The Role Of Mitochondrial Omi/htra2 Protease In Protein Quality Control And Mitophagy

2013

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THE ROLE OF MITOCHONDRIAL OMI/HTRA2 PROTEASE IN PROTEIN QUALITY CONTROL AND MITOPHAGY

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Department of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2013

Major Professor: Antonis S. Zervos

ABSTRACT

Omi/HtrA2 is a mitochondrial serine protease with a dual and opposite function depending on its subcellular localization. Most of the previous studies focused on Omi/HtrA2's pro-apoptotic function when the protein is released to the cytoplasm. It is becoming apparent that the main function of Omi/HtrA2 is within the mitochondria, where it has a pro-survival role. However, its mechanism is still poorly understood. To this end, we used the yeast two-hybrid system to dissect the Omi/HtrA2 pathway by identifying novel interactors and substrates. Our studies revealed a novel function of Omi/HtrA2 in the regulation of a deubiquitinating (DUB) complex. In addition we found that Omi/HtrA2 participates in mitophagy by regulating Mulan E3 ubiquitin ligase, which recruits GABARAP (gamma-amino-butyric acid receptor-associated protein) to the mitochondria.

Abro1 is the scaffold protein of the DUB complex known as BRISC (BRCC36 isopeptidase complex) that is specific for Lys-63 deubiquitination. This complex is similar to the BRCA-1 complex, a known and important player in DNA damage response. Using the yeast two-hybrid screen and a bait consisting of the unique carboxy-terminus of the Abro1 protein, we identified three transcription factors which are members of the activating protein 1 (AP-1) family, namely ATF4, ATF5 and JunD. The AP-1 family member ATF4 is ubiquitously expressed, like Abro1, and important in cell cycle regulation and survival, thus we further analyzed this interaction. Abro1's interaction with ATF4 was specific and present only when cells are under cellular stress. When Abro1 protein level is increased it provides protection against stress-induced cell

death, but interaction between Abro1 and ATF4 is necessary to achieve this protection. The significance of this interaction was the translocation of Abro1 from the cytoplasm to the nucleus. These results establish a new cytoprotective function of cytoplasmic Omi/HtrA2 as a regulator of the BRISC DUB complex.

Under normal conditions Omi/HtrA2 is localized in the intermembrane space (IMS) of the mitochondria. We have recently identified that the mitochondrial Mulan E3 ubiquitin ligase is a substrate of Omi/HtrA2 protease. Mulan, along with MARCH5/MITOL and RNF185, are the only three mitochondrial E3 ubiquitin ligases identified thus far. The function of Mulan has been linked to cell growth, cell death and autophagy/mitophagy. In addition, we showed that Omi/HtrA2, through regulation of the Mulan protein level, controls mitophagy, especially during mitochondrial stress. To understand Mulan's function and its control by Omi/HtrA2, we set out to identify E2 conjugating enzymes that form a complex with Mulan E3 ligase. We isolated four specific interacting E2's, namely Ube2E2, Ube2E3, Ube2G2 and Ube2L3. To identify substrates for each unique Mulan-E2 complex we used fusion baits in a second yeast two-hybrid screening. One of the interactors isolated against the Mulan-Ube2E3 bait was the GABARAP protein, a member of the Atg8 (autophagy) family. The mammalian Atg8 family is composed of seven members that have been linked to important roles in autophagy/mitophagy. We characterized this interaction both *in vitro* and *in vivo* and its role in mitophagy. Our results suggest that Mulan participates in various pathways, depending on the nature of its partner E2 conjugating enzyme. In addition, we identified the pathway by which Mulan participates in mitophagy by recruiting GABARAP to the mitochondria.

I want to dedicate this hard work to the people who mean the most to me, the people who have been more than supportive in these past five years; they are my very small, but very loud and loving, family. My brother Raffaello has always been proud of me and always told his friends how his sister was a “doctor.” My father, Alvaro, always told me I had to be better than him, a man who has a master’s degree in chemistry. Finally, but most importantly to me, I want to dedicate this work and degree to my mother, Maria Aparecida Troncon, a woman who is like no other. She has always supported me, and with small gestures like having snacks ready when I came to visit after work or coming with me to lab on the weekends so I would not be alone, she told me every day that she was proud of me. Every time she met someone she told them I was her doctor and she said it with the biggest smile on her face. Unfortunately she passed away on December 17th, 2012, just months shy of the completion of my degree. I remember how proud she was when I received my bachelor’s and I can only imagine the size of her smile when I walk across the stage this time as a Ph.D.

I love you mom, you are my rock and my strength; without your constant support and dedication I would have not reached this point. I know you are smiling at me from above.

Last but not least I want to dedicate this to the man who is my better-half. Thank you for all your love and support, Guillermo. I love you.

ACKNOWLEDGMENTS

The completion of this work has dependent on a lot of people, and it could not have been completed without their constant support. First, I would like to acknowledge my advisor and mentor, Dr. Antonis S. Zervos, whose encouragement and ideas helped me complete the present work. These past four years in his laboratory have been a great experience and have prepared me for a future in the field. I have learned a lot from him, not only on the bench doing experiments, but also as a better presenter, lecturer, writer, thinker, and all around person. For all of this I thank him greatly.

Another person who has been irreplaceable in the completion of this work has been Dr. Lucia Cilenti. Her constant support, expertise, knowledge, and guidance allowed me to reach a conclusion. She is an amazing researcher and a role model for research women. Her advice was always with my best interest in mind and her critique was never in a demeaning way, but always so I could be a better scientist. Without her I would not be able to write this today. Not to forget Dr. Meenakshi Balakrishnan, a fellow colleague who not only was a supportive in the lab but also outside.

The members of my graduate committee have also been imperative in the completion of my work: Dr. Sidiqqi, Dr. Self, Dr. Teter, and previous members Dr. Ireton and Dr. Chan. Their comments and advice were always with my advancement in mind and without it I would not have been able to move forward in my work as quickly. Two other great scientists who helped me achieve my goal were Dr. Chai and Dr. Chen who trained me to be a precise scientist.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abro1	Abraxas brother 1
AP-1	activator protein 1
ATF	activating transcription factor
Atg	autophagy-related
BARD1	BRCA1 associated RING domain 1
BiP	immunoglobulin heavy chain-binding protein
BRCA1	breast cancer 1
BRCC36	BRCA1/BRCA2-containing complex, subunit 3
BRE	brain and reproductive organ-expressed
BRISC	BRCC36 isopeptidase complex
bZIP	basic Leucine-zipper
Ca	calcium
CCCP	carbonyl cyanide m-chlorophenyl hidrazone
DD	death domain
DED	death effector domain
DISC	death-inducing signal complex
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
ERAD	ER-associated degradation
fosB	FBJ murine osteosarcoma viral oncogene homolog B
GABARAP	gamma-amino-butyric acid receptor-associated protein

GATE-16	Golgi-associated ATPase enhancer of 16kDa
Gly	glycine, G
HAX-1	HS1-associated protein X-1
HECT	homologous to E6-AP carboxy-terminus
HtrA	high temperature requirement A
IAP	inhibitors of apoptosis proteins
IMS	intermembrane space
JAMM/MPN+	Josephins (MJDs) and the mettaloproteases JAB1/MPN/Mov34 metalloenzyme
KO	knock out
LC3	microtubule-associated protein 1, light chain 3
Lys	lysine, K
Maf	Avian musculoaponeurotic fibrosarcoma
MARCH5	membrane associated RING-CH5
MERIT40	mediator of Rap80 interactions and targeting 40 kDa
Mfn	mitofusin
MI/R	myocardial ischemia/reperfusion
MKRN1	Makorin ring finger protein 1
mnd2	motor neuron degeneration 2
MOM	mitochondrial outer membrane
MPT	mitochondrial permeability transition
Mulan	mitochondrial ubiquitin ligase activator of NF-κB
NBR1	neighbor of *BRCA1* gene 1

Nix ` protein X	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
OMM	outer mitochondrial membrane
OUT	ovarian tumor proteases
PD	Parkinson’s disease
PDZ	postsynaptic density of 95kDa, disk large, and zonula occludens 1
PE	phosphoethanolamine
PINK1	phosphatase and tensin homolog-induced putative kinase protein 1
PQC	protein quality control
RAP80	receptor associated protein 80
RING	really interesting new gene
RNF	ring finger protein 185
ROS	reactive oxygen species
siRNA	small interfering RNA
SQSTM1	sequestosome 1
THAP5	thanatos associated protein 5
TNF	tumor necrosis factor
TRIM	tripartite motif-containing
Ub	ubiquitin
UBC	ubiquitin conjugating
Ubl	ubiquitin-like molecule
UCHs	ubiquitin C-terminal hydrolases
UPR	unfolded protein response

UPS	ubiquitin-proteasome system
USP	ubiquitin-specific proteases
VDAC1	voltage-dependent anion channel 1
XIAP	X chromosome-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

Protein homeostasis is essential for a cell's ability to survive and thrive. This process requires precise control of protein synthesis, processing, and protein degradation. Misfolded and damaged proteins are processed through protein quality control (PQC) mechanisms, in which the proteins are degraded through the ubiquitin proteasome system (UPS) or autophagy.

Since its first discovery in the late 1970s, ubiquitin has gained much attention as a marker for protein destruction, but it has been noted recently that ubiquitin also plays a role in other physiological functions (1). Some of the cellular processes in which ubiquitin has been linked to include: antigen processing, apoptosis, biogenesis of organelles, cell cycle and division, DNA transcription and repair, differentiation and development, immune response and inflammation, neuronal and muscular degeneration, morphogenesis of neural networks, modulation of cell surface receptors, the secretory pathway, response to stress and extracellular modulators, ribosome biogenesis and viral infection (for current reviews see (1-4)). Dysfunction of ubiquitination is implicated in multiple pathological conditions and human diseases. Disorders involving UPS dysfunction include Alzheimer's disease, Parkinson's disease, Huntington's disease, prion-like lethal disorders, cystic fibrosis, cancer and many more (2, 5-8).

Apoptosis

Apoptosis is a process whereby the cell decides to “commit suicide”. There are two pathways of apoptosis: the intrinsic pathway and the extrinsic pathway (Figure 1) (9). The extrinsic pathway involves transmembrane receptor-mediated interactions that include members of the tumor necrosis factor (TNF) receptor gene superfamily (10). Members of the TNF family contain a cysteine-rich extracellular domain as well as a cytoplasmic domain, called the death domain (DD), which plays an important role in transmitting the information from the cell surface to intracellular pathways (11). The best characterized models of the extrinsic apoptotic pathway are the FasL/FasR and TNF-α/TNFR1 models, in which there is a clustering of receptors that binds to the trimeric ligand. Cytoplasmic adapter proteins with corresponding DDs are then recruited to the receptors. In the FasL/FasR model, the adapter protein is FADD (12-14). FADD will then interact with procaspase-8 (the inactive form of caspase-8) through dimerization of the death effector domain (DED), forming a complex collectively known as the death-inducing signal complex (DISC). DISC can then in turn activate more procaspase-8, thus leading to the triggering of apoptosis (15).

On the other hand, the intrinsic pathway initiates apoptosis by intracellular signals that do not derive from receptor-mediated stimuli. Instead, the signals come from within the cell and are mitochondrial-initiated events which can be either positive or negative signals. Negative signaling involves the absence of certain growth factors, cytokines or hormones that when withdrawn, the suppression of apoptosis is lost (16). Positive signaling are stimuli that when present lead to apoptosis; examples include radiation, toxins, and free radicals (16). Both positive and negative stimuli cause a loss

in mitochondrial transmembrane potential through the opening of mitochondrial permeability transition (MPT) pores in the inner mitochondrial membrane. With the opening of these pores there is also the release of pro-apoptotic proteins from the intermembrane space (IMS) which can be divided into two subgroups (17). The first subgroup includes the proteins cytochrome *c*, Smac/DIABLO and Omi/HtrA2 which can activate the caspase-dependent mitochondrial pathway of apoptosis (18-20). The cytochrome *c* achieves this by binding and activating Apaf-1 and procaspase-9, which collectively form the apoptosome (21, 22). Smac/DIABLO and Omi/HtrA2 promote apoptosis by inhibiting the inhibitors of apoptosis proteins (IAP) activity (19, 23). The second subgroup includes AIF, endonuclease G and CAD which are also released from the mitochondria later and only after the cell has committed to death (24).

The control and regulation of the mitochondrial apoptotic events are orchestrated by the Bcl-2 family of proteins (25). These proteins are in charge of the mitochondrial membrane permeability and can play a role as either pro-apoptotic or anti-apoptotic. There is also cross-talk between the two pathways, for example, mitochondrial damage in the Fas pathway is mediated through the cleavage of Bid by caspase-8 (26-28). Activation of the execution pathway occurs when both the intrinsic and extrinsic pathways end and is considered the last step of the apoptotic pathway. Execution caspases (caspase-3, caspase-6, and caspase-7) are activated and begin the final steps of apoptosis by cleaving various substrates such as PARP and plasma membrane cytoskeletal proteins (29). The most important execution caspases is caspase-3, which can be activated by any of the initiator caspases that are part of the intrinsic or extrinsic pathways, which include caspase-8, caspase-9 or caspase-10. The activated caspase-3

cleaves ICAD which is normally bound to the endonuclease CAD, inhibiting its function (30). Activated CAD will then degrade the chromosomal DNA, causing chromatin condensation, as well as cytoskeletal reorganization. The last step of apoptosis is phagocytic uptake of the apoptotic cell, which includes the translocation of phosphatidylserine to the outer leaflet of the cell.

Omi/HtrA2

Omi/HtrA2 is a serine protease and a member of the HtrA family in Eukaryotes (31). Along with Omi/HtrA2, there are also HtrA1/L56 and HtrA3/PRSP, which are all homologous to the bacterial *HtrA* endoprotease, a proteolytic enzyme that removes damaged and denatured proteins at elevated temperatures (32). Omi/HtrA2 is synthesized as a precursor at 458 amino acids and is processed in the mitochondria to its mature form through the removal of the first 133 amino acids, exposing a AVPS motif (33). Unlike the other members of the HtrA family, Omi/HtrA2 is not secreted and it is exclusively located in the IMS of the mitochondria under normal conditions (31, 34, 35). Omi/HtrA2, like the rest of HtrA proteins, contains a catalytic domain as well as a PDZ domain (postsynaptic disk large and zonula occludens 1) known to be involved in protein-protein interaction (Figure 2) (32).

After its release to the cytoplasm, Omi/HtrA2 binds and degrades several proteins including the X chromosome-linked inhibitor of apoptosis protein (XIAP) which results in the activation of caspase-9 (31). Omi/HtrA2 can also induce apoptosis in a caspase-independent manner but the mechanism of this function remains unknown (36-39). A surprising and unexpected new function of Omi/HtrA2 was uncovered when it

was found that its inactivation is responsible for the *mnd2* (motor neuron degeneration) phenotype in mice. These animals carry a spontaneous mutation on Ser276Cys located in the protease domain, and homozygous animals exhibit muscle wasting, neurodegeneration, involution of the spleen and thymus, and a shortened life span, typically dying by 40 days of age (40-42). Mutations in the *HtrA2* gene have also been linked to Parkinson's disease although these data were contradicted by later studies (43, 44). Another study has recently linked Omi/HtrA2 and the prevention of lymphocyte apoptosis through the suppression of activated Bax (45).

The phenotype of Omi/HtrA2 KO animals suggests that this protease has two different and opposite functions based on its subcellular location. In the mitochondria, Omi/HtrA2 has a cytoprotective function; whereas once it is released to the cytoplasm, it has a pro-apoptotic role. Most of the studies so far have focused on its pro-apoptotic function and very little if anything is known about what it does in the mitochondria. Its bacterial homologue DegP is a molecular chaperone under low temperatures which helps fold proteins and assemble oligomers, but in high temperature environments, it acts as a protease (33). Since DegP acts as a chaperone, it has been assumed that Omi/HtrA2 has some sort of similar function while in the mitochondria. Thus far, Omi/HtrA2 has been found to interact and cleave HAX-1, WARTS, Ped/Pea-15, XIAP, cIAP1, cIAP2, APP, THAP5, HSPA8, amyloid-β protein and WT1 (46-56). There has also been contradicting information regarding the functional interaction between Omi/HtrA2 and parkin or PINK1 (57, 58).

Ubiquitination

Ubiquitin (Ub) is a small 76 amino-acid, 8kDa, peptide that can be covalently attached via the carboxyl group of its carboxy-terminus residue (Gly76) to a lysine in the substrate (59, 60). Ubiquitination of a target protein occurs through sequential reactions involving three classes of enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes (Figure 3) (61). The E1 activates the ubiquitin peptide by forming a high energy thioester bond between a cysteine in the E1's active site and the carboxy-terminus of the ubiquitin via the hydrolysis of one ATP molecule. The active ubiquitin is then transferred to an E2 through the formation of a new thioester bond. Finally, the ubiquitin is directly or indirectly transferred to the target protein's ε-amino group of a lysine (5). The addition process can happen once (monoubiquitination) or repeatedly, forming a poly-ubiquitin chain where each additional ubiquitin is linked to one of the seven Lysines (K or Lys) - K6, K11, K27, K29, K33, K48, and K63 - present in the terminal ubiquitin molecule (1). The fate of the target protein is dictated by the length and the type of the Ub-chain formed; the most well understood and studied are Lys-48 chains which are responsible for targeting proteins to the proteasome for degradation (62).

There are two E1s, more than 30 E2s, and over 1000 E3s identified in the Eukaryotes (63). E2 conjugating-enzymes are classified as Class I through IV, where Class I E2s consist of only the catalytic domain; Class II and III have an extension at either the amino- or carboxy-terminus, respectively, and Class IV have both extensions (64). The E3s can be further divided into two major classes: HECT (homologous to E6-AP carboxy-terminus) and RING (really interesting new gene) families. The HECT

ligases catalyze the formation of an ubiquitin-thioester intermediate, followed by the transfer of the ubiquitin to the target protein (61). RING ligases do not form an intermediate and instead function as a scaffold protein bringing the substrate and E2 into close proximity (65). Some reports also recognize a class of E4 enzymes, specifically those of the U-box type, which are associated with the elongation of the ubiquitin chain rather than the addition of the first ubiquitin (65). Proteins that are ubiquitinated for destruction contain a “death domain” where the target lysine is recognized by a specific heterodimer formed between the E2 and E3, creating great substrate diversity (66). These domains can be hidden if the protein is correctly folded, only appearing for interaction with the heterodimer if the folding is wrong, or it is present at all times in short-lived proteins, such as those that play a role in the cell-cycle (67).

Once a protein is tagged for destruction via ubiquitination, it is transported to the 26S proteasome by shuttling factors; this process predominantly occurs in the cytosol where the majority of the factors are present (5, 67). The 26S proteasome is assembled from two 19S regulatory complexes (the lid and base) as well as a 20S central particle (65). Protein degradation is not the only function of ubiquitination. One of the first identified functions was its involvement in cargo internalization as well as cargo targeting to specific vacuoles (68, 69). Another role of ubiquitination is sequestering of the proteins to prevent their activity (70).

Due to the large number of possible target substrates of the UPS, it is no surprise that this process is directly and indirectly involved in multiple pathways and that its deregulation can be detrimental to the organism (5). Neurodegenerative diseases caused by deregulation of the UPS include Alzheimer’s disease (AD), Parkinson’s

disease (PD), amyotrophic laterial sclerosis (ALS), Huntington's disease (HD), spinocerebellar ataxias (SCAs) and Kennedy's syndrome (5). Other disease states include, but are not limited to, Cystic fibrosis (CF), autoinflammatory and autoimmune diseases, prostate, breast, and colorectal cancer (5, 71-75).

There are also ubiquitin-like molecules (Ubls) which provide new protein-protein interaction interfaces for protein recognition as well as for modifying their binding affinities (76).

Deubiquitination

The opposite of ubiquitination is deubiquitination, the process in which the covalently attached ubiquitin is removed or edited (77). As with ubiquitination, deubiquitination is closely regulated. The human genome encodes for about 80 deubiquitinating enzymes (DUBs) that can be subdivided into five different families (78). These families are: ubiquitin carboxy-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins (MJDs) and the mettaloproteases JAB1/MPN/MOV34 (JAMMs) (76).

Removing the ubiquitin from the target protein plays an essential role in two processes: protein degradation by the 26S proteasome and ubiquitin biosynthesis/recycling (Figure 4) (65). Deubiquitinating enzymes are very tightly regulated through their recruitment to the target proteins, chain specificity, and intrinsic catalytic activity (79). Deubiquitination can assist in rescuing the substrate from destruction as well as changing the protein's location or activation state (80). Therefore, this process is very important in many cellular functions, including regulation of receptor

trafficking, cell migration, intracellular signaling, cell cycle progression and transcriptional control (81). Studying deubiqutinating enzymes and mechanisms is therefore just as important as the ones involved in ubiquitinating.

Autophagy

Eukaryotic cells use two systems for protein degradation: the ubiquitin-proteasome system (UPS) for short-lived and abnormal/misfolded proteins and the autophagy-lysosome pathway for bulk degradation of long-lived proteins as well as damaged organelles (82, 83). The word “autophagy” is derived from the Greek for “self-eating”, originally proposed over 40 years ago by Christian de Duve and can be subdivided into three categories depending on its cargo: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (84, 85). When autophagy is activated a pre-autophagosomal double membrane structure is created to surround what is destined for degradation. The outer membrane then fuses with the lysosome to form a single membrane vesicle called the autolysosome, where the inner membrane and contents are degraded (Figure 5) (82). The pathway is important in many physiological functions, such as cell development, cell death, pathogen infection, and degradation of ubiquitinated proteins; any imbalance in this process is detrimental for the cell and can lead to diseases such as cancer or Parkinson’s disease (86, 87). The process was believed not to be selective, but recent studies have shown that there are proteins that function to target ribosomes, lipid droplets, certain proteins, pathogenic bacteria, and specific organelles for destruction (88-95). Autophagy differs from the UPS system in that it doesn’t only eliminate single proteins, but it is just as selective

(96). Another belief that is no longer held to be true was that the UPS and autophagy pathways were distinct, but new evidence shows that there is a cross-talk between the two in which lack of autophagy leads to accumulation of ubiquitinated proteins. The opposite is also true: when the preoteasome is inhibited, it leads to the activation of autophagy, suggesting that the two pathways are indeed linked (97-99).

Autophagy has been linked to protein, lipid, and glycogen degradation (100). In cells under starvation conditions, in which the amount of free amino acids for protein synthesis has been compromised, there is an increase in autophagy that increases amino acid turnover.

There is also evidence that ubiquitination is essential in the autophagy process. Proteins, such as p62/SQSTM1 and NBR1, contain both an ubiquitin-associated (UBA) domain as well as the WXXL motif called the LC3 interacting (LIR) domain, raising the possibility that these proteins are working as adaptors in ubiquitination as well as autophagy (94, 101). In addition, this link might be able to explain how autophagy can be so specific.

Mitophagy

Mitochondria are the primary cellular site for energy production, apoptosis, Ca^{2+} buffering and macromolecule synthesis; it also is the major source of reactive oxygen species (ROS) that may oxidize their own lipids, proteins and DNA (102). Damaged mitochondria release pro-apoptotic factors such as Omi/HtrA2 and cytochrome *c* which lead to cell death through the intrinsic apoptotic pathway (103). By removing these damaged mitochondria through mitophagy, the cell protects itself from apoptosis. In

mammalian cells, mitophagy is preceded by mitochondrial fission to help break down the mitochondria into more manageable pieces as well as for quality control segregation (104). There appears to be two steps necessary for mitophagy to occur: activation of the autophagic machinery followed by the selection of the dysfunctional mitochondria (105-107). Mitophagy is not only present in cells under stress, but also in cell differentiation. Erythrocytes do not have mitochondria or other organelles due to their removal through mitophagy with the assistance of Nix (NIP3-like protein X, also known as BNIP3-like protein (BNIPL3)) (108).

A recently identified pathway, and the only one thus far, requires the function of PTEN-induced putative kinase protein 1 (PINK1) and the E3 ubiquitin ligase parkin (109). To further validate their importance in mitophagy, the gene that encodes for parkin has been shown to be mutated in Parkinson's disease (110). Parkin is an E3 ubiquitin ligase which is expressed in many tissues suggesting widespread physiological function, however, its deregulation is linked to neuronal loss in substantia nigra (111). During cellular stress, parkin translocates from the cytosol to the mitochondria. The same accumulation of parkin is seen when cells lose their ability to fuse or lose mitochondrial potential, but this does not occur in healthy cells. If the stress is prolonged, mitophagy is seen (88). With this information, it was hypothesized that parkin mediates mitochondrial quality control. The recruitment of parkin to the mitochondria is mediated by the activity of PINK1 (Figure 6) (111-117).

PINK1 translocates and is rapidly degraded in healthy mitochondrion; however, accumulation of PINK1 is seen in injured/damaged cells, indicating a mechanism for sensing mitochondrial damage (111). When the mitochondrion is damaged, there is a

halt in PINK1 destruction, which leads to the accumulation of the protein and the recruitment of Parkin specifically (111, 116, 118). The mechanism which degrades PINK1 or how its accumulation leads to the recruitment of parkin is still unclear.

The E3 ubiquitin ligase activity of parkin increases when it is translocated to the mitochondrion, which is believed to play an important role in the ubiquitylation of mitochondrial substrates, one known substrate being the voltage-dependent anion channel 1 (VDAC1) (88, 116). Using the uncoupling agent carbonyl cyanide m-chlorophenyl hidrazone (CCCP) to induce mitophagy, leads to an increase in the Lys63-linked chains in the outer mitochondrial membrane, an event normally associated with signaling and not proteasomal degradation (115, 116, 119, 120). The ubiquitinated proteins then interact with ubiquitin-binding adaptor proteins, such as p62, which then can interact with the autophagosomal protein LC3 (101, 105).

Another substrate of parkin that is relevant to mitophagy is mitofusin, which when degraded can inhibit re-fusion of mitochondria. We have also recently shown that mitofusin is a substrate of Mulan (121-123). Atg8 is a family of proteins composed of LC3, GATE-16/GABARAP, all of which have been shown to be critical in the maturation of the autophagosome by interacting with phosphatidylethanolamine on the autophagic membrane at different time points. While LC3 is important in the elongation of the phagophore membrane, both GATE-16 and GABARAP are essential in a later stage of maturation (124).

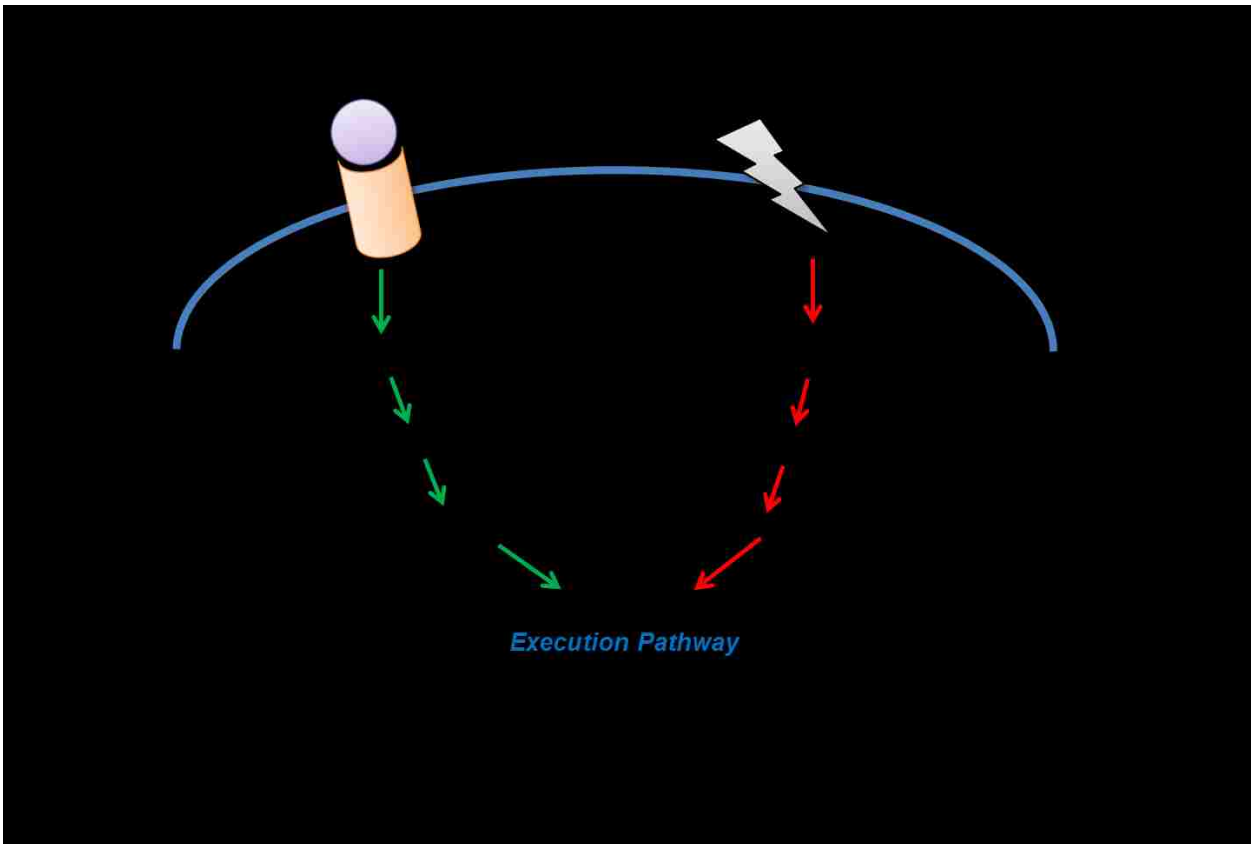

Figure 1. Schematic representation of the two major apoptotic pathways: intrinsic and extrinsic.

There are different signals needed to activate each of the two pathways, a cascade of molecular events follows leading to apoptosis. Mitochondria permeability transition (MPT).

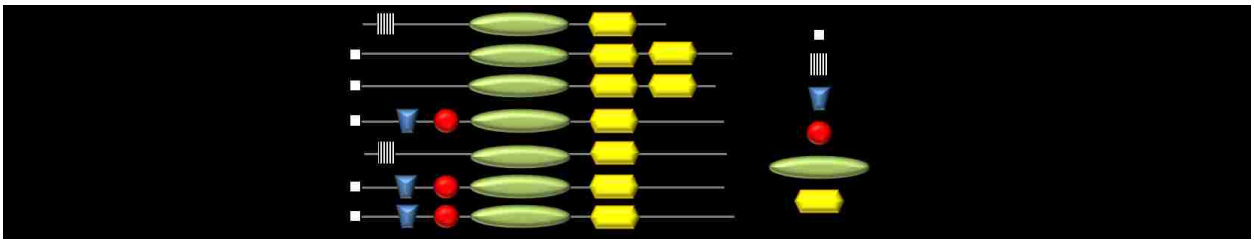

Figure 2. Domain organization of HtrA family members.

Full-length Omi/HtrA2 consists of 458 amino acids serine protease located in the inter-membrane space (IMS) of the mitochondria. The mature form of Omi/HtrA2 lacks the first 133 amino acids. The mature form contains a Trypsin-like proteasome domain as well as a PDZ domain. The protease domain is composed of two six-stranded beta-barrels and the active site contains the amino acid triad His-Asp-Ser. Both domains have been shown to be important in Omi/HtrA2's function.

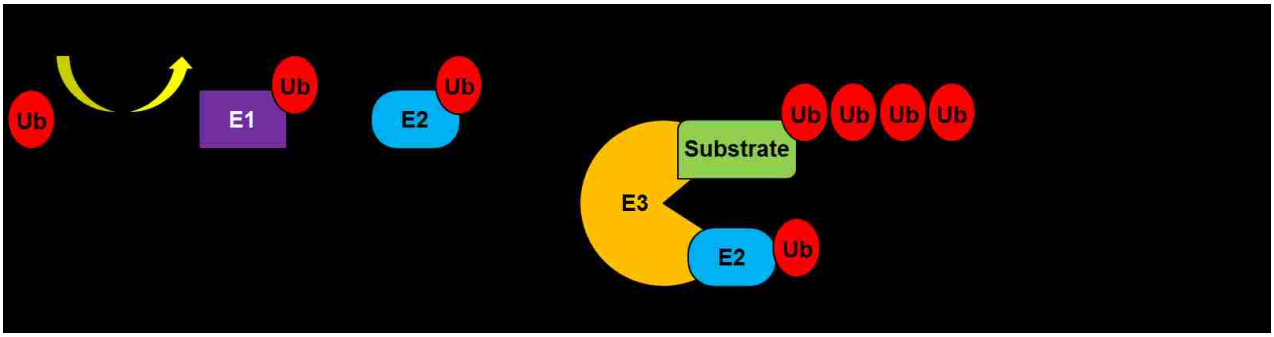

Figure 3. Protein ubiquitination.

The process of ubiquitination is achieved by three enzymes: activating ubiquitin enzyme (E1), conjugating ubiquitin enzyme (E3) and ligase ubiquitin enzyme (E3). While there is a single E1, there are many E2s and several E3s. The activation of ubiquitin occurs through a thioester bond formation which requires ATP. The ubiquitin when attached to E2 can be directly transferred to a substrate when they are brought closer together by the E3 (as seen with the RING family) or it must first be transferred to the E3 itself (HECT family). The number and type of ubiquitin chain varies and determines the fate of the substrate.

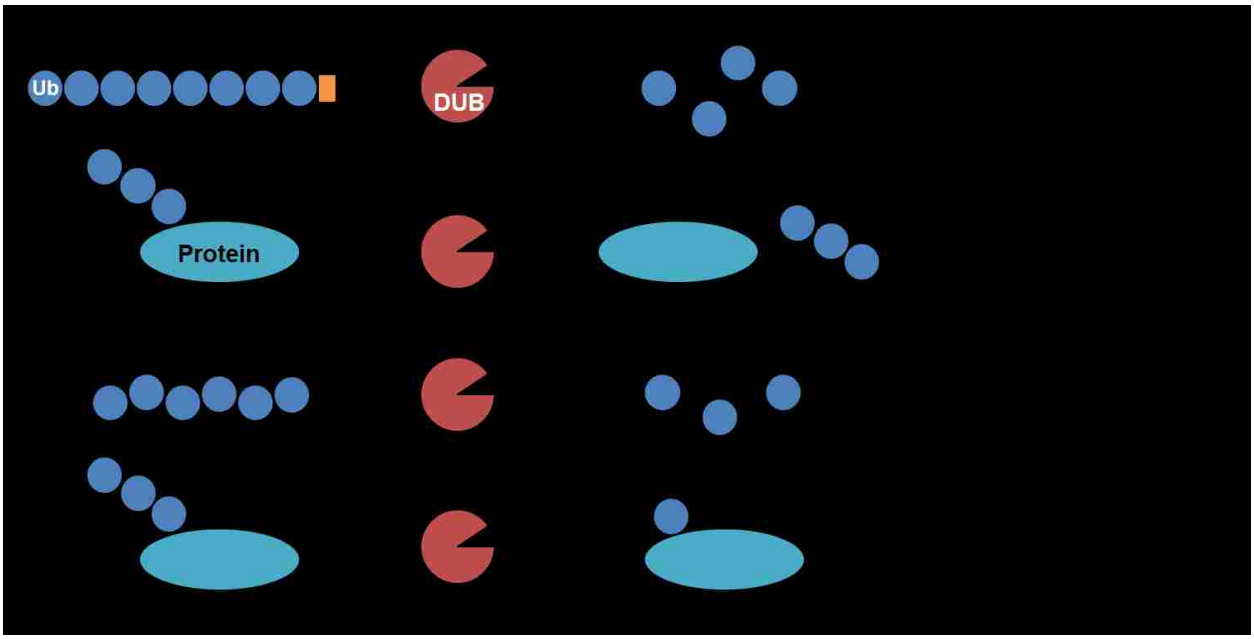

Figure 4. Function of the deubiquitinating enzymes.

Deubiquitinating enzymes (DUBs) play many different roles to keep the cell in balance. **(A)** Ubiquitin (Ub) is encoded as transcribed and translated as a linear fusion, consisting of multiple subunits. DUBs will process this chain into free Ubs that can then be used. **(B)** When a protein is Lys-48 polyubiquitinated for degradation it is translocated to the 26S proteasome in the cytoplasm. Other chains, such as Lys-63, act as non-degradative signaling that can change a protein's function or location. These processes can be stopped and reversed by the removal of the targeting chain by the DUB. **(C)** Before the protein is tagged for destruction, the chain is removed by a DUB. This is important in keeping a balance in free Ub present in the cell. **(D)** Different chains and different number of Ubs have different function. Some DUBs can edit the chains, therefore changing the protein's final outcome.

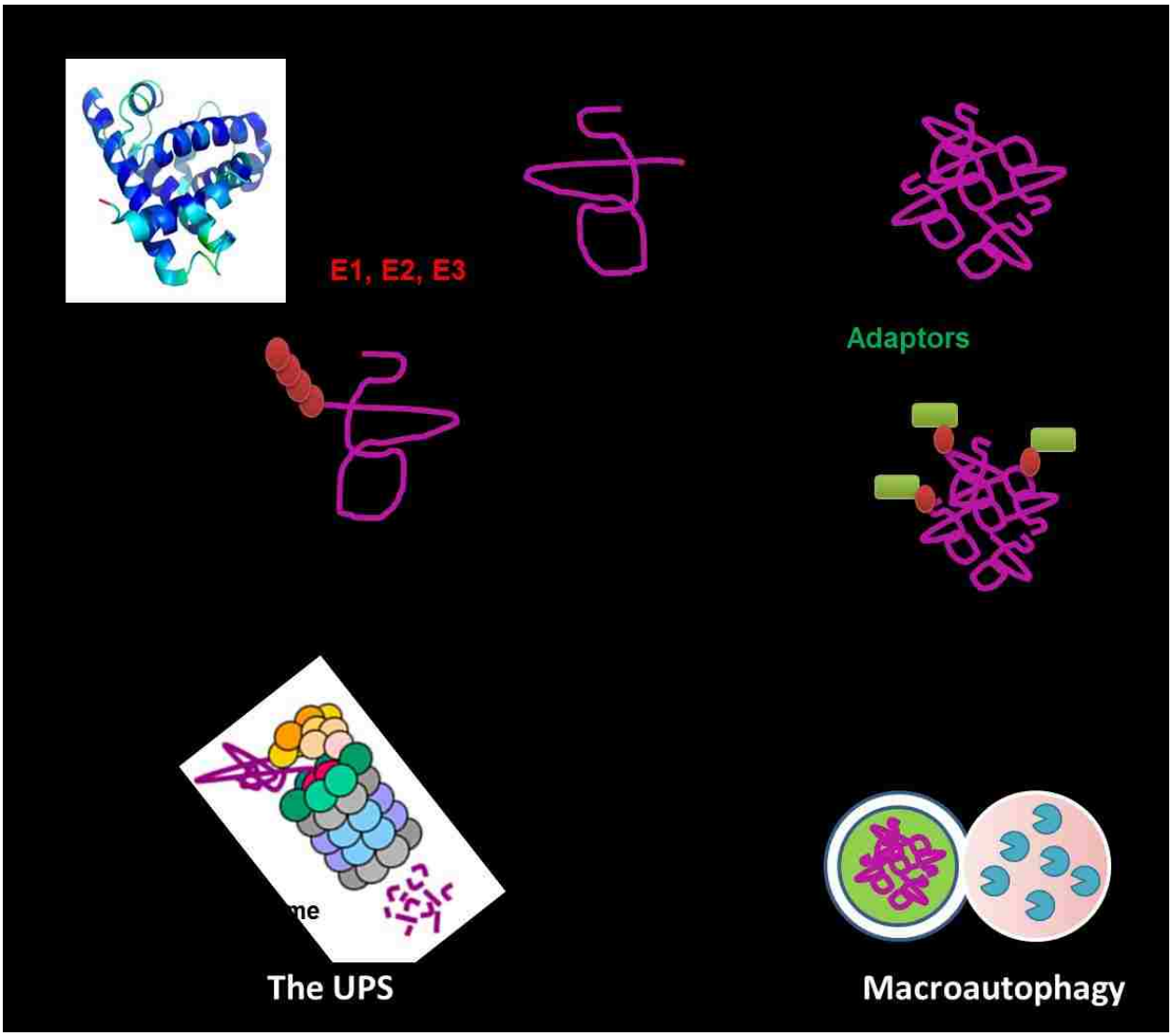

Figure 5. Protein quality control.

Nascent proteins must be folded to their final tertiary structure, and this can be achieved with the help of chaperones. When native proteins are misfolded or unfolded the chaperones can refold them, but if the chaperons are overwhelmed or cannot do this task, the proteins can become aggregates or are tagged for destruction by the ubiquitin-proteasome system (UPS). Protein aggregates are degraded by a process known as macroautophagy which is initiated by adaptor proteins, followed by the formation of the autophagosome and which is then delivered to the lysosome.

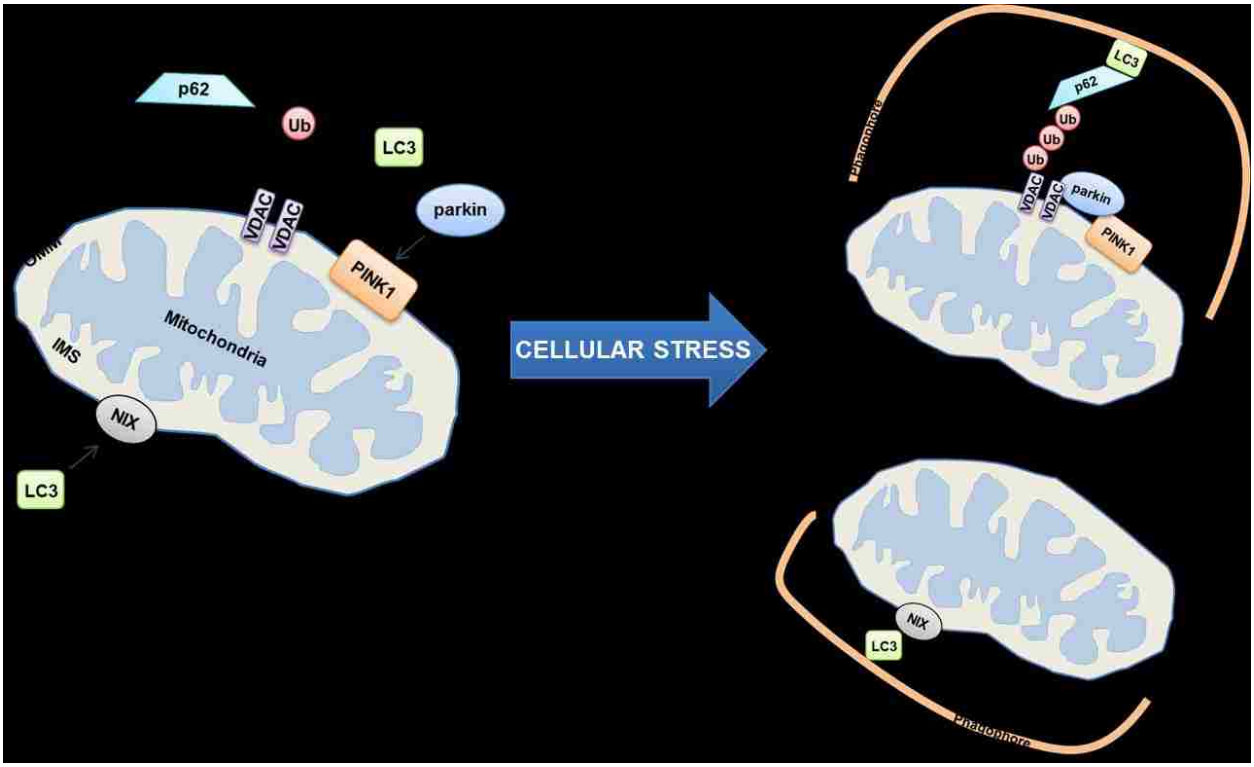

Figure 6. Current model of mitophagy.

Currently there are two proposed pathways for mitophagy: the E3 ligase parkin is recruited to the mitochondrion by PINK1, which is accumulated in mitochondria under stress. The ligase then ubiquitinates its substrates, one of which is believed to be VDAC1, followed by the recruitment of the adaptor protein p62. The adaptor protein then binds to the ubiquitin chain and through its LIR domain it interacts with LC3 in the phagophore. The phagophore closes and matures into the autophagosome. The second pathway involves Nix, a mitochondrial protein found in the outer mitochondrial membrane (OMM) that contains an LIR sequence. By being embedded in the mitochondria, Nix bypasses the need for substrate ubiquitination and it can directly target the mitochondrion to the autophagosome.

CHAPTER 2: ATF4 INTERACTS WITH ABRO1/KIAA0157 SCAFFOLD PROTEIN AND PARTICIPATES IN A CYTOPROTECTIVE PATHWAY

As previously published in Biochimica et Biophysica Acta (BBA) - Molecular Cell Research

Volume 1823, Issue 12, December 2012, Pages 2149–2156

Introduction

Our studies are focused on Abro1, which is the scaffold protein of the BRISC deubiquitinating enzyme (DUB) complex, and its potential regulation through specific protein-protein interactions during cellular stress. Abro1 shares 39% similarity with another protein, Abraxas, which is a scaffold protein for the BRCA1-A (breast cancer 1) DUB complex (125). Both complexes share three subunits: MERIT40/NBA1, BRE/BRCC45 and BRCC36/BRCC3, a member of the JAMM/MPN+ (JAB1/MPN/Mov34) DUB metalloproteases (77, 126-129). The similarity between Abro1 and Abraxas is present at the amino-terminus and this part of the protein interacts with the common subunits. We therefore assume that the distinct function of the BRCA1-A and BRISC complexes is conferred by the unique carboxy-termini of these proteins. Abraxas' carboxy-terminus interacts with BRCA1, which targets the BRCA1-A complex to specific DNA damage foci while the function of the Abro1 carboxy-terminus domain is unknown (125, 127, 130-132). Our previous studies have shown that this domain can interact with a human cardiospecific transcription factor, THAP5 (thanatos associated protein 5) (47). THAP5 is predominantly expressed in the human heart whereas Abro1 is expressed ubiquitously, suggesting the existence of other as yet unidentified proteins that interact with the unique carboxy-terminus of Abro1 and affect its function. Furthermore, there is a disproportional number of subunits

between the better characterized BRCA1-A complex consisting of six subunits and the BRISC complex consists of only four subunits. This is in spite of the fact that both scaffold proteins, Abro1 and Abraxas, are similar and comparable in size (126, 127).

In the present study, we used the yeast two-hybrid system to isolate proteins that interact with the unique carboxy-terminus of Abro1. We screened a mouse embryonic cDNA library that provides a broad representation of various tissue specific mRNAs. We isolated JunD, ATF4 and ATF5 as specific interactors. These interactors are all distinct members of the AP-1 family of transcription factors (133). They carry a basic-leucine zipper motif at their carboxy-terminus and our studies show this domain to interact with Abro1. We selected ATF4 for more detailed studies since this protein is expressed ubiquitously and it has previously been shown to be a stress modulator with an important function in heart disease (134, 135). Our results clearly show that Abro1 and ATF4 interact both *in vitro* as well as *in vivo* and this interaction is observed predominantly in the cell nucleus. Furthermore, during ER stress the protein level of ATF4 dramatically increases leading to a concurrent up-regulation of the ATF4-Abro1 complex. Our previous studies show that Abro1 provides cytoprotection against oxidative stress-induced cell death (136). We now demonstrate that ATF4 is necessary for this function of Abro1, since reducing the ATF4 protein level significantly impairs its anti-apoptotic role. Finally, our results suggest that Abro1 can be involved in multiple specific interactions with various transcription factors. These interactions can either be tissue specific or in response to a particular stress. The potential function of these interactions could be the translocation of Abro1 scaffold protein and the BRISC complex to various subcellular compartments including the cell nucleus. This in turn potentially

allows the BRISC DUB complex to act on specific local Lys63-linked polyubiquitinated substrates leading to cytoprotection.

Materials and Methods

Yeast two-hybrid screen

The EGY 48 yeast strain with the LexA-β galactosidase reporter construct (PSH 18-34) was used to perform a yeast two-hybrid screen on a mouse embryonic cDNA library as previously described (32, 46, 47). The bait used was the unique carboxy-terminus sequence of the Abro1 protein (amino acids 199-415) cloned in the pGilda vector (Clontech) and expressed as a LexA fusion protein. Several interacting proteins were identified in this screen, including a full-length cDNA for JunD and partial cDNAs for ATF4 and ATF5 (a complete list of the interactors found is summarized in Table 1). The full-length cDNAs for ATF4 and ATF5 were isolated from the mouse embryonic cDNA library using the following specific primers:

mouse ATF4 full length:

Fw 5'-GCTTCGAATTCATGACCGAGATGAGCTTCCTG -3',

Rw 5'-CACCGCT CGAGTTACGGAACTCTCTTCTTCCCCC-3';

mouse ATF5 full length:

Fw 5'-GCTTCGAATTCATGTCACTCCTGGCGACCC-3',

Rw 5'-CACCGCTCGAGCTAGGTGCTGCGGGTCCTC -3'.

For the bZIP constructs of ATF4, ATF5, JunD, fosB and Maf the following primers with *EcoRI* and *XhoI* restriction sites were used:

mATF4 aa280:

Fw 5’-GCTCGAATTCGACCCACCTGGAGTTAGTTTG-3’;

mATF5 aa195:

Fw 5’-GCTTCGAATTCCCCGCCCGCCCAGCCCCTTATC-3’;

mJunD aa241:

Fw 5’-GCTTCGAATTCCCGGACGTGCCGAGCTTCGGC-3’;

mfosB aa121:

Fw 5’-CCGGAATTCGGTGGGCCTTCAACCAGCAC-3’,

Rw 5’-CTGCTCGAGTTATGGCAAATCTCTCACCTCGC-3’;

mMaf aa286:

Fw 5’-CCGGAATTCATGTCGGTGCGCGAGCTGAAC-3’,

Rw 5’-CTGCTCGAGCTACCGTTTTCTCGGAA GCCGTTG-3’.

Primers were designed with an *EcoRI* site at the 5’ end and *XhoI* site at the 3’ end. PCR products were digested and cloned in frame into the prey pJG4-5 vector as previously described (46). The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis using LexA-antibodies (for baits) or HA-antibodies (for preys).

Cell culture

HEK293T cells were grown in DMEM supplemented with 10% fetal calf serum (Atlanta Biologicals), 2mM L-glutamine, 1.5g/l sodium bicarbonate, 1mM sodium pyruvate, 50U/ml penicillin, and 50µg/ml streptomycin (Invitrogen) at 37°C and 5% CO_2.

Interaction between Abro1 and ATF4 proteins in HEK293T cells

HEK293T cells were plated in 100mm dishes and transfected with 8.0µg of either EGFP-Abro1$_{1-415}$ plasmid or pEGFP-C1 empty vector (Clontech) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Fourteen hours after transfection, half of the cells were treated with 20µg/ml of tunicamycin for 10 hours and the other half were used as control (untreated). Cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% NP-40, 0.25% sodium deoxycholate) containing the protease-inhibitor cocktail (Roche). The lysates were cleared by centrifugation for 10 min at 10000*g*, and total lysates were collected and subjected to immunoprecipitation. Approximately 200µg of total protein cell lysate was pre-cleared by mixing with protein G-Agarose beads (Roche) for 1 hour followed by incubation with ATF4 (Santa Cruz Biotechnology) or Abro1 (Bethyl) polyclonal antibodies for 2 hours at 4°C. Protein G-Agarose beads were then added and allowed to bind overnight at 4°C. Immunoprecipitates were collected by brief centrifugation for 3 min at 800*g* and washed three times with 500µl RIPA buffer. After the final wash, 25µl of RIPA buffer and 25µl of 2X SDS-PAGE sample buffer were added. All procedures were performed at 4°C. Samples were boiled for 5 min and resolved by SDS-PAGE. Proteins were then electro-transferred onto a PVDF membrane and probed with either a mouse monoclonal GFP (Santa Cruz Biotechnology) or a rabbit ATF4 antibody followed by a goat anti-mouse or Rabbit TrueBlot (eBiosciences) HRP-conjugated secondary-antibodies. The immunocomplex was visualized using a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Sub-cellular localization of Abro1 and ATF4 proteins

To investigate the sub-cellular localization of Abro1 protein in the presence or absence of ATF4, the cDNA encoding the full length Abro1 protein was cloned into pEGFP-C1 vector (Clontech) and the cDNA for the full length ATF4 protein was also cloned into the mRFP-C1 vector (Clontech). HEK293T cells were grown on glass cover slips to 70% confluence and they were then transfected with 1µg of EGFP-Abro1 and mRFP-ATF4 constructs using Lipofectamine 2000 Transfection reagent. Half of the transfected cells were treated with 20µg/ml tunicamycin for 10 hours and the other half used as control. Both control and treated cells were washed and fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and incubated in room temperature with DAPI (Molecular Probes) in order to stain the nucleus. The cover slips were then washed and placed on microscope slides using Fluoromount-G as the mounting solution. Slides were observed using a Leica TCS SP5 II confocal laser-scanning microscope (Leica). The expression and stability of the GFP-Abro1 and RFP-ATF4 proteins was verified by Western blot analysis using ATF4 or Abro1 specific antibodies. For subcellular fractionation, HEK293 cells were grown to 80% confluence and they were either left untreated (control), treated with 20µg/ml tunicamycin or 0.2mM H_2O_2 for 10 hours. Cells were then collected and nuclear/cytoplasmic fractionation was carried out using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (THERMO Scientific) according to the manufacturer's protocol. A total of 40µg of protein from each corresponding fraction was resolved by SDS-PAGE and analyzed by Western blotting using ATF4, Abro1, β-Actin and Histone H1 specific antibodies.

Regulation of Abro1 and ATF4 proteins by tunicamycin

HEK293T cells were plated in 60mm dishes and when they reached 90% confluence they were treated with various concentrations of tunicamycin (0, 2.5, 5, 10, 20 and 50µg/ml) for 10 hours. Total cell lysates were prepared as described above and 40µg of protein was resolved by SDS-PAGE and analyzed by Western blotting using ATF4 or Abro1 specific antibodies.

Inhibiting ATF4 protein level and Annexin V assay

HEK293T cells grown in 35mm dishes were co-transfected with 2.0µg pEGFP-C1 empty vector or pEGFP-Abro1 plasmid together with siRNA specific for ATF4 (siATF4) siGENOME SMARTpool or scrambled siRNA (siCON) (Thermo Scientifc). Some of the transfected cells were treated with 0.2mM of H_2O_2 for 10 hours. The percentage of apoptotic cells in the transfected population was estimated by staining with phycoerythrin-conjugated Annexin V at room temperature for 15 min in 1X binding buffer followed by analysis on a FACSCalibur flow cytometer (47, 136). Cells were also used to prepare cell lysates for Western blot analysis using ATF4 and Abro1 specific antibodies.

Results

Isolation and characterization of Abro1 specific interactors

We employed a yeast two-hybrid system to isolate LexA-Abro1$_{199-415}$ interactors. We used a mouse embryonic cDNA library in order to screen as many diverse proteins

as possible, including any tissue-specific interactors. Furthermore, using a cDNA library constructed from primary cells avoids a potential problem often seen in cDNA libraries prepared from transformed cell lines which often have deregulated expression of genes involved in cell growth as well as cell death. The bait in this screen was the LexA-Abro1$_{199-415}$, which represents the coil-coil domain as well as the unique carboxy-terminus of Abro1 protein that is completely different from its homologue, Abraxas (Figure 7A). The screen was performed as previously described (32, 46, 47). Approximately one million independent yeast colonies were screened, one hundred interactors were isolated and after further analysis several LexA-Abro1$_{199-415}$ specific interactors were identified (Table 1). One of the specific interactors was a cDNA encoding the full length JunD protein, while two other cDNAs represented partial polypeptides of ATF4 (amino acids 21-349) and ATF5 (amino acids 20-283). All three interactors are members of the AP-1 family of transcription factors (133). Other interactors included Ubiquitin C (UbC) and BRCC36. BRCC36 is a known interactor of Abro1 and its isolation provides independent proof that the yeast two-hybrid screening was successfully performed. Using specific primers and rapid amplification of cDNA ends we were able to isolate the full-length cDNAs of ATF4 and ATF5, which were then cloned back into the pJG4-5 vector and their interaction with LexA-Abro1$_{199-415}$ was verified. As with the partial polypeptides, full-length ATF4 and ATF5 proteins were able to interact with LexA-Abro1$_{199-415}$ in yeast (Figure 7B). *Figure 7C* shows that all constructs used in the experiments expressed the recombinant proteins in yeast.

ATF4, ATF5 and JunD are the only members of the large AP-1 family of proteins that were isolated in our screen (133). They are distinct members but they all carry a

similar bZIP domain at their carboxy-terminus (Figure 7D). To investigate the specificity of the interaction between these three proteins and LexA-Abro1$_{199-415}$, as well as to investigate if the bZIP domain is involved in this specific intereraction, we chose two other members of the AP-1 family as controls, namely fosB and Maf (137). ATF4, ATF5 and JunD share 59% similarity at the bZIP domain, whereas fosB and Maf are only 33% similar (Figure 7D) (138). The bZIP domain of ATF4, ATF5 and JunD clearly interacted with the unique carboxy-terminus of Abro1 equally well or even stronger than the full-length proteins (Figure 7B). As predicted there was no detectable interaction between LexA-Abro1$_{199-415}$, and the bZIP domain of either fosB or Maf (Figure 7B).

Interaction of Abro1 with ATF4 in mammalian cells during stress

We focused our studies on ATF4 because this protein, like Abro1, is expressed ubiquitously, known to be involved in generalized stress response and participates in cardiovascular disease (139). To investigate whether Abro1 can interact with ATF4 *in vivo*, in the presence or absence of cellular stress, HEK293T cells were transfected with a construct encoding full-length Abro1 fused to GFP (GFP-Abro1) or empty pEGFP-C1 vector. Tunicamycin was used to induce ER stress and twenty-four hours after transfection total cell lysates were collected and a GFP antibody was used to precipitate the GFP-Abro1 protein. The presence of any ATF4 protein in the complex was monitored by Western blot analysis using an ATF4-specific polyclonal antibody. *Figure 8A* shows that endogenous ATF4 interacts with GFP-Abro1 in HEK293T cells under normal conditions as well as during ER stress. After tunicamycin treatment, another form of the ATF4 polypeptide with a higher molecular weight also appears to associate

with Abro1 (140). In addition, we performed the reverse experiment using the ATF4 antibody to precipitate endogenous ATF4 and any associated GFP-Abro1 protein. *Figure 8B* clearly shows that endogenous ATF4 interacts with the GFP-Abro1 protein. There was not any detectable interaction when GFP protein was expressed alone (Figure 8A and B).

Subcellular localization of Abro1 and ATF4 proteins during stress

Abro1 is predominantly a cytoplasmic protein (127) while ATF4 is found both in the nucleus and the cytoplasm (141). To better understand the biological role of the ATF4-Abro1 interaction, HEK293T cells were co-transfected with GFP-Abro1 (amino acids 1-415) and mRFP-ATF4 (amino acids 1-349). Using a Leica TCS SP5 II confocal laser-scanning microscope we observed that in the absence of stress, GFP-Abro1 was predominantly in the cytoplasm whereas mRFP-ATF4 was in the cell nucleus (Figure 8C, top panel). When transfected cells were treated with tunicamycin, some of the GFP-Abro1 protein translocated to the nucleus where it co-localized with the mRFP-ATF4 protein (Figure 8C, bottom panel). In cells co-transfected with GFP-Abro1 and empty mRFP, the GFP-Abro1 protein remained in the cytoplasm after tunicamycin treatment (results not shown). To quantify these results, a total of 300 co-transfected cells were counted between 15 fields of view for each control or following tunicamycin treatment. The average of co-transfected cells with GFP-Abro1 in the cytoplasm was 60%, which decreased to 28% in cells treated with tunicamycin. Cells with GFP-Abro1 in the nucleus increased from 40% in the control to 72% in tunicamycin treated cells.

To verify that the results are not due to the overexpression of these two GFP/RFP-fusion polypeptides, we investigated the potential translocation and co-localization of endogenous Abro1 and ATF4 proteins. For this experiment, untransfected cells were treated with tunicamycin or H_2O_2 to induce cellular stress. The cytoplasmic and nuclear fractions were isolated and analyzed by SDS-PAGE followed by Western blot. *Figure 8E* shows that there was a small amount of Abro1 protein in the cell nucleus of control untreated cells. This level of nuclear Abro1 protein increased in cells treated with tunicamycin or H_2O_2. ATF4 was almost exclusively present in the cell nucleus; H_2O_2 treatment increased ATF4 protein level while tunicamycin had a much greater effect. Furthermore, the mobility of the ATF4 polypeptide in the SDS-PAGE gel was slightly different in cells treated with tunicamycin or H_2O_2. This is probably due to posttranslational modification suggesting tunicamycin and H_2O_2 could regulate ATF4 protein through different mechanisms.

Regulation of ATF4 and Abro1 protein levels by tunicamycin

Translocation of Abro1 to the nucleus seems to occurs when ATF4 protein is overexpressed and at the same time cellular stress is induced. Therefore we investigated whether the endogenous level of ATF4 or Abro1 is regulated in cells treated with various concentrations of tunicamycin. Figure 9A shows a progressive and significant increase in the ATF4 protein level in cells treated with various concentrations of tunicamycin. In contrast to ATF4, Abro1 protein level remained the same and was not regulated by tunicamycin (Figure 9B).

Abro1 induced cytoprotection requires the presence of ATF4 protein

Abro1 has been previously shown to have a cytoprotective role following oxidative stress (136). To investigate if ATF4 is involved in this process we transfected cells with GFP-Abro1 in the presence of ATF4 siRNA or scrambled control siRNA. Under normal conditions the basal level of apoptosis in cells transfected with GFP-vector alone was 12.5% versus 4% in cells transfected with GFP-Abro1 (Figure 10A). When the cells were treated with H_2O_2, 71% of the cells transfected with GFP-vector alone were apoptotic compared to 44% of cells transfected with GFP-Abro1. Inhibiting the ATF4 protein level using siATF4 significantly reduced the Abro1 cytoprotective ability and the number of apoptotic cells increased from 44% to 64% (Figure 10A). These results clearly demonstrate that ATF4 is necessary and essential for the cytoprotective function of Abro1 following oxidative stress. *Figure 10B* shows the ATF4 protein levels under the various conditions as well as the effect of siATF4. Furthermore, induction of apoptosis by H_2O_2 in HEK293T cells does not affect the endogenous protein levels of Abro1 (Figure 10C) and ATF4 (results not shown).

Discussion

Ubiquitination is a post-translational modification discovered in the 1980s that has gained a lot of attention for its biological significance and its potential role in the development of human disease (for a recent review see (142)). The modification is through the addition of ubiquitin, a small 76 amino-acid protein of about 8.5kDa in size. There are a total of eight different polyubiquitin linkages, and depending on the lysine

used on the ubiquitin for attachment, the number of ubiquitins in a chain or type of chain made, it can bestow a different fate to the substrate protein (143). Ubiquitination is involved in diverse processes such as protein degradation, cell-cycle progression, receptor transport, immune-response and viral infection (70, 144). The most common and better-understood form of polyubiquitination involves the Lys48-linked chain. Substrates that are Lys48-linked are known to be targeted for degradation by the 26S proteasome (143). Another form of ubiquitination is Lys63-linked, which has a non-proteolytic role but regulates a protein's function, its subcellular localization or protein-protein interaction in response to cytoplasmic or nuclear cues (145). Ubiquitination can be reversed by deubiquitination, which is mediated by DUBs. There are over 100 putative DUBs identified in mammalian cells, subdivided into five-distinct families (78, 146). As with ubiquitination, deubiquitination is also highly regulated, it has been implicated in many cellular functions and its deregulation can lead to many human diseases (78, 144, 146).

We have recently uncovered an important role for Lys63-ubiquitination as a cytoprotective mechanism in the heart (136). This mechanism is mediated by the BRISC DUB complex, which contains a member of the JAMM/MPN+ family of DUBs, BRCC36. Another complex that employs this JAMM/MPN+ DUB is BRCA1-A. The BRCA1-A complex has been extensively studied and is found in the nucleus where it interacts with histones and DNA to assist with double-stranded DNA damage repair (125, 126, 132). Unlike the BRCA1-A complex, very little is known about the normal function of the BRISC complex or its role in cellular stress. Both complexes rely on similar scaffold proteins, Abro1 and Abraxas, to recruit the various polypeptides that

make up the complex. The similarity of these two scaffold proteins is restricted to the amino-terminus of the proteins, whereas their carboxy-terminus is quite distinct. The BRCA1-A complex consists of the following polypeptides: RAP80, BRE, MERIT40, Abraxas, BRCC36 as well as the BRCA1-BARD1 heterodimer. The BRISC complex is made up of four subunit polypeptides: BRE, MERIT40, BRCC36 and Abro1. Three of these subunits (BRE, BRCC36 and MERIT40) are common in both complexes and interact with the similar amino-terminus domain of Abro1 and Abraxas. The unique carboxy-terminus of Abraxas binds to RAP80 and the BRCA1-BARD1 heterodimer, where the interaction is regulated by phosphorylation (125, 127). RAP80 allows the complex to interact with ubiquitinated histones in DNA damage foci, while BRCA1-BARD1 interaction helps localize the complex (131, 147). Abro1 has its own unique carboxy-terminus and our recent studies have identified this domain to interact with the human zinc finger protein THAP5 (47). THAP5 expression is highly restricted to the heart and brain, and furthermore it has no mouse or rat homologue (47). Therefore, we assumed that, besides THAP5, there might be other protein(s) that could interact with Abro1 to modulate its activity or its subcellular localization. We used a yeast two-hybrid screen to isolate and characterize Abro1 specific interactors from a mouse embryonic cDNA library. From hundreds of potential interactors, we narrowed down our search to only a few true protein-protein interactors. These included the JunD, ATF4 and ATF5 proteins that are members of the AP-1 family of transcription factors as well as the BRCC36, a known component of the BRISC complex. JunD, ATF4 and ATF5 proteins are known to form homo- and heterodimers that bind to specific DNA sites to regulate the transcription of target genes (133).

We focused our studies on the ATF4-Abro1 interaction because this transcription factor is expressed ubiquitously (similarly to Abro1), it is known to be involved in cellular stress and to potentially play a role in the development and progression of heart disease (134, 135). The interaction of ATF4 with Abro1 was verified in yeast as well as in mammalian cells. The carboxy-terminus of ATF4 was shown to be responsible for the interaction with Abro1. This domain is known to carry the bZIP motif that is involved in protein-protein and protein-DNA interactions (148). Furthermore, this motif is similar in JunD and ATF5 proteins that were also isolated in our screen. We verified the specificity of this interaction by using two other members of the AP-1 family (fosB and Maf) that have limited similarity at their bZIP motif with that of ATF4, ATF5 and JunD, and because of this they were unable to interact with Abro1. To investigate the biological significance of the Abro1-ATF4 interaction in normal cells and during cellular stress, cells were treated with tunicamycin (an ER stressor) and the subcellular localization of these two proteins was observed. Under normal conditions Abro1 is predominantly cytoplasmic and ATF4 is mostly nuclear but in the presence of tunicamycin Abro1 is translocated into the nucleus where it co-localized with ATF4. Since the translocation of Abro1 occurred when ATF4 was overexpressed and stress was applied, we investigated whether the endogenous ATF4 protein level might be regulated by tunicamycin. The ATF4 protein level was found to substantially increase and be directly proportional to the concentration of tunicamycin used. Abro1, on the other hand, did not respond to the tunicamycin treatment and its protein level remained unchanged. Furthermore, tunicamycin treatment not only increased the ATF4 protein level but also its post-translational modification. At higher tunicamycin concentrations

various forms of ATF4 with higher molecular weight appeared. We assume that these represent polyubiquitinated forms of the polypeptide since it is known that ATF4 is ubiquitinated and degraded by the proteasome (149, 150). The biological relevance of the ATF4-Abro1 interaction was further investigated using a previously described assay where overexpression of Abro1 protects cells from apoptosis induced by oxidative stress (136). Oxidative stress also increases the amount of nuclear Abro1 protein. In this assay, the cytoprotective effect of Abro1 was dramatically reduced when the ATF4 protein level was inhibited by siRNA. The interaction of Abro1 with ATF4, as well as ATF5 and JunD, suggests that various nuclear proteins can bind to this scaffold protein in the nucleus. These proteins could act as a “driver” for the Abro1 polypeptide in a similar manner to the BRCA1-BARD1 interaction with Abraxas to direct the BRCA1-A complex to the DNA damage foci (131). In addition, we cannot exclude the possibility that the interaction of ATF4, ATF5 and JunD with Abro1 can also modulate the DUB activity of the BRISC complex. Based on our data, as well as previously published work, we were able to assign three discernible functional domains to the BRISC and BRCA1-A complexes: (a) the scaffold domain, represented by Abro1 and Abraxas; (b) the catalytic domain which includes the common polypeptides BRCC36, MERIT40 and BRE; and (c) the “driver” domain which in BRCA1-A includes the BRCA1-BARD1 heterodimer and RAP80, and in BRISC it can include one of the following four polypeptides: ATF4, ATF5, JunD and THAP5 (Figure 11). The presence of several “driver” proteins that could interact with Abro1 at any time suggests that this process could be further regulated. The identity of the “driver” proteins that interact with Abro1 could be determined by the particular tissue/cell line or the type and severity of the

cellular stress. In addition, we have previously shown that the translocation of Abro1 to the cell nucleus by THAP5 leads to the deubiquitination of specific substrates (136). Our results suggest that either Abro1 translocation carries the complete BRISC complex to the nucleus or the complex assembles soon after nuclear localization of Abro1. It will be important to identify the Lys63-linked polyubiquitinated substrates of BRISC and to investigate how their deubiquitination results in cytoprotection against oxidative stress-induced cell death.

Table 1. A list of Abro1$_{199-415}$ interactors isolated in our yeast two-hybrid screen using a mouse embryonic cDNA library.

Abro1$_{199-415}$ Interactors	Accession Number	Number of Independent Clones
ATF4	NM_009716.2	8
JunD	NM_010592.4	4
BRCC36	NM_001018055	4
ATF5	NM_030693.2	2
UbC	NM_019639	1

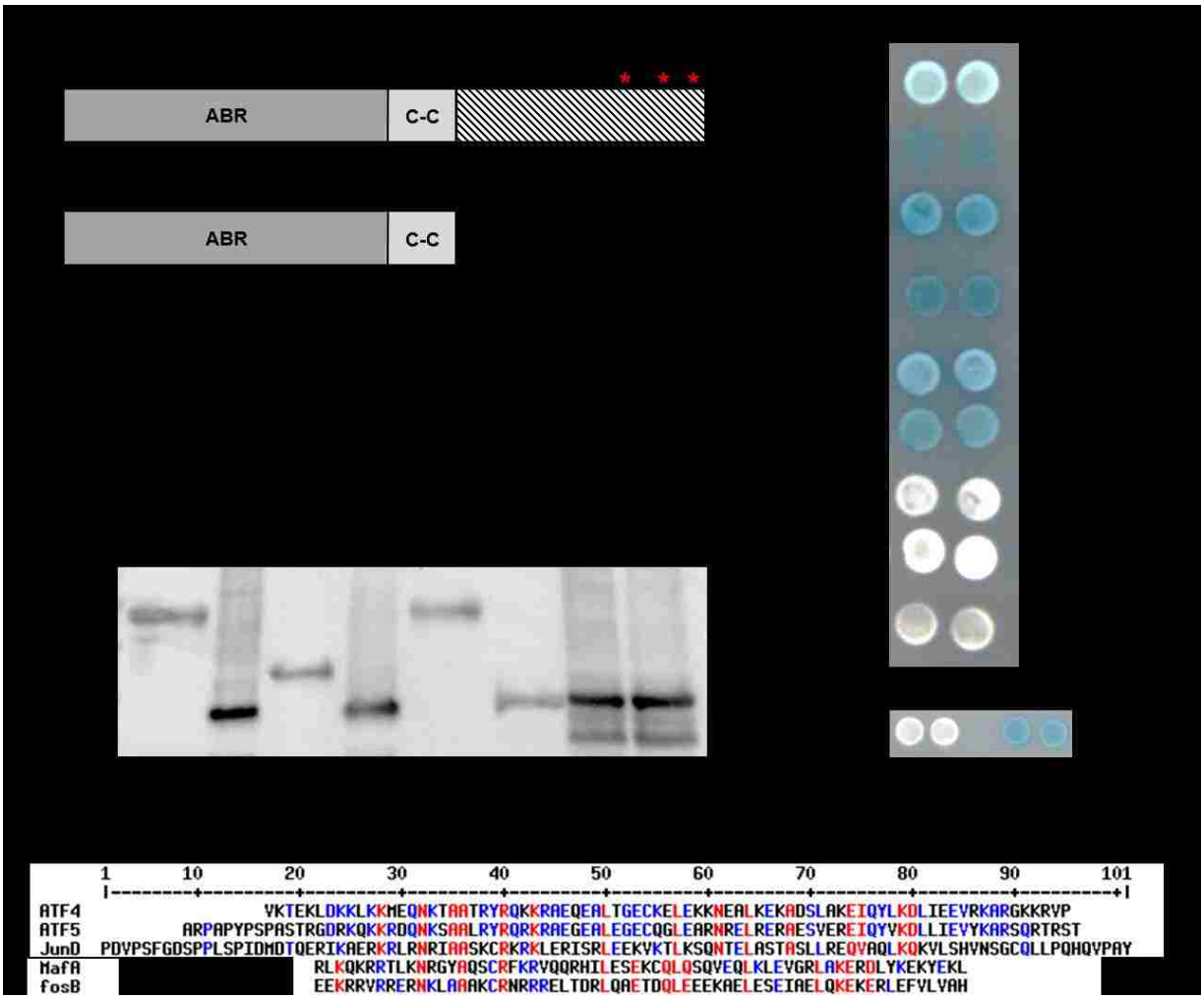

Figure 7. ATF4, ATF5 and JunD interact with LexA-Abro1$_{199-415}$ using their bZIP domain.

(A) A schematic representation of the Abraxas and Abro1 proteins showing their similar ABR and coil-coil domains as well as their unique carboxy-termini. Abraxas contains three phosphorylation sites (denoted by the *) that regulate its interaction with BRCA1-BARD1 which are absent in Abro1 [1]. The unique carboxy-terminus of Abro1 (amino acids 199-415) fused to LexA was used as the bait in the yeast two-hybrid screen. **(B)** Blue yeast colonies result from a positive protein-protein interaction between the bait and prey. **(C)** Single yeast colonies were grown overnight, induced with galactose/raffinose media the next day for four hours and then used in a Western blot analysis to verify the expression and stability of the various recombinant proteins in yeast. **(D)** Alignment of the amino acid sequences of the AP-1 family members used in our experiments to illustrate the similarity in their bZIP domains. [MultAlin software (http://multalin.toulouse.inra.fr/multalin)]

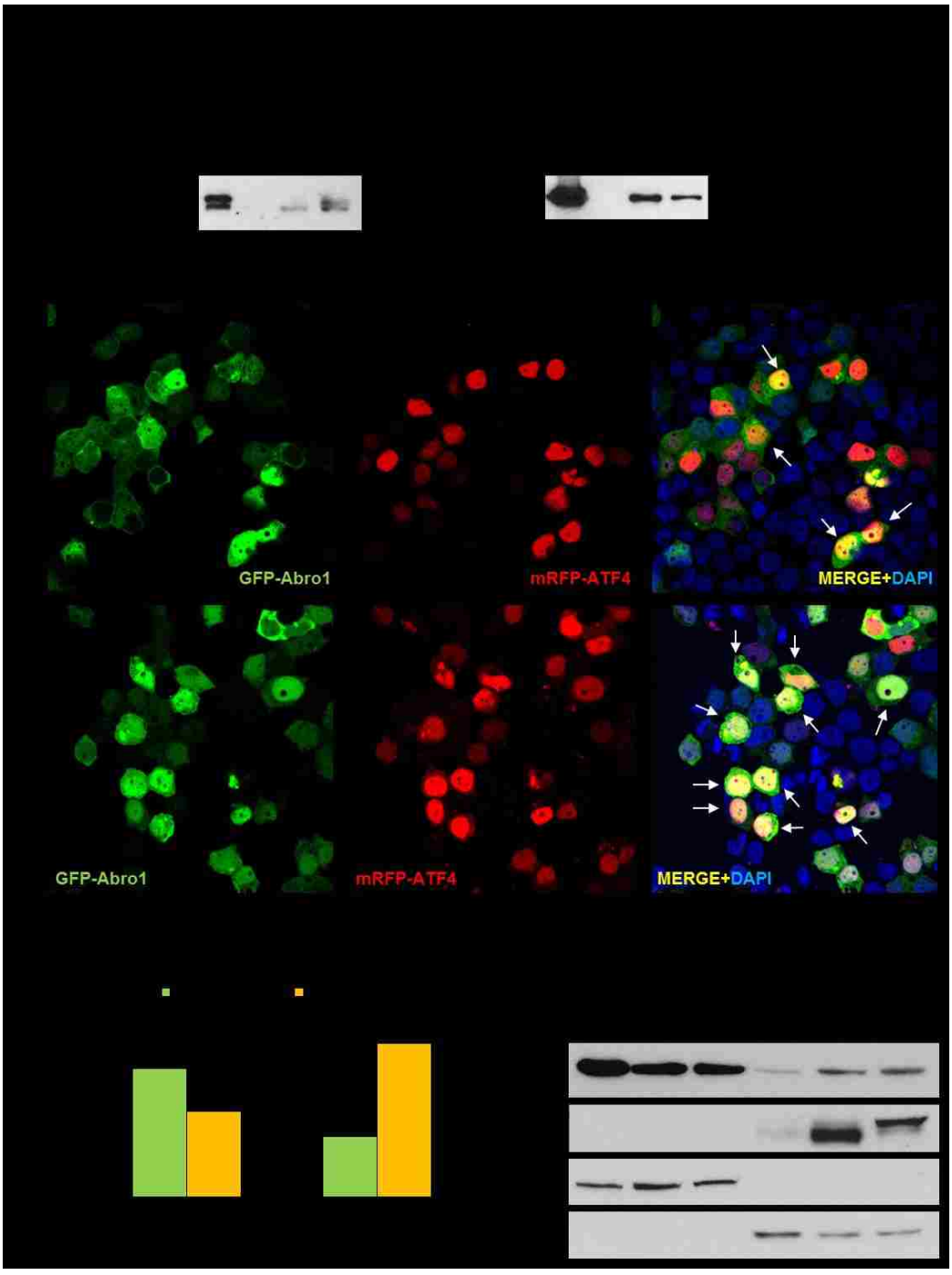

Figure 8. Interaction and subcellular localization of Abro1 and ATF4 in mammalian cells.

(A) HEK293T cells were plated in duplicate and transfected with either GFP-vector or GFP-Abro1. Twenty-four hours after transfection, one of the plates was treated with tunicamycin and the other plate was used as control. GFP-specific antibody was used to precipitate GFP or

GFP-Abro1. The immuno-complex was resolved on SDS-PAGE followed by Western blot analysis and the presence of ATF4 in the precipitated complex was detected using ATF4 specific antibodies. **(B)** The reverse experiment was also performed where ATF4 antibodies were used to precipitate the endogenous protein complex and GFP antibodies were then used to identify any GFP-Abro1 protein bound to ATF4. **(C)** HEK293T cells were plated in duplicate on glass-cover slips and co-transfected with GFP-Abro1 and mRFP-ATF4. Half of the transfected cells were treated with tunicamycin and the other half used as control. Cells were stained with DAPI nuclear staining and visualized using a confocal microscope system. White arrows indicate cells that show co-localization of GFP-Abro1 with mRFP-ATF4 in the cell nucleus. **(D)** Three-hundred cells were counted in total between 15 different fields of view. Co-transfected cells were categorized as having GFP-Abro1 in the cytoplasm or nucleus. A percentage of the total counted cells is represented here. **(E)** HEK293T cells were grown and either left untreated for control, treated with tunicamycin or H_2O_2. Cells were then collected and nuclear/cytoplasmic fractionation was carried out, followed by analysis through SDS-PAGE and Western blot using ATF4 or Abro1 specific antibodies. β-actin and Histone H1 antibodies were used as cytoplasmic and nuclear markers respectively. *, $p < 0.05$ *versus* cytoplasmic control.

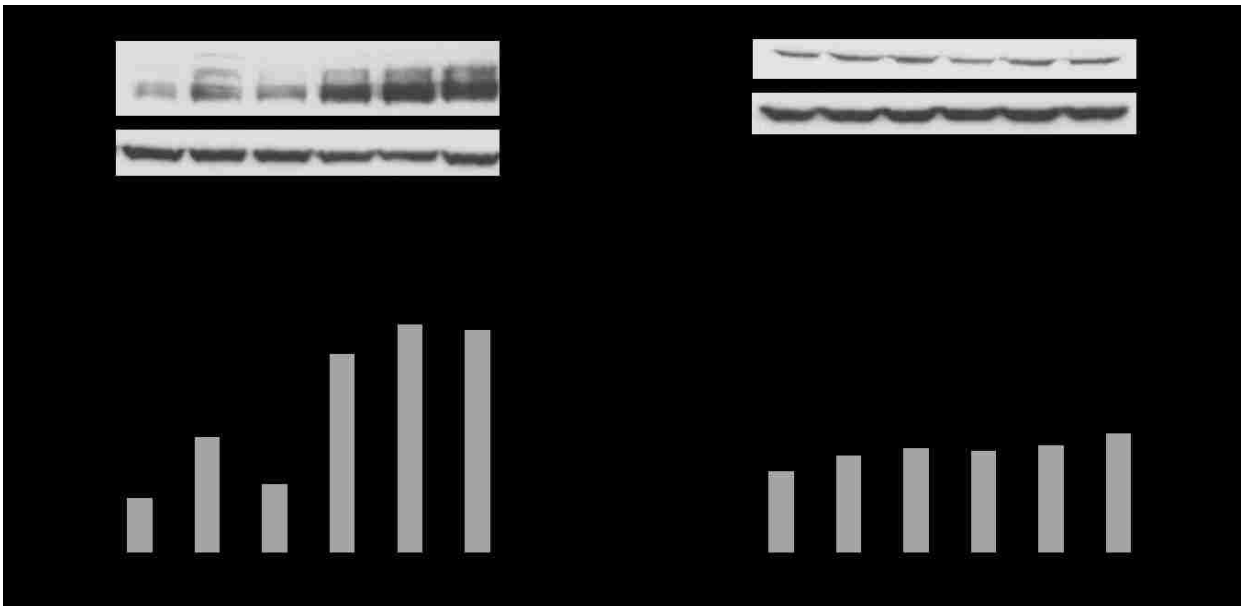

Figure 9. Regulation of ATF4 and Abro1 protein levels by tunicamycin.

HEK293T cells were treated with various concentrations of tunicamycin. Total cell lysates were collected and analyzed by SDS-PAGE and Western blotting using ATF4 **(A)** or Abro1 **(B)** specific antibodies. β-actin was used to verify equal loading of proteins in each lane. Lower panels show ATF4/β-actin or Abro1/β-actin ratio calculated after densitometry analysis.

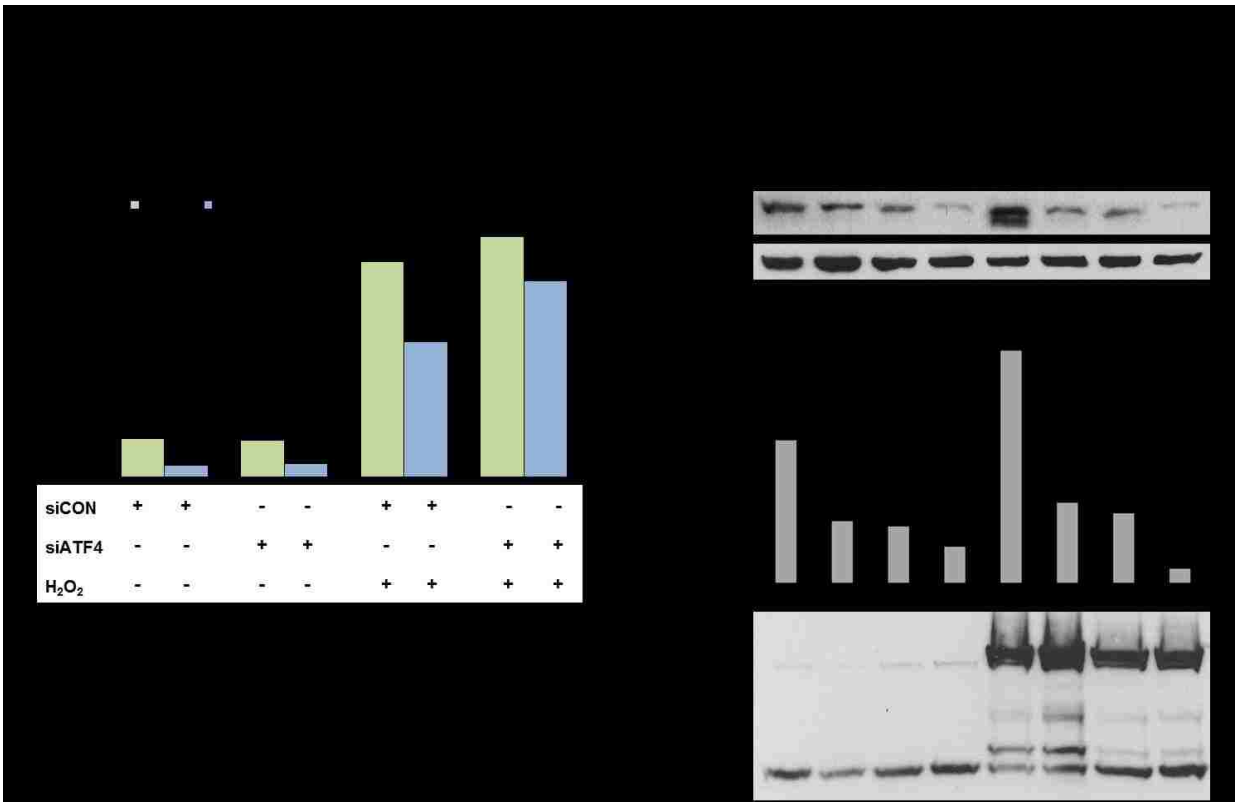

Figure 10. ATF4 is required for Abro1-mediated cytoprotection during oxidative stress.

HEK293T cells were co-transfected in duplicate with GFP-Abro1 or GFP-vector alone as well as with control siRNA (siCON) or ATF specific siRNA (siATF4). Half of the cells were treated with H_2O_2 to induce oxidative stress and cell death. **(A)** The percentage of apoptotic cells in the population was monitored by Annexin V staining and FACS. **(B)** Western blot analysis of the endogenous ATF4 protein level; bottom panel shows ATF4/β-actin ratio calculated after densitometry analysis. β-actin was used to verify equal loading of proteins in each lane. Results shown are means ± S.D. of three independent experiments. **(C)** Western blot analysis for the endogenous level of Abro1 as well as GFP-Abro1 proteins in the samples used for **(A)**. *, $p < 0.05$ *versus* GFP-vector; ‡, $p < 0.05$ *versus* GFP-Abro1 siCON + H_2O_2.

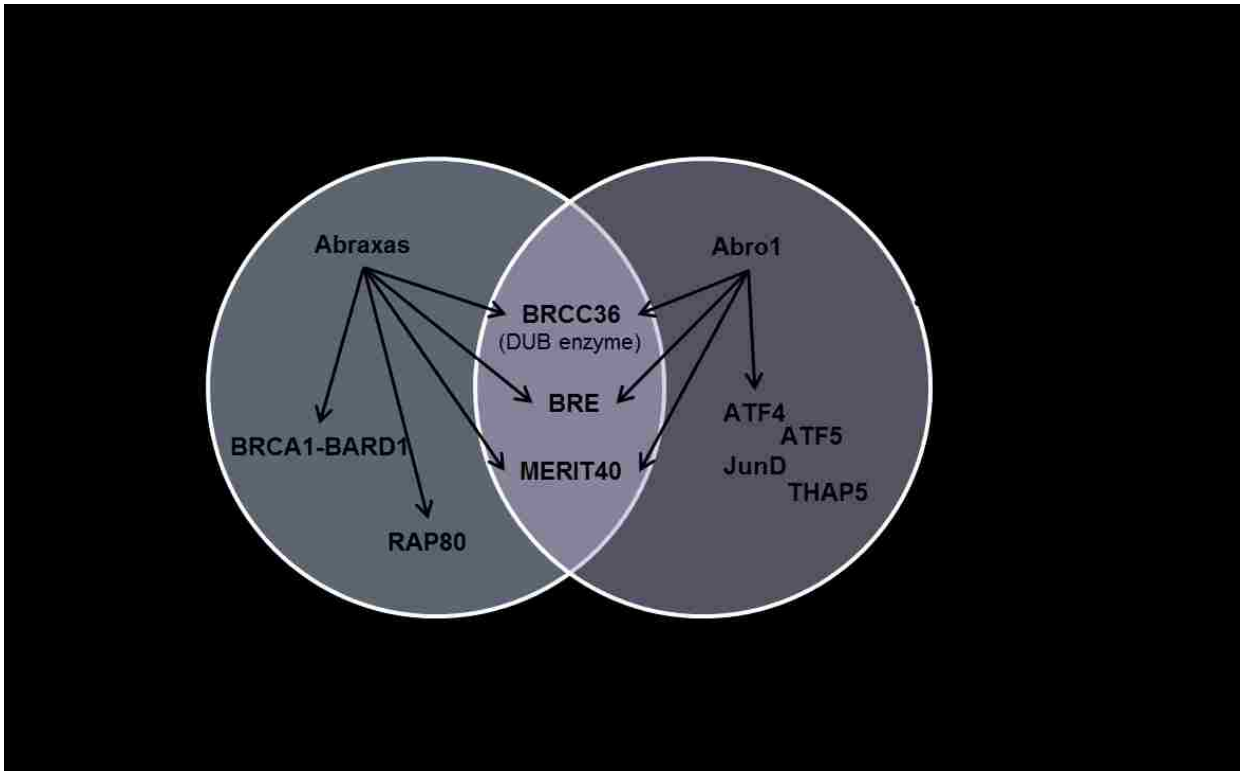

Figure 11. Schematic diagram showing the current composition of the BRISC and BRCA1-A DUB complexes.

Both BRISC and BRCA1-A complexes share three common subunits (BRCC36, BRE, MERIT40) that compose the catalytic core. BRCA1-A complex contains two unique proteins, RAP80 and the BRCA1-BARD1 heterodimer, that are used to target the complex. Our work also identified four unique proteins (THAP5, ATF4, ATF5 or JunD), three of which are members of the AP-1 family, which can be used to target the BRISC complex to the cell nucleus under conditions of cellular stress.

CHAPTER 3: MULAN E3 UBIQUITIN LIGASE IS INVOLVED IN MULTIPLE PROTEIN-PROTEIN INTERACTIONS AND PARTICIPATES IN MITOPHAGY BY RECRUITING GABARAP

Introduction

Autophagy is generally believed to be a process in which nonspecific degradation of cytoplasmic content, including organelles, occurs (87). Recent studies have revealed that there is specificity in autophagy that allows the degradation of specific cargo. Such selective types of autophagy include mitophagy, the process whereby damaged mitochondria are removed from the cell (109). Mitophagy is crucial for many physiological processes such as development and differentiation, and its deregulation has been implicated in Parkinson's disease, as well as various infections and cancers (86, 87). Mitophagy has been shown to be mediated by the E3 ubiquitin ligase parkin, which is recruited to the mitochondria by PINK1 (PTEN-induced putative kinase protein 1). Recent studies have identified yet another mitochondrial E3 ubiquitin ligase, Mulan/Hades/GIDE (mitochondrial ubiquitin ligase activator of NF-κB; hereafter referred to as Mulan) to play a role in mitophagy (123, 151). We have identified Mulan as a specific substrate of the mitochondrial Omi/HtrA2 protease. Furthermore, inactivation of Omi/HtrA2 protease leads to the accumulation of Mulan protein and increased mitophagy (123).

Mulan is one of three E3 ubiquitin ligases that are present in the mitochondria, the other two being MARCH5/MITOL (membrane associated RING-CH5) and RNF185 (152, 153). Mulan is a 352-residue polypeptide that crosses the mitochondria outer membrane (OMM) twice, positioning its amino-terminus - which includes the RING

finger domain - to the cytoplasm and forming a large domain in the inter-membrane space (IMS) of the mitochondria (Figure 11A). Both the mitochondrial localization signal, as well as an intact RING finger domain have been shown to be necessary for Mulan's function (154). Mulan's location suggests that its substrates will be proteins that are either integral or associated with the mitochondria, and whose function can be affected by ubiquitination. Mulan has been shown to directly or indirectly regulate various proteins including NF-κB, JNK, p53, Akt and Mfn2, which suggest that it plays a role in cell growth as well as apoptosis (151, 154-158). In order to investigate the mechanism by which Mulan participates in mitophagy, we set out to identify E2 ubiquitin conjugating enzymes that form specific complexes with Mulan (151). We used the cytoplasmic domain of Mulan (amino acids 259-352) that includes its RING finger domain, and identified four E2 conjugating enzymes, namely Ube2E2, Ube2E3, Ube2G2 and Ube2L3, as specific interactors. We then used fusion baits consisting of Mulan's RING finger domain attached to the full length of each of the four specific E2's that were identified. These fused baits were used to screen for possible substrates of the heterodimer Mulan-E2 complex. In this screening, several distinct interactors were identified for each of the four Mulan-E2 complexes.

One of the isolated heterodimer interacting proteins was GABARAP ($GABA_A$ receptor-associated protein), a known member of the Atg8 that includes LC3 (also known as MAP1LC3). The proteins of the Atg8 family are integral players in autophagy/mitophagy, (159), and are part of the ubiquitin-like conjugating cascade. They share similarities, including their interacting motif which is essential for selective autophagy, however each Atg8 protein also has many distinct features (160, 161).

GABARAP is known to bind to many different proteins and it plays a role in the elongation of the autophagosome (162, 163). Our data show that the interaction of GABARAP requires the presence of Ube2E3 as well as a specific amino acid motif located in the RING finger domain of Mulan. This motif is known as LC3-interacting region (LIR) motif, and is present in other proteins that interact with LC3 and/or GABARAP (101, 162, 164, 165). The consensus for the LIR motif is (W/F/Y)xx(L/I/V) (166). Our present study characterized the mechanism of Mulan's function in mitophagy as the recruiter of GABARAP. Mulan interacts with GABARAP but not LC3B, suggesting that this interaction is involved in the same pathway but operates downstream of PINK1/parkin/LC3.

Materials

Yeast Two-Hybrid Screening of Mulan Interactors

The EGY 48 yeast strain with the LexA-β galactosidase reporter construct (PSH 18–34) was used to perform a yeast two-hybrid screen on a mouse embryonic cDNA library as previously described (32, 46, 47). The bait used was the cytosolic carboxy-terminus sequence of the Mulan protein (amino acids 259-352) cloned in the pGilda vector (Clontech) and expressed as a LexA fusion protein. Both a mouse embryonic and a HeLa cDNA library were used. Several interacting proteins were identified in this screen, including cDNAs for Ube2E2, Ube2E3, Ube2G2 and Ube2L3 (a complete list of the interactors found is summarized in Table 2). To clone the full-length cDNA, primers

were designed with an *MfeI/EcoRI* site at the 5′ end and *XhoI* site at the 3′ end. PCR products were digested and cloned in frame into the prey pJG4-5 vector.

Yeast Two-Hybrid Screening of Mulan-E2 Fusion Interactors

To make the hybrid fusion proteins, primers were designed with the addition of a five amino acid linker (Gly-Gly-Ser-Gly-Gly) as previously described (Table 4) (167). These primers contained the last 21 nucleotides of the Mulan protein and the first 16 nucleotides of the specific E2 flanking the linker sequence. After the first PCR reaction the products for $Mulan_{259-352}$ and the full-length E2 conjugating enzyme were isolated, gel purification, and added together as the template for the second PCR reaction (Figure 12A). The resulting product of the PCR was *MfeI/EcoRI*-$Mulan_{259-352}$-G-G-S-G-G-Specific E2-*XhoI*. The product was then digested using *MfeI*/*EcoRI* and *XhoI* and cloned in frame into the bait pGilda vector. The LexA-fusion proteins were then used as the bait in the screening of a HeLa cDNA library. The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis using LexA-antibodies (for baits) or HA-antibodies (for preys).

Cell Culture

HEK293T cells were grown in DMEM supplemented with 10% fetal calf serum (Atlanta Biologicals), 2mM L-glutamine, 1.5g/l sodium bicarbonate, 1mM sodium pyruvate, 50U/ml penicillin, and 50µg/ml streptomycin (Invitrogen) at 37°C and 5% CO_2.

Interaction Between Mulan and GABARAP Proteins in HEK293T Cells

HEK293T cells were plated in 100mm dishes and transfected with 8.0µg of total DNA between EGFP-GABARAP and either mRFP-Mulan (amino acids 1-352) plasmid or mRFP-C1 empty vector (Clontech) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Fourteen hours after transfection, one plate was treated with 2µM MG132 for 10 hours and the other half were used as control (untreated). Cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% NP-40, 0.25% sodium deoxycholate) containing the protease-inhibitor cocktail (Roche). The lysates were cleared by centrifugation for 10 min at 10000*g*, and total lysates were collected and subjected to immunoprecipitation. Approximately 200µg of total protein cell lysate was pre-cleared by mixing with protein G-Agarose beads (Roche) for 1 hour followed by incubation with GFP (Santa Cruz Biotechnology) polyclonal antibodies for 2 hours at 4°C. Protein G-Agarose beads were then added and allowed to bind overnight at 4°C. Immunoprecipitates were collected by brief centrifugation for 3 min at 800*g* and washed three times with 500µl RIPA buffer. After the final wash, 25µl of RIPA buffer and 25µl of 2X SDS-PAGE sample buffer were added. All procedures were performed at 4°C. Samples were boiled for 5 min and resolved by SDS-PAGE. Proteins were then electro-transferred onto a PVDF membrane and probed with either a rabbit polyclonal Mulan (Novus Biologicals), followed by a Rabbit TrueBlot (eBiosciences) HRP-conjugated secondary-antibodies. The immunocomplex was visualized using a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

LIR-motif Studies

Two amino-acids found in the LIR-like motif of Mulan were mutated, specifically Tyr327Ala and Leu330Ala. To achieve this, two primers were designed where the codons were changed to encode for the Alanine. The LexA-Mulan$_{259-352}$-Ube2E3 was used as the template for the first PCR reaction, where the primers were paired with the LexA forward or reverse primer. In the second round of PCR, a small amount of each product was used as the template along with external primers creating restriction sites. The PCR product was purified and digested with *MfeI* and *XhoI*, then cloned in frame in pGilda vector as described above. Single colonies were selected and sent for sequencing to verify the mutation. Expression of the mutant protein was also verified with Western blot analysis using the LexA-antibody.

Results

Isolation of Mulan$_{259-352}$ E2 interactors

Mulan is a mitochondrial E3 ligase which contains two transmembrane domains, an inter-membrane domain as well as a short cytosolic amino-terminus and a longer carboxy-terminus where the RING domain that is necessary for its function is located (Figure 11A) (154, 155). This cytosolic domain was cloned in-frame in the pGilda vector and expressed as a LexA-Mulan$_{259-352}$ fusion protein that used in a yeast two-hybrid screening. Both a mouse embryonic and a HeLa cDNA library were used and four specific interactors were isolated. All four are members of the E2 conjugating enzyme

family (Table 2) and include the Class I Ube2G2 and Ube2L3 as well as the Class III Ube2E2 and Ube2E3 (Figure 11B).

Creating the Mulan$_{259-352}$-E2 Fusion Bait & Isolation of Substrates

For ubiquitination to occur, the E3 ligase must interact with an E2 conjugating enzyme that provides the substrate specificity. E3 ligases are subdivided into five large families according to the presence of E3 signature motifs: HECT, RING, U-box, PHD and LAP (168). RING family ligases, such as Mulan, act as a scaffold that bring the E2~Ub in proximity of the substrate for the direct transfer of the active ubiquitin (169). Based on this information, we used a modified yeast two-hybrid system (167) to find specific ubiquitination substrates for each of the Mulan-E2 heterodimer complexes.

We created fusion bait proteins where the cytosolic portion of Mulan with the RING domain was fused to one of the four isolated E2 conjugating enzymes using a five amino acid linker (Figure 12A). Expression and stability of each LexA-Mulan$_{259-352}$-E2 bait was tested by Western blotting using anti-LexA antibodies (Figure 12B). Fused baits were then used to screen a HeLa cDNA library and the results from these experiments are shown in Table 3.

Isolation of Mulan$_{259-352}$-Ube2E3 Substrates

We used the LexA-Mulan$_{259-352}$-Ube2E3 fusion bait to screen a HeLa cDNA library. During the screening, we isolated four novel interactors of the heterodimer: GABARAP ($GABA_A$ receptor-associated protein), GRP78 (also known as Hsp5a and immunoglobulin heavy chain-binding protein (BiP)), as well as two members of the

Class I E2 conjugating enzyme family, Ube2D2 and Ube2D3 (Table 3). Ube2D2 and Ube2D3 are members of the Ubch5 family, known as initiation E2 enzymes. These enzymes are important in the addition of the first ubiquitin; they do not play a role in ubiquitin chain elongation (170). It has been shown that E3 ligases can interact with multiple E2 enzymes which can explain this interaction (171). The protein GRP78 is an ER chaperone that has been shown to localize in the cytosol and ER membrane in cells undergoing ER stress where it can interact with caspase-7, -12 and other cytosolic proteins (172). When cells are stressed due to the unfolded protein response (UPR), GRP78 translocates into the mitochondria, where Mulan is located. (173). The GABARAP protein is a member of the Atg8 family and has been linked to autophagy (174). Since Mulan has also been recently linked to mitophagy, we were very interested to further investigate this interaction (151). The isolated clone of GABARAP encompassed amino acids 36-417, lacking the amino-terminus. To test if the interaction would still occur with the full-length protein, we cloned $GABARAP_{1-417}$ into the pGJ4-5 prey vector and monitored the interaction with the fusion protein. The full-length protein was unable to interact with the fusion bait (data not shown); however, we continued to use this as a substrate of the complex because previous researchers have encountered the same problem when using the full-length GABARAP in yeast two-hybrid screenings but were successful in seeing interaction of the proteins in mammalian cells (175).

Heterodimer Interaction Specificity

To investigate if the interaction between GABARAP and GRP78 was specific to the heterodimer $Mulan_{259-352}$-Ube2E3 complex, and not to the individual proteins, we

cloned Ube2E3 full-length in frame in the pGilda vector and used the LexA-Ube2E3, LexA-Mulan$_{259-352}$ as well as the fusion LexA-Mulan$_{259-352}$-UbeE2E3 as baits. There was no interaction between the single bait proteins and the identified substrates, suggesting this interaction is specific for the Mulan-Ube2E3 heterodimer (Figure 13A).

Isolation of Mulan$_{259-352}$-Ube2E2 Substrates

A yeast two-hybrid screening of a HeLa cDNA library was carried out using the LexA-Mulan$_{259-352}$-Ube2E2 as bait and the following possible substrates were isolated: RNF61/MKRN1, RNF103 and RNF14/ARA54. RNF14 and RNF103 proteins are known interactors of Ube2E2 (176, 177) (Figure 13C). RNF14's RING domain was shown to interact with the UBC domain of Ube2E2 both *in vitro* and *in vivo*, while RNF103 was shown to bind Ube2E2 in a yeast two-hybrid system (176, 177). None of the three interactors bound to LexA-Mulan$_{259-352}$. Since the interaction was mediated by the E2 and not by Mulan-E2 heterodimer, RNF14 and RNF103, were not further studied. The third protein isolated as an interactor of LexA-Mulan$_{259-352}$-Ube2E2 was RNF61, also known as Makorin ring finger protein 1 (MKRN1) and it is expressed ubiquitously from fetus to adulthood (178). MKRN1 contains a RING finger domain which gives it the E3 ubiquitin function and it substrates include p53 and p21, both important proteins involved in cell cycle and apoptosis (179). Its interaction with p53 leads to a p53-depenedent cell death pathway by suppressing p21 (179).

E3 conjugating enzymes Ube2E2 and Ube2E3 have very similar amino acid sequence (Figure 13D) so we verified that the interaction of LexA-Mulan$_{259-352}$-Ube2E3 with GABARAP as well as GRP78 was specific. Both GABARAP and GRP78 did not

interact with LexA-Mulan$_{259-352}$-Ube2E2 (Figure 13E), illustrating the specificity of the heterodimer complex.

Isolation of Mulan$_{259-352}$-Ube2L3 and Mulan$_{259-352}$-Ube2G2 Substrates

Next, we used the fusion bait LexA-Mulan$_{259-353}$-Ube2L3 to screen the HeLa cDNA library. We found ARIH2/TRIAD1, an E3 ligase, to interact with our fusion bait (Figure 13B). This protein also interacted with LexA-Ube2L3 bait. ARIH2/TRIAD1 is known to interact with one of the two RING domains of Ube2L3 (180). Due to this information, we did not proceed into mammalian studies with this protein. Lastly, we were unable to screen the HeLa cDNA library with the LexA-Mulan$_{259-353}$-Ube2G2 fusion bait due to self-activation. For a complete list of all interectors isolated refer to Table 3.

LIR-like Motif Specificity

It has been well documented that proteins which act as mediators between ubiquitinated proteins and LC3 and/or GABARAP, such as p62, contain a linear motif with the core consensus (W/F/Y)xx(L/I/V) (101, 166, 181). We found Mulan to contain the characteristic LC3-interacting region (LIR) at Y(327)xxL(330) in the amino-terminus which is exposed to the cytosol (Figures 14A and 14B). Unlike other autophagic adapter proteins, Mulan's physiological location in the OMM bypasses the need for ubiquitination to help deliver the damaged mitochondrion to the autophagosome. To demonstrate that the interaction between the heterodimer Mulan$_{259-352}$-Ube2E3 and GABARAP is through this motif, we generated a mutant at amino acids Y327A and L330A. When the mutant LexA-Mulan$_{259-352}$MUT-Ube2E3 was used as the bait, we did

not see any interaction with the GABARAP protein (Figure 14C). This data demonstrates that the interaction between Mulan and GABARAP is LIR specific. To further show that the interaction is specific to GABARAP and not the Atg8 family members, we used LC3B as a prey. There are four isoforms of LC3, with LC3B being the only one that is induced during vacuolation (182). LC3B did not interact with the LexA-Mulan$_{259-352}$-Ube2E3 heterodimer or the mutant form, once again showing specificity of the GABARAP interaction.

Discussion

Mulan's function has been linked to organelle dynamics, apoptosis, mitochondrial fission and mitophagy, through its interaction with NF-κB, JNK, p53, Akt and Mfn2 (154-156). Mulan is an E3 ubiquiting ligase and needs to interact with a conjugating E2 enzyme in order to carry out its function. The identity of these E2 conjugating enzyme(s) is unknown. In this paper we identify the conjugating E2 enzymes that interact specifically with Mulan. Furthermore, we used a modified yeast two-hybrid system in which the Mulan-E2 heterodimer was a bait to identify specific substrates for each complex. Specificity of substrates is due to the heterodimer formed between the E3 (in this case Mulan) and E2. GABARAP was one of the proteins isolated during the screening for substrates of Mulan-Ube2E3.

The bulk degradation of cytosolic material by autophagy was believed to be nonspecific, but recent data suggests that there is specificity to this process through the identification of target proteins (96). Several proteins important in ubiquitination have

been linked to targeted autophagy, which is a beginning to understanding the crosstalk between proteasomal and autophagy degradation (183). Mulan functions as an E3 ubiquitin ligase and is found in the outer mitochondrial membrane, where its RING finger domain is facing the cytosol and can interact with E2 conjugating enzymes as well as substrates (155). Many E3 ligases are part of a multi-subunit heteromeric complex which generally includes an E2, adaptor proteins, and the substrate. They could also contain anchor proteins for a particular compartment as well as regulatory proteins (76). Previously, there were no known E2 interactors of Mulan identified; in this report we were able to isolate the Class I Ube2G2 and Ube2L2 as well as the Class III Ube2E2 and Ube2E3 as interactors of Mulan. The E2s are classified according to the addition of an extension at the amino-terminus and/or carboxy-terminus to the catalytic core UBC domain. Class I E2s consist of only the catalytic domain, Class II and Class III have the extension at either the amino- or carboxy-terminus respectively, and Class IV have both extensions (64). There are also different classes of E3 ubiquitin ligases including the RING family of which Mulan is a part of. RING-type E3s act as scaffold proteins, bringing the E2 and substrate in close proximity for the transfer of the ubiquitin directly from the E2 to the substrate (184).

Since the interaction between the E2 and E3 are very weak and transient, many groups have used high throughput screening to identify novel interactions between E2 and E3s, (167, 185, 186). In our work, a yeast two-hybrid system was used to isolate E2 interactors of Mulan. We were able to identify eight total novel substrates of Mulan by using this technique (Table 3). Isolated substrates can be divided into those that were only able to interact with the complete heterodimer or those that interacted with both the

heterodimer and the specific E2. Of the isolated interactors, three are previously known interactors of the specific E2 being used, providing evidence that the screening was successful. The multiple complexes formed between Mulan and the various E2s sheds light on how the specific recruitment of the E2 determines the function and the substrates of Mulan. In addition, Mulan-E2 fusion baits were used to identify specific interactors of the heterodimer complex. One of these interactors was GABARAP, a protein that has been linked to autophagy, which suggests the mechanism of Mulan's function in this process. GABARAP is a ubiquitously expressed protein that was originally isolated as an interactor of the $GABA_A$ receptor. GABARAP can also interact with the cytoskeleton, emphasizing the role of this protein in the reuptake of the $GABA_A$ receptor. It was also noted that the GABARAP protein contained similarities to the LC3, a component of the MAP complex important in autophagy (175, 187).

Autophagy occurs when a double-membrane vesicle (autophagosome) is formed around unwanted content in the cell, such as damaged organelles or protein aggregates (188). The vesicle is then delivered to the lysosome, where the content is degraded and the product is recycled as nutrients (100). Mitophagy is the process where damaged mitochondria are removed from the cell. In addition to supplying energy to the cell, mitochondria are also a major source of reactive oxygen species (ROS) (102). The removal of excess or damaged mitochondrion then becomes an essential process to protect the cell from damage. Defects in the autophagy progress can cause the accumulation of ubiquitinated proteins leading to neurodegeneration (189, 190). Omi/HtrA2 is a serine protease with high homology to the bacterial HtrA endoprotease, a protein with proteolytic activity which removes damaged and misfolded proteins at

high temperatures (32). The mature form of Omi/HtrA2 is found in the IMS of the mitochondria, but it is released to the cytosol under cellular stress where it can participate in caspase-dependent and caspase-independent apoptosis pathways (31). While in the IMS, Omi/HtrA2 has been implicated in pro-survival functions. Mice lacking the active form of the protease (known as *mnd2* mice) show a Parkinsonian phenotype and die within 30 days after birth (40). Furthermore, mice lacking Omi/HtrA2 function in non-neuronal tissues are observed to undergo premature aging (191). Omi/HtrA2 thus seems to play an important role in cell survival. Recently, lack of functional Omi/HtrA2 was linked to loss of functional mitochondria as well as an increase in Mulan protein level (123). The IMD of Mulan can be degraded by Omi/HtrA2 *in vitro* (5).

Parkin, which is also an E3 ligase, participates in mitophagy is recruited to mitochondria by PINK1 (192). Parkin then ubiquitinates mitochondrial proteins such as p62/SQSTM1 which act as an adapter and through the specific LIR-motif interact with members of the Atg8 family, such as LC3 (120). This interaction allows for the formation of the phagophore around the damaged mitochondrion, giving it specificity for the content of the vacuole (165, 193). The LIR-like motif is also found in Mulan (amino acids 327-330), which we show to be responsible for the interaction between Mulan and GABARAP. Furthermore, we show that this interaction between GABARAP and Mulan-Ube2E3 is specific; since when the highly homologous Ube2E2 enzyme was used, the complex was unable to interact with LC3B. In our present study we characterized the mechanism of Mulan's function in mitophagy as the recruiter of GABARAP. GABARAP participates in the formation of the autophagosome at a different time point than PINK1/parkin/LC3. Mulan interacts with GABARAP but not LC3, suggesting that this

interaction is involved in the same pathway but operates downstream of PINK1/parkin/LC3.

Table 2. A list of the four E2 that were isolated through the yeast two-hybrid screen as Mulan$_{259-352}$ specific interactors.

Mulan$_{259-352}$ Interactors	Accession Number
Ube2E2	NM_152653
Ube2E3	NM_006357
Ube2G2	NM_019803
Ube2L3	NM_003347

Table 3. A list of Mulan$_{259-352}$-E2 fusion interactors isolated in our yeast two-hybrid screen using a HeLa cDNA library. (*) denotes previously identified interactors of the corresponding E2.

Mulan$_{259-352}$-Ube2E3 Interactors	Accession Number
GABARAP	NM_007278
GRP78	NM_005347
Ube2D2	NM_003339
Ube2D3	NM_181891
Mulan$_{259-352}$-Ube2E2 Interactors	**Accession Number**
RNF103 *	NM_001198951
RNF14	NM_183401
RNF114 *	NM_018683
Mulan$_{259-352}$-Ube2L3 Interactors	**Accession Number**
ARIH2 *	NM_006321

Table 4. A list of the primers used to create the LexA-Mulan$_{259-352}$-Specific E2, LexA-Specific E2 fusion baits, LexA-Mulan$_{259-352}$ and LexA-Mulan$_{259-352}$(MUT). Underlined is the linker sequence, in *italics* is the restriction enzyme site and in bold is the mutated nucleotide.

Primer Name	Restriction Enzyme	Sequence
Ube2E3 Fusion Fw	-	ACCCCTGTACAACAGC GGTGGTTCCGGTGGT ATGTCCAGTGATAGGCAAAGG
Ube2E3 Fusion Rv	-	CCTTTGCCTATCACTGGACAT ACCACCGGAACCACC GCTGTTGTACAGGGGTATCAC
Ube2E3 Fw	*MfeI*	GCG *CAATTG* ATGTCCAGTGATAGGCAAAGG
Ube2E3 Rv	*XhoI*	CTG *CTCGAG* TTATGTTGCGTATCTCTTGGTCCAC
Ube2E2 Fusion Fw	-	ACCCCTGTACAACAGC GGTGGTTCCGGTGGT ATGTCCACTGAGGCACAAAG
Ube2E2 Fusion Rv	-	CTCTTTGTGCCTCAGTGGACAT ACCACCGGAACCACC GCTGTTGTACAGGGGTATCAC
Ube2E2 Fw	*MfeI*	GCG *CAATTG* ATGTCCACTGAGGCACAAAG
Ube2E2 Rv	*XhoI*	CCG *CTCGAG* CTATGTGGCGTACCGCTTG
Ube2G2 Fusion Fw	-	ACCCCTGTACAACAGC GGTGGTTCCGGTGGT ATGGCGGGGACCGCGCTCAAGAAGC
Ube2G2 Fusion Rv	-	CTTGACCGCGGTCCCCGCCAT ACCACCGGAACCACC GCTGTTGTACAGGGGTATCAC
Ube2G2 Fw	*EcoRI*	GCG *GAATTC* ATGGCGGGGACCGCGCTC
Ube2G2 Rv	*XhoI*	CTG *CTCGAG* TCACAGTCCCAGAGACTTCTG
Ube2L3 Fusion Fw	-	ACCCCTGTACAACAGC GGTGGTTCCGGTGGT ATGGCGGCCAGCAGGAGGCTG
Ube2L3 Fusion Rv	-	CAGCCTCCTGCTGGCCGCCAT ACCACCGGAACCACC GCTGTTGTACAGGGGTATCAC
Ube2L3 Fw	*EcoRI*	GCG *GAATTC* ATGGCGGCCAGCAGGAGGCTG
Ube2L3 Rv	*XhoI*	CCG *CTCGAG* TTAGTCCACAGGTCGCTTTTCC
Mulan$_{259-352}$ Fw	*MfeI*	GCG *CAATTG* CGGAAGCAGTATCTGCAGCG
Mulan$_{259-352}$ Rv	*SalI*	CTCC *GTCGAC* TTAGCTGTTGTACAGGGGTATCA
Mulan$_{259-352}$(MUT) Fw	-	GCACCGAGTGC **GCC**CGCGCC**GCG** CCAGAGCCC
Mulan$_{259-352}$(MUT) Rv	-	GGGCTCTGG **CGC**GGCGCG**GGC** GCACTCGG

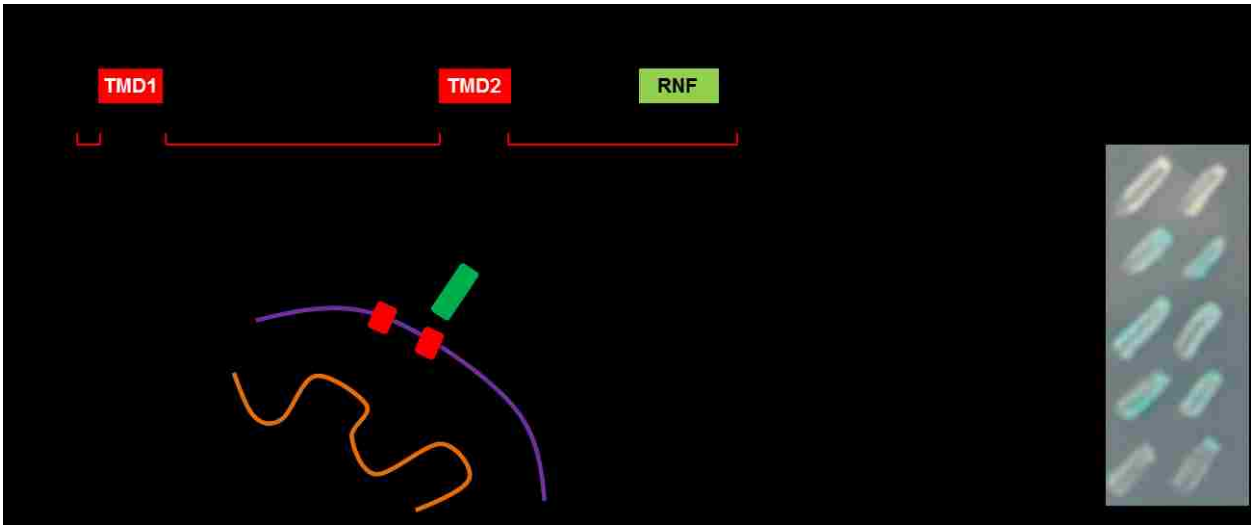

Figure 12. Mulan's RING finger domain interacts with E2 conjugating enzymes found in the cytoplasm.

(A) Schematic diagram of the Mulan protein E3 ligase and its topology in mitochondria. Mulan is a 352-residue protein with two transmembrane domains (TMD ■), an intermembrane space domain as well as a short cytosolic amino-terminus and a longer carboxy-terminus where the RING domain (RNF ■) is located. The cytosolic carboxy-terminus was used as the bait in a yeast two-hybrid screen. **(B)** Interaction of four specific E2 conjugating enzymes with LexA-Mulan$_{259-352}$ isolated in the yeast two-hybrid screening. Blue yeast colonies result from a positive protein-protein interaction between the bait and prey.

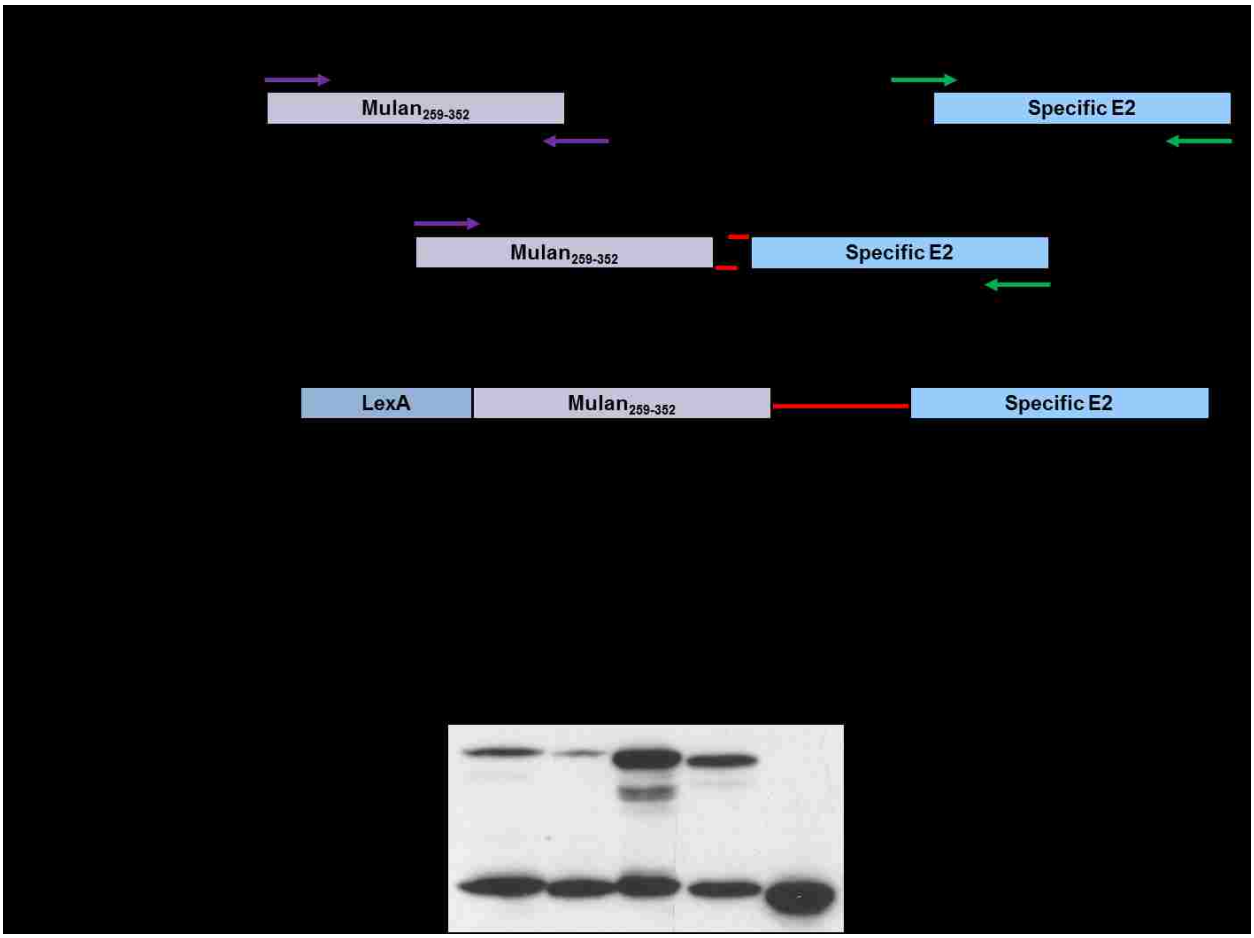

Figure 13. Fusion baits used during a modified yeast two-hybrid screening.

(A) Schematic representation of the method used to create the LexA-Mulan$_{259-352}$-E2 fusion baits. More information can be found in the *Methods* section. **(B)** Expression and stability of the various recombinant fusion baits used in the yeast two-hybrid screening. Single yeast colonies were grown overnight, induced with galactose/raffinose media the next day for four hours and then used in a Western blot analysis.

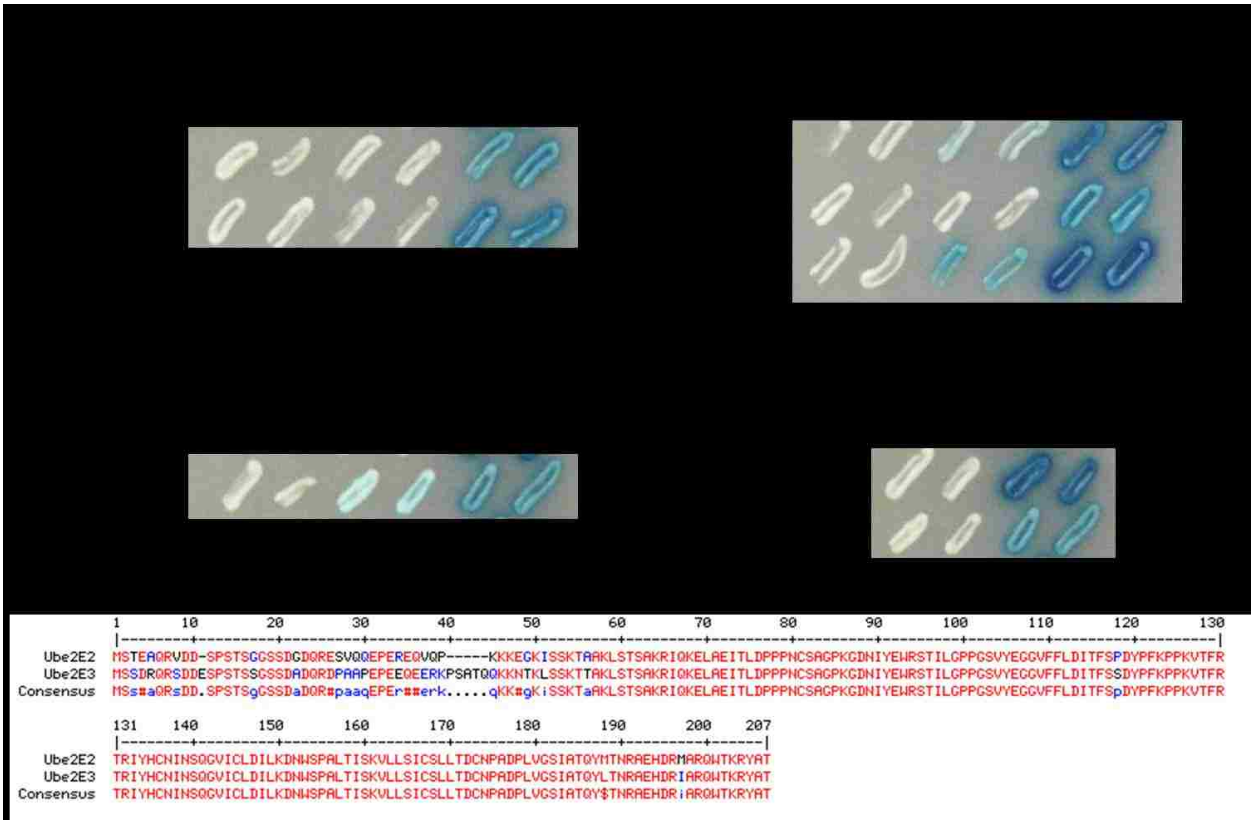

Figure 14. Specificity of interaction between isolated preys with different fusion bait proteins.

(A) Specificity of interaction between isolated preys with different fusion bait proteins. The interaction of GABARAP and GRP78 was only present when Mulan and Ube2E3 were expressed together. **(B)** The fusion bait was used in a screening. Interaction of ARIH2 with the heterodimer is through Ube2L3. **(C)** The fusion bait was used in a screening. The interaction of RNF103 and RNF114 is through UbeE2. RNF14's interaction is specific to the heterodimer. **(D)** Alignment of the amino acid sequences of Ube2E2 and Ube2E3 used in our experiments to illustrate their similarities. [MultAlin software (http://multalin.toulouse.inra.fr/multalin)]. **(E)** To further characterize interaction specificity both fusion baits $Mulan_{259-352}$-Ube2E2 and $Mulan_{259-352}$-Ube2E3 were used. Both GABARAP and GRP78 only interacted with $Mulan_{259-352}$-Ube2E3 and not the highly homologous $Mulan_{259-352}$-Ube2E2.

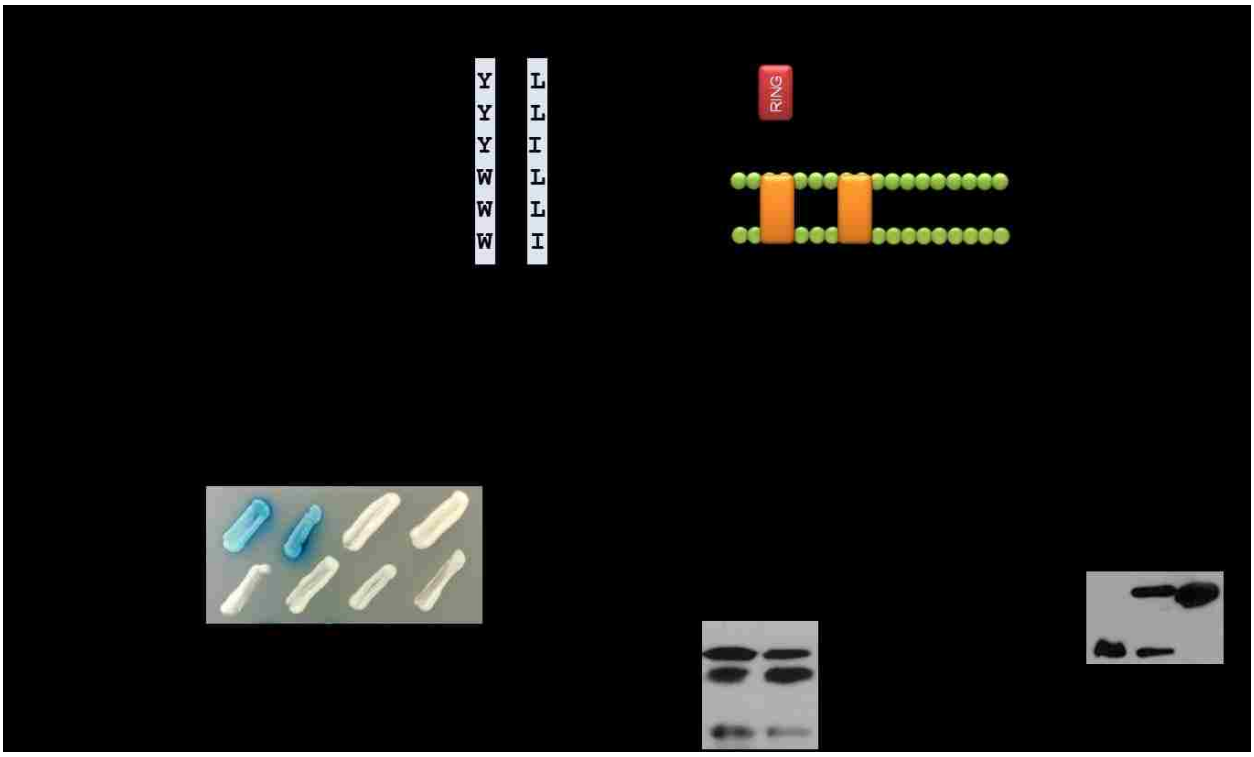

Figure 15. LIR motif of Mulan is necessary for the interaction with GABARAP.

(A) Comparison of typical LIR motif present in proteins that interact with the Atg8 family members, which is also found in Mulan. The highlighted amino acids indicate the conserved residues of the LIR in all proteins shown. **(B)** Schematic representation of the Mulan protein in the mitochondria outer membrane, along with the location of the putative LIR. **(C)** Specificity of interaction between the Atg8 family members GABARAP and LC3 with the $Mulan_{259-352}$-Ube2E3 heterodimer and LIR mutant. By mutating the conserved residues to Alanine the interaction with GABARAP is lost. **(D)** Expression of both the $Mulan_{259-352}$-Ube2E3 wild type and LIR mutant in yeast. **(E)** Expression the preys, GABARAP-HA and LC3B-HA.

CHAPTER 4: CONCLUSION

Omi/HtrA2 is a serine protease with a well-documented function in apoptosis. Recent genetic studies suggest it might have a secondary function as a regulator of mitochondrial homeostasis (31, 40). In the preceding chapters we described a new approach used to elucidate the cytoprotective function of Omi/HtrA2. The study of this mitochondrial protease has been the focus of our studies for the past ten years. Omi/HtrA2 was originally isolated in our lab and since then we have developed many tools for studying it, including a specific inhibitor (ucf-101), and identified Hax-1, THAP5, and Mulan as specific interactors and substrates of the protease (31, 46, 47, 123).

We have recently characterized Abro1 as a protein that is modulated following myocardial ischemia/reperfusion (MI/R) injury, where the protein levels are increased and provide cytoprotection. Abro1 is a novel scaffold protein, part of the BRISC complex that plays a role in Lys63-deubiquitination (126, 129). This protein was identified as a specific interactor of THAP5, a transcription factor isolated in our lab as a substrate of Omi/HtrA2 (194). Abro1 is closely related to another protein, Abraxas, which is also a scaffold protein in the deubiquitinating (DUB) complex known as BRCA1 (125-127, 130). The unique carboxy-termini of these two scaffold proteins give them their distinct functions (131). To further understand the mechanism of Abro1 in cytoprotection, we used the unique carboxy-terminus of Abro1 as a bait in a yeast two-hybrid screen, and isolated three members of the AP-1 family: ATF4, ATF5 and JunD. These transcription factors are important in cell growth, regulation, and cell cycle control

(133). We further characterized this interaction to be unique to Abro1 and restricted to only these three members of the very large AP-1 family.

The BRISC complex has a similar composition to BRCA-1, another DUB complex (126, 127). The two complexes share three proteins in common: BRCC36, BRE and MERIT40 (126, 127). These proteins interact with the highly homologous amino-terminus of Abro1 and Abraxas. BRCA-1 is known to act on damaged double stranded DNA; however, little is known about the function of the BRISC complex (125, 126, 132). BRCA-1 is recruited to DNA damage sites through the interaction of its RAP80 subunit with ubiquitinated histones (125, 131). The BRISC complex does not contain the RAP80 protein; however, we hypothesized that THAP5 or the isolated AP-1 family members (ATF4, ATF5, and JunD) could function in a similar manner to drive the complex to various subcellular locations. Overexpression of Abro1 provides cytoprotection, but only in the presence of ATF4. Under these conditions, cytoplasmic Abro1 was translocated to the nucleus with the help of the transcription factor ATF4. These findings provide new information on the mechanism of cytoprotection by Abro1 and its indirect regulation by Omi/HtrA2 protease.

To further characterize the cytoprotective function of Omi/HtrA2 within the mitochondria, we studied the expression of the three known mitochondrial E3 ubiquitin ligases: Mulan, MARCH5/MITOL and RNF185 (152, 153, 155). We found that the IMS domain of Mulan can be cleaved by Omi/HtrA2 *in vitro* (123). In animal models that lack the Omi/HtrA2 protein or carry an inactive protease, there is accumulation in Mulan protein level as well as increased mitophagy (123). For an E3 ligase to perform its function, it needs to interact with an E2 ubiquitin conjugating enzyme (2). Since Mulan is

a RING protein, the RING domain was used to isolate and identify these E2s (195). In these studies, we isolated four distinct E2s as specific interactors of Mulan. These included the class I E2 conjugating enzyme members Ube2G2 and Ube2L3 as well as the class III E2 conjugating enzymes Ube2E2 and Ube2E3 (64, 171, 185, 186). The different E2s suggest Mulan can have multiple functions depending on the nature of its interacting E2 partner. To further characterize its function, we searched for substrates of Mulan when associated with a specific E2. Specifically, a fusion bait protein was generated where the Mulan RING domain was joined to the full length specific E2 using a small five-amino acid linker. This approach allowed us to screen for proteins that interact with the Mulan-E2 fusion but not with the individual Mulan or E2 proteins alone. Unique proteins to each heterodimer complex were isolated, and since our interest is mitophagy, we focused on the interaction between Mulan-Ube2E3 and GABARAP.

We characterized the interaction of Mulan with GABARAP both *in vitro* and *in vivo*. These studies showed that Mulan's cytosolic portion contains an LIR motif that is necessary for its interaction with GABARAP. This interaction is similar to the one identified between the mitochondrial protein PINK1, the cytosolic E3 ligase parkin, and the Atg8 family member LC3, which leads to selective mitophagy (113, 115-117, 196, 197). LC3 and GABARAP are members of the same family of proteins and interact with phosphoethanolamine (PE) in phagophores (124, 159, 160, 198, 199). Adaptor proteins which can bind Atg8 family members have been identified, allowing for selectivity in the content of the phagosome (94, 101, 105, 115, 200-202). These adaptor proteins all share a similar LIR motif (166). The PINK1/parkin/LC3 process is initiated in mitochondria that are damaged or have lost their mitochondrial potential due to cellular

stress (109, 111-117). When mitochondria are damaged beyond repair, they undergo fission followed by mitophagy (104, 153, 203). Our proposed pathway of Mulan-Ube2E3/GABARAP may work in conjunction with the PINK1/parkin/LC3 pathway or in a later stage of the phagophore formation. There have been reports that the GABARAP protein increases at the time in which the vesicle is being closed (124, 159). Additionally, the PINK1/parkin/LC3 mitophagy-pathway *in vivo* is only seen when there is a catastrophic mitochondrial dysfunction and not as a response to the gradual accumulation of damaged mitochondria (197). This could explain the unique role of the Mulan-Ube2E3/GABARAP pathway but further studies are necessary to verify and expand our results.

The discovery and identification of Mulan as a participant and possibly a major player in mitophagy may help explain in part the role of Omi/HtrA2 as a pro-survival protein while in the mitochondria (53, 204). In cells that have damaged mitochondria, Omi/HtrA2 does not cleave Mulan, which leads to the accumulation of this protein and increased mitophagy (123). A physiological characteristic of PD patients is the accumulation of damaged mitochondria, which is also seen during normal aging (205). Animals that lack the Omi/HtrA2 protein or carry an inactive protease develop motor neuron disease with a parkinsonian phenotype, premature aging and a shortened lifespan (40, 191). The contribution of Mulan in these processes and whether other mitochondrial proteins are involved is unclear. We expect that mitochondrial damage and deregulated mitophagy are involved, and that Omi/HtrA2 protease plays a central role in this process. In addition, whether any human disorders similar to those observed

in Omi/HtrA2 KO animals are caused by the inactivation or deregulation of the Mulan-mitophagy pathway remains to be elucidated.

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