

Dissertation

**Protein misfolding and cell death:
From yeast to fly**

Submitted by

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Declaration

I hereby declare that this doctoral thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to my research thesis. I followed the guidelines of “Good Scientific Practice” for the completion of this thesis work.

Please note that parts of my thesis have already been published or in the process of submission:

“The cell death protease Kex1p is essential for hypochlorite-induced apoptosis in yeast”.

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“Spermidine protects against α -synuclein neurotoxicity”.

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Abbreviations

Activating transcription factor	ATF
Advanced glycation end products	AGE
Autophagy-related gene	Atg
Autosomal recessive juvenile parkinsonism	AR-JP
Amyloid lateral Sclerosis	ALS
Apoptosis signal-regulating kinase 1	ASK1
Adenylate energy charge	AEC
Alzheimer's disease	AD
Apoptotic protease activating factor 1	Apaf1
Basic leucine zipper	bZIP
B-cell lymphoma 2	BCL-2
Bruchpilot	Brp
B-cell lymphoma-extra-large	Bcl-xl
cAMP response elements	CRE
c-Jun N-terminal kinase	JNKs
CCAAT/-enhancer-binding protein homologous protein	CHOP

Cysteine	Cys
Central nervous system	CNS
Chaperone-mediated autophagy	CMA
Cathepsin	Cts
Dopamine	DA
Dopamine transport	DAT
Dihydroethidium	DHE
Double distilled water	ddH ₂ O
DnaJ homolog subfamily C member 3	DnaJC3
4',6-diamidino-2-phenylindole	DAPI
α -difluoromethylornithine	DFMO
Enhanced chemiluminescence	ECL
Endoplasmic reticulum	ER
Extracellular regulated kinase	ERK
ER-stress response elements	ERSE
Eukaryotic translation initiation factor 2 α	eIF2 α
Endoplasmic-reticulum-associated protein degradation	ERAD
Endothelial cells	EC

Endonuclease G	EndoG
Fasciclin domain	FAS
Familial parkinson's disease	FPD
γ -Aminobutyric acid	GABA
Glutathione	GSH
Human immunodeficiency virus	HIV
Hypochlorous acid	HOCl
Huntington's disease	HD
Inhibitor of apoptosis	IAP
Idiopathic parkinson's disease	IPD
Low-density lipoprotein	LDL
Low-density lipoprotein receptor	LDL-R
Low-density lipoprotein scavenger receptor	LDL-SR
Lewy bodies	LB
Lysine	Lys
Leucine-rich repeat kinase 2	LRRK2
Microtubule-associated protein 1A/1B-light chain 3	LC3
Methionine	Met

Myeloperoxidase	MPO
Mitogen-activated protein kinase kinase kinase	MAP3K
Manganese	Mn
Native low-density lipoprotein	nLDL
Nuclear factor (erythroid-derived 2)-like 2	NRF2
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Nitric oxide synthases	NOS
N-acetyl cysteine	NAC
Optical density	OD
Oxidized low-density lipoprotein	oxLDL
Ornithine decarboxylase	ODC
Programmed cell death	PCD
Phosphorylated activating transcription factor	pATF
Probable G-protein coupled receptor 78	GPR78
Parkinson's disease	PD
Reactive oxygen species	ROS
Reactive oxygen intermediates	ROI
Reactive nitrogen species	RNS

Relative fluorescence unit	RFU
Site-2 protease	S2P
Site-1 protease	S1P
Serine/threonine-protein kinase1, 2	ULK1,2,
Superoxide dismutase [Cu-Zn]	SOD1
Substantia nigra pars compacta	SNpc
Saccharomyces cerevisiae	S.cerevisiae
TNF receptor-associated factor 2	TRAF2
Tryptophan	Trp
Terminal deoxynucleotidyl transferase dUTP nick end labeling	TUNEL
Unfolded protein response	UPR
West nile virus	WNV
Wild type	WT
X-box binding protein 1	XBP1
X-box binding protein 1	XBP-1
Yeast caspase-1	yca1

Summary

Folding of the proteins has a critical role in maintaining cellular homeostasis. Misfolding of different proteins (e.g. modification, oxidation, etc.) can result in onset of various diseases. The text begins with an introduction to protein folding and talks about the links between protein misfolding and cardiovascular complications as well as neurodegenerative diseases followed by a glimpse on how different model organisms contribute to research. The methodology of the whole work is introduced in “material and methods” section. Results are split into two sections: In section one, we will show how *Saccharomyces cerevisiae* encounters cell death upon contact with hypochlorous acid (HOCl). Several deletion mutants of apoptotic machinery are tested. Among all, we could identify a kex1p-dependent apoptotic pathway that possibly involves specific oxidation-based protein modifications reminiscent of those present in mammalian proteins upon HOCl stress. We found that upon deletion of this protease, yeast could survive the toxic concentrations of HOCl that previously were able to induce death markers. We could also show that this death pathway is through mitochondria. This was confirmed by using yeast strain lacks mitochondrial DNA. We hypothesize that kex1p-glycosylated proteins are prone to be modified by highly reactive hypochlorous leading to programmed cell death (PCD). HOCl is able to induce the expression of calcium channels including ryanodine receptor 2 (Ryr2) and voltage-dependent channel (Cacna1h) in *HL-1 cardiomyocyte* as well as cathepsin S (CtsS) and TNF α . In section two, we will discuss how autophagy can help *Drosophila melanogaster* to modify α -synuclein aggregation. We will show that 5mM spermidine supplementation can rescue and restores Mn⁺² – Induced death, locomotion impairment, as well as autophagy impairment in α -synuclein expressing flies. In the final part, we show by Immunostaining of the whole brain of a fly that the level of bruchpilot protein - an important active zone protein - decreases upon α -synuclein expression and restores by spermidine treatment.

Zusammenfassung

Die korrekte Faltung der Proteine spielt eine wichtige Rolle bei der Aufrechterhaltung der zellulären Homöostase, während Fehlfaltungen (z.B. durch Modifikationen, Oxidation, etc.) zu verschiedenen Krankheiten führen können. Der Text beginnt mit einer Einführung in die Proteinfaltung und behandelt weiters die Zusammenhänge zwischen Proteinfehlfaltung und kardiovaskulären Komplikationen sowie neurodegenerativen Erkrankungen, gefolgt von einem kurzen Einblick in verschiedene Modellorganismen. Die Methodik der ganzen Arbeit wird im Abschnitt „Material und Methoden“ erläutert. Das Kapitel „Ergebnisse“ gliedert sich in zwei Teile: In einem Abschnitt werden wir zeigen, wie Hypochlorsäure (HOCl) zu programmiertem Zelltod in *Saccharomyces cerevisiae* führt. Dazu wurden verschiedene Deletionsmutanten getestet, wobei wir einen kex1p-abhängigen apoptotischen Mechanismus identifizieren konnten, der Ähnlichkeiten mit Säuger-spezifischen Oxidations-basierten Proteinmodifikationen aufweist. Wir fanden heraus, dass Deletion der Kex1p Protease zu einer Resistenz von Hefe gegenüber toxischen Konzentrationen von HOCl führt. Aufgrund von Experimenten mit Zellen, denen mitochondriale DNA fehlt, konnten wir beweisen, dass der Weg des HOCl-induzierten Zelltods über Mitochondrien führt. Wir vermuten, dass durch kex1p glykosylierte Proteine anfälliger für Modifikationen durch die hochreaktive Hypochlorsäure sind, was letztlich in programmiertem Zelltod (PCD) endet. HOCl ist in der Lage, die Expression von Calciumkanälen, einschließlich des Ryanodin-Rezeptors 2 (Ryr2) und des spannungsabhängigen Kanals (Cacna1h), sowie von Cathepsin S und TNF α in *HL-1 Kardiomyozyten* zu induzieren. In Abschnitt zwei werden wir diskutieren, wie Autophagie im Modellorganismus *Drosophila melanogaster* die α -Synuclein Aggregation beeinflussen kann. Wir werden zeigen, dass Supplementierung mit 5 mM Spermidin über die Stärkung von Autophagie vor Mn⁺² – induziertem Tod schützt und Beeinträchtigungen der Bewegungsfähigkeit in α -Synuclein exprimierenden Fliegen verbessern kann. Im dritten und letzten Teil zeigen wir mit Hilfe von Immunfärbungen des gesamten Gehirns einer Fliege, dass das Level von Bruchpilot - einem wichtigen Aktive-Zone Protein - bei α -Synuclein-Expression zurückgeht und durch Spermidin Behandlung wieder erhöht werden kann.

1. Introduction

1.2 Protein folding, misfolding

Proteins are the most abundant molecules in living organisms. Almost all the functions in a single cell or in multicellular organisms are done through these amino acid chains. Their importance in cellular functions allows them to reach concentrations equal to 300-400 mg/ml in a single cell and 100 mg/ml in the lumen of endoplasmic reticulum (ER) (1, 2). Being synthesized from DNA encoded information on ribosomes, there are about 100,000 different types of proteins each one consisting of a chain of amino acids which controls their diversity. Since limitless number of amino acids can form a single protein, it will not be hard to imagine that there are more proteins than there are atoms in the universe. However, before being able to perform their functions, they have to go through different steps to obtain right conformational or folding. In fact, by folding processes, favorable enthalpy status of the biological system will be achieved (3, 4). According to Dobson et. al., protein folding is described as the process in which partially or completely unfolded proteins acquire their native structures (2). Henceforward, misfolding can be described as a condition of a protein in which the non-native or abnormal number of interaction affects its overall architecture in a biologically significant manner. It is known that the folding starts to take place when the protein is still attached to the ribosome (1, 5). This process gets expanded while the structure is being re-located to other compartments of the cell (e.g. membrane) in rough ER. ER is the site for regulation and modification of the proteins to ensure proper function. To execute this key role, they use foldases, chaperones and cofactors. Since unfolded proteins are major contributors towards many pathophysiological and/or physiological complications in human body, learning how this complex and sophisticated pathway works is of the utmost importance. Factually, most of the research in molecular biology and studying the signaling pathways is associated with at least a protein member involved in this regulatory mechanism. By using such simulation methods, it was revealed that there is a transition state that acts like a quality control mechanism and check point for proper protein folding(4). It is up to the quality control of the system to activate pathways that are involved in either refolding or degradation of such

proteins which are called ubiquitin-proteasome pathways. It is notable that only half of the synthesized proteins in a single cell meet the requirements of this quality control system (6), hence giving a crucial role to these check points and mechanism. In other words, a cellular folding capacity can be described for the cell that in normal condition meets the protein folding of the system. Since ER plays a major role in folding of the proteins, any perturbation in this equilibrium can lead to ER stress. In fact, the ER has a crucial role in the defense against these redundant structures. Accumulation of unfolded proteins leads to activation of certain cellular processes and factors consisting of proteins and chaperones (3). There would be different ways for a cell to encounter these unfavorable conditions; in mild conditions activation of these several mechanisms help the cell in recycling or refolding the proteins (adaptive responses). However, if the stress continues, it activates another mechanism that either eliminates these naïve particles in an ATP dependent manner (autophagy) or eliminates the cells themselves to spare nutrient energy for others (programmed cell death, PCD) (7).

1.3 Calnexin and Calreticulin: quality control system for protein folding

As mentioned above, due to the high concentration of proteins within the ER and its role in controlling the protein quality, each cell has developed multiplex system to control the balance and keep the proteostasis of the cell to make sure that physiological and pathophysiological pathways will not be perturbed. Little is known about how this system functions, however, it's known that molecular chaperones play a major part (1, 2). Among all chaperones, it has been suggested that calnexin and calreticulin play a major role to maintain the proper protein folding (6, 8). Calnexin, with a 573 amino acid chain and 65 kDa, is considered as a type I integral membrane protein and is similar to a Ca^{2+} -binding protein of the ER lumen (6). Deletion of calnexin leads to impairment of protein trafficking in an ATP-dependent manner though no nucleotide binding site has been defined (9). Yeast has been shown to conserve a consensus sequence for calnexin within the luminal domain of the ER that is involved in protein glycosylation (10). It has been shown that using conventional protein glycosylation inhibitors ,e.g. tunicamycin, abolishes the association of calnexin with misfolded

proteins (11). Although it's a membrane protein, its role in folding of secretion of proteins has been defined using HepG2 cell line (6). Furthermore, it has been proven that it interacts with monomeric as well as complex polypeptides (12). They (consider using noun instead of pronoun THEY) interact partially and transiently with nascent membrane and soluble proteins. This binding helps the system to prevent aggregation of proteins until they gain their proper folding. If this binding doesn't occur, the interaction remains permanent and escorts them to the ubiquitin-proteasome system (6, 8, 12). This calnexin-calreticulin associates with another factor namely ER soluble thiol-disulfide oxidoreductase (ERP57) and together they are involved in oxidative folding of newly synthesized proteins (8). Upon biosynthesis of new proteins on the ribosome, they get glycosylated, and within the lumen of the ER they get transferred and obtain their folding on this complex; they get re-checked again in calnexin cycle (13). Sometimes proteins have to go through this cycle a few times to obtain proper folding. Most of these proteins are then transferred to the secretory pathway and via the Golgi apparatus they reach their final destination including other organelles, cell surface and extracellular space (3, 13). In case of any misfolding or unfolding, they will be accompanied by the association of BiP chaperon and get degraded by proteasome-mediated degradation known as ER-associated protein degradation (ERAD) (**Figure-1**) (14). Calreticulin has been defined as the calnexin's homologue. With 400 amino acids, it has a molecular weight of 46kDa (8). It doesn't have a redundant role in the system as it has been proven that calreticulin is more abundant in the lumen of ER. Calnexin is more likely to be present in all compartments of the cell (8, 15).

1.4 Unfolded Protein Response (UPR) from yeast to mammals

Overall, the cells are not defenseless facing misfolded proteins; through evolution, they have gained specific factors and mechanisms that make them capable of facing these scenarios. Since the ER is the main compartment where newly synthesized proteins obtain their folding, it is not unanticipated that they have also developed defense mechanisms against UPR. A cell facing such proteins will go through specific scenarios. At the normal level or initial periods of facing misfolded proteins, a single cell tries to adapt or change its environment.

Either degradation of those proteins through specific pathways (autophagy, etc.) and/or alternatively

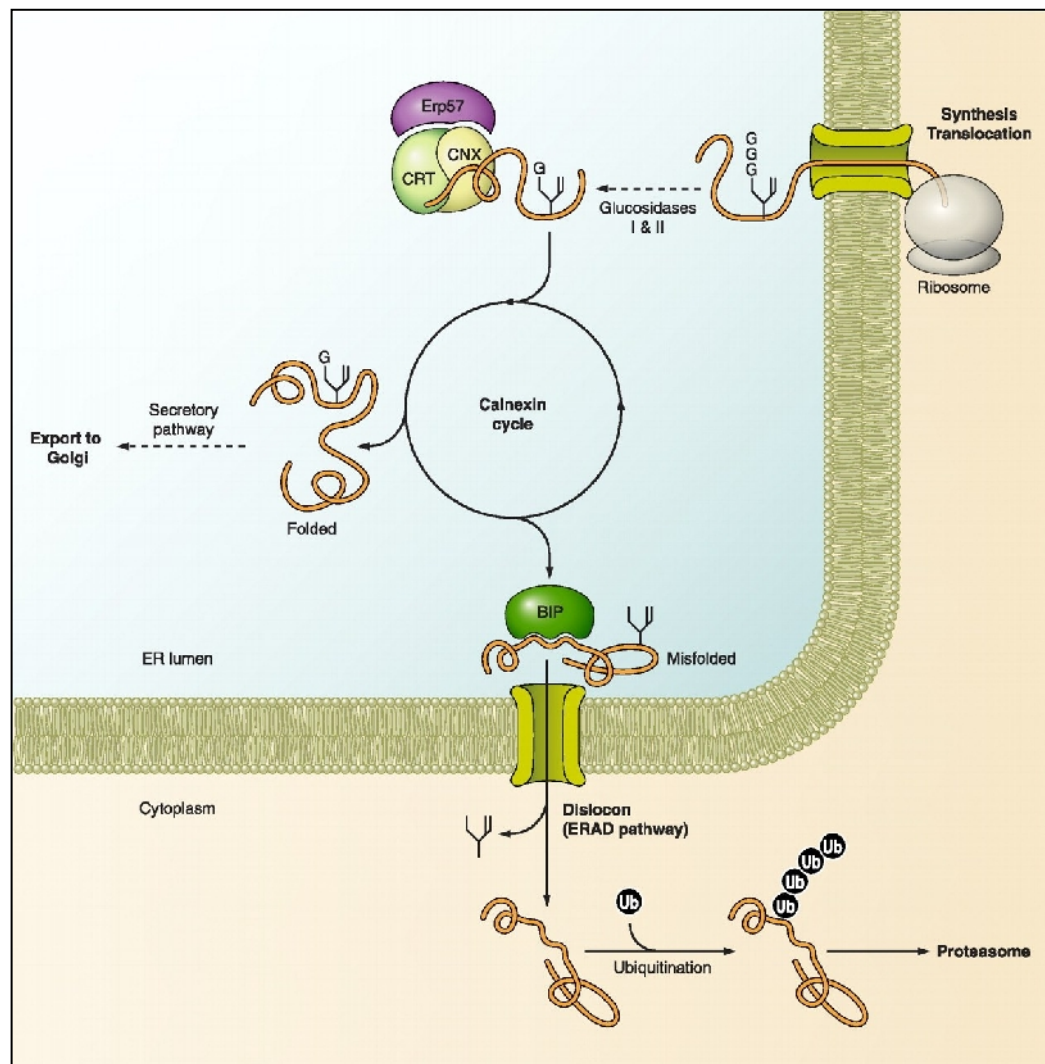


Figure - 1: The calnexin cycle. The folding of glycosylated proteins at the ER is regulated by the calnexin cycle. After nascent chains enter the ER, lumen proteins are glycosylated by the oligosaccharyltransferase. The two terminal glucose residues are rapidly trimmed. Mono-glycosylated N-glycans mediate initial association of folding polypeptides with the ER lectin chaperones calnexin (CNX), and/or calreticulin (CRT), and exposure to ERp57 through a protein complex. It is likely that most glycopolypeptides are released from calnexin/calreticulin/ERp57 in a native, transport competent state. Glycopolypeptides released from calnexin displaying major folding defects are attracted by BiP and are dislocated across the ER membrane for ubiquitination (U) and proteasome-mediated degradation through a pathway known as ERAD [adapted from Hetz et. al.(13)].

trying to decrease the loading of the ER trough ERAD (3, 5, 16). The latter can be achieved by inhibiting the rate of protein translation and/or increasing ER chaperones, hence activating downstream pathways. This function is up to

molecular chaperones that bind to hydrophobic epitopes of incorrectly folded proteins to prevent nascent and unfolded protein interaction (2, 3). In general there are three main stress sensors on the ER including type I membrane protein kinase endoribonuclease (IRE1) that is present in yeast and mammals and two other sensors including type I transmembrane protein kinase (ERK/PERK) and activating transcription factor 6 (ATF6) that are exclusive to the mammalian system (17). All their protein sensors have their N-terminus in the lumen of the ER and the C-terminus in the cytosol hence enabling them to connect the cytosol to the ER which is crucial in an effective UPR(3). The correct functioning of these proteins is so important that their inability to sense the UPR will lead to cellular injury and cell death(18). All these sensors use different ways to get activated.

IRE1 is considered to be the fundamental factor and the most conserved ER sensor in eukaryotic systems. In general, IRE1, and all three sensors are kept inactive by their N-terminus interaction with glucose-regulated protein 78kDa (GRP78) (19). Upon stress, this sensor gets activated by oligomerization leading to activation of UPR. IRE1 activation induces the endoribonuclease activity that cleaves the mRNA which encodes mammalian X-box binding protein (XBP1) and HAC1 in yeast, both leading to the production of their transcription factors (20). Then binding of the XBP-1 to ER-stress response elements (ERSE I, II) leads to up-regulation of UPR regulated genes (21). The homology of IRE1 to the mammalian one is not clear, though expression of human/yeast in yeast shows that their function is interchangeable, but their domain has limited homology. In yeast, there are two other effector molecules (other than IRE1) for transferring the signal from the ER to the nucleus, namely *Rtg1p* and *Hac1p*. *R1g1p* functions as the activator of UPR active genes and *Hac1p* acts as a link between IRE1 as the sensor and *R1g1p* as the effector (22).

PERK plays a major role in helping the ER to reduce its protein overload. It induces the phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) that in turn decreases or completely turns off protein translation of the whole cell (23). Downstream this pathway is also ATF2 and NRF2 which plays a crucial role in antioxidant response to external stress(17).

ATF6 however acts through different levels. This pathway acts through its two different factors that are ATF6 α and ATF6 β . Upon stress they translocate from ER to Golgi where different proteases including site-2 protease (S2P) and sphingosine 1-phosphatase (S1P) work on them to cleave them into the cytosolic and an ER domain (24). These proteases, unlike their terminology, have different roles and backgrounds. S2P is considered as a metalloprotease that always acts on transcriptional regulators and cleaves transmembrane substrate in all domains of life. On the other hand, S1P is a signaling sphingolipid that has particular roles in association with lipoproteins in the cell which makes it the major spotlight when it comes to the role of UPR in cardiovascular diseases (25). Nevertheless, ATF6 upon cleavage acts into completely different ways through its cytosolic domain. In one way, it acts through its domain that is called basic leucine zipper domain which encodes bZIP transcription factor and is involved in cell development. Evolutionary it is conserved in eukaryotes and a variety of eukaryotic proteins contain this specific domain e.g. ATF1 (26). Downstream ATF6's cytosolic domain there are three individual elements cAMP response elements (CRE) and ER stress element I and II that get activated upon triggering of the sensor. ER stress elements I and II then induce variety of other effectors like chaperones, isomerases involved in ERAD, Hsp and P58^{IPK}/DnaJC3 (DnaJ homolog subfamily C member 3). The latter is a tetratricopeptide repeat family of proteins and is the negative regulator of PERK (9).

1.5 UPR and regulation of cell death

1.5.1 UPR via Autophagy

One of the alternative ways (other than reducing protein translation or increasing chaperones) that the cellular system uses to maintain proteostasis is autophagy (27, 28). Autophagy is described as the cellular self-eating process in which upon induction a double-membrane autophagosome engulfs redundant - but yet valuable- biomolecules and recycle them via fusion with the lysosome. It's a beneficial process when the cell is under nutrient deprivation (e.g. high nutrient consumption by tumor cells in cancer) or hypoxia and serves as an efficient way to use all cellular resources when there is a high demand (29). However when the

amount of protein- and organelle turn-over and demand or duration of stress exceed the capacity of the cell, it is able to induce non-apoptotic cell death (30). Autophagy is a druggable-target and a variety of chemicals have been described to be able in its induction or inhibition (28). This characteristic makes it an important field in the therapy of diseases that bear ER stress and UPR e.g. cancer (31). Away from its important role on individual cells, there are numerous studies that show that triggering this pathways has a crucial role in increasing life span of many organisms and has anti-aging effects (32, 33). Knowing the autophagy regulators and effectors simplify the topic of how ER stress and autophagy are linked together. Four main steps have been described for this process that includes induction, nucleation, elongation and completion (34). Unc-51-like kinase 1/2 (Ulk1/2) complex gets activated upon its induction that in turn induces the formation of Beclin 1-VPS34 complex (nucleation). Beclin1 complex in turn is able to up-regulate autophagy-related genes (Atg) 3, 5, 7, 10, 12 as well as microtubule-associated protein 1A/1B-light chain 3 (LC3) through ubiquitin-like protein conjugation and the double-membrane structure starts to form (elongation). In the completion phase all of the mentioned factors, except LC3-II, get degraded before the fusion of the autophagosome gets completed. The ultimate step would be its fusion with the lysosome. Worth mentioning that mTOR kinase (target of rapamycin) is a well-known and master regulator of autophagy that inhibits the kinase activity of ubiquitin kinase (ULK) and thus autophagy (35).

It's well clear that UPR is able to induce autophagy (36). It has been shown that ER stress sensors PERK-eIF2 α and IRE1-TRAF2-JNK are the key axis for linking UPR to autophagy (37, 38). Furthermore PERK-eIF2 α induces the activation of Atg12 that in turn converts LC3 to a lipidated state and thus enables autophagosome formation. TNF receptor-associated factor 2 (TRAF2) that is a member of the TNF receptor family, acts as a bridge between IRE1 and c-Jun N-terminal kinases (JNK) and is involved in autophagy. The connection of IRE1-TRAF2-JNK in autophagy is clear as absence of autophagy has been shown in a mouse model lacking IRE1 α and TRAF2 and using JNK inhibitor (38). As apoptosis and autophagy work in harmony together, there is a biological need for a switch to decide between these two pathways. Beclin-1 acts as a knob for both pathways; Beclin-1 is the mammalian orthologue of the yeast Atg6 and BEC-1 in

the nematode *C. elegans* (39). Upon ER stress cleaved Beclin-1 relocates to mitochondria where it inhibits Bcl-X_L activity that results in an increase in the pro-apoptotic regulators Bax/Bak with a positive feedback of emancipating more Beclin-1. Augmentation of free Beclin-1 can induce autophagy, however, the exact mechanism is not clear.

1.5.2 UPR and Apoptosis

As discussed above upon accumulation of misfolded proteins and induction of stress, resident stress proteins in the ER sense the undesired situation and activate several mechanisms which vary from inhibition of translation to induction and release of chaperones to bind to the hydrophobic domains on the misfolded proteins (40). If the stress continues or the UPR fails to control the level of misfolded to correctly folded proteins then the ER sensors activate apoptosis to eliminate the cell itself. Execution of single cells will ultimately save the organism as a whole. In general all three sensors that have been discussed and are involved in adapting the cell to the unkind condition are capable of inducing apoptosis if needed (3). CCAAT-enhancer-binding protein homologous protein (CHOP) acts as a hub for in this pathway. CHOP is considered as a pro-apoptotic protein that acts via down regulation of anti-apoptotic proteins like Bcl-2 (2). It has been shown that CHOP deletion in mouse embryonic fibroblasts protects cells from ER stress by decreasing the loading of the protein synthesis (41). Excessive level of stress has also been shown to induce apoptosis through NF- κ B, Bcl-2 family and JNK pathway. Once the disproportionate level of misfolded proteins is sensed then tumor necrosis factor receptor-associated factor 2 (TRAF-2) gets released from its ER membrane anchor (pC-2), a member of Inhibitors of apoptosis (IAP), to form a heterodimeric complex together with IRE1. Well ahead apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPK kinase kinase (MAP3K) family, joins this complex and hence builds a complete apoptotic executor through pro-apoptotic genes including Fas and FasL. The complete tetramer activates XBP-1, one of the four cis-acting elements (AARE1, AARE2, ERSE1 and ERSE2. ATF4, pATF6), ultimately leads to transcriptional activation of CHOP. The release of TRAF-2 from the IAP anchor also plays an important role in induction of apoptosis through activation of caspase. It leads to cleavage of

caspase-12, caspase-9 and caspase-3. Overload calcium efflux can also upsurge the activation of caspase-12 by TRAF2 through calpain and caspase-7(42) (Figure -2).

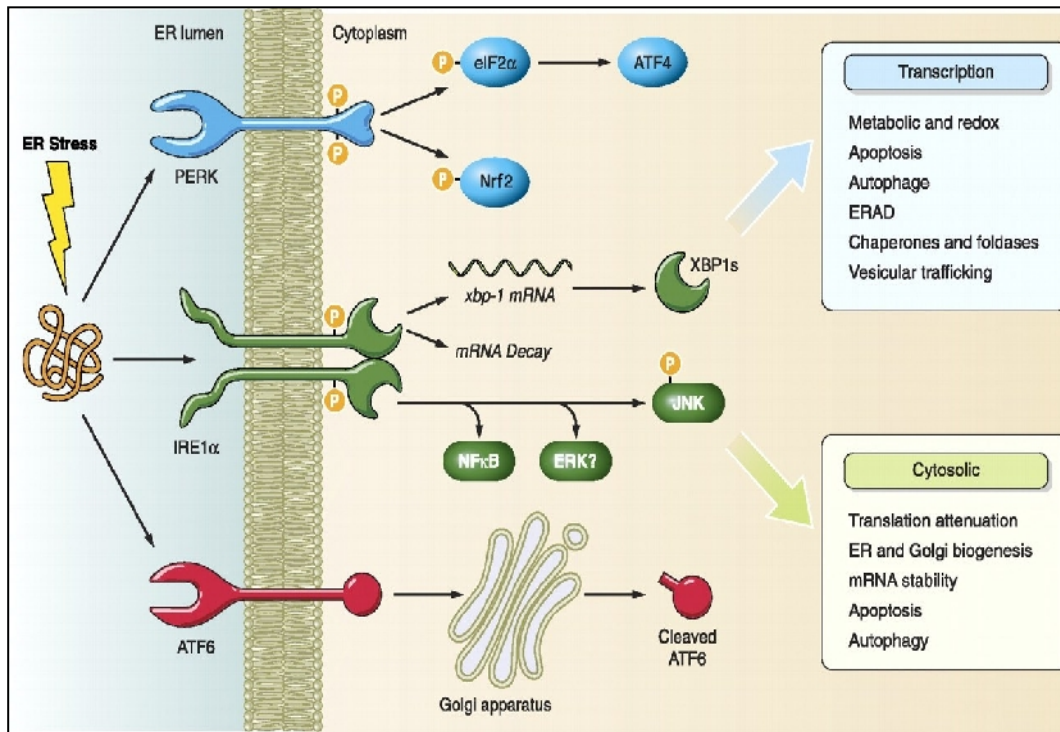


Figure - 2: UPR and cellular death scenarios. The major unfolded protein response (UPR) signaling pathways induced by endoplasmic reticulum (ER) stress are depicted, and some connections to the cell death machinery are illustrated [adapted from Hetz et. al. (13)] .

As mentioned above ATF6 and PERK also act through CHOP. Dissociation of GRP-78 or BiP from ATF6 serves for its glycosylation and transfers it to the Golgi that in turn is involved in CHOP activation. Of note ATF6 (N) can also have a positive feedback on up-regulation of XBP-1 mRNA precursor. PERK however works in a more sequential way. Phosphorylation of eukaryotic Initiation Factor 2 (eIF2) leads to activation of ATF4 as another member of four activators of CHOP (17).

1.6 UPR in implication of diseases: by Heart and in Brain

UPR has been shown to be involved in the initiation and progression of many diseases (17). In eukaryotes, It has been shown that ER stress leads to oxidative stress and cell death (17). The role of UPR in lipid metabolism, glucose

metabolism and inflammatory responses in human body have been described(13). The recent advances in biological techniques and using genetically manipulated models have enabled us to dig deep in and explore more of the individual role of each part of this system. Hetz et. al.(13) has summarized the physiological importance of each individual member of UPR in disease, varying from Crohn to neurodegenerative diseases. For instance, the role of a IRE1 α knockout mouse in auto-immunity responses as well as neurodegenerative diseases has been implicated (43). One should not forget that the functionality of the UPR is so complicated and thus makes it so hard to define specific roles for each individual player. As a case in point, it has been shown that PERK deletion contributes to exacerbation of Amyloid Lateral Sclerosis (ALS) in mice(13), while knocking out XBP1 in the same model organism protects or improves the phenotypic characteristics of ALS and it has no adverse effect in neuronal development.

1.6.1 Lipoproteins, oxidation, ER stress and cell death: eminent danger for the heart

ER stress has been shown to occur in atherosclerotic plaques in 3 cell types: smooth muscle cells, cardiomyocyte and endothelial cells (19). According to American Heart Association atherosclerosis has been defined “as a general term for the thickening and hardening of arteries”. It’s being considered No.1 killer and responsible for 800,000 deaths only in the United States. Hardening of arteries occurs inadvertently by aging. It starts with damaging of arteries wall known as endothelium. Lipoproteins, responsible for the transfer of lipids in blood stream and membrane fluidity, infiltrates through cell monolayer of endothelium. By getting modified or upon oxidation they accumulate in the cell wall of arteries causing atherosclerosis that also can be a consequence of apoptosis (19, 44, 45). Through year’s high blood pressure, unhealthy diet with high cholesterol and high triglycerides diet, smoking, diabetes and Inflammation from various diseases or (inflammation in general) can cause the damage of endothelial cell through the above-mentioned pathway. In other words any increase in the level of reactive oxygen species (ROS) or perturbation in redox state of the cell can lead to oxidation of proteins and lipids that ultimately lead to induction of apoptosis in vascular cells (7). It has been shown that proteasome inhibition exacerbates the

cell death via apoptotic dependent pathway in mouse model that may suggest the involvement of UPR (46). High level of oxidized lipids and proteins has been shown to be presents in human atherosclerotic plaques and shown to be responsible for death and impairment of endothelial cells (47). Oxidized low density lipoproteins (LDL) are the major components of lipid part at the site of plaque formation (47). The term LDL- refers to in-vivo modified form of LDL. In certain diseases the amount of LDL- in plasma increases (48). It's worth pointing out that atherosclerosis plaques and LDL are known to cause not only coronary disease but also cerebrovascular diseases and peripheral artery disease, making its research field important (44). Circular dichroism analyses have shown an increase in the proportion of α -sheet structure to β -sheets in LDL- (49). In general, LDL consists of a lipid core surrounded by apopB-100 moiety that is a pentapartite each with a different folding including α 1, β 1, α 2, β 2, α 3 parts (48). A separate role has been described for each of these parts; α 1 is responsible for connection of the protein part to the lipid core, while β 1 is involved in electrostatic interactions with phospholipids α 2 and α 3 stabilize these interactions. LDL-R has been defined as a general receptor for LDL on normal cell. Upon oxidation or modification, the uptake receptor for ox-LDL or LDL- on the endothelial cells (EC) and macrophages changes to scavenger receptor (LDL-SR) for EC and CD36 for macrophages. This has a major impact on the initiation of the disease since SR and CD36 receptors don't have the feedback inhibition which is critical for preventing the accumulation of these particles within endothelial as well as macrophages (50). Additional to that, ox-LDL has been shown to be resistant to proteolysis (49). The ApoB-100 part of the LDL can get oxidatively modified in variety of ways. Why and how this biological phenomenon happens has been well studied and described before in the literature. It could happen by metal ions in early stages and by lipooxygenase in late stages of atherosclerosis and superoxide, homocystein, myeloperoxidase as well as nitric oxide synthase (NOS) (45). In general, Lysine (Lys), Tryptophan (Trp), Methionine (Met) and Cysteine (Cyst) are the amino acids from the ApoB-100 part that gets modified by HOCl (51). Henceforward the oxidized LDL gets protease resistant and can induce its cytotoxicity in a variety of ways (see below).

The cell mediated oxidation of LDL can also occur in atrial wall where an increase in rate of nitric oxide synthase (NOS), myeloperoxidase (MPO) or

NADPH oxidase contributes to its modification (52, 53). NOS is known to increase the production rate of peroxynitrite that contributes to modification of tyrosine residues and initiation of lipid peroxidation. Since there is a fast kinetic for the reaction of $O_2^{\cdot-}$ and $\cdot NO$, the importance of this pathway has not drawn much attention (48).

1.6.2 Hypochlorous acid (HOCl): two sides of a coin

Hypochlorite (NaOCl) or hypochlorous acid (HOCl) is one of the most interesting biomolecules in the body. It is a highly reactive oxidant that was traditionally used as disinfectant or décolorant. Commercially, it was first synthesized in a french town called Javel by a French chemist, Claude Louis Berthollet, in 1775. It has been widely used as a disinfectant in drinking water. The first existing report shows that Minneapolis might be the first city in the world that started to study the effect of hypochlorite on the drinking water on public health (54).

But more than its commercial importance, it has a pivotal role in human physiological system that has put it in the focus of scientific research for decades. It is being biosynthesized enzymatically by MPO from the reaction of hydrogen peroxide (H_2O_2) and chloride anion (Cl^-). This reaction takes place during the neutrophil's respiratory burst encountering an infection in the body (55, 56). In fact by using $[6c^{13}]$ labelled tyrosine, it has been shown that pathogenic bacteria indeed get directly oxidized by hypochlorous acid in phagosomes (55). It also has been widely used as a medical disinfectant in a variety of ways (57-59). The first reported disinfectant role was in 1915 when the beneficial use of HOCl was described in wound disinfection as well as being efficient in water disinfection against spore and non-spore forming bacteria (57). Throughout years, it's role against different bacteria species was well described and studied (55, 58-62). Yet from mid 80s viral disinfecting role was added, describing it effective against variety of viruses from human to murine (63-66). As it has been shown that 100 ppm solution of hypochlorous acid within 15 seconds is able to reduce the population of *feline calicivirus*, *human influenza virus*, *measles virus*, *canine distemper virus*, *human herpes virus*, *human adenovirus*, *canine adenovirus* and

most interestingly only 30 seconds to disinfect *human immunodeficiency virus* (HIV1) (64, 65). It should be noted that there are different factors affecting its potential reactivity like concentration, the nature of the molecule, temperature, pH and commixture of any other compound or even the duration of the exposure (56, 66-71). In studying the effect of a compound, a natural question is always the concentration at which the compound is used. It has been well shown that there is a linear correlation between concentration and killing potency of HOCl (67). In that direction, most studies agree that 100 ppm is enough; however for bacterial disinfection 15 second of such concentration has been shown to be effective, as shown for *Staphylococcus aureus*, *E.Coli* and *salmonella*, different from 30 second for viral efficacy, as it was discussed for HIV1 and other human related viruses (see above) (64, 65, 69). To be more precise, there are different outcomes by using the same exposure time when the same concentration is applied. In *E.Coli* it has been shown that within 5 minutes 20 μ M and 50 μ M have different effects being able to inhibit cell growth by 50 to 100 percent respectively. It's not surprising to mention the linear correlation between time and reactivity potential as application of 50 μ M has higher reactivity for protein disturbance after 30 minutes exposure comparing to 5 minutes (68). Temperature and pH have different effects. In a phosphate buffer, the temperature is having the same effect in such a way that by increasing it there is highest disinfecting effect. In the same effect pH acts differently as by decreasing it, its infection potency gets higher as shown on *Listeria monocytogenes* (67). Not to forget that since HOCl can react readily with most of the compounds within organisms or in their media, there has been evidence showing the inconsistent results at the same concentration of HOCl as the composition of the reaction media changes (56). Noticeably, the presence of ammonium, as a source of nitrogen, in the media can compete with other compounds (e.g. Phosphatidylserine) altering the predictable effect of HOCl (72).

Nonetheless, since HOCl has been well described as a potent oxidant, being able to react with any substance, there are plentiful amounts of research on how it reacts differently with biomolecules (e.g. proteins, DNA, amino acids, etc.) (68, 73-75). Then again, its reaction goes toward different ways; after the discovery of its physical properties in 1950s, the inactivation of the DNA in response to HOCl is one of the major HOCl-research fields since DNA is considered the main component of a cell and controls development and proper

functioning in all model organisms. It has been shown in infection from *parovirus* and *west nile virus* (WNV) that HOCl might not be able to completely inactivate the DNA replication but at least it prevents the virions from going to the host cells (63, 75). In bacteria, the effect seems to be more direct. Studies on *E-Coli* have shown that DNA is the first biomolecule being affected upon 1 minute exposure to 50 μ M HOCl (68). Not only being able to inactivate the DNA completely, HOCl also has been shown to be able to down-regulate specific genes which are involved in biosynthesis of the certain amino acids (see below) (60, 62, 76). One still has to consider that after challenging certain model organisms like *E.faecalis* with HOCl, there is still some amount of PCR-detectable DNA which makes the use of it as an effective irrigator in root canal therapy controversial (59). The inactivation of DNA does not necessarily lead to sole inhibition of replication since the down-regulation of certain genes can lead to impairment of electron transport, sulfur transport, or metabolism that result in oxidative stress as shown in *P.aeruginosa* (60). Its noteworthy to point out that excessive amount of certain biomolecules like iron can lead to more HOCl-toxicity as it has been shown that in presence of excessive iron, generation of hydroxyl radicals after exposure of *E.Coli* to HOCl lead to more toxicity via fenton-type reaction (61). It should not be forgotten that the ability of HOCl to modify other molecules is the source of its destructive potential. However, not always the rate of reaction is the same for all the molecules. For instance, it has been shown that methionine and cysteine, apart from other amino acids, react more readily with HOCl (75).

Several researches suggest intermediate molecules that can intervene in the process of HOCl toxicity (73, 74, 77, 78). In fact, among the first evidences suggesting such intermediate agents is a study conducted on human fibroblasts (78). The research suggests production of genotoxic agents upon reaction of HOCl in mild concentrations with benign xenobiotic e.g. aniline that ultimately leads to DNA strand break and cell death. Last but not least is the evidence done with calf thymus DNA showing that reactivity of HOCl is not via its direct reaction with DNA but rather upon reaction of HOCl with NO_2^- , nitrating or chlorinating agents will be formed that are highly reactive with molecules like DNA or RNA and can trigger cell death pathways (72, 74).

Protein modification with HOCl in heart tissues leads to apoptosis and cell death. This phenomenon can be the major cause for cardiovascular complications.

So studying such mechanisms by which proteins are modified is of a great importance. The possibility of proteins to be changed, modified, and hence inactivated by HOCl was first described in 1947. By using egg albumin, sodium hypochlorite, and titration method, they were able to show that the protein gets inactivated after 10 minutes in a pH of 9.8 and as the medium goes more toward the alkaline state, the amount of such inactivation increases (71). It has been shown in *E.Coli* that 19 different proteins gets modified upon reaction with HOCl (79). Not always, the modification of proteins has detrimental effect. For instance, it has been shown that modification of serum albumin by HOCl has an anti-viral effect on WNV. This inactivation acts through the third domain of its envelope-glycoprotein that plays an important role in the entry of the virus into the host (75). It should not be forgotten that research on *E-coli* has showed that no protein degradation or modification occurs below 5mM HOCl (68). Henceforward there are numerous studies showing the ability of its reaction and modification of lipoproteins that lead to PCD in various model organisms (62, 80, 81). The modification of lipoproteins that leads to apoptosis is not restricted to mammals. It has been shown that there are protein epitopes in yeast that are oxidized upon challenging with HOCl, leading to cell death via apoptotic executors (56). In time, researchers tried to unveil more detailed and/or mechanistic pathways toward HOCl toxicity rather than showing simple reaction with biomolecules. Naturally, bacteria are the first organism for such purpose. Among first evidences suggesting a different pathway is a research conducted on *E.coli* suggesting energy status that is being altered by HOCl (82). Oxidative phosphorylation as well as other pathways and executors involved in energy release are also among the main target of these studies varying from Bacteria to yeast (81-85). In *E.coli*, it has been shown that HOCl can hydrolyse cytosolic phosphoanhydride bonds which ultimately leads to reduction of adenylate energy charge (AEC) and protein motive force (82). There are also evidences suggesting the effect of HOCl on metalloproteins in bacteria. As most of these proteins are involved in different steps of oxidative phosphorylation, if being modified by HOCl, an alteration in energy level is expected. For instance, succinate dehydrogenase activity has been shown to be impaired in *E.coli* upon HOCl exposure (84). Mitochondria as the source of the cellular energy are also susceptible to HOCl. The mitochondrial

pathway for HOCl toxicity has been suggested and shown that functional mitochondrion is necessary for HOCl toxicity (56).

Moreover, It has been shown in bacterial models that inactivation of all subunits of membrane ATPase synthase lead to a change in electrophoretic properties and hence cellular inactivation (83). This could be the first evidence suggesting plasma membrane-localized proteins are also being affected by HOCl. In fact, since proteins on the plasma membrane can be involved in more complex functions of the cell (e.g. receptors, etc.), it seems that the effects of HOCl on those proteins were always more interesting in different model organisms from bacteria, yeast and in mammalian models (56, 62, 83, 85).

Evolutionary, cells have versatile innate protection mechanisms toward damaging factors. Looking for such a mechanism towards HOCl toxicity in different model organisms was always interesting for researches around the world and Kex1p is not the only one being discovered (76, 81, 86-88). Among innate-protection the first scientific report is the one describing the resistance of a spore-bearing bacteria (bacillus) to HOCl (88). Since the oxidative damage remains the main and the most important consequence out of the challenging a cell with HOCl, studying the protective effect of antioxidants sound like a routine. Among those are studies that show glutathione (GSH) or N-acetylcysteine (NAC) being effective in restoring growth inhibition effect caused by HOCl (56, 89). The role of antioxidants in this direction will be discussed further below in the text. By development of new sophisticated gene manipulation techniques in the 90s, researchers were able to use knock-out of the genes they were interested-in and study their direct effect in desirable molecular scenario. Identification of the role of superoxide dismutase 1 (SOD1) is one example of using such techniques; as it has been described that a yeast SOD1 knock-out is hypersensitive to HOCl, describing the SOD1 peroxides scavenging activity pro-survival (81). There have been trials to identify any transcriptional elements being responsive to modify HOCl toxicity using bacterial genome or even cloning foreign genes (76, 86). In search for a protective genetical pathway perhaps the identification of hypochlorite transcription factor in *E.coli* (YjiE) was a step forward. In developing mechanisms against HOCl toxicity, this transcript forms di- and tetrameric ring like structures protecting the DNA against inactivation. It has been shown that this specific factor is also responsible for regulation of other genes as well, though its own regulation is not changed. It up-

regulates the genes involved in biosynthesis of amino acids including cysteine, methionine and down-regulates the ones involved in acquisition of iron and sulfur in response to HOCl, both pathways are the ones that are among protective pathways bacteria have developed during evolution (76). Still other ways exist in cellular battle against HOCl. As mentioned above hypochlorous acid is the major source of ROS along with reactive oxygen intermediates (ROI) and reactive nitrogen species (RNS) (56). These different highly reactive species disturb cellular redox states and proceed to reaction and modification of molecules with the reactive species in the cell and leading to cellular damage. There have been numerous studies showing the effect of antioxidants against HOCl toxicity in various model organisms; In mammals for instance, it has been suggested that 15 different flavonoids as well as rosea extract can be effective in protecting HeLa cells and erythrocytes from HOCl, or in yeast by showing NAC or GSH are effective against hypochlorite (56, 90, 91). Also an innate mechanism for keeping the balance of redox-state has been described in bacteria; namely *S.bacillithiolation*. It has been shown that such mechanisms are able to protect enzymes and proteins against oxidation by HOCl (92). Other innate mechanisms for HOCl in bacteria have also have been described. Methionine is one of the amino acids that react more readily with HOCl. It has been shown in 4 different bacteria including *E. coli*, *Staphylococcus aureus*, *Helicobacter pylori* and *Mycobacterium smegmatis* that both methionine sulfoxide reductase A and B are able to reduce the oxidized methionine into the normal state henceforth being protective against HOCl oxidation (93).

There is still need to shed more light on how HOCl implicates its toxicity and if there are any other pathways in developing protection against it. The knowledge will be an asset not only to the better use of its disinfection ability but also in the battle against its role in cardiovascular complications.

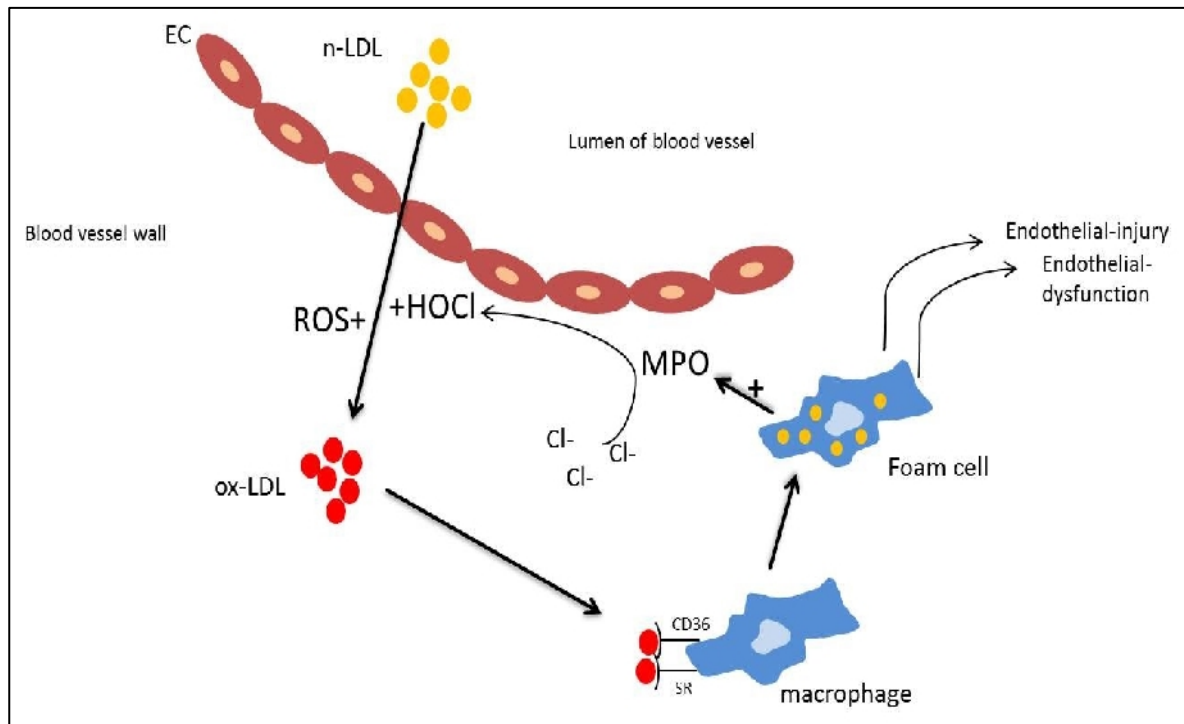


Figure - 3: The role of HOCl in endothelial injury. Native LDLs are internalized into blood vessel wall where they get oxidized by ROS or HOCl. Internalization of ox-LDL by macrophages result in foam cells that have positive feed-back in HOCl production and also inducing cardiovascular complication.

1.6.3 Oxidation, modification and misfolding of the proteins in neurodegenerative diseases: Parkinson and Parkinsonism

Accumulation of unfolded proteins in the neurons and nervous system can lead to common diseases that have amyloid origin. Parkinson's disease (PD) is one of those amyloid originated diseases. Its prevalence varies from 1% at the age of 65 to 5% for 85 according to American Health Association. The main causes of the disease are the selective loss of nigrostriatal dopaminergic neurons and the accumulation of protein as well as occurrence of the intracytoplasmic protein rich inclusion that are called Lewy bodies. These inclusions are formed from the accumulation of misfolded α -synuclein bound to ubiquitin in damaged cells. These Lewy bodies ultimately lead to the dopaminergic cell loss and leads to a collection of clinical symptoms. Resting tremor, slow movement (bradykinesia), rigidity and also dementia are among the most occurring symptoms in humans. Depending on the origin and causes, it is classified to familial Parkinson disease (FPD) for hereditary form of the disease and idiopathic Parkinson disease (IPD) that describes the

occurrence of the disease due to exposure to environmental neurotoxins such as head trauma, toxins or drugs e.g. Mn^{2+} (94). Regardless of the origin and causes, in both cases dopaminergic cell loss occurs within substantia nigra pars compacta (SNpc) that leads to perturbation of acetylcholine, glutamate and γ -Aminobutyric acid (GABA) balance in parts of brain that are responsible for the clinical symptoms of a patient diagnosed with PD (95).

Alpha-synuclein (α -synuclein) protein, as the main cause of the disease, is encoded by SNCA gene that gets mutated in both sporadic and familial form of the PD. Three missense mutations (A53T, A30P, and E46K) in α -synuclein gene has been shown to be associated with its rapid progression (96). It encodes a small 140 aa protein with an amphipathic N-term, hydrophobic central core and a negatively charged C-term that is expressed in the nervous system and is enriched in presynaptic nerve terminals. Synaptic transmission, neuronal plasticity and vesicular transport are among the physiological role of this protein. In normal conditions it's in a soluble and unfolded form but in the misfolded condition it forms fibrils that contain more stable aggregates called amyloidogenic β -sheet structures. The aggregation of α -synuclein in turn interacts with lipids in biomembranes and interferes with their function that may cause mitochondrial, lysosomal and proteasomal damage all involved in proteostasis of the nervous system. For decades PD had been described as the consequence of sporadic disorder caused by environmental stress. After years of technological innovation revealed the role of genetic causes and few genes have been described as the origin of the disease that may be called familial PD. By these discovery the direct link of ER stress with PD has been described. Few gene mutations have been described to have a connection with autosomal recessive juvenile parkinsonism (ARJP), one of the examples of familial PD that its onset is before 40, and includes: PARK2, PINK1/PARK6 and DJ-1(PARK7) (97).

PARK2 is described as the encoder gene of parkin, a part of the ubiquitin-proteasome system. Parkin with functional homology to ubiquitin E3 ligases, is involved in proteasomal degradation of cytosolic proteins by tagging their respective cytosolic part. It has been shown that mutation in PARK2 leads to the loss of E3 ubiquitin ligase activity (98). It should be noted that parkin mutation differs from other genetic causes of PD in terms of lacking lewy bodies and having early

onset. This loss of function may occur due to oxidative and dopaminergic stress both as good cases of UPR (98, 99).

PINK1 is the second most common cause of ARJP. It is involved in the regulation of mitochondrial calcium dynamics as it encodes a serine-threonine kinase that is localized on it. Of note PINK1 and parkin hermonization serves in maintaining a healthy mitochondrial status and DJ-1 or PARK7 serves as a collaborator with two above mentioned proteins by traslocating to mitochondria upon oxidative stress in the same direction (98). Using different model organism the role of mitochondrial dysfunction has been studied and shown to be present in α -synuclein pathology as welll as above mentioned muations (100). For instance by using a PINK1 *Drosophila melanogaster* (*D.melanogaster*) mutant it has been shown that parkin is recruited to the damaged mitochondria and promotes autophgay (101). Also it has been shown that complex I deficinecy is present in PD patients.

An additional role of α -synuclein will be its role in dopamine (DA) regulation. Dopamine is considered as a neurotransmitter in the brain and it has a varitey of pivotal roles in central nervous system (CNS). Infact loss of dopaminergic neurons is one of the key features in PD. DA regulates tyrosin hydroxylase (TH) activity, dopamine transporter activity (DAT) , vesicular storage and efficient neurotransmitter release. Among all, TH activity regulation is critical since it has a rate limiting effect on DA production. In PD, LB formation may lead to hyperactivity of TH that has a positive feed back on DA biosynthesis. In early stages of PD cytosolic over-production of DA can result in its oxidation under oxidative stress that leads to production of superoxide anions and reactive DA quinine. Increased DA oxidation and modification also has been implicated in mitochondrial dysfunction.

Not only genetical mutations are the major source for dopaminergic neurodegeneration. Environmental stress has also been shown to be able to induce oxidative stress and mitochondrial dysfunction hence promoting PD. Manganese (Mn) is one of the historical factors whose role in parkinsonism has been proven in miners. It's a naturally occuring element that has several biological roles (102). Being essential for animals it is known to be the cofactor for several biomolecules and enzymes e.g. arginase, Mn-SOD, etc. Its role in the onset of parkinsonism has been proven in 1837 by british academic James Couper. From

back then it has been proved that inhalation and/or intoxication by oral intake can lead to its deposition in parts of the brain that are highly active and are prone to oxidative stress. Mechanistically Mn is able to interfere with fenton reactions and increase cytosolic glutamate concentration that all lead to an increase in ROS production and oxidative injury (102). Clinically and pathologically it has been proven that intoxicated from of Mn is able to cause idiopathic features of PD (103).

1.6.4 Autophagy, impairment and induction, in neurodegenerative diseases : a simple observation or an ultimate solution?

Impairment of efficient turnover of defective proteins is evident in occurrence of neurodegenerative diseases hence making the autophagy (as a protein turnover enhancer) a possible therapy module. It has been shown that neurons bear this machinery and all its components (104). There are varieties of substances that are able to induce autophagy and ameliorate the cellular impairment in different model organisms. For instance it has been shown in a mouse model that rapamycin, the selective inhibitor of TORC1, lowers the pathophysiological characteristics of Huntington's disease (HD), Alzheimer' disease (AD) and PD confirming the impairment of autophagy in those diseases (105). Other than rapamycin, as an antifungal antibiotic produced by the bacterium *Streptomyces hygroscopicus*, autophagy-inducer elixirs can be among substances known to be favorable nutrients. Curcumin (a famous Asian – African ginger family) is a good example among those nutrients which has been shown to induce autophagy through TORC1 and AMPK and being neuroprotective in HD and AD (106).

In PD, however, it has been shown that LBs comprise 90 different proteins that includes parkin and α -synuclein as the major parts and the key initiators (97). Of note it's not clear whether these kind of accumulations are cytoprotective or pathogenic. A chaperon-mediated folding system is involved in the processing of the proper folding of the LBs. They will get guided to gain a proper folding and if this is not possible, they will be transferred to the degradation system. Subsequently any failure in their degradation can lead to formation of aggresomes in neuronal regions as an evident phenomenon in neurodegenerative diseases

(107). The involvement of chaperones, ubiquitin and autophagy in clearance of these aggregates have been reported in several studies (108-110). For instance it has been shown that knocking out Atg 5,7 genes in a mouse model lead to widespread neurodegeneration inclusion bodies via impairment of autophagy. Particularly in case of PD, it has been shown that overexpression of α -synuclein in-vitro and in-vivo lead to inhibition of autophagy (111). This kind of impairment has been shown to happen at the early stage of autophagy where Atg9 is involved in the biosynthesis of autophagosome (112). The autophagic regulatory role of α -synuclein has been confirmed by Winslow et. al. where they show the up-regulation of autophagic markers upon RNAi-mediated knockdown of synuclein activity (111). More detailed studies by only knocking down atg7 in midbrain in a mouse model has been shown to cause the accumulation of α -synuclein in presynaptic regions leading to a reduction in both DA content and pronounced axonal dystrophy (113).

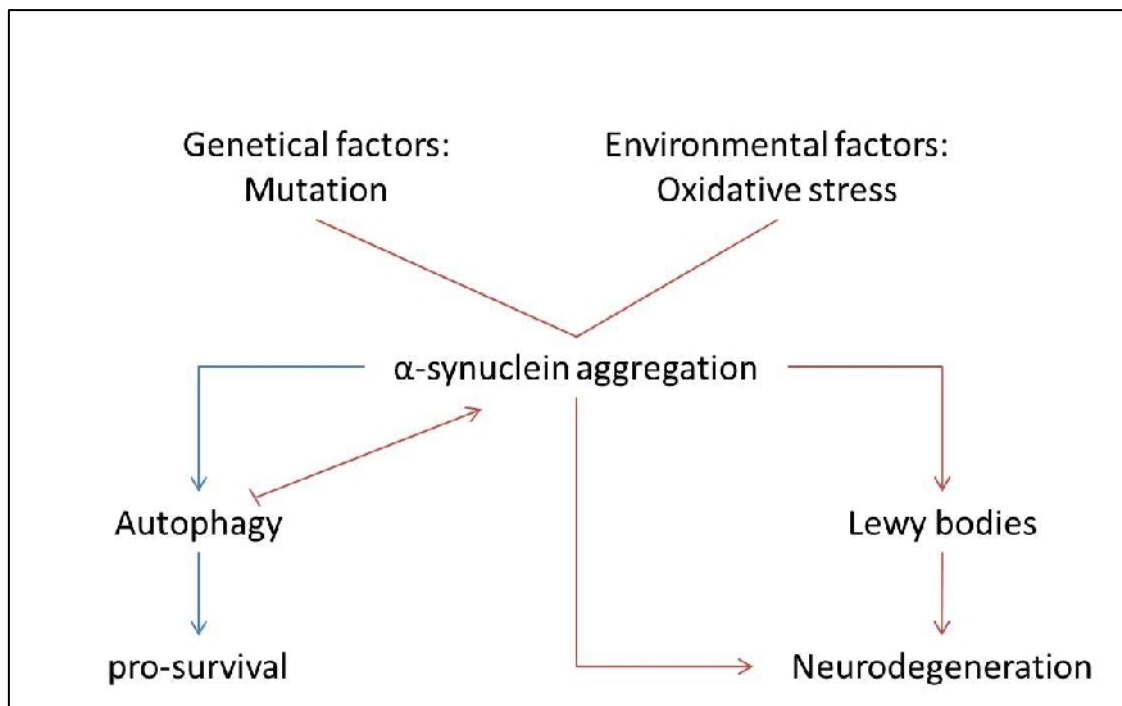


Figure - 4: The role of α -synuclein in neurodegeneration.

Not only overall pathway of autophagy has been studied in pathology of PD but α -synuclein has also been shown to affect the chaperon mediated autophagy (CMA). It has been shown that it binds to CMA receptors with high affinity and blocks the protein degradation (114). For instance, using α -synuclein transgenic mice it has

been shown that there will be more aggregation in CMA blocked regions (99). Lysosomal dysfunction has been proposed for the late autophagy steps in PD where mutation in ATP13A2 gene encoding lysosomal P-type ATPase. Several other PD related mutations have also been shown to be able to impair autophagy including Leucine-rich repeat kinase 2 (LRRK2) and PARKIN where ubiquitinated autophagy has been impaired (115).

Altogether, at the present time, the involvement of autophagy is a clear fact rather than an obscure myth. This fact makes autophagy important in pathogenesis, therapy and even diagnosis (see below) of neurodegenerative diseases. On this route polyamines and specifically spermidine has a long history and has major potentials. The first observation of crystalline substances, defined as spermine, in human semen is even older than discovery of nucleic acid (116). They have been identified in 1678 by the Dutch scientist Anton van Leeuwenhoek and it was not until 250 years later that its structure has been studied (117). It also had been shown that spermine forms after days of standing in semen and is exclusive for human samples as fish semen lacks these structures (116). Polyamines are present in both prokaryotic and eukaryotic systems and are being biosynthesized. Studying the effect of these natural occurring substances on organisms is an old field. It has already been shown that it induces growth of *Haemophilus parainfluenzae*, *Neisseria perflava* or *Pasteurella tularensis* (118, 119). It has also been shown that it accumulates in developing chicken embryo. With its emerging physiological role, its possible role in different diseases also became more eminent and was shown to be secreted into the urine of patients with cancer. It was until the late 80s that it started to be used for therapeutical purposes (116). It is worth emphasising that the cellular content of polyamines is tightly regulated in the cell by biosynthesis, oxidation or modification. It has been shown that in high concentrations it conversely can inhibit the growth of gram positive bacteria (120). By the discovery of α -difluoromethylornithine (DFMO), the inhibitor of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine biosynthesis, the inhibition of autophagy has emerged as a possible curative module (121). Model organisms being used for studying the effect of spermidine in promoting health span. For instance, it has been shown that spermidine increases the longevity in yeast as well as flies through autophagy-dependent pathways (122). Scientific community has already started to look at it as “the life potion”

when on 1970 the first international congress on polyamines took place in New York (116).

1.7 Looking for a suitable model organism: Yeast or Fly

Selecting the best model organism is an important subject of debates in the field of biology. The five members of the so-called “council of model organisms” described by Fields et. al. are bacterium (*Escherichia coli*) mouse (*Mus musculus*), fruit fly (*Drosophila melanogaster*), nematode worm (*Caenorhabditis elegans*) and baker’s yeast (*Saccharomyces cerevisiae*) (123). Shedding light into human biological systems is the main subject of bioresearch. Due to the complicated nature of biological systems, it is more applicable to select rather simpler models that could mimic the pathways related to the human body. It is also important to take care to select a model that can resemble more completely the pathway or factors of interest. Each of the above-mentioned systems has contributed to a big discovery in biology or at least in understanding the biomodules better. Nonetheless, few general criteria make an individual model organism appealing to be used in the laboratory. Fruit fly and baker’s yeast are among those model organisms that are now widely used among research groups. There are key features that have evolved these model organisms from being simple commoners to celebrities:

- Being nonpathogenic and bearing the possibility to amplify them in normal condition and in a short time; this aspect makes it possible to have them in experimental suitable amounts in a very short time. Additionally they don’t need to be cultured in clean room conditions (e.g. working with cell line), the fact that makes them cheaper to work with financially. Being easy to handle in culture also makes it painless to develop or adapt cutting edge molecular techniques and protocols for these two organisms.
- Having a large community to be able to work with the same genetic strain; specifically in yeast and fruit fly that exceed more than 1000 laborites all around the world and some of them provide protocols online. With an additional unique policy of those webpages the knowledge of a variety of cutting edge tools can move and be shared fast within the same community.

- Having the most studied genome; between eukaryotes, both yeast and fruit fly have their complete genome sequenced. Meanwhile genomic manipulation is feasible and straightforward relative to other organisms. This makes the model organism a powerful tool for expressing the gene of interest (even if it does not express it) and studying its effect.
- Conservation of the most mammalian biological pathways and regulators through evolution; in that manner wild type yeast and fruit fly, even without being genetically manipulated, are powerful tools for studying the complicated mammalian pathways including apoptosis, autophagy, protein-protein interactions misfolding of proteins, etc. in a simpler way. In fact, some of the mammalian pathways that gained the attention of most scientists today have been described and studied in yeast or fruit fly (124, 125). Autophagy, the cellular self-digestion process, is one of the famous examples and until now 15 separate genes (-Atg's) have been described responsible in its various steps using yeast (126).

1.7.1 Yeast as a model to study apoptosis: more than “a miracle”

Saccharomyces cerevisiae (*S. cerevisiae*) however has a different contribution to biology in different ways. Long before even the writing has been invented, Egyptians were using its' ability to bake bread calling it “a miracle phenomenon”, without knowing the organism. It was not until 1857 that Louis Pasteur described the organism, setting a point in the history of biology. Not only being useful in research laboratories, yeast is widely used in our daily life. Its usage varies from proving nutrition to healthcare. This unicellular organism also serves to be a unicellular factory in a variety of ways from production of insulin to even in biofuel production (127, 128). However, yeast has the major contribution to research by executing apoptosis:

Apoptosis can be described as an evolutionary programmed trait that ultimately leads to management of resources between elements of a multicellular organism or a colony that, all in the long run, serves to the life span of the organism. It has pivotal roles in development, hemostasis and disease prevention in animal kingdom. Its malfunction has been long shown to be involved in cell death as a trigger for development of many diseases e.g. atherosclerosis, neurodegeneration, etc. (129). Yeast has been shown to exhibit the hallmarks of

an apoptotic cell that includes chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (130). In fact apoptosis has been shown to serve in preservation nutrients in a yeast multicellular colony (131). In time, many research groups tried to express the main mammalian apoptotic regulators in yeast. It has been shown that expression of Bax, caspases, P53 and Apaf1 complex in yeast lead to cell death and co-expression of Bcl-2 and Bcl-X_L prevent this killing effect. In effect, Bax in *S. cerevisiae* induces DNA cleavage chromatin condensation and other apoptotic markers. The lack of these death markers in a wild type strain could suggest a possible existence of possible apoptotic machinery. However, it was not until 1997 that Madeo et al. could detect the indication of an apoptotic death in a CDC48 point mutated strain (124). This discovery led to defining the first member of caspase family, namely yeast caspase-1 (yca1). Onwards yeast has been used in a variety of apoptotic scenarios using the defined apoptotic executors that include the serine protease OMI (Nma111p), cathepsin D (Pep4p), endonuclease G (Nuc1p) or Bax (Ybh3p), mitochondrial nuclease (Nuc1) or proapoptotic proteases (nma111 and kex1) (**Table – 2**) (124, 132-135). Additionally, yeast PCD is connected to complex PCD features reminiscent of mammalian cells such as mitochondrial fragmentation, mitochondrial outer membrane permeabilization or histone H2B phosphorylation (136-138). Of note, two physiological scenarios deriving in yeast PCD, chronological and replicative aging, have been effectively applied to investigate phylogenetically conserved aging pathways occurring in post mitotic mammalian cells and stem cells, respectively (139-141). Thus, this functional correspondence allows using *S. cerevisiae* to model lethal pathways and scenarios of higher eukaryotes including those elicited in mammalian cells. Though yeast might not completely be able to mimic the whole mammalian apoptotic machinery but it can serve as a simpler model to study those. Induction of life span by supplementation of spermidine is one of those examples when this simplicity helped to elucidate controversial aspects of life.

Additional to the whole conserved signalling pathway of apoptosis, well defined laboratory techniques have been described to study those pathways as mentioned above that researches could rely on. Carmona-Gutierrez et. al. has well defined those assays that include: AnnV/PI co-staining, 4',6-diamidino-2-phenylindole (DAPI) staining, Terminal deoxynucleotidyl transferase dUTP nick

end labelling (TUNEL) test, Dihydroethidium (DHE) staining and clonogenic survival assay (130). In fact, some of the above mentioned pathways are considered as strong assays that also could differentiate between apoptosis (described as the programmed cell death) and necrosis (defined as the sudden cell death) (130). AnnV/PI co-staining is one of those assays that subtract necrotic cells via simultaneous staining with PI that dyes ruptured plasma membrane. Also DHE staining could serve as the additional way to well-define those cells that are already impaired. These specificities become even drastic when looking into more complex pathways like autophagy when respective markers are more elusive and subtraction of dead cells becomes more crucial.

1.7.2 Neurodegenerative studies in *D. melanogaster*: From nervous yeast to bradykinetic flies

Using *D. melanogaster*, the common fruit fly, has momentous traces in science from describing the sex-linked inheritance to discovery of mutations caused by ionizing radiation. Fruit fly served as an advantageous tool in the field of neuroscience. It has been successfully used as a model for understanding human neurodegenerative diseases in means of pathological occurrence and/or methodological therapies (142). About 75 % of human disease genes have been shown to have a homologue in fly. Its neuronal system is relatively easy to manipulate but at the same time complex and relevant enough for modeling cognitive, behavioral and neurological disorders. Although other model organisms like yeast has been used in neuroscientific studies, they could not ever provide complete physiological conditions as in mammalian brains. The presence of blood brain barrier (BBB) and conserved neurotransmitter systems make it a comprehensive model superior to other organisms like yeast. Nevertheless there are also well-defined techniques for behavioral studies including learning and memory. In laboratory it provides advantages as discussed above. Additionally since its genome has only few redundancies, it makes it easier to study complicated loss-of- function analysis. Contrary to other models like mice, there are no ethical constraints.

They are numerous studies that try to model PD in other model organisms and unveil genetic or molecular features of the disease. For instance using non-human model organisms the role for α -synuclein in lipid and vesicle dynamics, proteasomal dysfunction, or oxidative stress has been proven (100, 143). Since neither yeast nor *D. melanogaster* have PD related genes and alpha-synuclein is only constricted to vertebrates, for detailed studies of PD autosomal-dominant genes overexpression (α -synuclein) or knock down/outs of autosomal recessive genes (e.g. Parkin, DJ-1 and PINK1) in model organisms should be used.

For proper modelling of PD, however, key features of the disease could be replicated. These key features include age-dependency of the disease, loss of DA neurons, motor neuron dysfunction and the presence of LB's and neuritis. *D. melanogaster* has been shown and well used to model PD and its overexpression has been shown to disrupt trafficking of protein in ER to Golgi network and enhancing cell death through alpha-synuclein protein aggregation. The fruit fly is also known to develop progressive DA neuronal loss via inhibition of mitochondrial complex I and inducing oxidative stress (100). It has been shown to be able to mimic most of the phenotypes of the disease like decreased survival and DA neuronal loss by using overexpressed wild type (WT), A53T, and A30P α -synuclein flies (144). Genetic origin of the disease also has been illustrated using this model. Leucine-rich repeat kinase 2 (LRRK2) is one of those gene factors that is known as the biggest genetic contributor to Parkinson's disease. It has been shown that its overexpression in *D. melanogaster* leads to reduction of locomotor ability (145). However, it should be noted that since there would be no interaction between the genes like LRRK2 and α -synuclein, this potential mechanism is still vague and needs to be studied in other model organisms. In spite of these drawbacks still the fly model has been successfully used to study few molecular interactions. For instance it has been shown that expression of MUL1 protein can compensate a PINK1 loss-of function phenotype whereas the opposite way has no effect suggesting that parkin and PINK1 have a common pathway and PINK1 acts upstream of parkin(146).

2. Materials & Methods

2.1 Reagents

The following reagents were purchased from the indicated suppliers: FITC-labelled Annexin V (Roche Applied Science, 11828681001), reagents for 'terminal deoxynucleotidyl transferase dUTP nick end labelling' (TUNEL, Roche Applied Science, 11684795910), dihydroethidium (DHE, Sigma-Aldrich, D7008), propidium iodide (PI, Sigma-Aldrich, P4170), sodium hypochlorite (NaOCl, Sigma-Aldrich, A7191), cocktail tablet (Roche Diagnostics, Vienna, Austria), BCA protein assay kit (Thermo Scientific, IL, USA), (Thermo Scientific, IL, USA), 4x NuPAGE LDS sample buffer (Invitrogen), sample reducing agent (Invitrogen), nitrocellulose membranes (Invitrogen), VectaShield (Vector Laboratories LTD., Peterborough, United Kingdom). HOCl solutions were prepared immediately before use and the concentration was determined spectrophotometrically (ϵ_{292} 350 M⁻¹cm⁻¹), L-glutathione and N-acetyl-L-cysteine (Sigma-Aldrich). The constituents of yeast culture media including yeast extract, bacto-peptone, bacto-tryptone and yeast nitrogen base w/o have been ordered at Becton, Dickinson and Company (USA) and amino acids at AppliChem (Germany). In case of HL-1 culture the substance are listed with the supplier in parenthesis: fibronectin and gelatin (Sigma-Aldrich, MO, USA), Claycomb medium (Sigma-Aldrich), FBS (Sigma-Aldrich), norepinephrine (Sigma-Aldrich), L-glutamine (Sigma-Aldrich) and streptomycin (Invitrogen, Lofer, Austria). In case of any instruments or substances that are not listed here, the supplier has been mentioned in the text. A list of solution and components has been provided and is followed.

2.2 Antibodies and dilutions

anti-caspase-3 (1:1000, Cell Signalling-9662), anti-caspase-9 (Cell Signalling-9504), goat anti-mouse IgG (1:100000, Biomol-8101102), goat anti-rabbit IgG (1:200,000 Biomol-6293), Super Signal West Pico Chemiluminescent substrate (Thermo Scientific), anti- β -actin (1:1000, Santa Cruz-SC47778), anti-BRP-Nc82 (1:200 CD Creative Diagnostics), anti-GABARAP (1:1000, MoBioTec/

Biomedica, PM037), anti-tubulin (1:2000, Sigma Aldrich-T9026), goat anti-rabbit Cy3 IgG (1:500, Dianova 111-167-003), goat anti-mouse Alexa 488 (1:500, Invitrogen-A11001), goat anti-mouse horseradish-peroxidase-conjugated, (1:5000, Dianova 115-035-166), goat anti-rabbit horseradish-peroxidase-conjugated (1:5000, Dianova 111-035-144).

2.3 Solutions and Recipes

SDS (sodium dodecylsulfate polyacrylamide) PAGE and immunoblotting

Electrophoresis buffer:

25 mM Tris/HCl, 192 mM glycine, 0.2 % SDS; pH 8.3

Running gel (12%):

250 mM Tris/HCl; pH 8.8, 0.2 % SDS, 12 % acrylamide, 0.32 % N,N'-methylenebisacrylamide, 0.1 % ammoniumperoxodisulphate, 0.01 % N,N,N',N'-tetramethylethylenediamine

Stacking gel:

250 mM Tris/HCl; pH 6.8, 0.2 % SDS, 5 % acrylamide, 0.13 % N,N'-methylenebisacrylamide, 0.1 % ammoniumperoxodisulphate, 0.01 % N,N,N',N'-tetramethylethylenediamine

Urea loading buffer:

200 mM Tris/HCl, 8 M urea, 5 % SDS, 1 mM EDTA, 0.02 % bromophenolblue, 15 mM DTT; pH 6.8

Blotting buffer:

20 mM Tris, 150 mM glycine, 0.05 % SDS, 20 % methanol

Washing Buffer (PBS):

25 mM potassium phosphate buffer, 0.9 % (w/v) NaCl, pH 7.0

Stripping buffer (for 1 litre):

15 g glycine, 1 g SDS, 10 ml Tween 20, pH to 2.2

HL-3:

NaCl 70 mM, KCl 5 mM, MgCl₂ 20mM, NaHCO₃ 10mM, trehalose 5mM, sucrose 115mM, HEPES 5mM, pH adjusted to 7.2

Laemmli buffer (1x):

50 mM TRIS/HCl pH 6.8, 2 % SDS, 10 % (v/v) glycerol, 0.1 % bromphenol-blue, 2 % β -mercaptoethanol

2.4 Chemical lysis

2.4.1 *S. cerevisiae*

Lysis buffer CP:

1.85 M NaOH, 7.5 % β -mercaptoethanol, (freshly prepared)

Urea loading buffer:

200 mM Tris/HCl, 8 M urea, 5 % SDS, 1 mM EDTA, 0.02 % bromine phenolblue, 15 mM DTT; pH 6.8

TBST

Tris-buffered saline containing Tween 20

Tris solution:

1 M Tris/HCL

2.4.2 *HL-1 cardiomyocyte*

Lysis buffer:

50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 10mM NaF, 1% [v/v] Triton X-100, 10% [v/v] glycerol, pH 7.4)

Stripping buffer:

58.4 g/L NaCl, 7.5 g/L glycine, pH 2.15

TBST (Tris-buffered saline containing Tween 20)

2.5 DHE (dihydroethidium) staining

PBS buffer:

25 mM potassium phosphate buffer, 0.9 % (w/v) NaCl , pH 7.0

DHE solution:

From the stock 2.5mg/ml in DMSO: 1:1000 dilution in PBS

2.6 Annexin V/PI co-staining

Incubation buffer:

10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, pH 7.4

Buffer B + sorbitol:

35 mM potassium phosphate buffer, 0.5 mM MgCl₂, 1.2 M sorbitol, pH 6.8

Lyticase:

1000 U/ml in TE

β-glucuronidase/arylsulfatase:

from helix pomatia; 4.5 U/ml

Propidiumiodid (PI):

100 µg/ml in H₂O

2.7 Culture media

2.7.1 *S. cerevisiae*

Full medium (YEPD):

1 % yeast extract, 2 % bacto-trypton, 4 % glucose, (300 mg/l geneticin disulfate)

Minimal medium (SMD):

0.17 % yeast nitrogen base, 0.5 % ammonium sulphate, 2 % glucose, 80 mg/l histidine, 200 mg/l Leucine, 30 mg/l of all other amino acids, 320 mg/l uracil, 30 mg/l adenine

2.7.2 *HL-1 cardiomyocyte*

Full medium:

Claycomb medium containing 10% (v/v) FBS, 0.1 mM norepinephrine , 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin

2.7.3 *D. melanogaster*

Cornmeal-molasses-yeast medium (for 1 liter):

0.8 l liters of ddH₂O, 11 grams of yeast, 51 grams of yellow cornmeal, 5 grams of agar, 67 milliliters of molasses, 15 milliliters of 10% p-Hydroxy-benzoic acid methyl ester in 95% ethanol

2.8 Primers

Oligonucleotides were purchased from MWG Biotech (**see Table 3**). Upon delivery aliquots with the concentration of 0.1 nmol/µl in ddH₂O were made and being stored at –20 °C.

2.9 RNA isolation from HI-1 and cDNA preparation

RNA isolation was done as already described (147), according to the protocol from Qiagen RNeasy kit (Qiagen, Hilden, Germany). Cells were cultured to 70% confluency in 12-well plates. Upon applying the indicated concentrations of HOCl and time points, 500-700 µl RLT buffer (freshly add 1 % β-mercaptoethanol) were added per well. The cells were collected by continuous scraping and repeated pipetting. The then lysates were transferred to QIA shredder and being centrifuged for 15-20 s at 14000 rpm. Then equal amount of RNase-free EtOH was added to the same eppendorf tube (eppi) and the lower solution was transferred to RNeasy Mini Spin column and centrifuged for another 15-20 s, at 14000 rpm. The solution in the lower part of the eppi that had been filtrated was discarded, the upper columns were kept and 700 µl RW1-buffer was added to each of them and centrifuge for another 15.20 s at 14000 rpm. The filtrate was again discarded and 500 µl RPE buffer was added to each upper columns and

centrifuged at high speed for 37 15-20 s. This was repeated for two times and each time the lower tube has been emptied. Afterwards 20-50 µl of RNase free water was added to the upper part and the tubes were kept for 10-15 min at room temperature and centrifuged at 14000 rpm for 2 min with collecting the purified RNA dilution in an RNase free tubes in the bottom. 2 µl of the RNA concentrations were measured at 260 nm using NanoDrop (Thermo scientific, Wilmington,USA). Using reverse transcription, two µg RNA in volume of 10 µl (adjusted with ddH₂O) was reversely transcribed by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA).

2.10 Real time PCR procedure

As already described (147), three µl of diluted cDNA (1:50 in ddH₂O) and 1µl forward and reverse primer and 5µl QuantiFastTM SYBR® Green were pipette into Light Cyclers 480 Multiwell Plate. The plate was centrifuged for 1 min at 800 rpm. Roche Light Cyclers 480 instrument was used for the reading of the plate. Of note, glyceraldehyde phosphate dehydrogenase (GAPDH) were used as house-keeping genes for relative gene expressions. **Table - 1** shows the Real time PCR program:

	Temperature	Time	Cycles
Denaturation	95° C	5 min	1
Amplification	95° C	10 s	40
	60° C	30 s	
Melting Curve	95° C	10 s	1
	60° C	20 s	1
	95° C	Continuous	
Cool Down	4° C	20 s	1

Table - 1: Real time PCR program.

2.11 *HL-1 cardiomyocyte Culture*

Culturing the cells were done as already described (147). HL-1 cells that were obtained and originated from murine atrial cardiomyocyte cell line (HL-1) were cultured in fibronectin (0.5%, w/v)/gelatin (0.02%, w/v)-coated flasks and provided with full Claycomb medium as the culture media and kept in the incubator with the constant temperature at 37°C under 5% CO₂. For experiments, cells were cultured in six-well plates and were grown to 70% confluency. A 12h of serum starvation was performed before performing each experiment with HOCl at indicated concentrations and time points.

2.12 Cell viability assay

Cell viability assay was done as already described (147). Upon treating the cells with HOCl in six-well plates each well was incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/ml; dissolved in serum-free medium) for 30 min at 37°C. Dark purple colored dye (formazan) was solubilized with 300 µl acidic isopropanol (0.04 M HCl in absolute isopropanol). Microplate reader (Victor Multilabel Counter) and excitation/emission at 570/630 nm was used to measure the reduction of fluorescence intensity of MTT.

2.13 Yeast Strains and growth conditions

Experiments were carried out as already described by Carmona-Gutierrez et. al. (56) in wild type BY4741 and the respective null mutants (Rho0; aif1; nuc1; MCA1 (yca1); pep4; nma111; ndi1; cpl1; kex1 (**Table - 2**) obtained from Euroscarf. All experiments were performed in synthetic minimal medium (SMD) medium containing and all amino acids. Incubation of liquid cultures (in flasks) was performed with 145 rpm at 28°C. For certain experiments that 96-deepwell plates were used cultures were incubated shaking with 320 rpm at 28°C. Cell densities were measured using an automated cell counter (CASY1, Roche Innovatis). It should be noted that the quantitative degree of the cytotoxic effect elicited by HOCl seemed to be slightly dependent on the batch of SMD growth media used.

For all assays, overnight cultures of corresponding strains were inoculated in 45 ml SMD medium using 250 ml flasks (or - for the mini-screen - inoculated in 15 ml using 100 ml flasks) at a density of 1.5×10^6 cells/ml. Next, cells were grown to a cell density of $7-9 \times 10^6$ cells/ml, subsequently distributed into 100 ml quadruple-indented flasks at 10 ml cell culture each (or - for the mini-screen - into 96 deep-well plates at 250 μ l cell culture medium each) and then either challenged with HOCl stock solution to reach the indicated final concentration or left untreated (control). Cells were incubated for 16 h after HOCl treatment and used for diverse assays. Alternatively, cells were incubated with L-glutathione or N-acetyl-L-cysteine (both dissolved in distilled water) at indicated concentrations prior to HOCl treatment.

2.14 Yeast survival Plating and tests for cell death markers

Clonogenic survival plating was performed as already described in Carmona-Gutierrez et. al. (56) Briefly, an aliquot of yeast cells were diluted in CASYton and the concentration of the culture was quantified by using CASY counter and detecting particles between 1.5 and 15 μ m. Two times with 200 μ l for each sample. Cell counts of HOCl-treated cultures and controls were measured, 500 cells plated on YEPD agar plates and incubated for two days at 28°C to allow colony formation. The colony forming units (CFUs) were analysed using an automated colony counter (LemnaTech). For each strain the CFUs determined for the control cultures were set to 100 % and the survival of the respective HOCl-treated cultures calculated relative to the corresponding control culture.

2.15 DHE (dihydroethidium) staining

DHE staining (indicative for ROS production) was performed as already described in Carmona-Gutierrez et. al. (56). Briefly, 5×10^6 cells were harvested by centrifugation (7 min. 4000 rpm), re-suspended in 250 μ l of 2.5 μ g/ml DHE in PBS, and incubated in the dark for 10 min. Relative fluorescence units (RFU) were determined by using a fluorescence reader (Tecan, GeniusPRO) and normalized

to the OD₆₀₀ of each sample. Alternatively, positive cells were counted using flow cytometer (BD FACSAria); 30,000 cells were used per experiment. Annexin V/PI co-staining (apoptosis/necrosis marker) and TUNEL staining (apoptosis marker) were performed and quantified by flow cytometry as described (134). 30,000 cells per sample were evaluated using BD FACSDiva software. Representative samples were subjected to qualitative analysis by fluorescence microscopy.

2.16 Immunofluorescence staining

Immunofluorescence staining was done as already described in Carmona-Gutierrez et. al.(56). For immunofluorescence studies, 1.5×10^7 cells were fixed with 4% formaldehyde solution and their cell wall digested for 30 min with glucuronidase/arylsulfatase and lyticase as described (134). After washing with sorbitol-containing buffer B, cells were blocked with 100 µl blocking solution (Ultra V block, lab vision, Fermount CA) for 20 min at 20° C. Cells were then spinned down and the resulting pellets re-suspended in 100 µl mAb antibody solution (clone 2D10G9, dilution 1:2, v/v in antibody diluent, Dako, Glostrup, Denmark; 25°C for 30 min); the mAb recognizes HOCl-oxidized proteins, but does not cross react with epitopes generated by oxidative reactions involving nitrating species, transition metals or lipid peroxidation reactions (148). After washing once with sorbitol-containing buffer B, cells were incubated with 100 µl cyanine 3-conjugated goat anti-mouse antibody (Jackson Dianova, 1:300 in antibody diluent) for 20 min at 25°C. Fluorescence was microscopically evaluated and quantified using a fluorescence reader (Tecan, GeniusPRO). The obtained intensity was normalized to the OD₆₀₀ of each sample.

2.17 Microscopy

2.17.1 *S. cerevisiae*

Microscopy was performed as already described in Carmona-Gutierrez et. al. (56) with a *Zeiss Axioskop* microscope using a *Zeiss Plan-Neofluar* objective lens with 40 x or 100 x magnifications and 1.30 numerical aperture in oil (using Zeiss Immersol[®]) at room temperature. Imaging medium was AnnexinV/PI incubation buffer. Fluorochromes were fluorescein (used as fluorescein isothiocyanate, FITC), PI and ethidium (after conversion from DHE). Fluorescence microscopic sample images were taken with a *Diagnostic Instruments* camera (Model: SPOT 9.0 Monochrome-6), acquired using the Metamorph software (version 6.2r4, Universal Imaging Corp.) and processed with IrfanView (version 3.97) and Adobe Photoshop (version CS2) software. Specifically, picture processing involved creation of AnnexinV and PI overlays were done using Metamorph and cropping of representative areas with IrfanView. In addition, contrast and brightness was adjusted with Adobe Photoshop (applying equal adjustment parameters to all pictures of an experiment).

2.17.2 *D. melanogaster*

Confocal laser microscopy was used to obtain whole fly brain images using Leica Microsystems TC SSP5 and Leica LCS AF software. 20x, 0.7 NA oil objective has been used and the distance between each stacks was set to 2.01 μm . Each image stack was taken with 1024x1024 resolution at 400 Hz using bidirectional 4x line averaging. In case of co-staining of the brains, two laser channels - argon and diode pumped solid state (DPSS) - were used. Leica LCS AF software was also used for post processing and quantification of the acquired images. Maximum projection option was used to obtain the maximum intensity of the entire z-stacks. By drawing region of interest (ROI) around the whole brain pictures the voxel values from separate channels, hence different antibodies were obtained.

2.18 Western blot analysis

2.18.1 *S. cerevisiae*

Preparation of cell extracts (1×10^7 cells) and Western blot analyses were performed as described (149). Cell extracts were produced by adding 0.1 M NaOH to $\sim 1.5 \times 10^7$ cells and incubated for 5 minutes. Cells were then incubated for 20 minutes in 100 μ l lysis buffer containing and heated up for 10 minutes at 95 °C before applied on the gel. Electrophoresis was performed on 4–12% gradient Bis-Tris gels (Sigma) and proteins were transferred to nitrocellulose membranes (149). Membranes were blocked with 5% (w/v) non-fat milk in TBST at 25°C (2 h) and incubated with primary antibodies (overnight at 4°C). Blots were incubated with a mAb clone 2D10G9 (dilution 1:25) (148). Peroxidase-conjugated goat anti-mouse IgG (Sigma) was used as a secondary antibody. Immunoreactive bands were visualized using the ECL-development system. Pre-absorption of the mAb was performed with a 20-fold molar excess of HOCl-modified or native albumin.

2.18.2 *HL-1 cardiomyocyte*

Preparation of the cell extracts were done as already described (147). HL-1 cells were lysed in 100 μ l of ice-cold lysis buffer containing a protease inhibitor cocktail tablet for 10 min on ice. Cells were collected by continuous pipetting and scraping and centrifuged at 10,000 rpm (4°C, 10 min) and the supernatant were collected. BCA protein assay kit was used to determine protein concentration. In each case 50 μ g of total protein was heated at 70°C for 10 min with 10 μ l of 4x NuPAGE LDS sample buffer and 2 μ l sample reducing agent. Proteins were separated by electrophoresis on NuPAGE 4-12% Bis-Tris gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) non-fat milk in TBST at 25°C (1 h) and incubated with overnight incubation of primary antibodies at 4°C. The following primary antibodies were used (diluted in 5% [w/v] BSA-TBST): anti-caspase-3 (1:1000), anti-caspase-9 (1:1000). After being washed membranes were incubated with HRP-conjugated goat anti-mouse IgG (1:100,000) or goat anti-rabbit IgG (1:200,000) (25°C, 2 h). Immunoreactive bands

were then detected using Bio-Rad ChemiDoc MP Imaging System using an enhanced chemoluminescence substrate (GE Healthcare). Normalization was performed by incubation of membranes stripping buffer for 1h at 25°C and following incubation with anti- β -actin (1:1000) as primary antibodies.

2.18.3 *D. melanogaster*

20 heads per genotype were decapitated by continuous vortexing and deep freezing in liquid nitrogen. Protein extractions were done manually by crushing them in 40 μ L of 2% SDS aqueous solution and using a micropistil. Samples were cooked with 40 μ L of 2x sample buffer (Laemmli) and heating at 95°C for 5 min. The samples were then centrifuged for 5 min at 16'000g and the supernatant was taken. 8 or 20 μ L that is equal to 2 or 5 heads were then subjected to 12% Tris-HCl gel separated by SDS PAGE and transferred to nitrocellulose membranes. The membrane was blocked with 5% milkpowder in PBS for 1h at 25°C (1 h). The membranes were incubated with primary antibodies (diluted in 5% milkpowder in TBS) as indicated in the figure legends overnight at 4°C. The membranes then were incubated with the respective secondary antibodies shaking for 1 h at 25°C. Immunoreactive bands were then detected using Bio-Rad ChemiDoc MP Imaging System using an enhanced chemoluminescence substrate (GE Healthcare).

2.19 *D. melanogaster* fly strains

All experiments were carried out under standard laboratory conditions (150) at 25°C, 65-70% humidity and a constant 12/12 hours of light/dark cycle. Except otherwise mentioned, flies were fed with standard semi-defined cornmeal/molasses medium according to the Bloomington recipe. The GAL4-UAS system was used for expressing the gene of interest. All the Gal4 driver lines and the line UAS- α -synuclein (Bloomington # 8146) were obtained from the Bloomington Stock Center (Indiana University, USA). Chromosome X-linked Gal4 - Elav X (Elav X) and Gal4 - Nsyb (Nsyb) enhancer trap lines were used to drive expression of the genes. All the experiments were done in fly strains in the

background of wild type iso-w1118 (W1118 or WT) obtained from Bloomington stock collection. In case of behavioural experiments (locomotion assessment), the flies were outcrossed to W1118 for more than five generations, in order to generate an isogenic genetic background. Notably W1118 flies are mutant for the white gene and thus have white instead of red eyes.

2.20 *D. melanogaster* aging experiments

To uncover the effect of α -synuclein expression upon aging in transgenic flies this setup has been used; Gal4 - Elav X virgins were crossed with male UAS- α -synuclein flies (ratio of 1:5 – 1:4) in big vials (diameter 4.5cm). Filter papers were used in the solid food to allow the fly larvae to complete their life cycle. To address and minimize any confiction effects of environment on the flies, the parents were flipped every third day to a new vial for making different cohorts of a same cross allowing a diverse collection of hatched flies. 1-3 day old F1 offspring flies were collected in big fresh vials to allow the flies to mate for another 24 hours at 25°C. Subsequently the flies were sex-separated and 20 flies each were transferred to small vials containing either standard corn food or fly food supplemented with 5 mM spermidine. Throughout the whole experiment, the flies in each individual vial were flipped into a fresh one every other day. The number of dead flies was counted and reported at the indicated time points. The experiments were carried out until all the flies in the control group died.

2.21 *D. melanogaster* survival experiments

To assess survival aptitude upon challenging the flies with manganese, two types of schemes (old and young) have been used. For both schemes and depending on the experiment, virgin flies from either Gal4 - Elav X or Gal4 - Nsyb were collected and crossed with male flies harboring the UAS- α -synuclein (ratio of 1:5 – 1:4) in big vials (diameter 4.5cm, with filter papers). The parents were flipped every third day to a new vial for making different cohorts of a same cross thus minimizing the environmental factors affecting the experiments. Upon hatching, 1- to 3-day-old flies (only F1 generation) were collected in males and females using CO₂ and were incubated at 25°C for 24 h (regeneration from CO₂). In case of the

young setup flies were incubated at 29°C for 24 h (expression) and subsequently transferred into fresh small vials (diameter 2.5) fixed on a petri dish with filter papers. These flies are called young flies throughout the text. The petri dishes were filled with the experimental solutions containing 10% sucrose (in ddH₂O) and either 20 mM MnCl₂ or 20 mM MnCl₂ with supplementation of 5mM spermidine (adjusted pH 5.9 - 6.1, freshly made), hence allowing the filters to be soaked with them. For the old setup the flies were kept for additional 14 days at 25°C after a regeneration phase, while being transferred to new vial every third day, and then being used on experimental setup and solution as indicated. These flies are called old flies throughout the text. In all the cases the petri dishes were monitored and refilled with new solution if needed to prevent filters to dry out. At indicated time points numbers of dead flies were counted and recorded.

2.22 *D. melanogaster* determination of locomotion ability

To determine whether locomotion ability is affected upon challenging the flies with 20 mM Mn²⁺ alone or with spermidine, climbing assay has been done. Flies from survival experiments' crosses (see above) and from indicated time points were used for this assay. Flies from each experimental regimen were divided into groups of 8–10 and transferred into vertical plastic tubes with a diameter of 1.5 cm. Since the flies tend to climb against the gravity, the tubes were tapped from the bottom and letting the flies to climb up the inner surface of the vials. Flies reaching a specific distance (7 cm for young setup or 4 cm for old setup) within 15 s or 30 s were counted. After each experiment the flies were transferred back to our experimental setup until all the time points were achieved. Since the difference in the intensity of eye colors between our experimental flies and the fact that they also tend to climb to the source of the light, experiments were conducted in the presence of red light that is non-recognizable for all red-colored eyed flies.

2.23 *D. melanogaster* whole-mount adult brain staining

Immunostaining of adult fly brain was performed according to Büttner et. al.(134). Adult flies from the indicated experimental time points were paralyzed on ice. They were decapitated and the brains were dissected in ice-cold hemolymph-like saline (HL3) solution. They were fixed immediately in 4% paraformaldehyde (PFA) in phosphate-buffered saline (1x PBS), pH 7.2, for 20 min. Subsequent 3-4 washing steps were performed in 0.3% PBS-T at 25°C before blocking unspecific antibody binding sites in 0.3% PBS-T/ 10% NGS for 1 hour. Brains were incubated with the desired primary antibody (according to the figure legends), in 0.3% PBS-T/ 5% NGS and sodium azide for 48h at 25°C. Afterwards the samples were washed with PBT for 1h and then the respective secondary antibodies were applied for 24h at 25°C. After an additional washing period for 1h with PBT the brains were mounted in VectaShield on glass slides. The brains were scanned by confocal microscopy (Leica SP8). Until all the samples are scanned, all the slides were stored at 4 °C for short term or at -20 °C for long term.

2.24 Statistical analysis

In all graphs error bars ($\pm$ s.e.m.) are shown for independent experiments. The results from different experiments were pooled and the number of independent data points (n) is indicated in the figure legends of the corresponding graphs. Significances were calculated using an ANOVA (one-way and two way as indicated) approach and were corrected by the Bonferroni post hoc test. In case of longevity experiments Mantel Cox and Wilcoxon tests were used to analyse the data. In case of *S. cerevisiae* when the experiments were carried out in parallel, a common overnight culture and common pre-cultures were used for each strain. In these cases, pre-cultures were divided into separate flasks (or wells in the mini-screen) prior to HOCl stress.

Standard Name and/or Alias	Genotype	Function of the protein	Homology to Mammalian System
BY4741 (wild type)	MAT a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0		
Rho0	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YJR144w::kanMX4	Lacks the mitochondrial DNA	N/A
aif1 (CPD1)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YNR074c::kanMX4	Caspase-independent apoptosis factor that translocates to the nucleus in response to apoptotic stimuli	Homologue to AIF in Mammals
nuc1	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YJL208c::kanMX4	Major mitochondrial nuclease, has RNase and DNA endo- and exonucleolytic activities; has roles in mitochondrial recombination, apoptosis and maintenance of polyploidy	Homologue to Endonuclease G in Mammals
MCA1 (yca1)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YOR197w::kanMX4	Putative cysteine protease ; contributes to clearance of insoluble protein aggregates during normal growth; may be involved in cell cycle progression	Homologue of Caspase in Mammals
pep4 (PHO9 , PRA1 , yscA)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YPL154c::kanMX4	Vacuolar aspartyl protease (proteinase A), required for the posttranslational precursor maturation of vacuolar proteinases; important for protein turnover after oxidative damage; synthesized as a zymogen, self-activates	Homologue to Cathepsin D in Mammals
kex1	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YGL203c::kanMX4	Protease involved in the processing of killer toxin and alpha factor precursor; cleaves Lys and Arg residues from the C-terminus of peptides and proteins	20% similarity to Cathepsin A in Mammals, <u>NO</u> homology
nma111 (YNM3)	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YNL123w::kanMX4	Serine protease and general molecular chaperone; involved in response to heat stress and promotion of apoptosis; may contribute to lipid homeostasis;	Homologue to Omi/HtrA2 family of serine proteases in Mammals
ndi1	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YML120c::kanMX4	NADH:ubiquinone oxidoreductase, transfers electrons from NADH to ubiquinone in the respiratory chain but does not pump protons, in contrast to the higher eukaryotic multisubunit respiratory complex I; phosphorylated;	Homologue to mitochondrion-associated inducer of death (AMID) in Mammals

Table - 2: Main yeast apoptotic factors and their respective description.

Primer	Sequence (F: Forward, R: Reverse)
cathepsin A (CtsA)	F: AGAGCGGTCAGGCCAAGACG R: CGCGGTTCCGGGCATCTCTG
cathepsin B (CtsB)	F: CGGCTCCGTTTGGCTTGGTGG R: AGGCTTGTCATGGGCACTGGTC
cathepsin D (CtsD)	F: TCGCCTAAGACCACGGAGCCA R: CGACTGTGAAACACTGCGGCGG
cathepsin G (CtsG)	F: CCGAGAAGCCAGGCCCCATT R: ACCCTCCACAAGCACTCAGCC
cathepsin S (CtsS)	F: GCATCGACGCCAGCCATTCTT R: TTGCCCCGTACAGGAGGGGTCG
cathepsin L (CtsL)	F: CGCAAGCCATCCGTCTCTCCA R: AGCCCACCAACAGAACCCCA
Ryanodine receptor 2 (Ryr2)	F: CCATGGCTGATGCGGGCGAA R: GCAGGGCCCCGTACTGACAGG
solute carrier family 8, member 1 (NCX1)	F: GCCCTGTTGTTGAATGAGCTTGG R: GCAATGCGCCTCTCCTCCTC
ATPase, Ca ²⁺ transporting, (Atp2a2/SERCA2a)	F: AGACGCTCAAGTTTGTGGCCCG R: GAGCAGCACAAACGGCCAGGA
calcium channel, voltage-dependent, T type, alpha 1H subunit (Cacna1h)	F: CACATCACCAAGGAGTACTGGC R: CAAATGAACGCTGAGCCTGA

Table - 3: Real time PCR program.

Remarks: The essence of the whole thesis is about studying the relationship between misfolding of the proteins and diseases (cardiovascular complications and neurodegenerative diseases) using different model organisms. All parts are written uniformly except the “Results and Discussion” which is split into two sections: Section 3 that includes the data from *D. melanogaster* and section 4 includes data from *S. cerevisiae* as well as *HL-1 cardiomyocyte*.

3. Results:

Misfolding of the proteins and neurodegenerative diseases in *D. melanogaster*

3.1 Spermidine rescues α -synuclein aggregation toxicity and prolongs life span in a *D. melanogaster* model of parkinson's disease

Manganese has been shown to have a synergic effect with α -synuclein aggregation that can be toxic in different model organisms (100, 101, 151). Hence the first experiments were carried out to validate that effect in a *D. melanogaster* model of PD. Using both old and young W1118 and α -synuclein expressing flies driven in separate experiments and upon applying 20mM manganese in a standard sucrose solution, we could be able to show that the level of survival decreases time dependently in both male and female flies and for both driver-lines (**Figure 5 and 6 A/B**). Notably, the level of this reduction markedly depends on the age and the sex of the flies; while the young female flies show a reduction of 50% after 4 days (**Figure 5B**), the same effect is seen after 5 days in old females (**Figure 6B**). Also in contrast to females, male flies are slightly more susceptible to the same concentration of manganese when they are old. Young male flies reach the median survival rate after 4 days and the overall life span is 7 days, the same median survival effect is reached after 3 days for old male flies with an overall life span of only 4 days where they are challenged with manganese (**Figure 5A and 6A**).

The effect of spermidine in reducing the stress induced damage (e.g. hydrogen peroxide) or prolonging the life span in different model organism has been discussed previously (152). But since contrary effects of polyamines (in general) have also been reported (116), experiments were carried out to see if the previously shown 5mM spermidine can rescue the manganese induced α -synuclein toxicity or increase the life span. As shown, unceremoniously of age and sex, spermidine can rescue the death effect seen at 108 and 120 hours for both young males and females, 72 and 84 hours for old males and 105, 120, 132 and 144 hours after Mn^{+2} exposure (**Figure 5 and 6 A/B**). Furthermore, preliminary experiments showed that female α -synuclein expressing flies can live longer on normal standard fly food supplemented with 5mM spermidine comparing to the control. That means spermidine can only able to increase the life span of female flies (**Figure 7 A/B**).

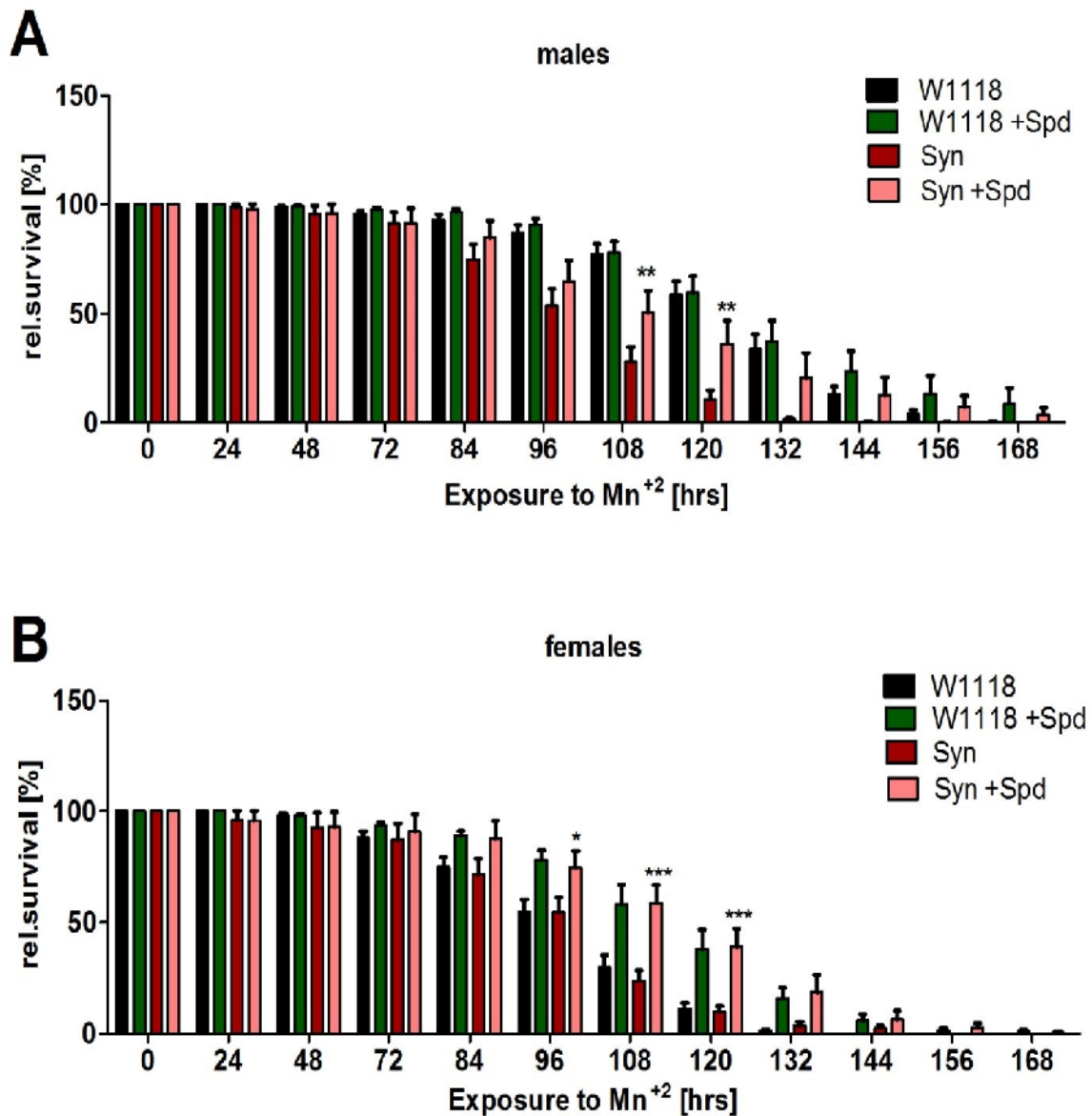


Figure - 5: Supplementation of spermidine rescues α -synuclein toxicity in young male and female flies upon manganese stress. 2-4 days old flies (either wild type or α -synuclein line) from survival's young scheme were sorted in males and females. They were then kept on 10% sucrose supplemented with 20 mM manganese, with and without supplementation of 5 mM spermidine. Dead flies were counted at indicated time points and survival was determined and depicted separately for males (A) and females (B). The data from each time point and group were then normalized to that of time point zero so all the groups start from 100%. The pan-neuronal driver line Gal4- Elav X was used to drive the expression of UAS- α -synuclein. Data represent means $\pm$ S.E.M. (n=9 and ~400-480 /sex and genotype; *** p<0.001 and ** p<0.01 vs the same non-Spd treated group).

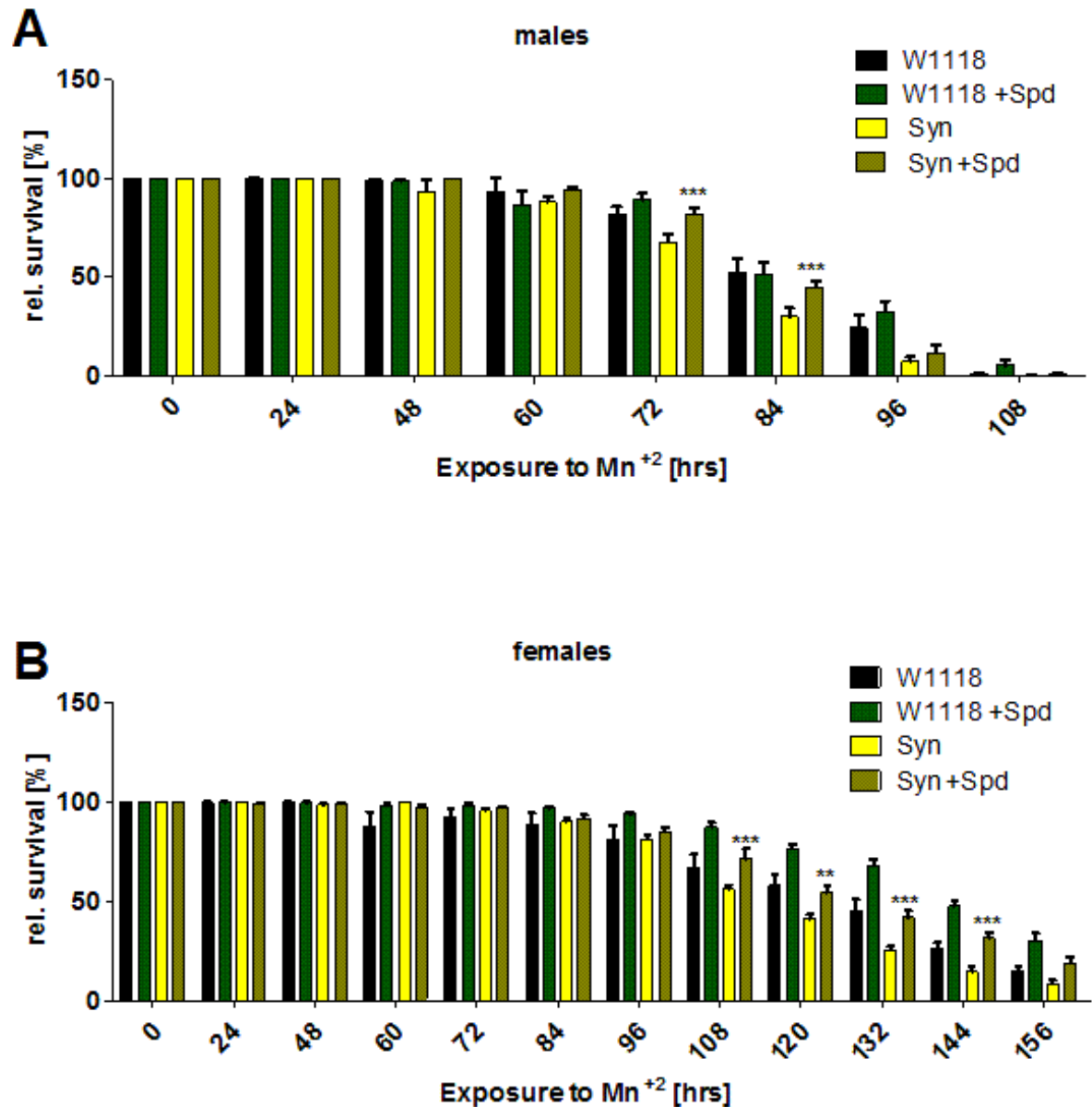


Figure - 6: Aged α -synuclein expressing flies are able to survive manganese stress upon spermidine treatment. 14-16 old out-sorted males and females (either wild type or α -synuclein line) flies from survival's old scheme were kept on 10% sucrose supplemented with 20 mM manganese, with and without addition of 5 mM spermidine. Dead flies were counted at indicated time points and survival was determined and shown separately for males (A) and females (B). The data from each time point and group were then normalized to that of time point zero so all the groups start from 100%. The pan-neuronal driver line Gal4- Nsyb was used to drive the expression of α -synuclein. Data represent means $\pm$ S.E.M. (n=9 and ~400-480 /sex and genotype; *** p<0.001 and ** p<0.01 vs the same non-Spd treated group).

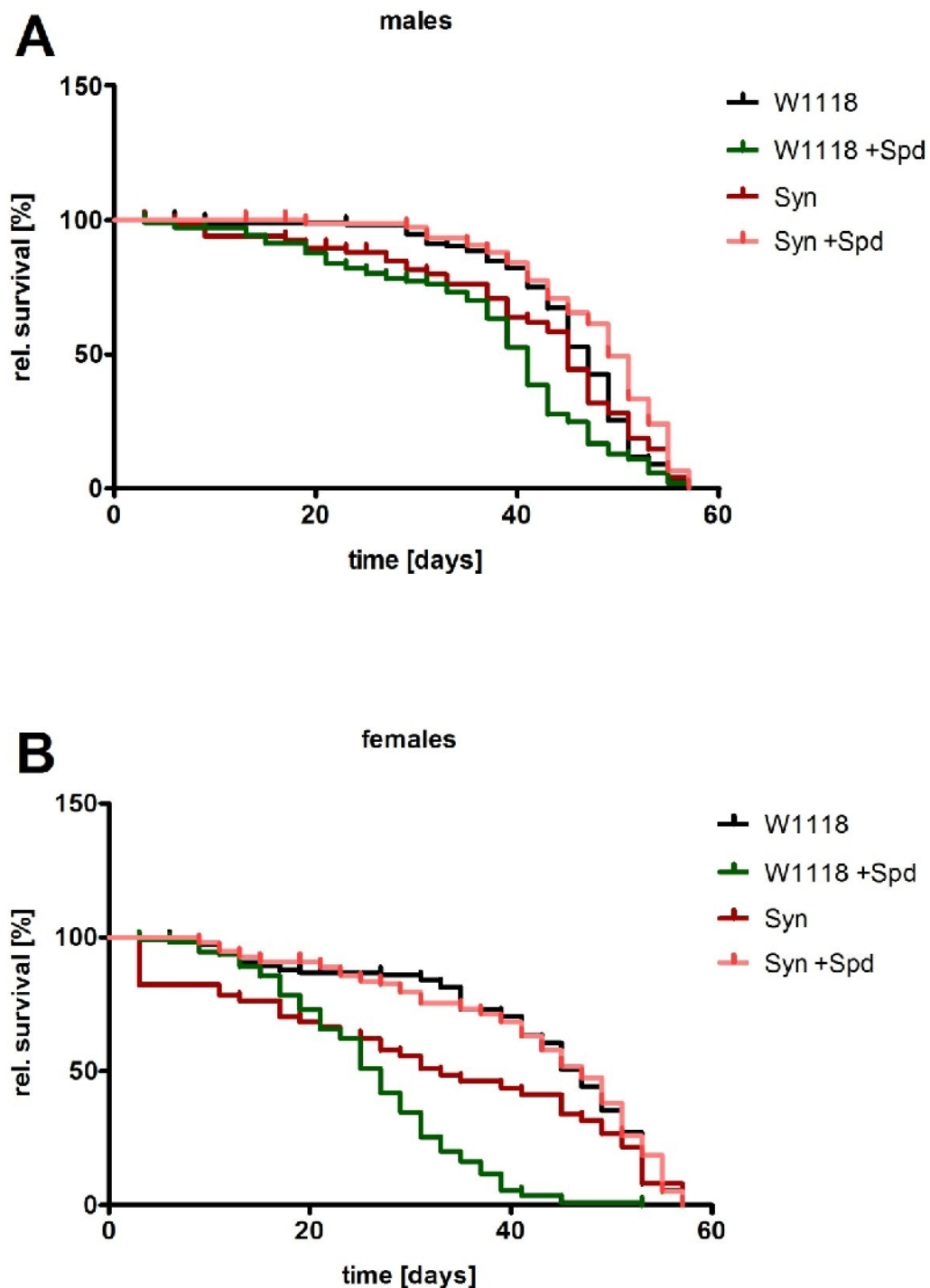


Figure - 7: Spermidine is able to increase the life span of α -synuclein female flies. 2-4 days old flies were separated in males and females and were aged on either on standard corn food or fly food supplemented with 5 mM spermidine. The number of dead flies were counted and reported at the indicated time points. Results are shown separately for males (A) and females (B). The pan-neuronal driver line Gal4- Elav X was used to drive the expression of α -synuclein. (n=3 and ~400-480 /sex and genotype).

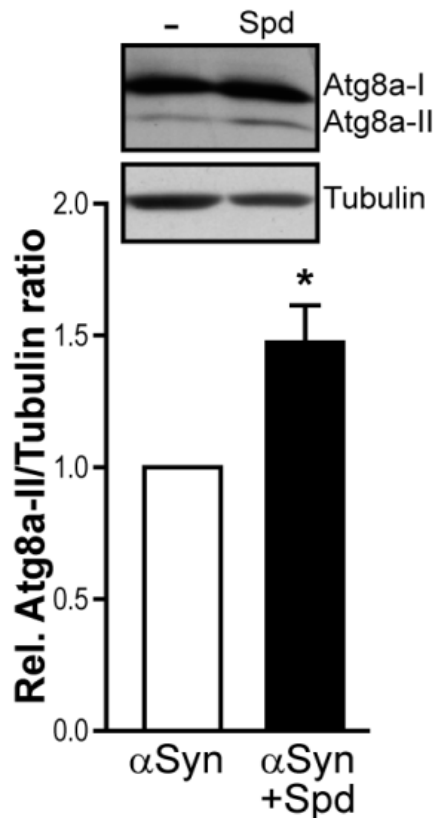


Figure - 8: Spermidine supplementation is able to induce autophagy markers in manganese treated flies. Whole cell protein lysates of wild type and α -synuclein expressing flies from old female upon 72h treatment with 10% sucrose supplemented with 20 mM manganese, with and without addition of 5 mM spermidine were used to run SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoreactive bands were visualized using horse-radish peroxidase-conjugated secondary antibody and ECL development upon application of mAbs Atg8a (GABARAP) and α -tubulin (the loading control). The pan-neuronal driver line Gal4- Nsyb was used to induce the expression of α -synuclein. Data represent means $\pm$ S.E.M. (n=3; * p<0.05 vs control).

3.2 Autophagy dysregulation acts as the main molecular pathway underlying Mn^{+2} toxicity in flies

Since accumulation of α -synuclein has been shown to be one of the pathophysiological hall marks of PD and is linked with inhibition of autophagy, experiments were carried out to investigate those effects upon Mn^{+2} exposure. To investigate this hypothesis, whole cell lysates were immunoblotted and checked for any change in subcellular level of Atg8a, the well-known marker of the autophagy pathway. Preliminary results revealed that application of 5mM spermidine leads to about 1.5 fold increase in endogenous formation of lipidated

Atg8a protein in α -synuclein expressing flies, which previously was inhibited upon 72 hours of Mn^{+2} exposure (**Figure 8**).

3.3 Mn^{+2} - induced locomotion defect is restored by spermidine in an age-independent fashion

Bradykinesia, described as slowness of movement, is one of the hallmarks of PD and can be the consequence of both α -synuclein accumulation as well as loss of neuropills in the region of the brain responsible for that upon manganese exposure. Since spermidine could reduce the aggregation of α -synuclein, we next decided to check if it can also rescue the phenotypic loss of locomotion ability. For that, flies were exposed to 20mM manganese with or without 5mM spermidine supplementation and their negative geotaxis activity (their ability to climb-up the wall of a cylinder after being tapped to its bottom) was assessed. Climbing index or locomotion ability is then calculated and described as the number of flies that could reach 7cm for young and 4cm for old flies after either 15 seconds or 30 seconds. Results revealed that 48h of manganese exposure cause about 50% decline in locomotion ability of the young males for 15 and 30 sec. locomotion ability of the young males (**Figure 9 A/B**). The same effect in old male flies is seen after 48h with a reduction of about 40% for 15 second or 30 second (**Figure 10 A/B**). In this case, however, 5mM spermidine treatment is more effective for old flies since it is able to increase the climbing index of the young flies by 10% (**Figure 9 A/B**) and 20% for old ones (**Figure 10 A/B**).

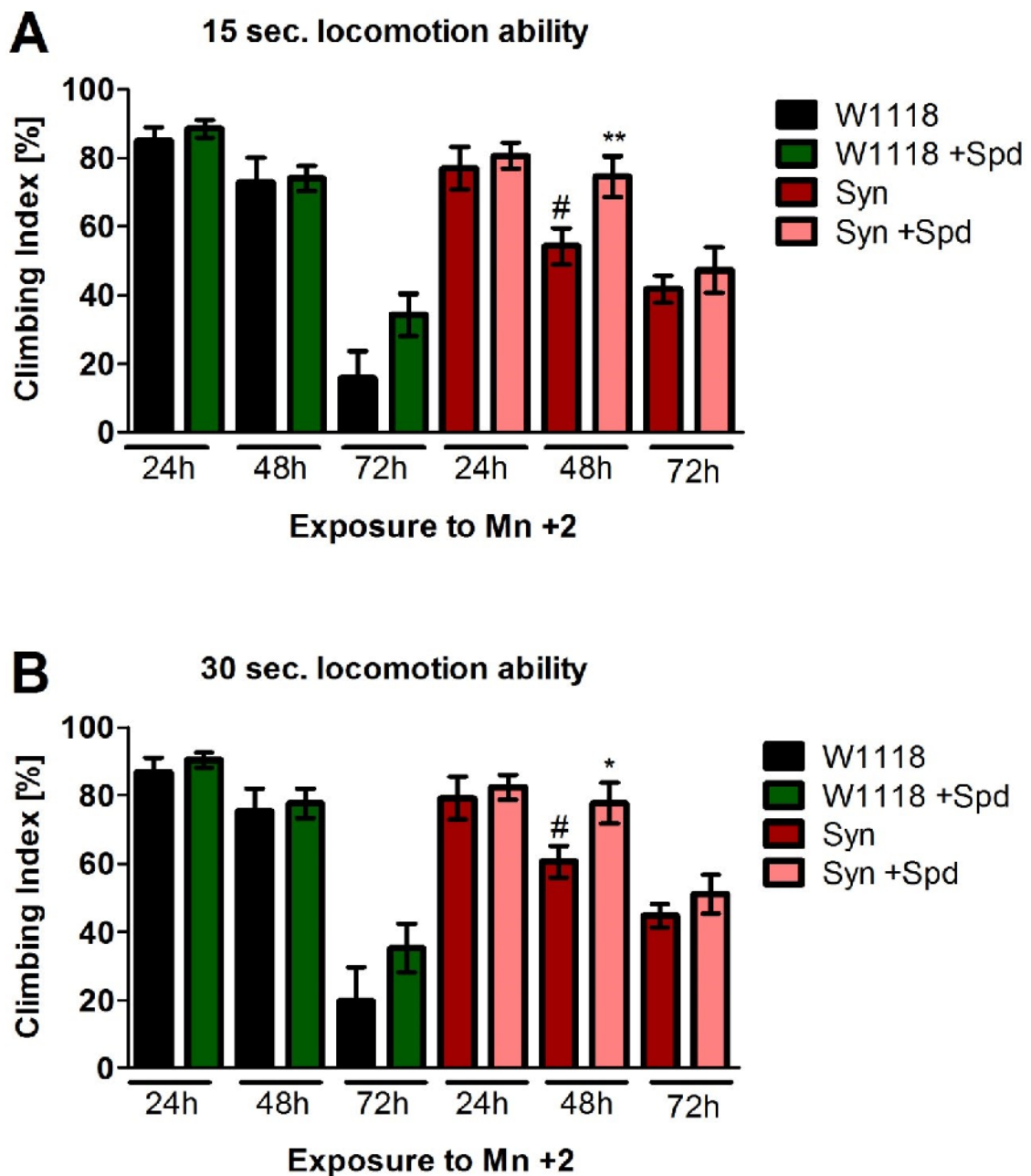


Figure - 9: Restoration of the locomotion ability in young male α -synuclein expressing flies upon supplementation of spermidine. 2-4 days old flies from survival's young scheme tested for the locomotion ability upon treatment with 10% sucrose supplemented with 20 mM manganese, with or without addition of 5 mM spermidine and their negative geotaxis activity (their ability to climb-up the wall of a cylinder after being tapped to its bottom) was assessed. Performance index (PI) was calculated and shown. The graphs represent the number of the flies that could surpass 7cm distance from the bottom of the vial within either 15 (A) or 30 sec (B). The pan-neuronal driver line Gal4- Elav X was used to drive the expression of α -synuclein. Data represent means $\pm$ S.E.M. (n=9 and ~400-480 /sex and genotype; * p<0.05 and ** p<0.01 vs the same non-Spd treated group; #<0.05 vs WT control with same exposure to Mn²⁺).

3.4 Spermidine rescues the α -synuclein linked decrease in Brp level

Since it has been shown that level of Bruchpilot protein (Brp) is tightly linked with the behavioural abilities such as locomotion in flies, we next hypothesized if the Mn^{+2} -induced-aggregation of α -synuclein and locomotion deficiency is accompanied by any changes in the expression level of Brp. Interestingly, the whole brain immunostaining with antibodies against Brp of old flies after 72h of 20mM Mn^{+2} exposure showed a marked decrease in the level of Brp expression and is reverted upon 5mM spermidine application (**Figure 11 B**). The same experiments were repeated to check the same effect when flies are hatched on 5mM spermidine (shown by *spd~*). Results revealed a lesser Brp restoration in this case (**Figure 11 B**). Control experiments also show that after 24h of Mn^{+2} exposure the level of α -synuclein signal increases and is being reverted by 5mM spermidine supplementation and to a lesser extent with flies that are being hatched on spermidine food (**Figure 11 A**).

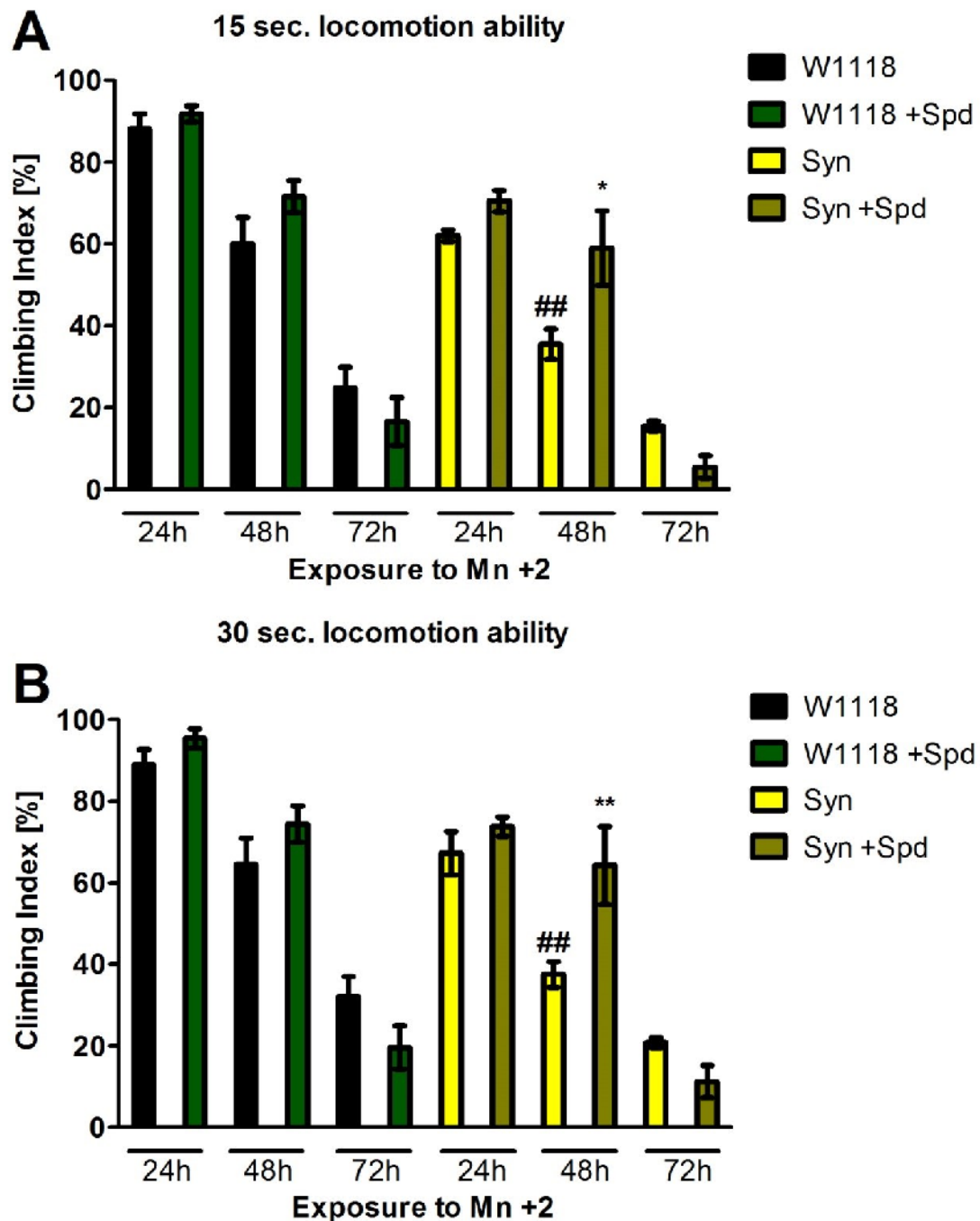
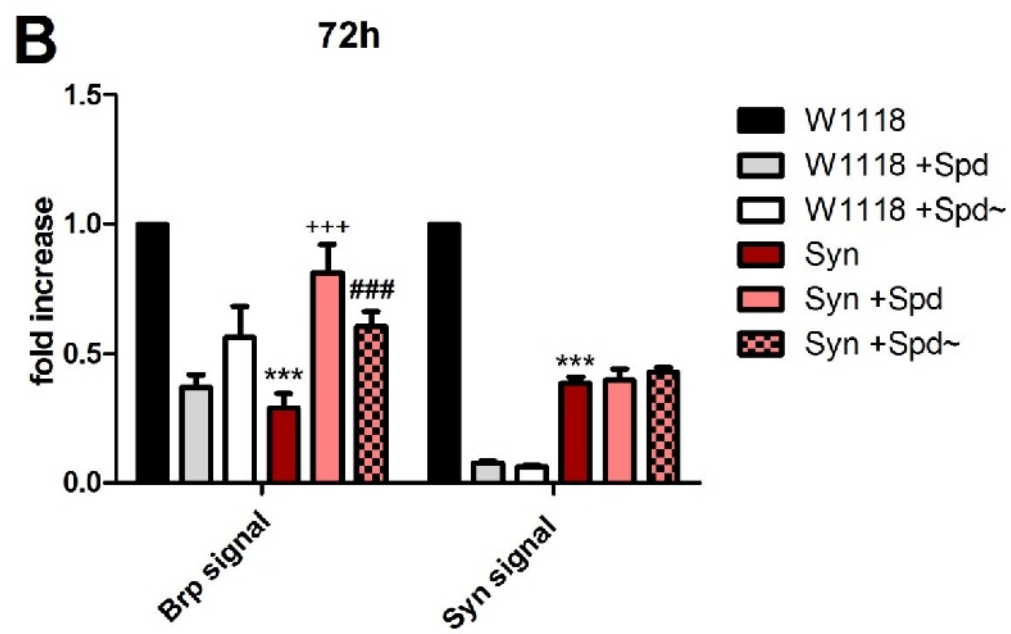
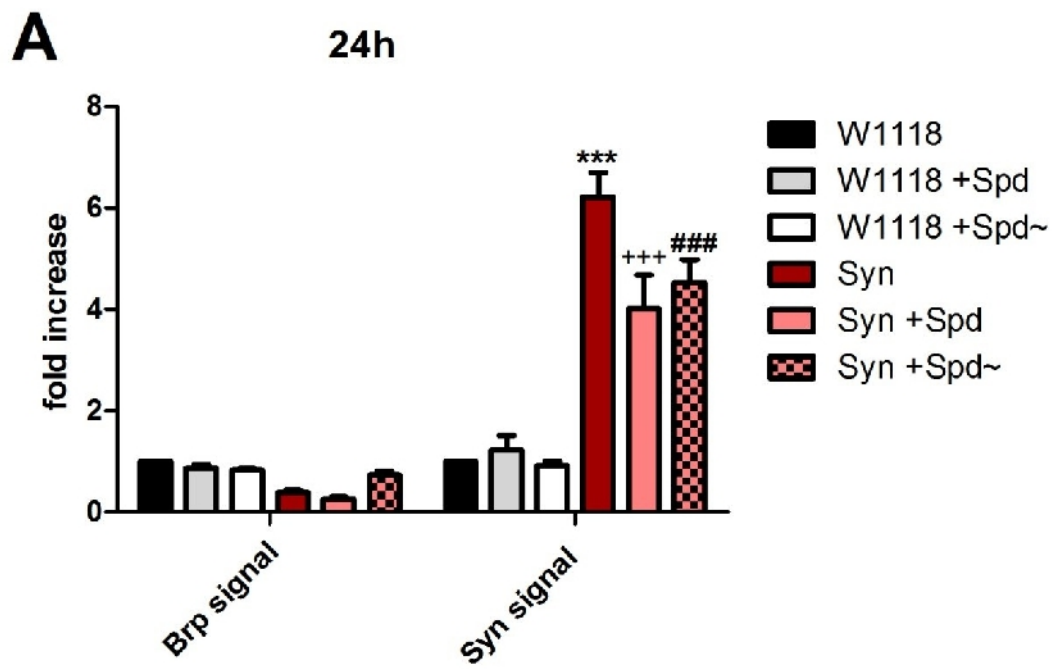


Figure - 10: Locomotion ability is restored upon supplementation of spermidine in aged male α -synuclein expressing flies treated with manganese. 14-16 days old flies from survival's old scheme tested for the locomotion ability upon treatment with 10% sucrose supplemented with 20 mM manganese with and without addition of 5 mM spermidine. Performance index (PI) was calculated and shown. The graphs represent the number of the flies that could surpass 4cm distance from the bottom of the vial within either 15 (A) or 30 sec (B). The pan-neuronal driver line Gal4- Nsyb was used to induce the expression of α -synuclein. Data represent means $\pm$ S.E.M. (n=9 and ~400-480 /sex and genotype; * p<0.05 and ** p<0.01 vs 01 vs the same non-Spd treated group; ##<0.01 vs WT control with same exposure to Mn⁺²).

exposure to Mn⁺²



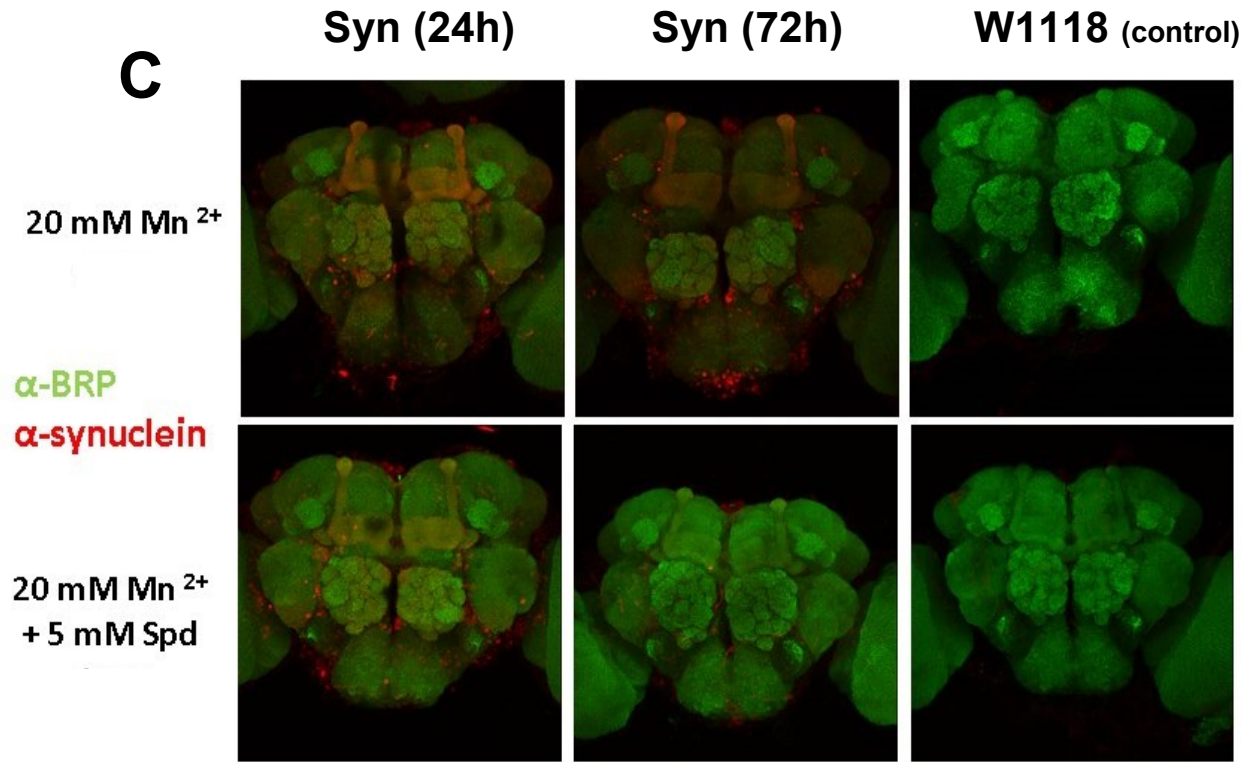


Figure - 11: Spermidine decrease the accumulation level of α -synuclein and increases the level of Bruchpilot protein (Brp) in adult brain sections of old females. (A and B) quantitative estimation of α -synuclein and Brp fluorescence in the absence (control) or presence of 5mM Spermidine after 24h and 72h of Mn²⁺ exposure. (C) Representative maximum projected images of laser confocal microscope for α -synuclein(red) and BRP-Nc82(green). Immunofluorescence staining was measured after incubation of whole brain with α -synuclein and BRP-Nc82 as a primary antibodies and cyanine-3-labelled goat anti-mouse IgG (red) and cyanine-3-labelled goat anti-mouse IgG (green) as secondary antibodies. The fluorescence intensity was quantified using Leica software and data are expressed as percentage of unstained cells. Data in (A) and (B) represent means $\pm$ S.E.M. (n=6; *** p<0.001 vs WT control; +++ p<0.001 vs W1118 flies treated with 5mM spermidine; ### p<0.001 vs W1118 that were born on spermidine supplemented food). (+spd~ represents flies that were born on food supplemented with 5mM spermidine).

3.5 Discussion

As discussed above, conformational changes in α -synuclein that ultimately lead to accumulation of α -synuclein cytotoxic oligomers can trigger cellular death scenarios as a part of UPR. The dysregulation of the proteasomal/lysosomal system is one of the major pathways to be the reason why α -synuclein cannot be cleared out from the cell, the effect that can lead to mitochondrial dysfunction and cell death (100). In effect, it has been shown that the level of different mammalian proteases like cathepsin B increases in response to lysosomal rupture due to α -synuclein accumulation (100, 143, 153). As shown by overexpressing human α -synuclein in rats as animal model as well as human neuroblastoma cells, MAPK pathway as well as NF- κ B nuclear translocation can act among the various pathways that α -synuclein induces its neurotoxicity (151, 154).

The effect of manganese on induction of ROS accumulation, which can lead to a cascade of cellular responses including autophagy (155), apoptosis (151, 156) and ultimate death (94) has been described in various model organisms. For instance, it has been shown that organotypic mouse brain slices can accumulate ROS that lead to cell death upon exposure to manganese up to 400 μ M. This effect is in fact due to α -synuclein oligomer damage to cell membrane caused by such an exposure (157). Nevertheless, different studies tried to figure out a molecular way to fight manganese toxicity. Essentially, it has been shown that enhanced exposure of manganese in *C.elegans* mutated for *djir1.1* can be reversed with *pdr1.1* mutation (158). Our data confirms the previous studies that show that the accumulation of the α -synuclein protein can be the consequence of metal ions exposure including manganese (94, 157, 159, 160). In fact, our results add further more by showing a rescuing effect of 5mM spermidine on Mn^{+2} toxicity. Moreover, unlike other above mentioned studies, we could be able for the first time to show that 5mM spermidine prolongs the life span of α -synuclein expressing flies.

As a general rule any perturbation in the normal level of metals can lead to a marked increase in fibril formation, oxidative stress and α -synuclein aggregation that leads to neurodegeneration (159). Mn^{+2} has been assessed as one of the environmental risk factor for PD cytotoxicity (161). In specific, it has been shown that learning ability of the rats declines by accumulation of α -synuclein and hyper-

phosphorylation of Tau due to lead (Pb) exposure, the effect that is mediated through ER-stressed induction of apoptosis (156). All together our results out of acute stress as well as longevity experiments are in accordance with previous studies by Bonilla-Ramirez et al. (94) that show that the chronic and acute exposure of Fe^{+2} , Mn^{+2} and Cu^{+2} are neurodegenerative in *D. melanogaster*. In their studies they have shown that manganese, exclusively, can induce parkinson's-like syndromes as well as decline in survival upon 15mM and 0,5mM Mn^{+2} exposure after 5 and 15 days respectively. Also our data advocate that manganese cytotoxicity is due to its accumulation in the brain since in the same study they have also measured the increase of manganese concentrations in their experimental fly brains. This confirms previous studies suggesting Malvolio (Mvl) protein to be the functional orthologue for the mammalian Divalent metal transport 1 (DMT1) and being able to transport the metal ions into the cells (162, 163). Of note the slightly difference between the survival of our data to theirs can be explained by different fly background (Canton-S vs W1118), exposure time and Mn^{+2} concentrations that they have used in their studies.

Indeed various studies yet have proposed that the cell's proteasomal system gets defected upon heavy ions exposure, the effect that could explain the synuclein intracellular and extracellular aggregation. For instance it has been shown that progressive spreading of LBs in mice model can have a role in progression of the molecular hall marks of PD. Lee et al. has described endocytosis of α -Synuclein protein that can traverse to the neighbouring cells and induce apoptosis in the recipient cell (155). Our results also clearly confirm that the accumulation is not limited within each cell but rather has pan regional effect, since the signal on the immunostaining pictures is homogenously spread out. That means the unfolded proteins, if not degraded, have a more general role in initiation of the death response within our model organism. In that regard, autophagy (as a subcellular digestion system) plays a pivotal role in maintaining the proteostasis of the cell hence deciding the life span of the whole organism (37). It has been shown that there is a tight connection between efficient turn-over of ubiquitinated waste within a cell and onset of neurodegeneration like PD, AD and HD (16, 43). Several mutations in various PD related genes including LRPK2, PINK1 and Parkin have been suggested to affect the clearance of ubiquitinated proteins (113,

158). Likewise, our immunoblotting with Mn^{+2} treated flies show that indeed the autophagy gets impaired upon accumulation of the α -synuclein protein. Moreover we could show that the effect of spermidine is paralleled with induction of autophagy and clearance of α -synuclein aggregates in the whole brain and hence increases the life span. In fact it's not surprising that 5mM spermidine is able to increase the life span of the wild type flies since previously it has been shown by Gupta et. al. (150). However it is for the first time that we were able to show (by adult fly brain immunostaining and immunoblotting) that spermidine at 5mM is able to clear the aggregation of α -synuclein through induction of autophagy in *D.melanogaster*.

Proper function of active zone proteins guarantee a balanced synaptic function and neurotransmitter trafficking (164-167). In effect, the neuronal plasticity and integrity guaranties the efficient and proper pre- and post-synaptic neurotransmitter release and is controlled by various proteins which are expressed uniformly through the brain structure of the flies (164, 165). Essentially, these proteins have been conserved through evolution and there is close homology between them (168). Bruchpilot (Brp) is among those proteins that assure the function of calcium channels within the active zone as well as efficient neurotransmitter release and is the functional homologue of the mammalian CAST protein (168, 169). The change of subcellular levels of Brp has been reported to be linked with accumulation of proteins that are involved in neurodegenerative diseases. For instance, it has been shown that the level of Brp decreases in flies using transgenic line UAS-A β arc (169). As a general fact, α -synuclein also assures the proper function of motor terminals and is involved in synaptic function and its overexpression leads to a marked decrease in neurotransmitter release (164, 165). Here we could show for the first time that upon expression of α -synuclein the level of Brp also decreases. Our results suggest that spermidine is able to restore the Brp reduction caused by expression of α -synuclein. As the matter of fact, colocalization of synuclein signal with the one from Brp affirms such asservation.

There is also a tight connection between active zone proteins and locomotion ability. Several studies have already shown that loss of dopaminergic

neurons as well as accumulation of Lewy bodies are responsible for locomotion disorder in flies (92, 144, 170-172). Büttner et al. (92) has previously proposed that expressing the human α - synuclein can lead to impairment of locomotion and a decline in survival in W1118 flies using pan neuronal Gal4- Nsyb driver. In fact, they have put mitochondria as the main route, showing Endonuclease G (Endo G) being required for α -synuclein toxicity along with locomotion defect. Anatomically, the mushroom body along with Kenyon cells within its axonal projection region are shown to have a more explicit role in controlling locomotion in *D. melanogaster* (173, 174). Mushroom bodies axonal routes form five different lobes (α , α' , β , β' , λ) that control the fate of the incoming neuronal messages to the flies' body motor neuron (170, 173). Accordingly, β' lobe has been shown to be the main part that controls the locomotion since its disruption leads to fly bradykinesia (170). Also 15 groups of dopaminergic neurons in anterior medial region of the fly brain are shown to be responsible for the locomotion ability (170). Our results uphold previous reports by showing that accumulation of α -synuclein within the whole brain structure of the fly along with loss of dopaminergic neurons is responsible for the marked locomotion decrease in our α -synuclein flies upon Mn^{+2} and its restoration of all mentioned molecular phenotypes. The presence of these finding in both young and old flies proposes a novel age- independent effect of synuclein toxicity on locomotion.

Summing up, our results show for the first time that supplementation of spermidine can rescue the death effect caused by expression of human α -synuclein in *D. melanogaster*. We showed that manganese treatment results in impairment of autophagy and decreased level of Bruchpilot protein (as a member of active zone proteins). Our results show that accumulation of α - synuclein protein is responsible for bradykinesia and decreased survival-rate and life span. These phenotypes can all be reverted by implementation of 5mM spermidine through an autophagy-dependent pathway.

4. Results:

HOCl induces apoptotic machinery in yeast

4.1 HOCl induces cell death, ROS production and specific protein modifications in *S. cerevisiae*

It has been shown before that the concentration of cytotoxic HOCl can reach up to 340 μM in physiological conditions (175). The first experiments were conducted and aimed for providing concentration dependent effect of HOCl on wild type yeast. Results revealed that concentrations between 60 to 340 μM can lead to an increase in the amount of ROS production and cause a decline in survival. As shown in **Figure 12** the major decrease in survival rate happens at concentrations between 250 and 310 μM and paralleled by a significant increase in ROS production at the same concentrations. By using two well know ROS scavengers, N-acetyl cysteine (NAC) and Glutathione (GSH), we were able to confirm that the source of cytotoxicity is due to production of ROS. Data show application of NAC at 5mM and GSH at 1 mM and 5 mM can fully block the ROS production and consequent cell death in HOCl treated wild type yeast comparing to untreated control (**Figure 13**). We also tried to elucidate the source of ROS production upon HOCl application at indicated concentrations. Using p0 strain that lacks mitochondrial DNA we could show that mitochondrion is the exclusive source of ROS production upon application of HOCl. **Figure 14** shows that by using the strain that lacks mitochondria functional DNA the ROS production and cell death are fully blocked.

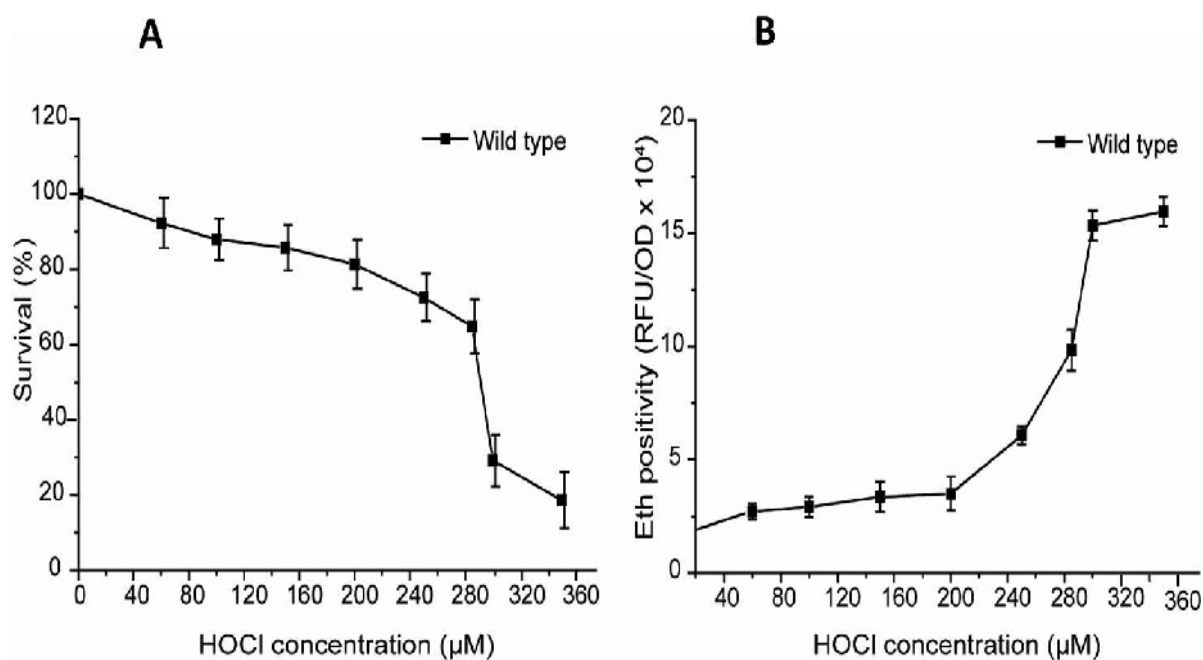


Figure - 12: Concentration-dependent cytotoxic effects of HOCl on *S. cerevisiae* in BY4741. Wild type cultures grown to exponential phase were treated with indicated HOCl concentrations and both (A) cell survival and (B) ethidiumfluorescence (Eth) was measured 16 h after exposure. Experiments in (A) were determined by clonogenic plating assay and values are given in % of untreated (control) cells. (B) Eth fluorescence (an indicator for ROS formation) was quantified using a fluorescence plate reader. Data represent means $\pm$ S.E.M. (n=8). RFU (Relative Fluorescence Unit); OD (Optical density).

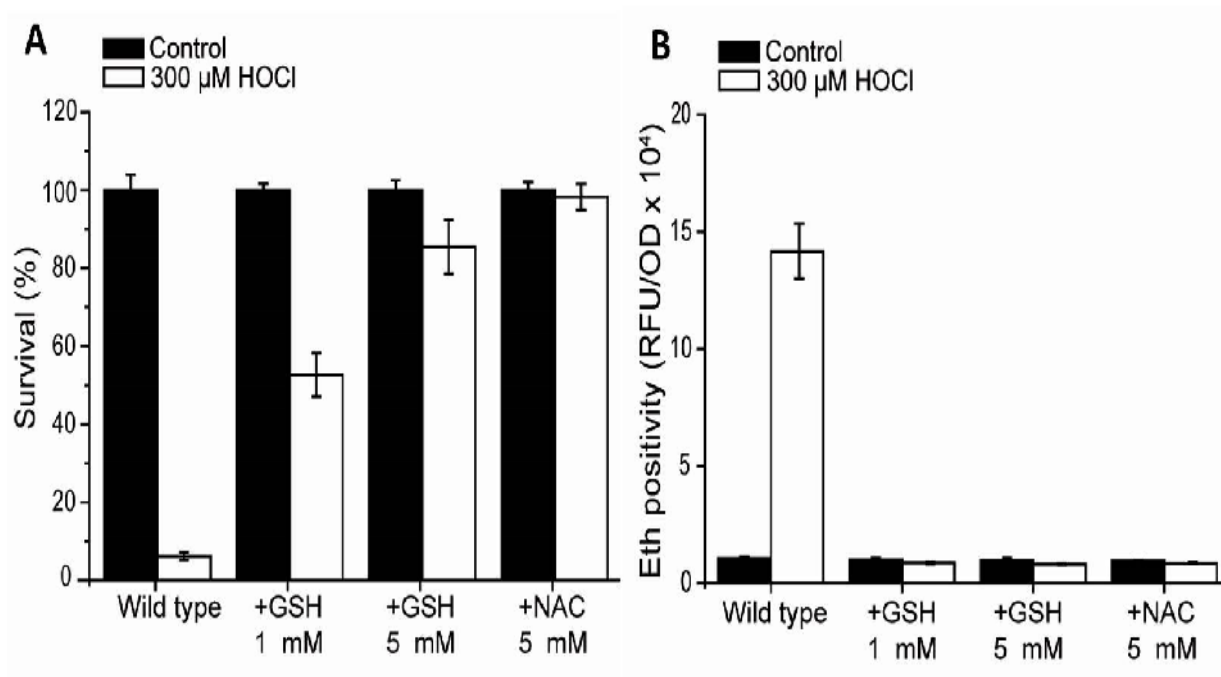


Figure - 13: Survival of the cells in the medium containing different ROS scavengers. Yeast wild-type (BY4741) cultures grown to exponential phase in the presence of L-glutathione (GSH) and N-acetyl-L-cysteine (NAC) at indicated concentrations and then treated with 300 μ M HOCl. (A) Cell survival was determined by cologenic plating assay. (B) Eth fluorescence was quantified using a fluorescence plate reader. Data represent means $\pm$ S.E.M. (n = 9). RFU, Relative Fluorescence Unit; OD (Optical density).

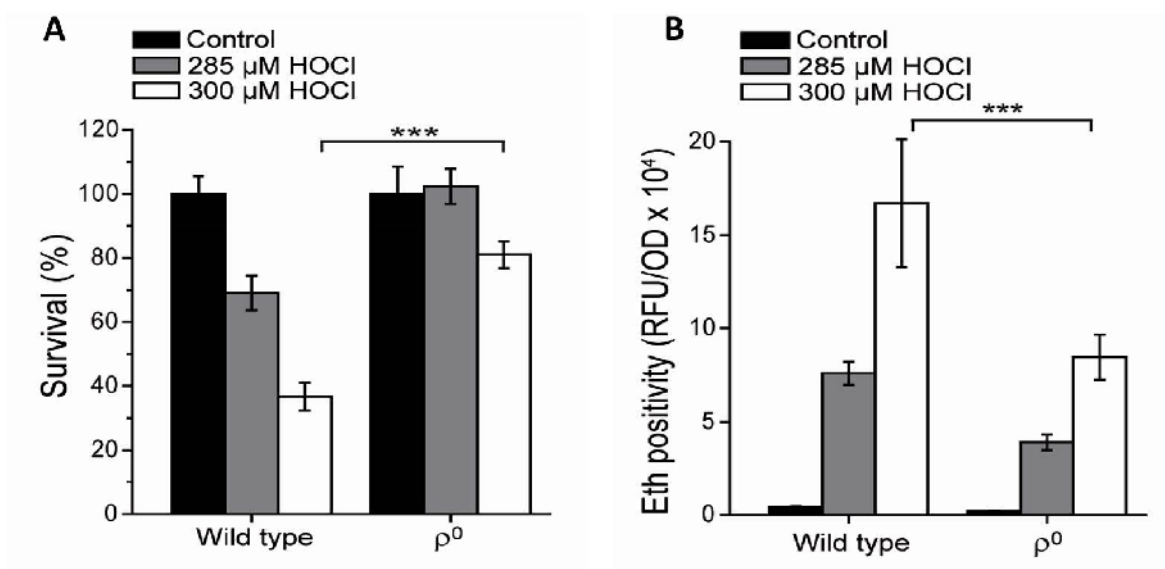


Figure - 14: Concentration-dependent cytotoxic effects of HOCl on *S. cerevisiae* and *rho0*. Wild type (BY4147) and *Rho0* (ρ^0 , lacking the mitochondrial DNA) were cultured and both (A) cell survival and (B) ethidium fluorescence (Eth) was measured 16 h after exposure to indicated HOCl concentrations. Experiments in (A) were determined by clonogenic plating assay and values are given in % of untreated (control) cells. (B) Eth fluorescence was quantified using a fluorescence plate reader. Data represent means $\pm$ S.E.M. (n=9; *** p<0.001). RFU (Relative Fluorescence Unit); OD (Optical density).

4.2 HOCl induces cell death through specific protein modifications that is followed by apoptosis in *S. cerevisiae*

It has been shown that protein modification can lead to apoptosis and cell death in heart tissues and be the major source of cardiovascular complications. Hence, studying the mechanism by which proteins gets modified is of a great magnitude. By performing immunocytochemistry using a specific mAb (148) that specifically recognize HOCl modified epitopes we could reveal for the first time that HOCl at indicated concentrations leads to an increase in modified protein epitope (Figure 19 B and C). These data show, for the first time, that HOCl has target proteins in yeast. To be able to shed more light on the mechanisms underlying the cytotoxic effect of HOCl we used Annex V/PI co-staining. These special assays could finally show us the amount of the cells that undergo

apoptosis (Annexin V positive cells) and the amount that are dying through necrotic death (PI positive cells). Results revealed that the main population of the cells are apoptotic with a marginal increase in the amount of necrotic cells (**Figure 15 C**). The apoptotic death scenario is being confirmed by using TUNEL test that revealed a significant rise of DNA fragmentation as the marker of apoptosis (**Figure 15 D**). We then could conclude that HOCl at highest concentration implicated its cytotoxicity mainly through apoptosis with marginal necrotic events that both are paralleled with DNA fragmentation.

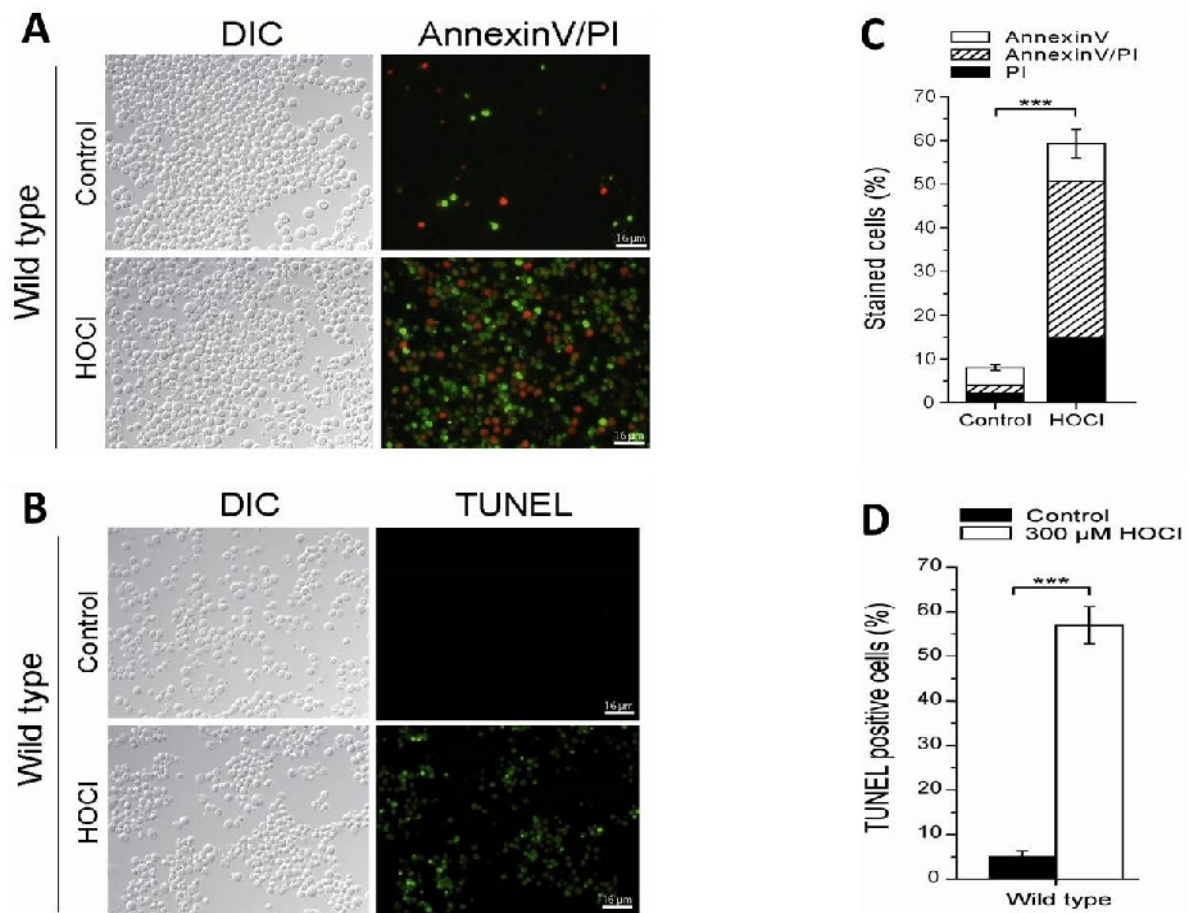


Figure - 15: HOCl induces cytotoxicity mainly through apoptosis and results in DNA fragmentation in *S. cerevisiae*. (A/B) Microscopic images and (C/D) the respective quantitative analyses using AnnexinV/PI co-staining (green = Annexin V-positive cells; red = PI-positive cells) and flow cytometry (green = TUNEL staining) of wild type cells (BY4741) in the absence (control) or presence of HOCl (300μM, 16 h). In each experiment 30,000 cells were evaluated using flow cytometry. Data represent means $\pm$ S.E.M. (n=6;*** p<0.001). DIC (Differential Interference Contrast). Scale bar represents 16 μm.

4.3 Deletion of the protease KEX1 but not of other lethal hydrolases partly reverts HOCl-induced cytotoxicity

It has been a fact that HOCl acts through production of ROS and inducing cell death (176). To gain further insights into the molecular regulators accounting for the execution of HOCl-induced cytotoxicity, we decided to determine loss of viability and ROS production levels in the deletion background of known apoptotic effectors, including the yeast metacaspase YCA1 (124), the pro-apoptotic proteases NMA111 and KEX1 (177, 178), the yeast homologues of the human death regulators cathepsin D and calpain (PEP4 and CPL1) (179), the mitochondrial nuclease NUC1 (180), the yeast BAX-homolog YBH3 (181) and the pro-apoptotic oxidoreductases NDI1 and AIF1 (182, 183). Upon HOCl treatment, all yeast cell disruptants showed either no changes or even reduced cell viability in comparison to wild type cells (**Figure 16A**). Furthermore, ROS levels were either unchanged or even increased when compared to wild type cells (**Figure 16B**). Only the deletion of Kex1 led to increased survival and decreased oxidative stress when compared to wild type and other yeast cell mutants studied here (**Figure 17 A-C**). From these studies (**Figure 16 and 17**) we conclude that Kex1p (but not other herein tested hydrolases) is required to execute the full cytotoxic activity of HOCl, as described by Carmona-Gutierrez et. al. (56)

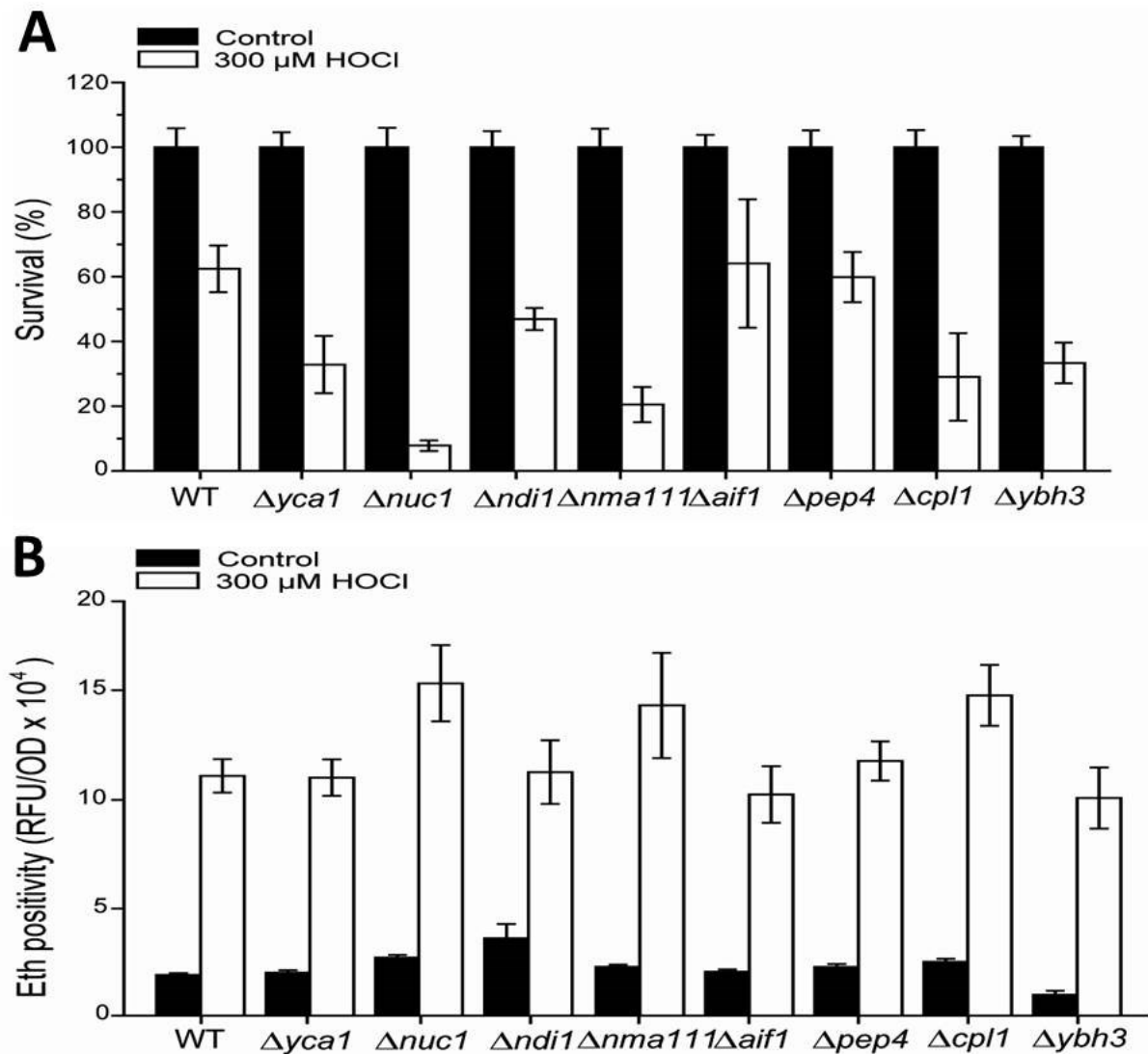


Figure - 16: Deepwell-Mini Screens for cell survival and ROS formation in various *S. cerevisiae* yeast mutants in response to HOCl-mediated oxidative stress. (A) Cell survival and (B) ethidiumfluorescence (Eth) of wild type cells (WT, BY4741) and indicated mutants ($\Delta yca1$, $\Delta nuc1$, $\Delta ndi1$, $\Delta nma111$, $\Delta aif1$, $\Delta pep4$, $\Delta cpl1$, and $\Delta ybh3$ all derived from BY4741 wild type cells) was measured in the absence (control) or presence of 300 μ M HOCl for 16 h. Experiments in (A) were determined by clonogenic plating assay and values are given in % of untreated (control) cells. (B) Eth fluorescence was quantified using a fluorescence plate reader. Data represent means $\pm$ S.E.M. (n=12). RFU (Relative Fluorescence Unit); OD (Optical density).

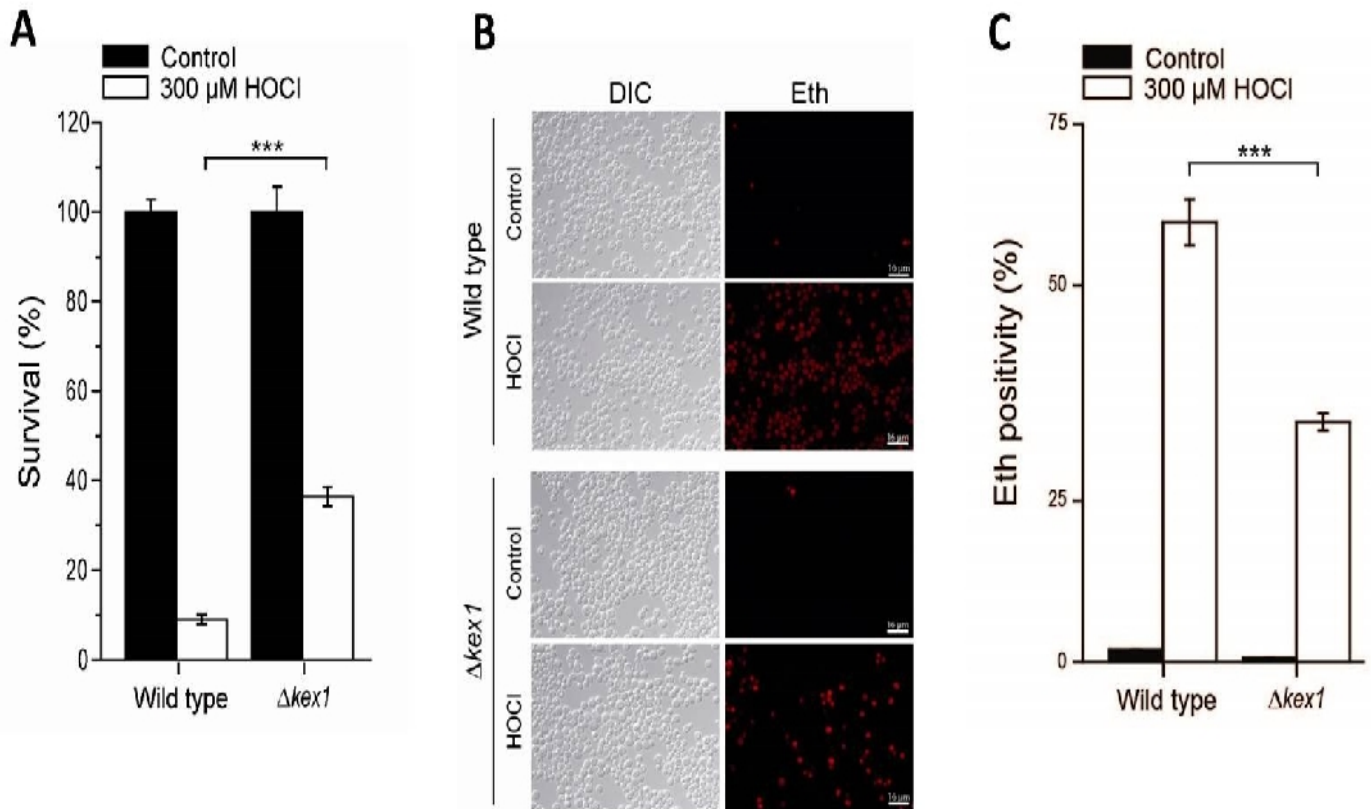


Figure - 17: Kex1p is a lethal factor in HOCl-induced cell death in *S. cerevisiae*. (A) Cell survival, (B) fluorescence microscopic images for ROS generation and (C) quantitative estimation of Eth fluorescence of wild type (BY4147) and Δ kex1 cells in the absence (control) or presence of 300 μ M HOCl for 16 h. Experiments in (A) were determined by clonogenic plating assay and values are given in % of untreated (control) cells. (B) Immunofluorescence staining (IF positivity) was measured after incubation of cells with mAb clone 2D10G9 as a primary antibody and cyanine-3-labelled goat anti-mouse IgG (red). DIC (Differential Interference Contrast); Scale bar represents 16 μ m. (C) Eth fluorescence was quantified using a flow cytometer and data are expressed as percentage of unstained cells. Data in (A) and (C) represent means $\pm$ S.E.M. (n=20; *** p<0.001).

4.4 KEX1-disruption diminishes apoptosis formation of HOCl-modified epitopes in response to HOCl-induced cell stress

It has been previously shown that Kex1p can be involved in other death scenarios (184). As described by Carmona-Gutierrez et. al. (56), here we could show that in Δ kex1 reduction of cell death and ROS generation (**Figure 17**) was accompanied with a specific and quantitatively similar decrease of the late apoptotic population as determined via Annexin V/PI co-staining (**Figure 18A**). This was confirmed by TUNEL staining, which showed a corresponding decline of

apoptotic DNA fragmentation in $\Delta kex1$ compared to the wild type cells when stressed with HOCl (**Figure 18B**). It should be noted that [as described in Carmona-Gutierrez et. al. (56)] since HOCl reacts with the components of the medium readily, similar results were produced from the same badge of liquid culture. Using immunochemical techniques we tested whether *kex-1* disruption is paralleled by altered protein oxidation during HOCl treatment. Using a specific mAb (148) immunocytochemistry revealed decreased staining for HOCl-modified epitopes in $\Delta kex1$ cells compared to wild type cells (**Figure 19 A/B**).

Preliminary data from immunoblot analyses further revealed that the extent of immunoreactive proteins present in total cell protein lysates is the same as in wild type and $\Delta kex1$ cells both in untreated and HOCl-stressed (**Figure 19C**).

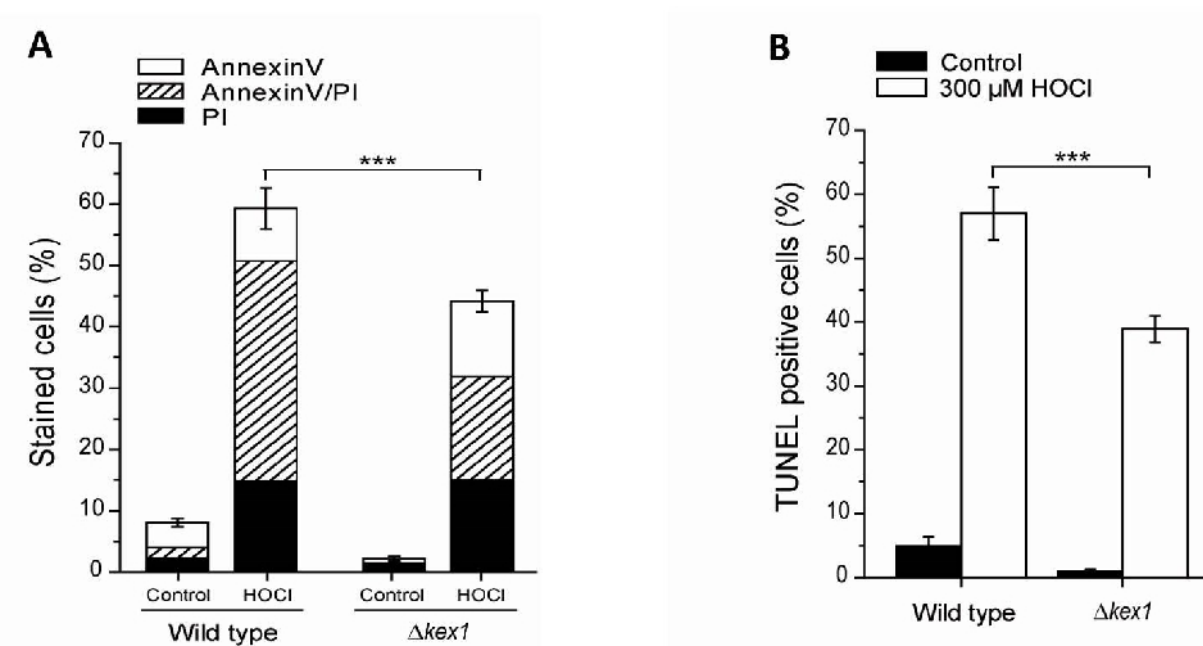


Figure - 18: Apoptosis induced by HOCl is relieved in $\Delta kex1$. (A) Phosphatidylserine externalization and loss of membrane integrity determined by Annexin V/PI costaining and (B) apoptotic DNA fragmentation using TUNEL staining of wild type (BY4147) and $\Delta kex1$ cells in the absence (control) or presence of 300 μ M HOCl for 16 h. In each experiment 30,000 cells were evaluated using flow cytometry. Values (given as % of unstained cells). Data represent means $\pm$ S.E.M. (n=4; *** p<0.001).

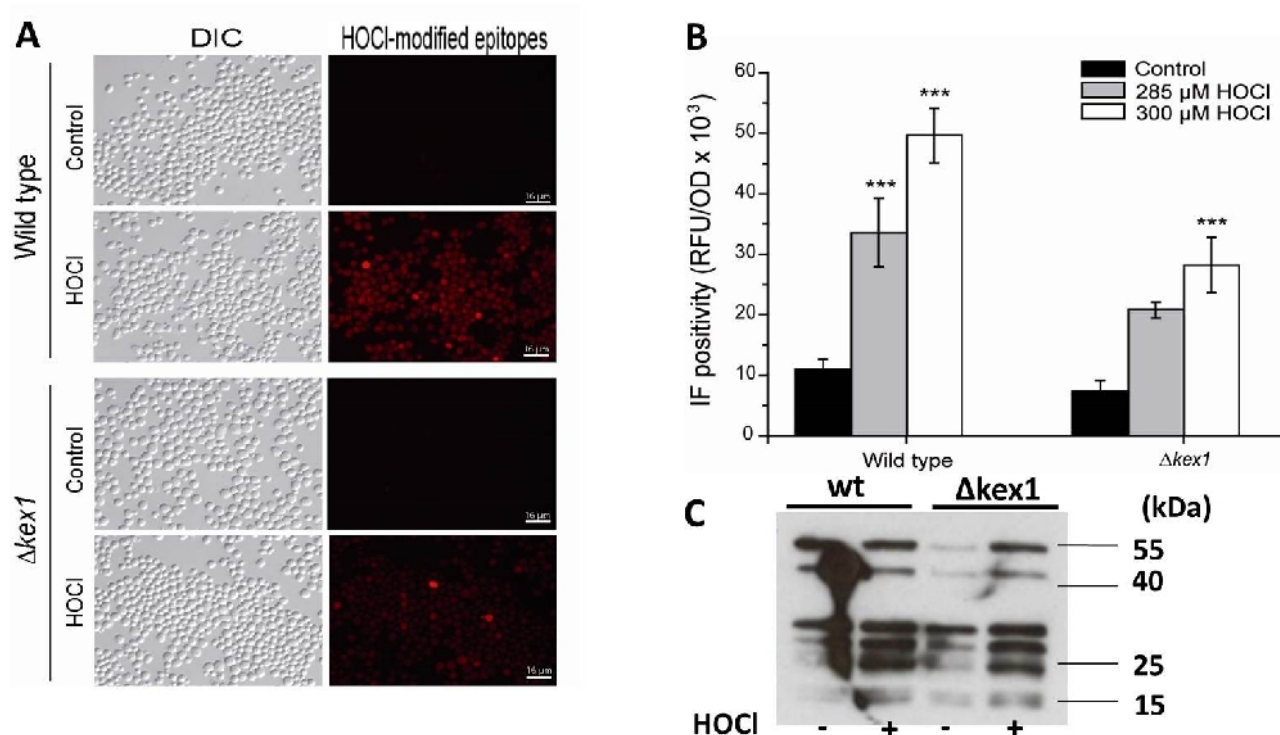


Figure - 19: Deletion of *kex1p* diminishes the extent of HOCl-modified epitopes in *S. cerevisiae*. Yeast wild type (BY4741) and $\Delta kex1$ cells were cultured in the absence (control) or presence of 300 μ M HOCl (A and C) or indicated HOCl concentrations (B) for 16 h. (A) One representative fluorescence microscopy image using mAb 2D10G9 (red) as a primary antibody is shown. RFU (Relative Fluorescence Unit); DIC (Differential Interference Contrast); Scale bar represents 16 μ m. (B) Quantitative estimation of immunofluorescence staining (IF positivity) after incubation of cells with mAb 2D10G9. Data were estimated using a fluorescence plate reader. Data represent means $\pm$ S.E.M. (n = 20; *** p<0.001). (C) Immunoblots of whole cell protein lysates of wild type (WT) and $\Delta kex1$ cells. Immunoreactive bands were visualized using mAb 2D10G9, peroxidase conjugates horse-radish peroxidase-conjugated secondary antibody and ECL development. Molecular mass markers are given in kDa.

4.5 HOCl-induced cell death is accompanied by an increase in cathepsin S and TNF α transcription and translation in *HL-1* cardiomyocytes

Since the alteration of different cathepsins in response to toxic HOCl have been described and the fact that cathepsin A is the mammalian homologue of yeast-kex1p, experiments were carried out in *HL-1* cardiomyocytes to see if HOCl can activate the same pathway. To do that, the first few experiments were conducted to find the proper concentrations that are not so toxic but still able to induce death pathway in cardiomyocyte (**Figure 20**). Results revealed that HOCl (at 300 and 500 μ M) is able to induce 1.5- and 3-fold up-regulation in TNF α expression (**Figure 21**). The same concentrations shown to be able to induce a 2- and 3-fold increase in cathepsin S translation while there is slightly a petit effect for cathepsin A and cathepsin L (**Figure 21**).

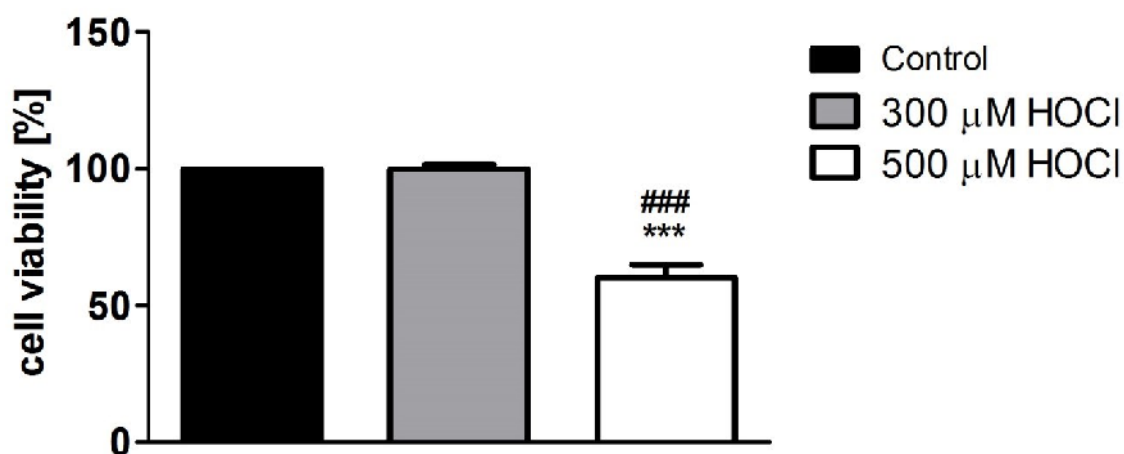


Figure - 20: HOCl induces its' cytotoxicity at higher concentrations in *HL-1* cardiomyocytes. Cell viability was measured following incubating cells with MTT (0.5 mg/ml) for 30 min after treatment with HOCl. Optical density levels of vehicle (0.1% DMSO)-treated cells were set 100% and values are expressed as mean $\pm$ S.E.M. (n = 3; *** p<0.001 vs untreated and ### p<0.001 vs 300 μ M HOCl).

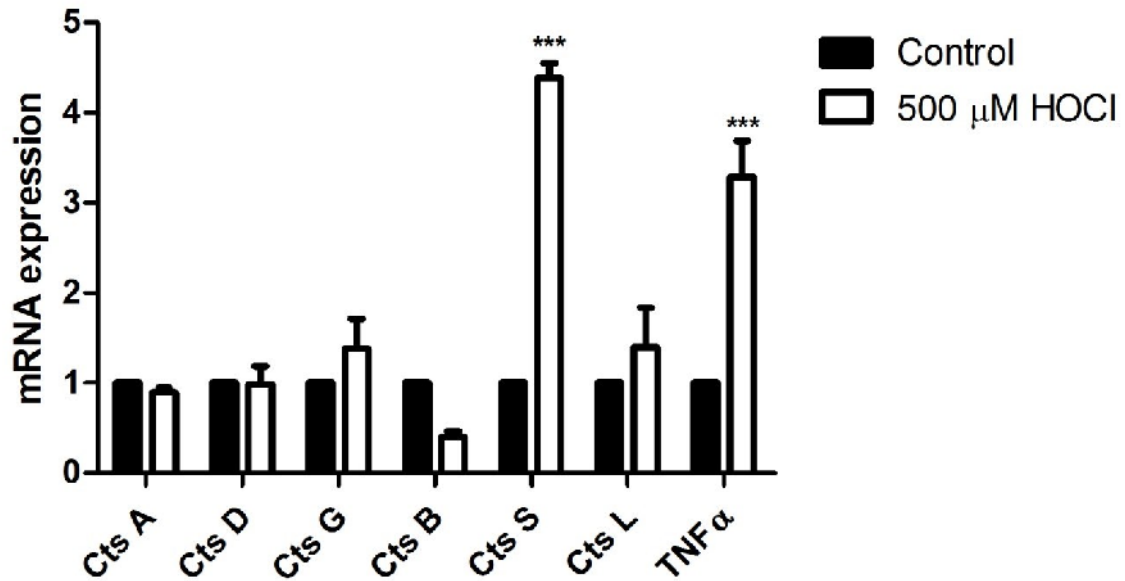


Figure - 21: HOCl increases cathepsin S and TNF α transcription and translation in *HL-1* cardiomyocytes. HL-1 cells were incubated with HOCl (300 and 500 μ M) for 12h to follow cathepsin A, D, G, B, S, L and TNF α mRNA expression by qPCR. After treatment with HOCl the cell culture medium was removed and cells were lysed to isolate RNA for qPCR analysis using specific primers. Data represent means $\pm$ S.E.M. (n = 3-5; *** p<0.001 vs. untreated control).

4.6 HOCl can perturb intercellular calcium channel orchestra in *HL-1* cardiomyocytes

Calcium homeostasis plays an important role in regulating the proper function of the heart. The role of myeloperoxidase-derived oxidants in altering the equilibrium between cytoplasmic and endoplasmic reticulum calcium level have been implicated. However, since the interaction between apoptotic proteins and these calcium channels e.g. SERCA (185) has been suggested, we aimed to check how HOCl acts in that manner. As previously tested (**Figure 20**) using slightly less toxic 300 and 500 μ M HOCl there is a 2-fold increase in *Cacna1h* expression upon applying both concentrations while the other regulators of calcium-level including SERCA2a, RYR2, and NCX1 are slightly down regulated (**Figure 22**).

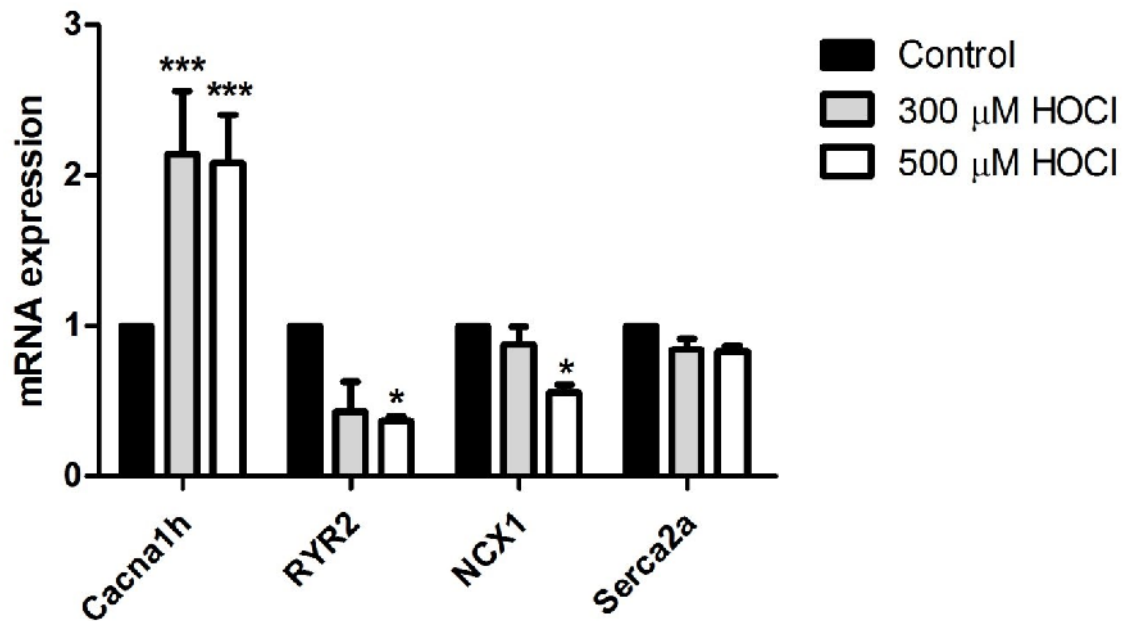


Figure - 22: Perturbed transcription and translation of calcium channels in HOCl treated *HL-1* cardiomyocytes. HL-1 cells were incubated with HOCl (300 and 500 μ M) for 12h to follow SERCA2a, RYR2, Cacna1h and NCX1 expression by qPCR. After treatment with HOCl the cell culture medium was removed and cells were lysed to isolate RNA for qPCR analysis using specific primers. Data represent means $\pm$ S.E.M. (n = 3-5; * $p < 0.05$ and *** $p < 0.001$ vs. untreated control).

4.7 Discussion

Studies performed with activated neutrophils as well as the cell-free myeloperoxidase-hydrogen peroxide-chloride system provided initial evidence for the cytotoxicity of HOCl in mammalian cells (176). Both, inhibition of myeloperoxidase as well as scavenging of HOCl, added as reagent or generated by the myeloperoxidase-hydrogen peroxide-chloride system, confirmed HOCl responsible for impaired cell viability and cell death in various mammalian cells (176, 186-190). HOCl may induce necrosis and/or apoptosis in a time- and concentration-dependent manner (191, 192) apparently via formation and decay of chloramines and generation of radical intermediates (193, 194). The various pathways involved in HOCl-mediated cell death include up-regulation of apoptotic genes (195), down regulation of anti-apoptotic proteins (188), activation of caspases (190), alterations of intracellular glutathione levels and signalling cascades as well as DNA strand breakage (196). Furthermore, in bacteria exposed to HOCl the patterns of metabolic dysfunction observed suggest mechanisms where death occurs by interruption of the energy-transducing capabilities of the cell by inhibiting plasma membrane-localized proteins (85).

In addition to the cellular systems, *S. cerevisiae*, a eukaryotic unicellular fungus has turned out an important model organism to study apoptosis (197) in response to environmental and chlorinative stress (198, 199). HOCl has been reported to elicit cyto- and/or genotoxic effects against *S. cerevisiae* (81, 85, 199) thus allowing to investigate antifungal activities of HOCl. Further studies have shown that toxicity of HOCl in wild type yeast strains was abolished by hypoxic and anoxic conditions and that anti-oxidant deficient *S. cerevisiae* strains were more sensitive to treatment with oxylchloro compounds including HOCl (81).

The herein described results characterize the cell death process occurring in the budding yeast *S. cerevisiae* upon exposure to lethal doses of HOCl, perhaps the most toxic of the potential phagocyte-generated oxidants commonly acting as antimicrobial agent. In contrast to earlier studies (81), we did not monitor growth upon challenge with HOCl, where decreased growth rates observed may be due to growth arrest and/or cell death. Instead, we determined actual survival rates, establishing a concentration-dependent deadly effect of HOCl on yeast. This

cytotoxicity is accompanied by increased levels of ROS confirming previous reports that show elevated intracellular oxidative stress to be causative and the endogenous antioxidant system to be preventive during HOCl toxicity (81). It is likely that the sources of these ROS are mitochondria, since cells devoid of mitochondrial DNA (ρ^0) are protected against HOCl-inflicted ROS production and cell death.

All together the results, out of AnnexinV/PI and TUNEL staining, could suggest that the death event caused by HOCl is mainly through apoptosis. Of note, this results is not in accordance with a previous study (81) that shows necrotic cell death is the main cellular death pathway followed by HOCl application. In fact, this discrepancy might be due to different reasons. First of all, our experiments are all performed in SMD medium that differs from the complete medium in composition. As HOCl can react with all the components of the medium, this different compositions of medium as well as different concentration that we have used could explain the different results. Secondly, our experiments include staining with both Annexin V and PI. This enables us to differ from the specific death scenario that results with application of HOCl. In fact the above mentioned study lacks the highly sensitive assay to define different death pathways that can underline the effect of HOCl at these high concentrations.

Indeed, exposure to increasing doses of HOCl in endothelial cells induces cell death that progressively shifts from apoptosis to necrosis (190). Similarly, many yeast cell death inducers known to cause apoptosis at moderate concentrations promote a necrotic phenotype when applied at higher doses (200). The mere concentration increase of typical apoptotic triggers may, in most but not all cases, promote an unregulated type of necrosis that likely arises from uncontrolled damage of cellular integrity. Thus, to test whether the necrotic phenotype displayed upon the high toxic HOCl dosage used (81) represents such accidental or rather programmed necrosis, experiments addressing typical markers of programmed necrosis could be tested under their conditions. Irrespective of this discrepancy that may have multi-factorial causes, HOCl cytotoxicity in *S. cerevisiae* induced in our hands is mainly linked to an apoptotic phenotype.

Moreover, It has been shown in bacterial model that inactivation of all subunits of membrane ATPase synthase leads to change of electrophoretic properties and hence cellular inactivation (83). This could be the first evidence suggesting plasma membrane-localized proteins being affected by HOCl directly or indirectly. In fact since proteins on the plasma membrane can be involved in more complex functions of a cell (e.g. receptors, etc.), it seems that the effect of HOCl on those proteins was always more interesting in model organisms from bacteria to yeast and mammalian model (56, 83, 85). (85). To address the possible pathways in HOCl toxicity, we tested well-known deletion mutants that can be involved in any protein modification (e.g. Ybh3p) (**see table 2**). Our results revealed that (between all mutants) *kex1p*, a protease involved in glycosylation protein modification in yeast, is a key mediator in HOCl-mediated yeast apoptosis as apoptotic cell death induced by HOCl is diminished in a $\Delta kex1$ mutant strain. In general, *kex1p* is also involved in encoding a serine-carboxypeptidase that ultimately involves in processing and maturation of the various proteins to the plasma membrane including phosphatidylserine, a process that also can be a part of glycosylation. Glycosylation is one of the various ways that cells possess for executing proper protein modification. It has been shown that HOCl reacts more readily with glycosylated proteins (e.g. LDL) (56) leading to formation of misfolded proteins and/or AGE products hence leading to cell death. On the other hand, it is well known that upon apoptosis phosphatidylserine is exposed to the outer leaflet of plasma membrane. Taking all of this into account, a mechanism can be proposed for HOCl toxicity describing the maturation phosphatidylserine by *kex1p* a target for HOCl reactivity leading to production and hence triggering cell (**Figure 23**). In fact, we could show that in the absence of *Kex1p* there are relatively less modified proteins by HOCl that might potentially lead to cell death.

Also it has been discussed before that Ca^{+2} receptors or channels could be involved in HOCl toxicity in mammalian model (191, 201). To address that we used *HL-1 cardiomyocytes* and we could reveal that HOCl is able to alter the expression of calcium channels including ryanodine receptor 2 (Ryr2) and voltage-dependent channel (*Cacna1h*). Since *kex1p* closest homologue in mammalian system is cathepsin A (CtsA), we also tested the expression of different cathepsins after using HOCl at indicated concentrations. Results revealed that, between all, only the expressions of CtsS along with TNF α are being significantly altered.

However, the exact mechanism should be investigated more to see whether inhibition of CtsS could alter HOCl toxicity on cardiomyocyte.

Altogether, this study establishes the PCD mode in yeast upon challenge with HOCl and unveils *kex1p* as a molecular executor in this process. Thus, it provides insight into the mechanisms underlying the microbicidal effects of HOCl towards fungal infections. However, these mechanisms might not correspond to those elicited upon HOCl-induced killing of prokaryotes. In fact, based on the patterns of metabolic dysfunction elicited by HOCl in bacteria, which involve processes that differ from eukaryotic cells like energy transduction or glucose transport, it has been previously questioned whether the microbicidal mechanisms of HOCl toxicity in *S. cerevisiae* parallels that observed in bacteria (85). Nevertheless, it has been recently shown that under certain circumstances bacteria can succumb to an apoptotic-like cell death (85, 202). To which extent this or other forms of PCD might be involved in the bactericidal action of HOCl remains to be investigated.

5. Conclusion and Outlook

It is a general fact that many factors including environmental stress, exogenous substances, mutations, etc. can lead to misfolding of the nascent proteins (1-3). The cellular system has developed its own way to fight that situation. Those pathways include refolding of those proteins or degradation of them through autophagy. If other ways do not work (in the last step) committing suicidal apoptosis to prevent damage to neighboring cells (2, 4). Both HOCl and metal substances including Mn^{+2} are shown to be able to affect the cellular proteostasis that result in ER stress and development of cardiovascular complications and neurodegeneration (3, 5).

In chapter 3 we were able to show that Mn^{+2} supplementation leads to α -synuclein accumulation through autophagy dependent pathway. We were able to show that spermidine can successfully activate autophagic pathway, confirmed with immunoblotting that leads to restoration of survival rate, life span and locomotion ability of parkinsonism-like-syndromes caused by α -synuclein accumulation in *D. melanogaster*. Immunostaining of whole brain suggests that the level of Brp (an active zone protein) decreases upon α -synuclein expression and accumulation and restores upon spermidine treatment.

In our work (chapter 4), we could show for the first time that HOCl has target protein epitopes in yeast that are modified by HOCl and result in apoptosis. We identified kex1p as the essential protease in HOCl-induced toxicity. It is known that HOCl reacts more readily with glycosylated proteins (e.g. LDL) leading to formation of misfolded proteins and/or AGE products hence leading to cell death (6). On the other hand, it is a general fact that upon apoptosis phosphatidylserine gets exposed to the outer leaflet of plasma membrane. Little was known about the reaction of HOCl with phosphatidylserine until it was shown that it is involved in production of short-lived chloramine (NH_2Cl) leading to apoptosis and cell death. Taking all of this into account, we could suggest a mechanism for HOCl toxicity that proposes the maturation of phosphatidylserine by kex1p as a good target for HOCl reaction leading to production of misfolded proteins and hence triggering cell death (**Figure 23**).

In conclusion, we could shed more light into new cellular pathways that modify cell death in different model organism. We have practically shown ways to activate those responses. We also could successfully introduce budding yeast and fruit fly as valid means to explore human HOCl-associated cellular demise and neurodegenerative diseases as the present study suggests.

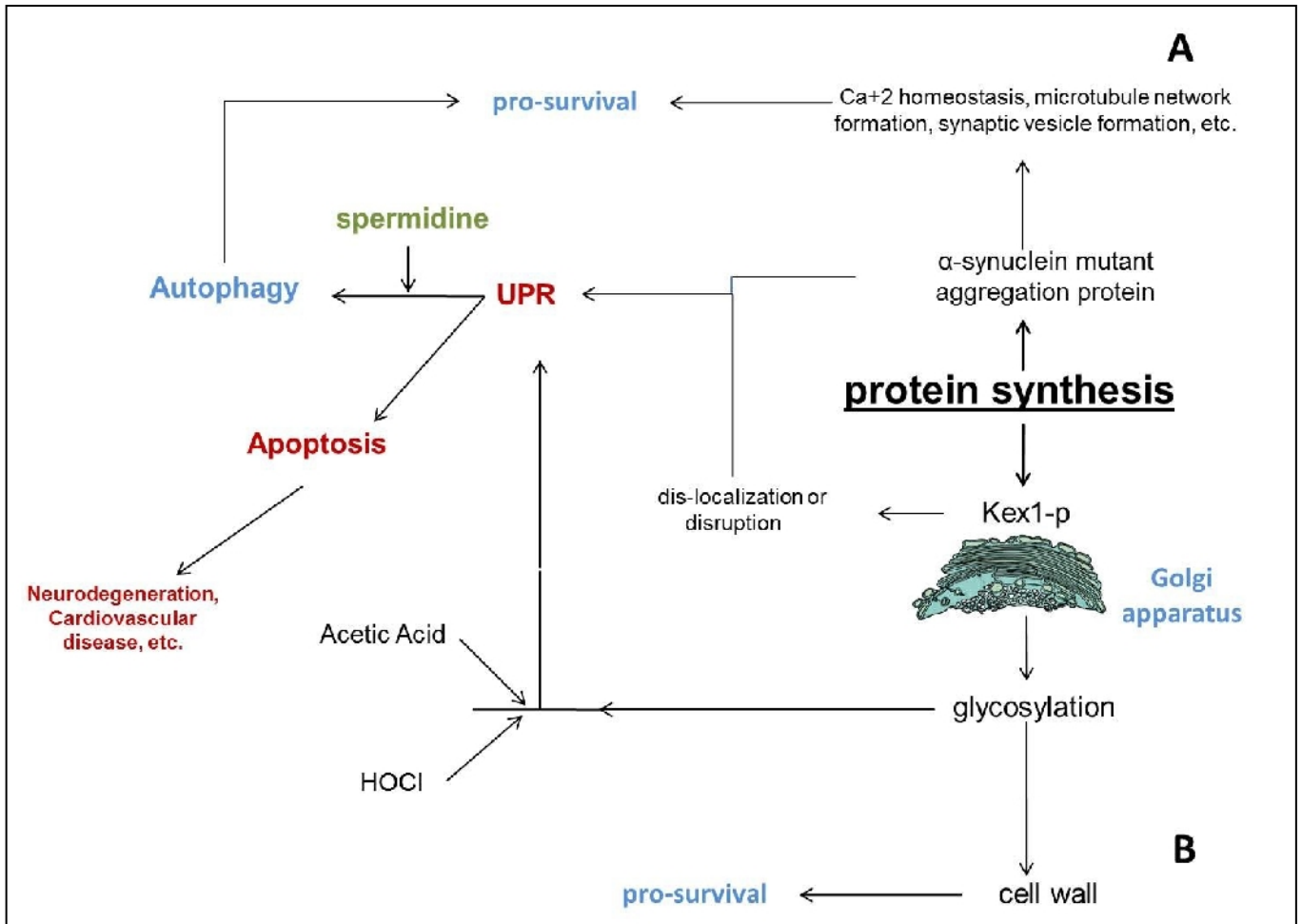


Figure - 23: The critical role of protein folding in the onset of different diseases. (A) The glycosylated by-products of kex1p are involved in cell wall assembly and integrity. However, overproduction of those proteins that are also prone to get modified by highly reactive hypochlorous acid play a major role in initiation of PCD that leads to cardiovascular diseases. (B) Normal levels of α -synuclein, which is tightly regulated by autophagy pathway, plays an important role in neuronal plasticity that upon aggregation leads to onset of PD and PD- related symptoms.

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7. Appendix

The cell death protease Kex1p is essential for hypochlorite-induced apoptosis in yeast

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The cell death protease Kex1p is essential for hypochlorite-induced apoptosis in yeast

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Abbreviations: CFU, colony-forming units; DHE, dihydroethidium; Eth, ethidium; FITC, fluorescein isothiocyanate; HOCl/OCl⁻, hypochlorous acid/hypochlorite; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species; *S. cerevisiae*, *Saccharomyces cerevisiae*

Following microbial pathogen invasion, the human immune system of activated phagocytes generates and releases the potent oxidant hypochlorous acid (HOCl), which contributes to the killing of menacing microorganisms. Though tightly controlled, HOCl generation by the myeloperoxidase-hydrogen peroxide-chloride system of neutrophils/monocytes may occur in excess and lead to tissue damage. It is thus of marked importance to delineate the molecular pathways underlying HOCl cytotoxicity in both microbial and human cells. Here, we show that HOCl induces the generation of reactive oxygen species (ROS), apoptotic cell death and the formation of specific HOCl-modified epitopes in the budding yeast *Saccharomyces cerevisiae*. Interestingly, HOCl cytotoxicity can be prevented by treatment with ROS scavengers, suggesting oxidative stress to mediate the lethal effect. The executing pathway involves the pro-apoptotic protease Kex1p, since its absence diminishes HOCl-induced production of ROS, apoptosis and protein modification. By characterizing HOCl-induced cell death in yeast and identifying a corresponding central executor, these results pave the way for the use of *Saccharomyces cerevisiae* in HOCl research, not least given that it combines both being a microorganism as well as a model for programmed cell death in higher eukaryotes.

Introduction

During inflammation, the activation of phagocytes both in vivo and in vitro can result in the generation and release of superoxide anion radical and hydrogen peroxide (respiratory burst) as well as in the secretion of myeloperoxidase. Myeloperoxidase, which is highly expressed in neutrophils, reacts with hydrogen peroxide in the presence of physiological chloride concentrations, catalyzing the formation of hypochlorous acid/hypochlorite (HOCl/OCl⁻). This short-lived and diffusible oxidant plays a major role as a potent microbicidal agent in the immune defense against invading microbial pathogens.¹ Upon phagocyte activation, HOCl concentrations can increase at least up to 340 μM.² However, excessive or permanent HOCl production may cause tissue damage, since HOCl can participate in secondary non-enzymatic reactions, including oxidation and/or chlorination. HOCl can react with a variety of target compounds, for instance with unsaturated fatty acids to form chlorohydrins or with amines resulting in reactive chloramines, which, in turn,

are powerful oxidants.³ Of note, lysine-derived chloramines can generate nitrogen- and, subsequently, carbon-centered radicals, which may amplify the formation of oxygen radicals.⁴ HOCl-modified target compounds are present during early and acute stages of inflammation.^{1,5,6} This suggests an implication of the myeloperoxidase-HOCl pathway in the pathogenesis of various inflammatory diseases, such as atherosclerosis or coronary heart disease.⁷ Thereby, a number of studies have reported that HOCl may activate the apoptotic machinery of cells involved in the initiation and progression of disease.^{8–10}

Establishing the mode(s) of action underlying HOCl-mediated cytotoxicity is thus important for two distinct reasons: (1) the mechanistic disclosure of HOCl's microbicidal effects and (2) the understanding of deleterious HOCl-modifications in human proteins. In order to examine the lethal mechanism(s) triggered by HOCl, we used the budding yeast *Saccharomyces cerevisiae*. On the one hand, HOCl has been previously reported to elicit cytotoxic effects against *S. cerevisiae*,^{11–13} thus allowing to investigate antifungal HOCl activities. On the other hand,

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S. cerevisiae also represents a well-established model organism to explore the core machinery executing programmed cell death (PCD), which, over the past decade, has been demonstrated to be phylogenetically conserved.^{14–16} In fact, the yeast genome encodes orthologs of crucial mammalian apoptosis and necrosis regulators, including one caspase (Yca1p), the serine protease OMI (Nma111p), cathepsin D (Pep4p), endonuclease G (Nuc1p) or BH3-only proteins (Ybh3p).^{17–22} Additionally, yeast PCD is connected to complex PCD features reminiscent of mammalian cells, such as mitochondrial fragmentation, mitochondrial outer membrane permeabilization or histone H2B phosphorylation.^{23–25} Furthermore, *S. cerevisiae* has been successfully used to explore cell death connections to, e.g., lipids or the cell cycle, as well as to investigate lethal pathways underlying pharmacologically relevant agents such as the anticancer drug cisplatin.^{26–30} Of note, two physiological scenarios deriving in yeast PCD, chronological and replicative aging, have been effectively applied to investigate phylogenetically conserved aging pathways of postmitotic mammalian cells and stem cells, respectively.^{31–33} For instance, the naturally occurring polyamine spermidine has been shown to exert anti-aging effects that share a common mechanism in yeast, flies and nematodes.^{34,35} Interestingly, recent evidence suggests that also the broader functional realm of spermidine (e.g., which includes, e.g., roles in reproduction) can be modeled in budding yeast.^{36,37} Altogether, this functional correspondence allows using *S. cerevisiae* to analyze lethal pathways and scenarios of higher eukaryotes including those elicited in mammalian cells.

In this study, we aimed at characterizing the cytotoxic effects of HOCl in *S. cerevisiae* and at identifying corresponding molecular executors. We show that HOCl-mediated cell death in *S. cerevisiae* is mainly of apoptotic origin and results in elevated levels of reactive oxygen species (ROS) and formation of HOCl-modified proteins. Thereby, the protease Kex1p appears to mediate HOCl-induced lethality, since its absence enhances cell viability and decreases levels of ROS as well as formation of HOCl-modified epitopes upon challenge with lethal doses of HOCl.

Results

HOCl induces ROS-mediated cell death. HOCl can exert cytotoxicity on both microbial and human cells. In order to explore both scenarios, we assessed the impact of HOCl on the budding yeast *S. cerevisiae*, which combines both being a microorganism and an established eukaryotic cell death model. Application of submillimolar concentrations of HOCl to exponentially growing yeast cells resulted in a significant and dose-dependent decrease of clonogenic survival (Fig. 1A). At 285 and 300 μM HOCl, survival rates were 30 and 60% lower compared with the untreated controls (Fig. 1A). This concentration range is similar to that calculated at sites of acute inflammation, where HOCl levels can increase up to 340 μM or even higher.² The determined loss of survival was paralleled by an increased production of ROS (Fig. 1B), as indicated by the enhanced superoxide-driven conversion of non-fluorescent dihydroethidium (DHE) into fluorescent ethidium (Eth). Conversely, the pre-treatment of yeast cells with the ROS-scavengers glutathione and N-acetyl-cysteine maintained

cell viability (Fig. 1C) and prevented ROS accumulation (Fig. 1D) upon HOCl challenge. Altogether, these results suggest that HOCl-triggered yeast cell death is mediated by ROS. In fact, it is likely that the sources of these ROS are mitochondria, since cells devoid of mitochondrial DNA (ρ^0) are protected against HOCl-inflicted ROS production and cell death (Fig. S2A and B).

It should be noted that there exists a quantitative difference between the survival levels upon HOCl treatment (300 μM) displayed in Figure 1A vs. Figure 1C (compare also Fig. 2A–C). In fact, the degree of HOCl cytotoxicity at a given concentration differed depending on the batch of SMD growth medium used, though a priori all batches should have had exactly the same composition. Conversely, the range of concentrations in which HOCl killed wild type yeast cells at a comparable quantitative level varied between 260–300 μM , depending on the specific medium batch (note the use of different HOCl concentrations in Fig. 1E and F vs. Fig. 2E and F). Within a batch and at a determined concentration, the qualitative and quantitative results remained stable. For this reason, we assume that some compound(s) in the medium cross-reacting with HOCl and slightly modifying its potency to kill yeast cells are sensitive to external factors that are difficult to control and might slightly differ from medium preparation to preparation. One of these compounds might be photosensitive, since the medium needed to be kept in the dark to maintain constant HOCl cytotoxic performance within one medium batch over time. Interestingly, it has been reported that ammonium ions, which are a component in the medium used in this study, influence HOCl cytotoxicity.³⁸ Also, moderate modifications in extracellular pH interfere with HOCl apoptotic induction.³⁹ These examples suggest that, indeed, slight changes in media composition may have an impact on the quantitative extension of HOCl-induced cytotoxicity, and that the corresponding concentration range must be assessed prior to the use of a specific medium batch. However, the actual nature of the described medium batch-dependent variance remains undetermined.

HOCl treatment results in specific protein modifications. In mammalian cells, HOCl cytotoxicity is accompanied by distinct protein oxidation products. Using a specific antibody that selectively recognizes HOCl-modified epitopes,⁴⁰ immunocytochemistry revealed increased staining in HOCl-treated wild type cells as compared with the untreated control (Fig. 2G). Omission of the primary antibody or its replacement with an IgG isotype control eliminated all staining, demonstrating that the observed staining was not due to a non-specific effect (data not shown). Also, competition with authentic HOCl-modified albumin (30 $\mu\text{g}/\text{ml}$) prevented antibody binding, demonstrating that the staining observed was specific for HOCl-modified proteins (data not shown). Thus, our data suggest that, similarly to mammalian cells, HOCl-mediated cell death and oxidative stress in *S. cerevisiae* is associated with oxidation-dependent specific modifications.

HOCl elicits yeast cell death mainly through apoptosis. In order to characterize HOCl-induced cell death in *S. cerevisiae*, we examined a typical phenotypic feature of apoptotic cells: externalized phosphatidylserine at the outer leaflet of the plasma

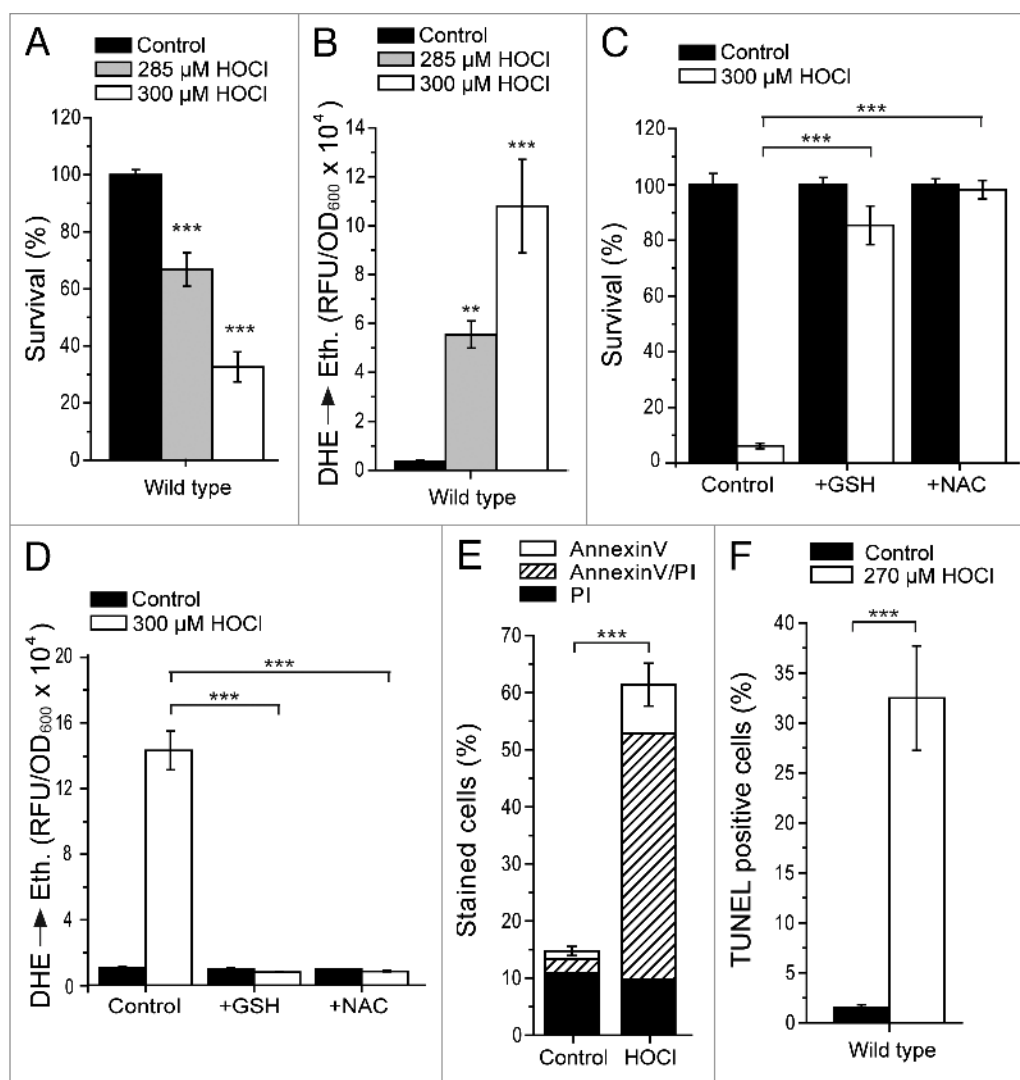


Figure 1. HOCl induces apoptotic cell death and specific protein modifications in *S. cerevisiae*. (A) Survival determined by clonogenicity of wild type cells after treatment with or without different concentrations of HOCl for 16 h. Data represent means $\pm$ s.e.m. ($n = 12$; ***, $p < 0.001$). Survival was normalized to the untreated control. (B) ROS production of wild type cells shown in (A) as determined via DHE $\rightarrow$ Ethidium conversion using a fluorescence plate reader. Data represent means $\pm$ s.e.m. ($n = 12$; **, $p < 0.01$; ***, $p < 0.001$). RFU, relative fluorescence unit. (C and D) Survival normalized to the untreated control (C) and ROS production (D) of wild type cells grown to exponential phase in the absence (control) or presence of 5 mM L-glutathione (GSH) or N-acetyl-L-cysteine (NAC) and subsequent treatment with or without 300 μ M HOCl for 16 h as determined via clonogenicity (C) and DHE $\rightarrow$ Ethidium conversion using a fluorescence plate reader (D). Data represent means $\pm$ s.e.m. ($n = 9$; ***, $p < 0.001$). RFU, relative fluorescence unit. (E) Phosphatidylserine externalization and loss of plasma membrane integrity of wild type cells after treatment with or without 270 μ M HOCl for 16 h as determined using Annexin V/PI costaining and quantified via flow cytometry. Data indicate means $\pm$ s.e.m. ($n = 6$; ***, $p < 0.001$). In each experiment 30,000 cells were evaluated. (F) DNA fragmentation of wild type cells after treatment with or without 270 μ M HOCl for 16 h as determined via TUNEL staining and quantified via flow cytometry. Data indicate means $\pm$ s.e.m. ($n = 6$; ***, $p < 0.001$). In each experiment 30,000 cells were evaluated.

membrane, which binds to FITC-conjugated Annexin V and thus can be quantified. In parallel, we monitored a characteristic marker of necrosis, loss of membrane integrity, by assessing membrane permeability with propidium iodide (PI). By co-staining yeast cells with FITC-Annexin V and PI (Fig. 1E), we were able to discriminate between early apoptosis (only Annexin V-stained cells), late apoptosis leading to secondary necrosis (double stained cells) and primary necrosis (only PI-stained cells).^{15,16} Compared with the untreated wild type cells, yeast cells exposed to a physiologically relevant HOCl concentration (here 270 μ M; see above) showed an increase of primarily apoptotic and more specifically

late apoptotic cells, though a small rise in the primary necrotic population was also detectable (Fig. 1E). This mainly apoptotic phenotype was further confirmed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining, which revealed exacerbated apoptotic DNA fragmentation upon HOCl treatment (Fig. 1F). In conclusion, HOCl cytotoxicity in yeast is mainly of apoptotic origin and only marginally involves primary necrosis.

Absence of the protease Kex1p but not of other lethal hydrolases partly reverts HOCl-induced cytotoxicity. The knowledge about the mechanistic details accounting for HOCl-triggered cell death in yeast is only limited, even though it seems clear

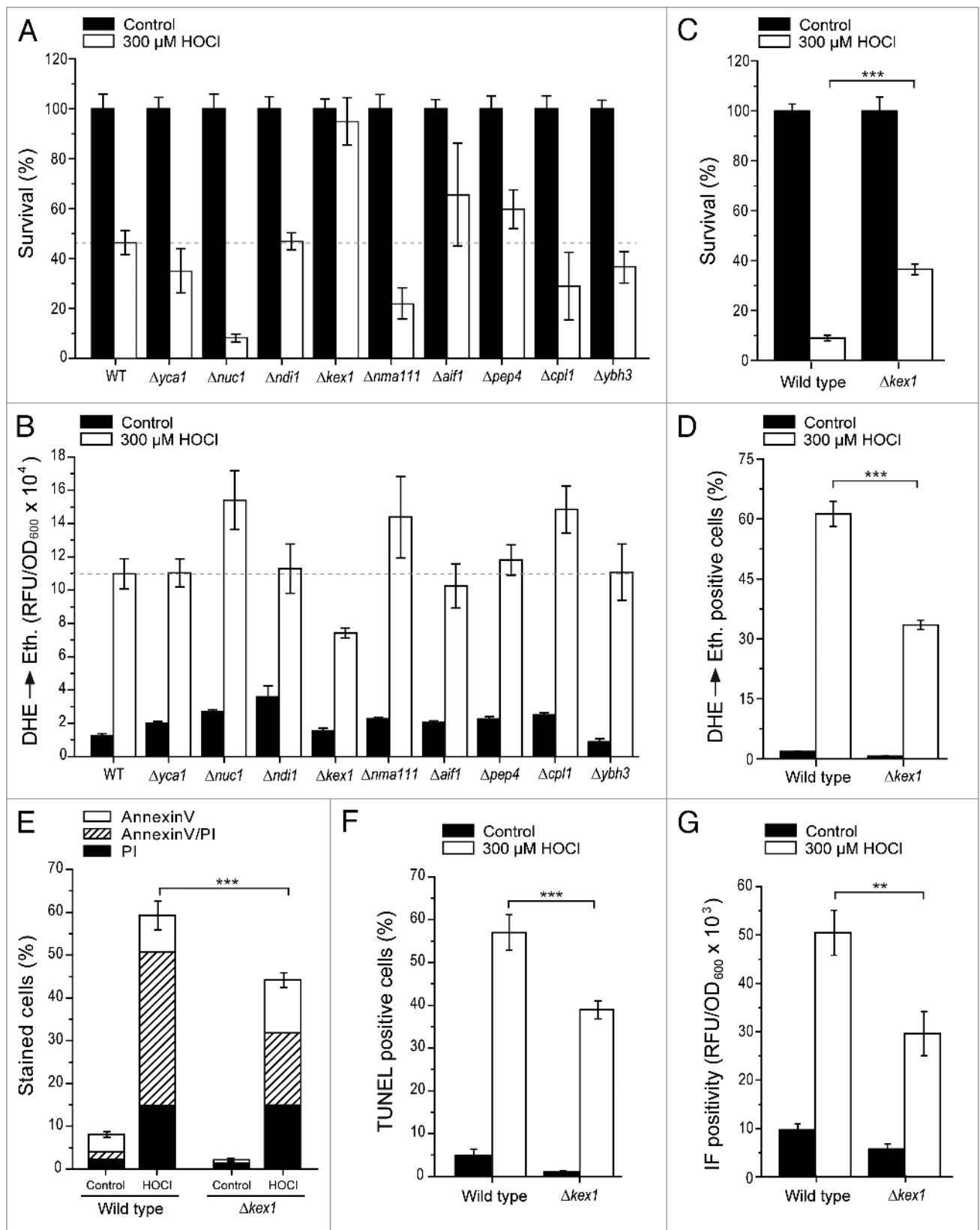


Figure 2. For figure legend, see page 7.

Figure 2 (See opposite page). The protease Kex1p is involved in HOCl-induced cytotoxicity. **(A and B)** Survival **(A)** and ROS production **(B)** of wild type, $\Delta yca1$, $\Delta nuc1$, $\Delta ndi1$, $\Delta kex1$, $\Delta nma111$, $\Delta aif1$, $\Delta pep4$, $\Delta cpl1$ and $\Delta ybh3$ cells after treatment with or without 300 μ M HOCl for 16 h as determined via clonogenicity **(A)** and DHE→Ethidium conversion **(B)**, respectively. Survival **(A)** was normalized to the untreated control. Experiments in **(B)** were quantified using a fluorescence plate reader. Data represent means $\pm$ s.e.m. (n = 8–16). The dashed line draws the survival **(A)** and ethidium fluorescence **(B)** levels of the HOCl-treated wild type, respectively. RFU, relative fluorescence unit. **(C–F)** Survival **(C)**, ROS production **(D)**, phosphatidylserine externalization **(E)**, loss of membrane integrity **(E)** and apoptotic DNA fragmentation **(F)** of wild type and $\Delta kex1$ cells after treatment with or without 300 μ M HOCl for 16 h as determined via clonogenicity **(C)**, DHE→Ethidium conversion **(D)**, Annexin V/PI co-staining **(E)** and TUNEL staining **(F)**. Data represent means $\pm$ s.e.m. (n = 8; ***, p < 0.001). Survival **(A)** was normalized to the untreated control. In each experiment for **(D–F)**, 30,000 cells were evaluated using flow cytometry. **(G)** Fluorescence intensity of wild type and $\Delta kex1$ cells after treatment with or without 300 μ M HOCl for 16 h and subsequent immunofluorescence (IF) staining using mAb 2D10G9 as a primary antibody and quantified using a fluorescence plate reader. Data represent means $\pm$ s.e.m. (n = 9; **, p < 0.01). RFU, relative fluorescence unit.

that oxidative damage represents a key component in the lethal process.¹³ To gain further insights into the molecular regulators accounting for the execution of HOCl-induced cytotoxicity, we decided to determine loss of viability and ROS levels in the deletion background of genes encoding known apoptotic effectors, including the yeast metacaspase Yca1p,¹⁷ the pro-apoptotic proteases Nma111p and Kex1p,^{18,41} the yeast homologs of the human death regulators cathepsin D and calpain (Pep4p and Cpl1p),²² the mitochondrial nuclease Nuc1p,²⁰ the yeast BH3-only protein Ybh3p²¹ and the pro-apoptotic oxidoreductases Ndi1p and Aif1p.^{42,43} Upon HOCl treatment, all disruptants except $\Delta kex1$ showed either no significant changes or even reduced cell viability in comparison to wild type cells (Fig. 2A). Accordingly, ROS levels in these strains, except $\Delta kex1$, remained either unchanged or were even increased as compared with the wild type control (Fig. 2B). Thus, Kex1p (but not other hydrolases tested in this study) is required to execute the full cytotoxic activity of HOCl.

KEX1-disruption diminishes HOCl-mediated apoptosis and the formation of HOCl-modified epitopes. Kex1p has been previously involved in yeast apoptotic scenarios.⁴¹ To further characterize the rescuing effect of $\Delta kex1$ upon HOCl-stress, we analyzed if its improvement in cell viability and reduction in ROS production would correlate with a possible diminishment of apoptosis. Indeed, reduction of cell death (Fig. 2C) and ROS generation (Fig. 2D; Fig. S1A) was accompanied by a specific and quantitatively similar decrease of the late apoptotic population as determined via Annexin V/PI co-staining (Fig. 2E; Fig. S1B). This was confirmed by TUNEL staining, which showed a corresponding decline of apoptotic DNA fragmentation in $\Delta kex1$ compared with the wild type control when stressed with HOCl (Fig. 2F; Fig. S1C). In addition, the formation of HOCl-modified epitopes, as detected via immunochemical techniques using the above mentioned specific antibody, was significantly reduced compared with wild type cells (Fig. 2G, Fig. S1D). Thus, Kex1p seems to promote HOCl-induced apoptosis apparently by acting upstream of ROS production and specific protein modification(s).

Discussion

The present study aimed at establishing a cell death model for HOCl cytotoxicity, both to investigate its microbicidal effects and to model its cytotoxicity in mammalian cells. To this end, we used the eukaryotic unicellular fungus *S. cerevisiae* (budding yeast), which combines being (1) a microorganism and (2)

an established model system to study PCD.^{15,16} While the antimicrobial properties of phagocyte-generated HOCl have been known for some decades,^{44,45} initial evidence for the cytotoxicity of HOCl in mammalian cells was provided by studies performed with activated neutrophils as well as the cell-free myeloperoxidase-hydrogen peroxide-chloride system.⁸ Both inhibition of myeloperoxidase as well as scavenging of HOCl, added as reagent or generated by the myeloperoxidase-hydrogen peroxide-chloride system, confirmed that HOCl is responsible for impaired cell viability and cell death in various mammalian cell types.^{8-10,46-49}

The results presented here characterize the cell death process occurring in *S. cerevisiae* upon exposure to lethal doses of HOCl. Previous reports have shown that budding yeast succumbs to cell death in response to chlorinative stress,^{11,50} and that HOCl elicits cyto- and/or genotoxic effects against *S. cerevisiae*.¹¹⁻¹³ Further work has demonstrated that toxicity of HOCl in wild type yeast strains is abolished by hypoxic and anoxic conditions, and that antioxidant-deficient *S. cerevisiae* strains are more sensitive to treatment with oxychloro compounds, including HOCl.¹³ In contrast to this earlier study, we more specifically monitored actual survival rates (via clonogenic tests), establishing a concentration-dependent deadly effect of HOCl on yeast. This cytotoxicity is accompanied by increased levels of ROS and can be counteracted by treatment with antioxidants, including glutathione. This confirms previous data showing elevated intracellular oxidative stress to be causative and the endogenous antioxidant system to be preventive during HOCl toxicity.¹³

Moreover, the present study defines the type of PCD induced by HOCl, which, according to our Annexin V/PI and TUNEL staining data, is mainly of apoptotic origin. However, a previous study¹³ suggested necrosis to be the death type preferentially occurring in HOCl-treated yeast cells based on the absence of TUNEL- and the prominent presence of PI-positive cells. This discrepancy might be due to various reasons. First, different culture conditions were used; while our study was performed in minimal (SMD) medium, Kwolek-Mirek et al.¹³ conducted theirs in complete (YEPD) medium, which differs in composition. This may have consequences for the extent and consequently type of toxicity (see below) at a particular concentration, especially given the high sensitivity of HOCl toxicity on slight media variance (see above). Second, the experiments performed with PI do not include a co-staining with an apoptotic stain like Annexin V that would allow discrimination between primary necrotic (PI+/Annexin V-) and late apoptotic (PI+/Annexin V+) cells, which both stain positive for PI. Thus, the authors¹³ cannot

exclude that part of the displayed PI positivity may actually correspond to a possible late apoptotic cell population. In fact, in our experiments, the main part of the stained population is late apoptotic and thus both PI- and Annexin V-positive. Third, the HOCl concentrations used in Kwolek-Mirek et al.¹³ triggered PI staining of almost the complete yeast cell population, which raises the question whether the chosen toxic HOCl doses were too high to observe a putative apoptotic phenotype.

Indeed, HOCl may induce necrosis and/or apoptosis in a time- and concentration-dependent manner,^{51,52} apparently via formation and decay of chloramines and generation of radical intermediates.^{53,54} For instance, exposure to increasing doses of HOCl in endothelial cells induces cell death that progressively shifts from apoptosis to necrosis.⁴⁶ Similarly, many yeast cell death inducers known to cause apoptosis at moderate concentrations promote a necrotic phenotype when applied at higher doses.¹⁶ The mere concentration increase of typical apoptotic triggers may, in most but not all cases, promote a non-regulated type of necrosis that likely arises from uncontrolled damage of cellular integrity. Thus, to test whether the necrotic phenotype displayed upon the high toxic HOCl dosage used in Kwolek-Mirek et al.¹³ represents such accidental or alternatively programmed necrosis, experiments assaying for typical markers of programmed necrosis would need to be performed under their conditions. Irrespective of this discrepancy, which may have multifactorial causes, HOCl cytotoxicity in *S. cerevisiae* as induced in our hands is mainly linked to an apoptotic phenotype. This is in line with several processes that have been involved in HOCl-mediated cell death, including upregulation of pro-apoptotic genes,⁵⁰ downregulation of anti-apoptotic proteins⁹ or DNA strand breakage.⁵⁵

Intriguingly, HOCl-induced yeast apoptosis is accompanied by formation of HOCl-modified proteins. HOCl and/or chlorinated proteins/chloramines have been shown to trigger the apoptotic machinery in different human cell lines and seem to preferentially activate the mitochondrial apoptotic pathway.^{49,56,57} Since the antibody used in this study to detect HOCl-modified epitopes in yeast also cross-reacts with HOCl-modified proteins occurring under inflammatory conditions in humans,⁵⁸ it is likely that similar epitopes are formed. We thus speculate that yeast and human cells share, at least in part, some molecular features of HOCl cytotoxicity. In this respect, follow-up studies will aim at identifying these modified proteins and elucidate their function in HOCl-triggered cell death execution.

While the induction of cell death by HOCl has been established in diverse cell types (human as well as microbial), the molecular players involved in each case remain elusive. Here, we aimed at identifying lethal hydrolases involved in HOCl-mediated yeast apoptosis. Interestingly, the single known yeast metacaspase Yca1p does not seem to represent the molecular executioner in this process. In contrast, HOCl cytotoxicity in mammalian cells has been linked to caspase activation.⁴⁶ Our data suggest, that—at least under conditions of fast proliferation and high glycolytic activity (represented by the model presented in this work)—HOCl exhibits its cytotoxicity through a caspase-independent mechanism. However, we also show that well-known caspase-independent effectors, such as the yeast homologs

of endonuclease G (Nuc1p), BH3-only proteins (Ybh3p), the nuclear serine protease HtrA-like protein (Nma111p) or the apoptosis-inducing factor (Aif1p) are dispensable for HOCl-induced cytotoxicity. Instead, we identify the pro-apoptotic serine protease Kex1p as a pivotal factor in this process. Disruption of *KEX1* causes a decrease in clonogenic cell death, ROS production and the apoptotic population upon challenge with HOCl, while at the same time it diminishes the extent of HOCl-modified proteins.

Kex1p has been previously described to be a major player in Yca1p-independent yeast apoptotic cell death induced by protein N-glycosylation defects, where the production of ROS is a determinant factor for lethality.⁴¹ Our results showing Kex1p-dependent increase of ROS production and formation of HOCl-modified proteins upon challenge with HOCl further suggest Kex1p as an executor of oxidation-mediated cytotoxicity. Thus, Kex1p-induced apoptosis seems to generally involve oxidative stress and possibly thereof resulting oxidized products, including modified proteins. HOCl-derived apoptosis could further comprise mechanistic elements present in cell death derived from defective protein N-glycosylation. At the same time, deletion of *KEX1* has also been shown to promote survival under different PCD-inducing conditions, namely acetic acid stress and chronological aging.⁴¹ Thus, Kex1p may exert a more general role in yeast PCD. In that case, HOCl-mediated toxicity would not necessarily need to recapitulate upstream lethal pathways involved in N-glycosylation defects but would converge, possibly with other inducers, at the stage of Kex1p execution. Future work will have to clarify whether HOCl-induced cell death shares an upstream pathway with any of the above-mentioned lethal scenarios involving Kex1p.

By establishing the PCD mode in yeast upon challenge with HOCl and unveiling Kex1p as a molecular executor in this process, this study provides insight into the mechanisms underlying the microbicidal effects of HOCl toward fungal infections. However, these mechanisms might not correspond to those elicited during HOCl-induced killing of prokaryotes. In bacteria exposed to HOCl, the observed patterns of metabolic dysfunction suggest mechanisms where death occurs by interruption of the energy-transducing capabilities of the cell via inhibition of plasma membrane-localized proteins.¹² These patterns thus involve processes (like energy transduction or glucose transport) that differ from eukaryotic cells. This has previously put in doubt whether the microbicidal mechanisms of HOCl toxicity in *S. cerevisiae* mirror those observed in bacteria.¹² Interestingly, our results show that mitochondria seem to play a significant role in ROS-dependent HOCl lethality. Considering an evolutionary point of view, it would be interesting to explore whether mitochondrial membrane-localized proteins (similar to the situation in bacteria) are a target of HOCl. In addition, it has been recently shown that under certain circumstances, bacteria can succumb to an apoptotic-like cell death.^{59–61} To which extent this or other forms of PCD might be involved in the bactericidal action of HOCl and how or if mitochondrial targeting in eukaryotes might recapitulate cytotoxic characteristics of HOCl in prokaryotes remains to be investigated.

In humans, activated phagocytic cells generate and release HOCl, a process, which (though firmly controlled) represents a

major contributor to inflammatory tissue damage. Interestingly, several characteristics of HOCl-mediated yeast cell death described in this study, including ROS generation, mitochondrial involvement, an apoptotic phenotype and increased levels of HOCl-modified proteins, parallel already known features of HOCl-mediated cytotoxicity in humans.^{8,10} Thus, our results also support the idea of employing *S. cerevisiae* as a model of HOCl-mediated cellular demise in human cells. In that respect, it will be interesting to test whether a similar protease, such as the herein identified yeast Kex1p, is involved in chlorinated protein-mediated apoptosis of inflamed human tissue. While there is no clear homolog of Kex1p in humans, the mammalian cathepsin A (like Kex1p a member of the serine protease group of hydrolases) shares about 20% overall similarity with Kex1p and represents a putative candidate for a factor involved in HOCl-induced apoptosis in human cells.

Summing up, this study establishes that the cytotoxic effect of HOCl on *S. cerevisiae* follows a Kex1p-dependent apoptotic pathway that possibly involves specific protein modifications reminiscent of those present in mammalian proteins upon HOCl stress. Whether or not these results compare with the situation in other eukaryotic and/or prokaryotic microorganisms needs further investigation. Similarly, future work will have to further address if budding yeast, in fact, represents a valid means to explore human HOCl-associated cellular demise, as the present study suggests.

Materials and Methods

Reagents. The following reagents were purchased from the indicated suppliers: FITC-labeled Annexin V (Roche Applied Science, 11828681001), reagents for “terminal deoxynucleotidyl transferase dUTP nick end labeling” (TUNEL, Roche Applied Science, 11684795910), dihydroethidium (DHE, Sigma-Aldrich, D7008), propidium iodide (PI, Sigma-Aldrich, P4170), L-glutathione (Sigma-Aldrich, G4251), N-acetyl-L-cysteine (Sigma-Aldrich, A9165) and sodium hypochlorite (NaOCl, Sigma-Aldrich, A7191). HOCl solutions were prepared immediately before use and the concentration was determined spectrophotometrically (ϵ_{292} 350 M⁻¹cm⁻¹).

Yeast strains and growth conditions. Experiments were performed in wild type BY4741 and the respective *aif1*-, *nuc1*-, *ycal*-, *pep4*-, *nma111*-, *ndi1*-, *cpl1*- and *kex1*-null mutants, all obtained from Euroscarf. The strain BY4741 ρ 0, previously described,^{62,63} was used to analyze mitochondrial dependency. All experiments were performed in synthetic minimal medium (SMD) medium containing 0.17% yeast nitrogen base (Difco), 0.5% (NH₄)₂SO₄ and 30 mg/l of all amino acids (except 80 mg/l histidine, 200 mg/l leucine), 30 mg/l adenine and 320 mg/l uracil with 2% glucose. Incubation of liquid cultures was performed with 145 rpm at 28°C. Cell densities were measured using an automated cell counter (CASY1, Roche Innovatis). As described in the results section, the quantitative degree of the cytotoxic effect elicited by HOCl seemed to be slightly dependent on the batch of SMD growth medium used. Thus, before using a new batch of medium, a concentration series was performed to determine

the concentration range at which to carry out the subsequent experiments.

For all assays, overnight cultures of corresponding strains were inoculated in 45 ml SMD medium using 250 ml flasks (or—for the mini-screen as well as for experiments with L-glutathione and N-acetyl-L-cysteine—inoculated in 15 ml using 100 ml flasks) at a density of 1.5×10^6 cells/ml. Next, cells were grown to a cell density of $7\text{--}9 \times 10^6$ cells/ml, subsequently distributed into 100 ml quadruple-indented flasks at 10 ml cell culture each (or—for the mini-screen as well as for experiments with L-glutathione and N-acetyl-L-cysteine—into 96-deep-well plates at 250 μ l cell culture medium each) and then either challenged with HOCl stock solution to reach the indicated final concentration or left untreated (control). Cells were incubated for 16 h after HOCl treatment and used for diverse assays. For experiments with antioxidants, cells were incubated with L-glutathione or N-acetyl-L-cysteine at indicated concentrations starting at the time of inoculation and thus prior to HOCl treatment.

Survival plating and tests for cell death markers. Clonogenic survival plating was performed as previously described.¹⁷ Briefly, cell counts of HOCl-treated cultures and controls were measured; 500 cells were plated on YEPD agar plates and incubated for two days at 28°C to allow colony formation. The colony-forming units (CFUs) were analyzed using an automated colony counter (LemnaTech). For each strain, the CFUs determined for the control cultures were set to 100%, and the survival of the respective HOCl-treated cultures calculated relative to the corresponding control culture.

DHE staining (indicative for ROS production) was performed as described.^{20,63} Briefly, 5×10^6 cells were harvested by centrifugation, resuspended in 250 μ l of 2.5 μ g/ml DHE in PBS and incubated in the dark for 10 min. Relative fluorescence units (RFU) were determined by using a fluorescence reader (Tecan, GeniusPRO) and normalized to the OD₆₀₀ of each sample. Alternatively, positive cells were counted using flow cytometry (BD FACSAria); 30,000 cells were used per experiment. Annexin V/PI costaining (apoptosis/necrosis marker) and TUNEL staining (apoptosis marker) were performed and quantified by flow cytometry as described.^{20,22} 30,000 cells per sample were evaluated using BD FACSDiva software. Representative samples were subjected to qualitative analysis by fluorescence microscopy.

Immunofluorescence staining. For immunofluorescence studies, 1.5×10^7 cells were fixed with 4% formaldehyde solution and their cell wall digested for 30 min with glucuronidase/arylsulfatase and lyticase as described.²⁰ After washing with sorbitol-containing buffer B [35 mM potassium phosphate buffer (pH 6.8), 0.5 mM MgCl₂ and 1.2 M Sorbitol], cells were blocked with 100 μ l blocking solution (Ultra V block, Thermo Fisher Scientific) for 20 min at 20°C. Cells were then spun down and the resulting pellets resuspended in 100 μ l mAb antibody solution (clone 2D10G9, dilution 1:2, v/v in antibody diluent, Dako; 25°C for 30 min); the mAb recognizes HOCl-oxidized proteins, but does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals or lipid peroxidation reactions.⁴⁰ After washing once with sorbitol-containing buffer B, cells were incubated with 100 μ l cyanine

3-conjugated goat anti-mouse antibody (Jackson Dianova, 1:300 in antibody diluent) for 20 min at 25°C. Fluorescence was microscopically evaluated and quantified using a fluorescence reader (Tecan, GeniusPRO). The obtained intensity was normalized to the OD₆₀₀ of each sample.

Microscopy. Microscopy was performed with a Zeiss Axioskop microscope using a Zeiss Plan-Neofluar objective lense with 100× magnification and 1.30 numerical aperture in oil (using Zeiss Immersol®) at room temperature. Imaging medium was AnnexinV/PI incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂ pH 7.4) + 0.6 M sorbitol for pictures shown in **Figure S1B** and water for pictures shown in **Figures 1F** and **Figure. S1A, C and D**. Fluorochromes were fluorescein (used as fluorescein isothiocyanate, FITC), propidium iodide (PI) and ethidium (after conversion from dihydroethidium, DHE) for pictures shown in **Figure S1A–C**. Fluorescence microscopic sample images were taken with a Diagnostic Instruments camera (Model: SPOT 9.0 Monochrome-6), acquired using the Metamorph software (version 6.2r4, Universal Imaging Corp.) and processed with Adobe Photoshop (version CS2) software. Specifically, picture processing involved creation of AnnexinV and PI overlays in **Figure S1B** and coloring (**Figs. 1F; Fig. S1A–D**) with Metamorph. In addition, contrast and brightness was adjusted with Adobe Photoshop (applying equal adjustment parameters to all pictures of an experiment).

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Statistical analyses. Error bars (± s.e.m.) are shown for independent experiments. When experiments were performed in parallel, a common overnight culture and common pre-cultures were used for each strain. In these cases, pre-cultures were divided into separate flasks (or wells in the mini-screen) prior to HOCl stress. The number of independent data points (n) is indicated in the figure legends of the corresponding graphs. Significances were calculated using an ANOVA approach and were corrected by the Bonferroni post-hoc test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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Supplemental Material to:

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Christine Rossmann, Andreas S. Zimmermann, Christoph
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Sattler, Ernst Malle and Frank Madeo**

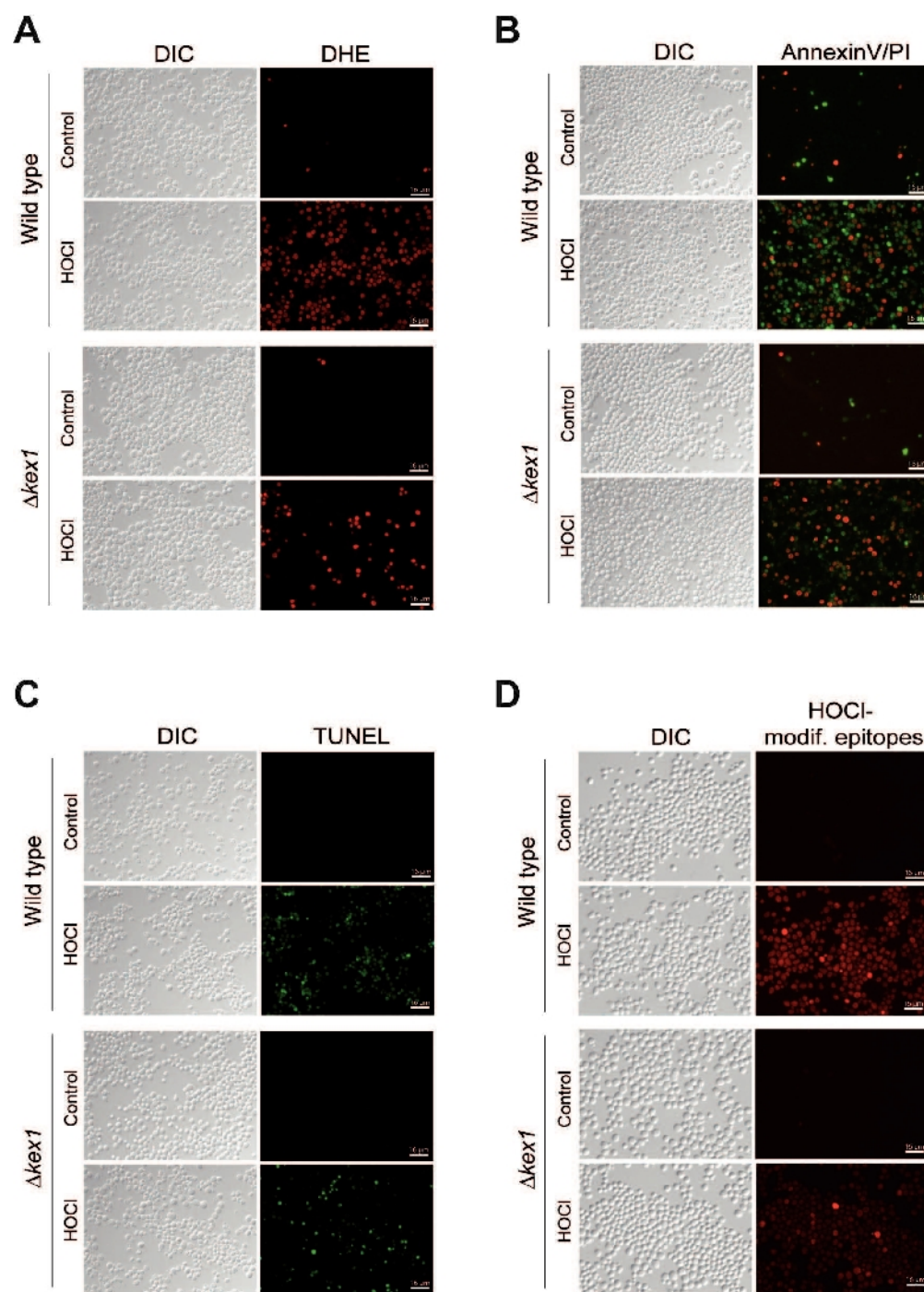
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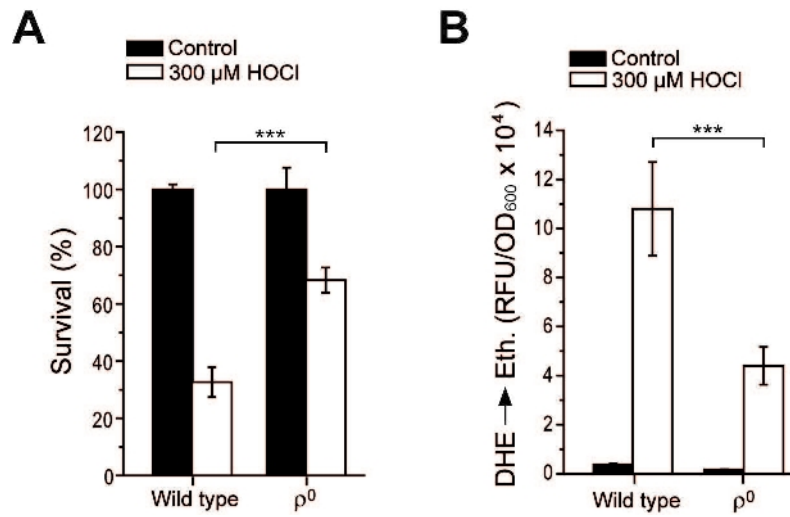
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Supplemental Material



Supplemental Figure 1

(A-D) Representative fluorescence microscopic images of DHE (A), Annexin/PI (B), TUNEL (C) and the immunofluorescence (D) stainings of the experiments shown in Figure 2 D-G, respectively. *DIC*, differential interference contrast. Scale bar: 16 μ m



Supplemental Figure 2

(A, B) Survival (A) and ROS production (B) of wild type and p^0 cells after treatment with or without 300 μM HOCl for 16 h as determined via clonogenicity (A) and DHE $\rightarrow$ Ethidium conversion (B), respectively. Survival (A) was normalized to the untreated control. Experiments in (B) were quantified using a fluorescence plate reader. Data represent means $\pm$ s.e.m. ($n = 12$; *** $p < 0.001$). *RFU*, relative fluorescence unit.