurkat cells treated with 25 µM cisplatin and 16% dead cells in NRD1 knockdown cells treated with 25 µM cisplatin. The cell death percentage was calculated considering the controls as 0% dead cells. Though, in untreated samples we observed quite a high percentage of Zombie positive cells, which should represent the dead population. The reason could be too long incubation with Zombie Violet^{TM} dye and thus more sensitive cells. With these findings we confirmed our hypothesis that the knockdown of NRD1 suppresses Jurkat cell viability. It would be also interesting to see the proliferation rate of NRD1 knockdown cells. Since TNF-α can promote tumor growth, in NRD1 knockdown cells we would expect lower proliferation rate. This we could do, i.e., with thymidine incorporation assay, by directly measuring DNA synthesis. Altogether, NRD1 may play a role in autophagy; however the exact mechanism remains unclear. It can be considered as a potential target in cancer therapy, although further investigations are required.

Another protein we were investigating is LPAP. In Jurkat cells we observed an induction of autophagy after silencing LPAP. Similarly we found an induction of autophagy in isolated T cells. It was shown that LPAP is up-regulated after an induction of autophagy, indicating that autophagy is a process that regulates the expression of LPAP. On the other hand, we showed an increased level of autophagy after LPAP knockdown which means, similarly like NRD1, LPAP is a protein which might regulate the process of autophagy. 200 nM siRNA turned out to be the most efficient dose for the knockdown of LPAP. With western blot analysis in Jurkat cells, we observed decreased expression of LC3 and slightly lower p62 expression. Primary cells are in general more difficult to transfect, since their membrane is difficult to penetrate or pass through (59). This turned out to be the case also in our experiments, since we were unable to knockdown the NRD1 in T cells. Another reason for a non-efficient knockdown could be an already low level of NRD1 in T cells. The LPAP silencing was more efficient, but still less than in other cells. In T lymphocytes we observed similarly as in Jurkat cells, induced autophagy after knockdown. Interestingly, there was also an up-regulation in NRD1, which was previously shown to be the case after pharmacological induction of autophagy. This data indicates that LPAP knockdown induces autophagy. It was shown that autophagy induces LPAP expression and in turn, LPAP suppresses autophagy probably as a negative feedback mechanism in T cells. Therefore, LPAP might be important in the homeostatic mechanism required to prevent prolonged or overactivated autophagy. Furthermore, LPAP is mostly expressed on T and B cells which are a part of the specific immune system. Recent

studies are reporting important roles of autophagy in immune response, like degrading microorganisms that invade intracellularly, controlling pro-inflammatory signals, secreting immune mediators or development and homeostasis of the immune system etc. (60, 61). Altogether, LPAP might have a role in the regulation of autophagy and it is a potential target in future cancer therapy.

6 CONCLUSION

Our understanding of the protein signaling in autophagy of cancer cells still has a lot of unanswered question. However, with every conducted research and experiment we get closer to a much deeper understanding of the even so complex process. In this thesis, we characterized NRD1 and LPAP, two proteins, whose expressions are regulated by autophagy. In Jurkat cells NRD1 knockdown decreased the level of autophagy, which turned out to be beneficial even after anticancer drug treatment, since the cell death was higher. In both freshly isolated human T cells and in Jurkat cells a different outcome was observed after LPAP knockdown; up-regulation of autophagy. It has to be further investigated in other cells, whether the autophagy inhibition or induction with either NRD1 or LPAP knockdown have the same effect on the cell viability. Both proteins, NRD1 and LPAP, turned out to be involved in the process of autophagy and their up- or down-regulation might be a potential target in upcoming anticancer therapy. After all, autophagy helps to protect organisms against some other diseases, including neurodegeneration, cardiomyopathy and inflammatory diseases. Inhibition of autophagy might, therefore, be useful for anticancer therapy, but have at the same time harmful effects on normal tissues.

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