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PhD thesis summary

Functional characterization of LACE1 ATPase and mitochondrial AAA proteases YME1L and AFG3L2 in mitochondrial protein homeostasis

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ABBREVIATIONS

AAA protease	ATPases associated with diverse cellular activities
AFG3L2	ATPase family gene 3-like 2
Bax	B-cell lymphoma -2-associated X protein
BN PAGE	blue native polyacrylamide gel electrophoresis
BN/SDS PAGE	blue native/ sodium dodecylsulphate polyacrylamide gel electrophoresis
CLPP	ATP-dependent caseinolytic protease - proteolytic subunit
CLPX	ATP-dependent caseinolytic protease – chaperone and sorting subunit
CLPXP	ATP-dependent caseinolytic protease
Drp1	dynamin related protein 1
HEK293	human embryonic kidney cells 293; ATCC® CRL-1573™
KD	knockdown
LACE1	lactation elevated 1
LON	long, undivided filaments upon UV irradiation, protease
Mgr1	Mitochondrial genome-required protein 1
Mgr3	Mitochondrial genome-required protein 3
mtHSP60	mitochondrial heat shock protein 60
mtHSP70	mitochondrial heat shock protein 70
OMA1	overlapping with the m-AAA protease
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
OXA1L	oxidase assembly 1-like protein
p53	protein 53
PARP	poly (ADP-ribose) polymerase
PINK1	phosphatase and tensin homologue (PTEN)-induced kinase
RNAi	RNA interference
SCA	spinocerebellar ataxia
SDS PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
Spg7	spastic paraplegia 7, paraplegin
wt	wild-type
YME1L	ATP-dependent metalloprotease yeast mitochondrial

ABSTRAKT

Udržení mitochondriální proteinové homeostázy je nezbytnou podmínkou pro průběh klíčových buněčných procesů a udržení buněčné integrity. Je zajištěna mnoha specifickými mitochondriálními proteázami s možnými chaperonovými funkcemi aktivních v různých mitochondriálních subkompartmentech.

V první části této disertační práce jsme se zaměřili na charakterizaci funkčního překrývání a spolupráce proteolytických podjednotek AFG3L2 a YME1L mitochondriálních komplexů m- a i-AAA lokalizovaných ve vnitřní mitochondriální membráně. Dvojitě utišená buněčná linie AFG3L2/YME1L vykazovala výraznou změnu ve zpracování isoforem OPA1, výrazné zvýšení proteázy OMA1 a snížení proteinu SPG7. Naše výsledky ukazují spolupráci a částečně i nadbytečné funkční překrývání proteáz AFG3L2 a YME1L v udržení mitochondriální proteinové homeostázy, a dále podtrhují jejich důležitost pro mitochondriální a buněčné funkce a integritu.

Cílem druhé části bylo charakterizovat buněčnou funkci proteinu LACE1 v mitochondriální proteinové homeostáze. Protein LACE1 je lidský homolog kvasinkové ATPázy Afg1. Z našich výsledků vyplývá, že LACE1 je mitochondriální integrální membránový protein, který existuje jako součást tří komplexů o přibližné molekulové hmotnosti 140, 400 a 500 kDa a zprostředkovává degradaci jaderně kódovaných podjednotek COX4, COX5A a COX6A komplexu IV. Použitím afinitní purifikace proteinu LACE1-FLAG jsme zjistili, že protein přímo interaguje s podjednotkami COX4 a COX5A komplexu IV, proteázou YME1L a proteinem p53. Pomocí ektopické exprese mutantních variant K142A v oblasti Walker A a E214Q v oblasti Walker B jsme prokázali, že intaktní ATPázová doména je nezbytná pro degradaci jaderně kódovaných podjednotek komplexu IV. Dále jsme zjistili, že protein LACE1 vykazuje významnou proapoptotickou aktivitu, která je závislá na proteinu p53 a je nezbytná pro jeho translokaci do mitochondrií indukovanou mitomycinem c. Naše výsledky ukazují, že protein LACE1 má nezastupitelnou úlohu v proteolýze proteinů podjednotek oxidativního fosforylačního systému, zprostředkovává mitochondriální translokaci p53 a jím indukovanou apoptózu nezávislou na jeho transkripci.

Klíčová slova: apoptóza, dýchací řetězec, komplex IV, mitochondrie, oxidativní fosforylace, proteáza AFG3L2, proteáza YME1L, protein LACE1, tumor-supresorový protein p53, translokace

ABSTRACT

Mitochondrial protein homeostasis is crucial for cellular function and integrity. It is ensured by many specific mitochondrial proteases with possible chaperone functions located across the various mitochondrial subcompartments.

In the first part, we have focused on characterization of functional overlap and cooperativity of proteolytic subunits AFG3L2 and YME1L of the mitochondrial inner membrane complexes m- and i-AAA in HEK293 cells. The double AFG3L2/YME1L knockdown cells showed severe alteration in OPA1 protein processing. All three analyzed knockdown cell lines showed marked elevation in OMA1 protease and severe reduction in SPG7. Our results reveal cooperative and partly redundant involvement of AFG3L2 and YME1L in the maintenance of mitochondrial structure and respiratory chain integrity.

The aim of the second part was to characterize the cellular function of LACE1 (lactation elevated 1) in mitochondrial protein homeostasis. LACE1 protein is a human homologue of yeast Afg1 (ATPase family gene 1) ATPase. Yeast Afg1 was shown to mediate degradation of mitochondrially encoded complex IV subunits and it was suggested to facilitate extraction of polytopic membrane proteins. We show that LACE1 is a mitochondrial integral membrane protein that exists as a part of three complexes of approximately 140, 400 and 500 kDa. We demonstrate that LACE1 mediates degradation of nuclear-encoded complex IV subunits COX4, COX5A and COX6A. Using affinity purification of LACE1-FLAG expressed in LACE1-knockdown background, we show that the protein interacts physically with COX4 and COX5A subunits, YME1L protease, and p53 protein. We demonstrate by ectopic expression of both K142A Walker A and E214Q Walker B mutants that an intact ATPase domain is essential for LACE-mediated degradation of nuclear-encoded complex IV subunits. We further show that LACE1 exhibits significant pro-apoptotic activity, which is dependent on p53, and is necessary for mitomycin c-induced translocation of p53 into mitochondria. Our work have identified that LACE1 has a role in protein turnover of subunits of the oxidative phosphorylation system, mediates mitochondrial translocation of p53 and its transcription-independent apoptosis.

Key words: AFG3L2 protease, apoptosis, complex IV, LACE1 protein, mitochondria, oxidative phosphorylation, p53 tumor suppressor protein, respiratory chain, translocation, YME1L protease

1. INTRODUCTION

1.1. The proteolytic system of mitochondria

Cell survival and thus survival of the whole organism depends on essential processes in mitochondria such as OXPHOS. The OXPHOS is build up of both nuclear and mitochondrial encoded protein subunits. Due to the potential function-threatening defects, the quality of these protein subunits is continuously under tight control of specialized proteases. Various proteases within these organelles regulate mitochondrial biogenesis and ensure the complete degradation of redundant or damaged proteins. Many of these proteases are highly evolutionary conserved and ubiquitous in prokaryotic and eukaryotic cells. They can be sorted into four functional classes i) processing peptidases, ii) ATP dependent proteases, iii) oligopeptidases, and iv) other mitochondrial proteases. Processing peptidases cleave off mitochondrial targeting sequences of nuclearly encoded proteins and process mitochondrial proteins with regulatory functions. ATP dependent proteases either act as processing peptidases with regulatory functions or as quality-control enzymes degrading non-native polypeptides to peptides. Oligopeptidases degrade these peptides and mitochondrial targeting sequences to AA. Other mitochondrial proteases are the remaining proteases present in mitochondria that haven`t been functionaly classified yet (Koppen and Langer 2007). The degradation of the substrate proteins is under the N-end rule pathway. This rule governs the rate of the protein degradation through recognition of the N-terminal residue of proteins. The last N-terminal AA of the protein determines its half-life. The rule applies to both eukaryotic and prokaryotic organisms but with different strength, rules, and outcome. The proteolytic system of mitochondria produces peptides and free AAs and continuously release them from mitochondria (Kambacheld, Augustin et al. 2005).

1.1.1. ATP-dependent AAA proteases

ATPases associated with diverse cellular activities (AAA proteases) are one of the main players in mitochondrial protein quality control and thus regulation of protein homeostasis by removing misfolded or no longer needed proteins. Moreover, AAA proteases participate in irreversible important cell steps such as apoptosis, etc. located into mitochondria. Their misregulation may lead to severe human diseases. AAA proteases are composed of mitochondrial targeting sequence and three main parts i) N-terminal domain that harbors one

(i-AAA protease) or two (m-AAA protease) helices (Koppen and Langer 2007, Lee, Augustin et al. 2011), ii) AAA domain with Walker A motif (also known as Walker loop or P-loop), and iii) Rossman fold. AAA proteases use chemical energy from ATP hydrolysis to make conformational changes themselves and in substrates and to degrade them (Erzberger and Berger 2006). So far, four main proteolytic complexes active within the various distinct mitochondrial subcompartments were identified. Both i- and m-AAA protease complexes are found in the IMM. Whereas the i-AAA protease is active in the IMS, the homologous m-AAA protease functions on the matrix side of the IMM. The remaining two complexes, CLPXP and Lon proteases are matrix localized.

1.1.1.1. i-AAA protease

Yeast mitochondrial escape 1-like (YME1L, also known as FtsH1, Yta1l in yeast, and presenilin-associated metalloprotease PAMP in mammals, EC 3.4.24.-) is nuclear encoded ATP-dependent metalloprotease that catalyzes the degradation of the proteins with a particular degron sequence in the IMS. It plays an important role in respiration activity, protection against oxidatively damaged membrane proteins, and modulation of mitochondrial morphology. I-AAA protease is built up of six copies of YME1 subunits formed into the homohexameric complex. This complex is anchored into the IMM with protruding exerts into the IMS. YME1 protease is composed of i) ATP-binding and hydrolysis domain and ii) zinc-dependent proteolytic domain with an active site. One of the substrates of YME1 protease is COX2 of complex IV. Yeast strains lacking YME1 show elevated rate of DNA escape from mitochondria, temperature-sensitive growth, and changes in the morphology of the mitochondrial compartment (Weber, Hanekamp et al. 1996). YME1 was shown to associate with additional non¬subunit adaptor proteins, e.g. mitochondrial genome-required protein 1 (Mgr1) (Dunn, Lee et al. 2006) and mitochondrial genome-required protein 3 (Mgr3) (Dunn, Tamura et al. 2008). The i-AAA protease is composed of i) N-termini domain, ii) AAA ATPase domain, iii) the conserve second region of homology (SRH) motif, iv) the proteolytic domain, v) and C-terminal Calponin homology domain (Koppen and Langer 2007). In HEK293 cells, YME1L modulates the membrane accumulation of non-assembled Ndufb6 and ND1 subunits of complex I and COX4 subunit of complex IV. The knockdown of YME1L leads to reduced cell proliferation and apoptotic resistance, altered mitochondrial ultrastructure, diminished rotenone-sensitive respiration, and increased sensitivity to oxidative damage (Stiburek, Cesnekova et al. 2012). These results show the essential role of YME1L in

the maintenance and turnover of IMM proteins. The clinical impact of YME1L protease is still not clear.

1.1.1.2. m-AAA protease

Nuclear-encoded ATPase family gene 3-like 2 protein (AFG3L2, EC 3.4.24.-) has molecular weight of 89 kDa. It is located into the IMM protruding to both AAA and metallopeptidase domains into the mitochondrial matrix. AFG3L2 protease, with proteolytic and chaperone functions, is responsible for maturation, maintenance, and quality control of membrane proteins on the matrix side of the IMM (Koppen, Metodiev et al. 2007). The AAA domain serves as sorting domain with chaperon-like activity. The m-AAA protease consists either of homo oligomer of six AFG3L2 subunits or hetero-oligomerize with spastic paraplegia 7 protein (also known as paraplegin, SPG7). The amount of AFG3L2 and SPG7 oligomers is tissue- and species-specific (Koppen, Metodiev et al. 2007). The m-AAA protease is responsible, independent on the OMA1 protease, for the cleavage of OPA1 protein. Both murine AFG3L1 and AFG3L2 are essential for mitochondrial fusion via stabilization of the L-OPA1 forms. Loss of AFG3L2 leads to elevated OPA1 processing by OMA1 protease (MacVicar and Langer 2016). Autosomal dominant inherited spinocerebellar ataxias (SCAs) especially SCA type 28 (SCA28) is manifesting by young-adult onset, slowly progressive trait, and limb ataxia resulting in problems with coordination and balance, dysarthria, ptosis, nystagmus, and ophthalmoparesis. A predominant amount of the patients inherited SCA28 disorder from their parents. This mutated variant leads to AFG3L2 homocomplex with proteolytic impairment, marked reduction of respiratory chain complex IV, and loosely affected complex III and complex V (Di Bella, Lazzaro et al. 2010). So far, 17 missense mutations have been identified in Europe patients. Homozygous AFG3L2 mutations were identified in a spastic ataxia neuropathy syndrome (Pierson, Adams et al. 2011). Mutations in catalytic centre of human SPG7 lead to complete abrogation of the enzymatic activity of m-AAA protease (Karlberg, van den Berg et al. 2009). Two other mutations were identified in two patients with HSP and confirmed by complementation experiments in yeast. These mutations Glu575Gln and Lys354Ala led to enzymatically inactive forms (Bonn, Pantakani et al. 2010).

1.2. Adaptor proteins

Adaptor proteins of the mitochondrial proteases exert their activity only in cooperation with the scaffold and docking proteins. They facilitate their enzymatic reactions. Adaptor proteins

are composed of a variety of protein-binding modules that modulate the enzyme`s active site. They usually contain Src homology 2 (SH2) and Src homology 3 (SH3) domains. Both domains distinguish different specific AA sequences. SH2 protein motif is about 100 AA long and serves to recognize substrates containing phosphotyrosine (Russell, Breed et al. 1992). SH3 domain is about 60 AA long and identifies proline-rich regions surrounded by other specific AAs (Pawson and Schlessingert 1993).

1.2.1. LACE1

Afg1 (ATPase family gene 1) is a yeast mitochondrial ATPase, a member of the SEC18-NSF, PAS1, CDC48-VCP, TBP family of ATPases (Lee and Wickner 1992). It is a well evolutionarily conserved protein with a robust mammalian homologue LACE1 (lactation elevated 1) (Khalimonchuk, Bird et al. 2007). The protein is composed of five domains and contains an ATP/GTP-binding Walker A motif (P-loop) (Walker, Saraste et al. 1982, Abrahams, Mak et al. 2002). Afg1 homologues structurally resemble mitochondrial FtsH/AAA family proteases but do not contain the conserved zinc protease domain (Sauer and Baker 2011). Yeast Afg1 deletion strain showed reduced respiratory growth rate and diminished activities of OXPHOS complexes III and IV. It was suggest that the protein facilitates the degradation of mitochondrially encoded complex IV subunits COX1, COX2, and COX3 (Khalimonchuk, Bird et al. 2007). RT (reverse transcription) PCR of mouse tissues showed marked tissue-dependent LACE1 expression with the highest mRNA levels in heart, kidney, and lactating compared with inactive breast tissues (Abrahams, Mak et al. 2002). LACE1 gene region was found to be downregulated in the natural killer cells (NK cells) neoplasms (Karube, Nakagawa et al. 2011). LACE1 was found associated with bipolar disorder (Knight, Rochberg et al. 2010).

2. AIMS OF STUDY

Mitochondrial proteases and their adaptor proteins are now seem to be more and more important not only for the mitochondrial structure and function, but for the whole cell and, in the case of a multicellular organism, for the whole organism. The thesis had two main goals:

1) Characterization of the functional overlap and cooperativy of proteolytic subunits AFG3L2 and YME1L of the mitochondrial inner membrane complexes m- and i-AAA in the maintenance of mitochondrial structure and respiratory chain activity using shRNAmir knockdown approach in the HEK293 cell line

2) Study the role of human LACE1 (lactation elevated 1) ATPase, the homologue of yeast Afg1 Atpase, in the mitochondrial protein homeostases using shRNAmir knockdown, protein overexpression and protein-protein interaction analyses

3. RESULTS AND DISCUSSION

3.1. Results and discussion related to aim A)

Mitochondrial protein quality control is crucial for the maintenance of correct mitochondrial homeostasis. It is ensured by several specific mitochondrial proteases located across the various mitochondrial subcompartments. Here we have focused on characterization of functional overlap and cooperativity of proteolytic subunits AFG3L2 and YME1L of the mitochondrial inner membrane complexes m- and i-AAA in the maintenance of mitochondrial structure and respiratory chain activity.

3.1.1. Knockdown of YME1L and/or AFG3L2 leads to increased accumulation of complex I, IV and V subunits

We have generated stable HEK293 cell lines with shRNA-downregulated expression of single YME1L (NM_014263) and AFG3L2 (NM_006796.1) as well as a double-KD cells expressing both YME1L and AFG3L2 shRNAs (Open Biosystems). Control cell line was prepared by transfecting HEK293 cells with commercially available scrambled (non-silencing) shRNAmir-containing expression vector RHS436 (Open Biosystems). Western blot analysis with antibodies to YME1L and AFG3L2 confirmed that all three produced cell lines contained less than 10% of residual target protein levels (Figure 4.1. A). We have previously demonstrated that YME1L protease affects stability of Cox4 and Ndufb6 respiratory chain subunits by mediating their proteolytic degradation (Stiburek, Cesnekova et al. 2012). However, not much is known of AFG3L2 protease function in oxidative phosphorylation system biogenesis and of the possible substrate overlap or cooperation between AFG3L2 and YME1L in this process (Stiburek, Cesnekova et al. 2012).

We have performed SDS-PAGE western blotting screen using mitochondrial fractions isolated from the KD cell lines to identify affected respiratory chain and ATP synthase subunits. The screen was clearly limited by the number of available antibodies, but we were able to identify several affected OXPHOS subunits in the KD cells (Figure 4.1. B).

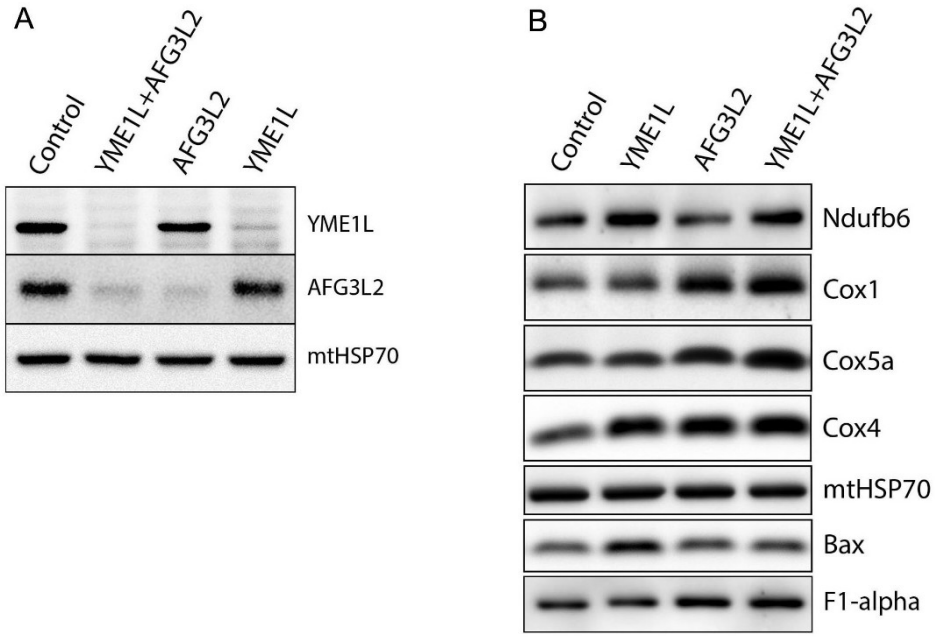

Figure 3.1. Knockdown of AFG3L2 and YME1L by shRNA leads to increased accumulation of complex I, IV and V subunits. (**A**) Knockdown of YME1L, AFG3L2 or both by stable shRNA expression leads to efficient reduction of corresponding protein levels. Whole-cell lysates from the shRNA knockdown cell lines were immunoblotted with antibodies to YME1L, AG3L2 or mtHSP70. The control cell line was prepared by transfecting HEK293 cells with the non-silencing (scrambled) shRNAmir vector. (**B**) Altered accumulation of respiratory chain subunits and Bax in YME1L and/or AFG3L2 KD cells. Whole cell lysates were immunoblotted with antibodies to Ndufb6, Cox1, Cox5a, Cox4, mtHSP70, Bax and F1-alpha.

In YME1L KD mitochondria, both Ndufb6 and Cox4 subunits were found to be increased, which is consistent with our previous report (Stiburek, Cesnekova et al. 2012). On the other hand, western blots of mitochondrial fraction from AFG3L2 KD cells revealed increased levels of Cox1, Cox4 and Cox5a subunits, as well as of the F1-alpha subunit of ATP synthase. Finally, mitochondria from the double knockdown YME1L/AFG3L2 cells showed markedly increased levels of Ndufb6, Cox1, Cox4, Cox5a and F1-alpha subunits (Figure 3.1. B). Additionally, immunoblot of single YME1L KD mitochondria showed increased amount of BAX protein (Figure 3.1. B.)

3.1.2. Loss of YME1L and/or AFG3L2 leads to marked accumulation of OPA1 protein forms, diminished Spg7 and elevated Oma1

Mitochondrial shape and cristae architecture depends on balanced accumulation of the dynamin-related GTPase Optic atrophy 1 (OPA1) protein forms. Mitochondrial inner-

membrane proteases were shown to mediate proteolytic processing of OPA1 protein, which directly influence mitochondrial fusion and cristae architecture (Anand, Langer et al. 2013). We have therefore analyzed the levels of OPA1 protein forms as well as of Spg7 and Oma1 proteases in whole-cell lysates of the knockdown cells. The OPA1 alterations found in single knockdown cells were limited to short OPA1 forms (S-OPA1), with increase in band c and band e in AFG3L2 KD cells, and increase in band c and band e accompanied by reduction in band d in YME1L KD cells (Figure 3.2.). In contrast, the YME1L/AFG3L2 double KD cells showed marked increase in all five detectable OPA1 bands, with the most prominent increase in L-OPA1 band b and S-OPA1 band d (Figure 3.2.).

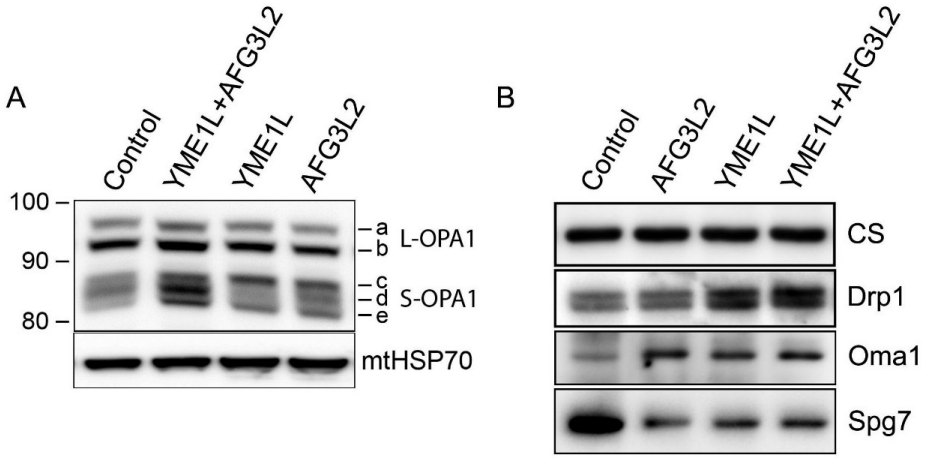

Figure 3.2. Loss of AFG3L2 and/or YME1L is associated with elevated OMA1, diminished SPG7, and markedly altered of OPA1 protein forms. Whole cell lysates were resolved using SDS-PAGE and western blotted with antibody to OPA1 (**A**); Drp1, Oma1 and Spg7 (**B**). Detection of mtHSP70 and citrate synthase (CS) was used to control for equal protein loading.

The main points related to aim A)

Loss of both AFG3L2 and YME1L leads to respiratory chain deficiency and impaired mitochondrial dynamics and ultrastructure. Our results show that:

- Knockdown of YME1L and/or AFG3L2 leads to increased accumulation of complex I, IV and V subunits and Exhibit reduced growth rate
- Loss of both YME1L and AFG3L2 leads to mitochondrial fragmentation and severely attenuated and disorganized cristae architecture
- Loss of YME1L and/or AFG3L2 leads to marked accumulation of OPA1 protein forms, diminished Spg7 and elevated Oma1
- AFG3L2/YME1L KD cells show reduced complex I holoenzyme and impaired activity of complexes I, III and IV and Accumulation of complex I and IV subcomplexes

3.2. Results and discussion related to aim B)

Mitochondrial protein homeostasis is crucial for cellular function and integrity and is therefore maintained by several classes of proteins possessing chaperone and/or proteolytic activities. In the present study, we focused on characterization of LACE1 (lactation elevated 1) function in mitochondrial protein homeostasis.

3.2.1. LACE1 is a mitochondrial integral membrane protein which exist as part of three protein complexes of approximately 500, 400 and 140 kDa

The yeast LACE1 homologue (Afg1) was shown to be an inner mitochondrial membrane protein (Khalimonchuk, Bird et al. 2007). MitoFates (http://mitf.cbrc.jp/MitoFates), a mitochondrial targeting prediction returned a high score for the presence of mitochondrial presequence in human LACE1 and identified MPP and Icp55 cleavage sites within the sequence (Fukasawa, Tsuji et al. 2015). By performing subcellular fractionation with subsequent immunoblot detection using anti-LACE1 antibody (Abcam), we found that the endogenous processed LACE1 (~50 kDa) is highly enriched in mitochondrial fractions of human embryonic kidney 293 (HEK293) cells (Figure 3.3. A). Immunodetection of mtHSP70 and β-actin was used to control for mitochondrial fractionation efficiency.

By subjecting mitochondrial fractions from HEK-293 cells to either sonic treatment or alkaline carbonate extraction with subsequent ultracentrifugation and immunoblotting, we found that the majority of endogenous LACE1 (~50 kDa) remained in the mitochondrial pellet fraction after both treatments (Figure 3.3. B). This indicates that, similarly to its yeast counterpart (Afg1), the endogenous LACE1 is an integral mitochondrial membrane protein. To verify whether overexpression of wild-type LACE1-FLAG (NM_145315) leads to the same mitochondrial targeting of the fusion protein, we have transiently transfected wild-type HEK293 cells with a C-FLAG-fusion human LACE1 ORF construct (GenBank® accession number NM_145743; OriGene Technologies). The cells were harvested 48 hours post-transfection and used to prepare mitochondrial fractions. Subjecting mitochondrial fractions to either sonic treatment or alkaline carbonate extraction with subsequent ultracentrifugation and immunoblotting we verify that the majority of LACE1-FLAG protein remained in mitochondrial pellet fractions (Figure 3.3. C). CLPP immunoblotting was used as an example

of soluble mitochondrial matrix protein, whereas PNPase immunoblotting was used as an example of membrane-associated intermembrane space protein.

To determine the native molecular mass of LACE1, the resulting LACE1-FLAG mitochondria (as described above) were solubilized with non-ionic detergent dodecylmaltoside (1% w/v) and resolved using two-dimensional blue-native/denaturing PAGE. The immunoblots were developed with anti-FLAG antibody. To enable estimation of the molecular mass of the LACE1 protein complex, the immunoblots were subsequently developed with antibodies against the NDUFA9 (NADH:ubiquinone 1 α subunit 9) subunit of complex I, SDH A subunit of complex II and COX2 subunit of complex IV. Under the conditions used, these antibodies recognized complex I holoenzyme (970 kDa), complex IV monomer (205 kDa) and complex II monomer (130 kDa) respectively. The direct size comparison revealed that the LACE1 protein complex has an apparent molecular mass of ~140 kDa under conditions of 1% dodecyl maltoside (Figure 3.3.D). Given the apparent molecular mass of LACE1 (~50 kDa), this result pointed to the existence of either LACE1 containing heteromeric complex or a homodimer of two LACE1 subunits.

Since AAA family members typically function as homohexamers, we performed the blue native PAGE Western blotting experiment using mitochondria solubilized with digitonin (1% w/v). In contrast with dodecyl maltoside, digitonin was shown to better preserve high-molecularmass inner membrane protein complexes, including respiratory supercomplexes, probably due to its lower delipidating properties (D'Aurelio, Gajewski et al. 2006). Detection of LACE1–FLAG on digitonin gels revealed, in addition to the previously identified ~140 kDa complex, two complexes of~400 and 500 kDa respectively (Figure 3.3.E). To find out whether similar species would be seen with affinity-purified LACE1–FLAG protein under the same solubilizing conditions,we have performed the blue native PAGE Western blotting experiment using LACE1–FLAG protein that has been affinity-purified (anti- FLAG) from isolated mitochondrial fractions. Indeed, all three previously identified LACE1 complexes could be observed on purified LACE1–FLAG protein gels (Figure 3.3. E). The lower amount of the ~500 kDa complex on the purified protein gel, when compared with solubilized mitochondria gel, is likely to be due to destabilization of the protein complex during the affinity purification process.

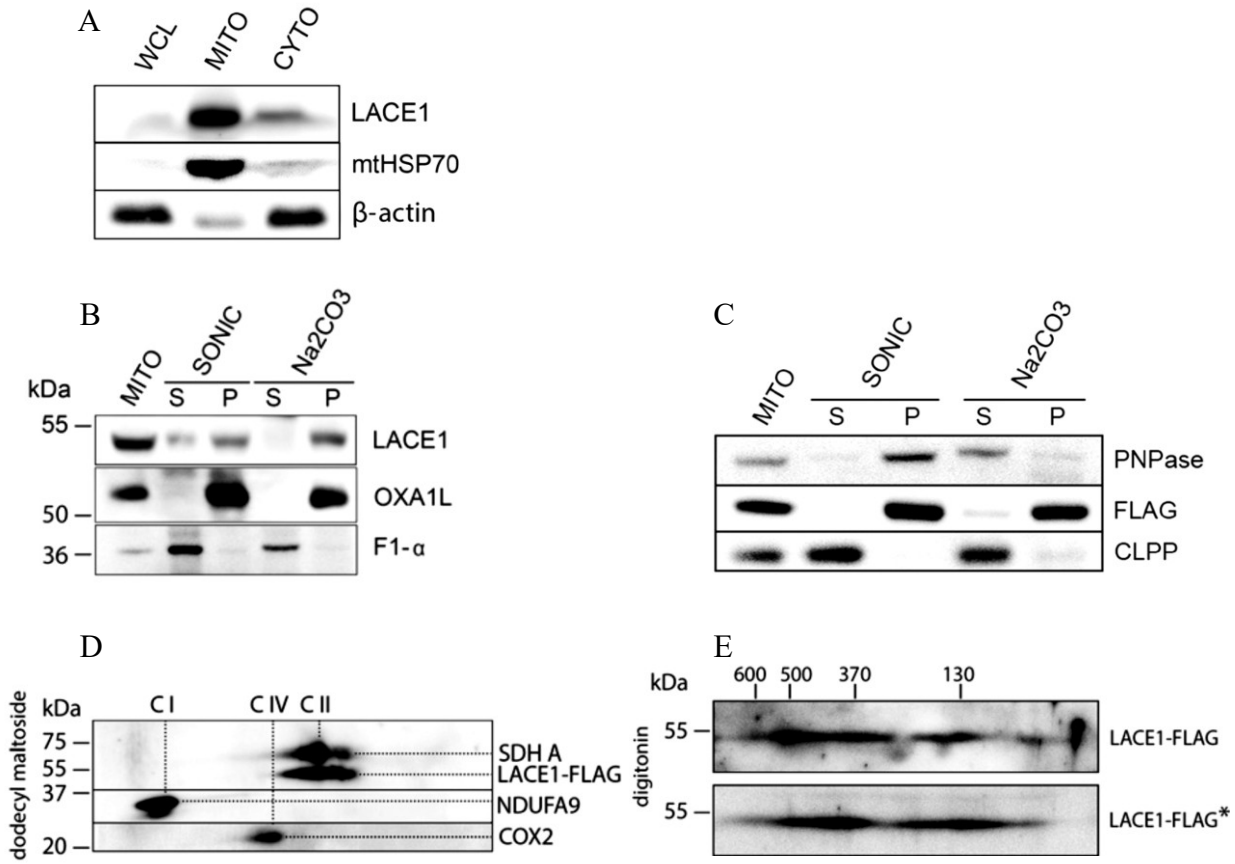

Figure 3.3. LACE1 is a mitochondrial integral membrane protein found in complexes of ~140, 400 and 500 kDa. (A) LACE1 is a mitochondrial protein. Whole cell lysate (WCL), mitochondrial (MITO) and cytosolic (CYTO) fractions were prepared from HEK293 cells and immunoblotted with antibody to LACE1 (Abcam, UK), to mitochondrial matrix heat shock protein mtHsp70 (Lonza, Switzerland) and to β-actin. (**B**) Human LACE1 is an integral mitochondrial membrane protein with an apparent molecular mass of ~50 kDa. Submitochondrial fractions were prepared from mitochondria from HEK-293 cells using either sonic disruption (SONIC) or extraction with 100 mM sodium carbonate (pH 11.5; Na2CO3). The resulting supernatant (S) and pellet (P) fractions and untreated mitochondria (MITO) were immunoblotted with antibodies against LACE1 (Abcam) and against OXA1L and F1-*α* subunit of ATP synthase. **(C)** LACE1-FLAG expressed in HEK293 cells is a mitochondrial integral membrane protein. Wild-type HEK293 cells were transiently transfected with a C-FLAG-fusion human LACE1 (NM_145315) ORF construct (OriGene). Submitochondrial fractions were prepared from isolated mitochondria using either sonic disruption (SONIC) or extraction with 100 mM sodium carbonate (pH 11.5). The resulting supernatant (S) and pellet (P) fractions and untreated mitochondria (MITO) were immunoblotted with M2 monoclonal antibody to FLAG epitope (Sigma Aldrich, Germany), and with antibodies to intermembrane space protein PNPase and matrix localized ClpP (Abcam, UK). (**D**) When solubilized with 1% dodecyl maltoside, LACE1 exists in a ~140 kDa complex. Mitochondria from HEK-293 cells transiently transfected with wt LACE1–FLAG construct were solubilized with 1% (w/v) dodecyl maltoside and resolved using two-dimensional blue native (5–15% polyacrylamide gradient)/10% denaturing PAGE. The resulting immunoblots were first incubated with antibody against FLAG epitope (Sigma–Aldrich), and subsequently with antibodies against the NDUFA9 subunit of complex I, COX2 subunit of complex IV and SDH A subunit of complex II. Under the conditions used, these antibodies recognize complex I (970 kDa), complex IV (205 kDa) and complex II (130 kDa) respectively. (**E**) Digitonin-solubilized LACE1–FLAG migrates in high-molecular-mass complexes of ~400 and 500 kDa respectively. Mitochondria from LACE1–FLAG-expressing cells (top) and anti-FLAG affinity-purified LACE1–FLAG (bottom*) solubilized with 1% (w/v) digitonin were resolved using two-dimensional blue native (6–15%) polyacrylamide gradient)/10% denaturing PAGE. The resulting Western blot was developed with anti-FLAG antibody. Subsequently, the Western blot was developed with antibodies against ATPase subunit α, Core 2, COX2 and SDH A. Under the conditions used, these antibodies recognize ATP synthase holoenzyme (600 kDa), residual free complex III dimer (500 kDa), F1-ATPase subcomplex (370 kDa), complex IV (205 kDa) and complex II (130 kDa) respectively. Molecular masses are indicated in kDa.

3.2.2. LACE1 variants with a T143V substitution in their Walker A motif fail to accumulate in mitochondria

To define cellular activities of the LACE1 protein, five different HEK-293 cell lines stably expressing *miR-30*- based shRNAs (shRNAmirs) targeting the human LACE1 transcript (GenBank® accession number NM_145315) were constructed. Control cell line was prepared by transfectingHEK-293 cells with commercially available scrambled (nonsilencing) shRNAmir-containing expression vector RHS436 (Open Biosystems, USA). Western blot analysis with antibody against LACE1 confirmed that two of the KD cell lines produced, denoted LACE1sh4 and LACE1sh5 (V3LHS_344953, V3LHS_344954; Open Biosystems), exhibit less than 10% of residual LACE1 protein (Figure 3.4. A).

To test the specificity of the RNAi KD obtained and to assess the significance of the putative P-loop motif of LACE1, we constructed three LACE1 variants in which either one or both of the conserved lysine and threonine residues of theWalker A motif (G/A)XXXXGK(T/S) were replaced by neutral AA (Deyrup, Krishnan et al. 1998). In the first mutant variant, Lys142 was replaced with alanine (K142A), whereas in the second variant Thr143 was replaced with valine (T143V). Finally, the last variant combined mutations of the previous two variants in a single ORF (K142A, T143V). The three LACE1 mutants and the wt LACE1–FLAG were transiently expressed in a LACE1-KD background. The control cell line expressing scrambled shRNA was transfected with the empty expression vector pCMV6-ENTRY. Despite the fact that the wt LACE1 as well as the K142A single mutant accumulated in mitochondria at high levels and in the correctly processed form, both of the variants harbouring the T143V substitutions were found to accumulate in mitochondria at ∼10-fold lower levels (Figure 3.4. B). At the same time, all mutant variants exhibited disproportionately high levels of the unprocessed protein form (*). To extend these structure–function data, we constructed aWalker B motif LACE1 mutant variant. In this variant, the conserved Glu214 of the Walker B motif (consensus sequence hhhhDE; h = hydrophobic residue) of LACE1 was replaced by glutamine (Hanson and Whiteheart 2005). Ectopic expression of this variant in a LACE1-KD backgroundmresulted in correct mitochondrial targeting and normal stability ofmthe E214Q LACE1 protein (Figure 3.4. B).

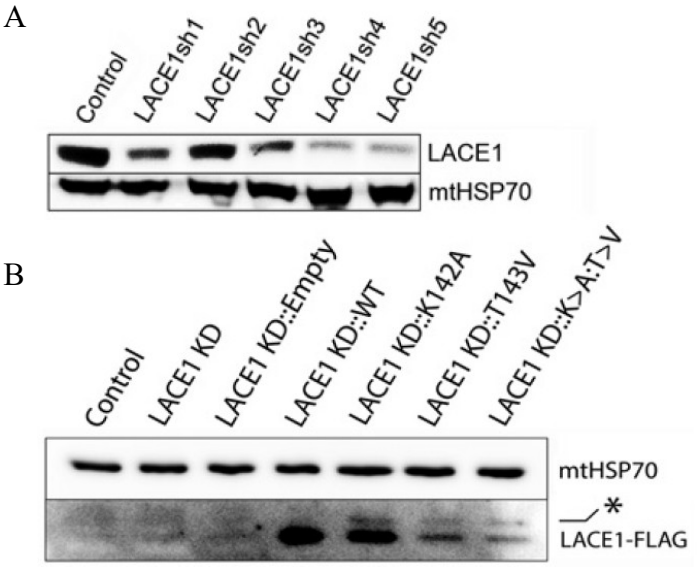

Figure 3.4. The T143V substitution in Walker A motif of LACE1 abrogates its mitochondrial accumulation (**A**) LACE1 protein is efficiently down-regulated in HEK-293 cells using the stable shRNAmir KD approach. Mitochondrial lysates (~10 μg of protein) from five different cell lines (denoted LACE1sh1–LACE1sh5) with stable vector-based expression of shRNAmirs targeting the LACE1 transcript were separated using SDS/PAGE (10% gel) and immunoblotted with antibodies against LACE1 and mtHSP70. The appropriate control cell line was generated by transfecting HEK-293 cells with non-silencing (scrambled) shRNAmir vector. Two of the cell lines, denoted LACE1sh4 and LACE1sh5, exhibited LACE1 protein levels of<10%of control values. (**B**) LACE1 variant with a T143V substitution in Walker A (P-loop) motif fail to accumulate in mitochondria of LACE1-KD cells. Three LACE1 mutant variants (K142A, T143V and K142A+T143V) in which one or two of the conserved residues of the Walker A (P-loop) motif (G/A)XXXXGK(T/S) were replaced with neutral amino acids were constructed using site-directed mutagenesis. These mutants were along with the wt LACE1 transiently expressed in a LACE1-KD background and the resulting whole-cell lysates were analysed by immunoblotting using anti-FLAG antibody. Antibody against mtHSP70 was used as a loading control. The asterisk (*) denotes the location of the LACE1–FLAG precursor protein band.

3.2.3. COX4, COX5A, YMEL and p53 co-purify with wt LACE1 expressed in LACE1-KD cells

To find out which of the observed effects associated with manipulation of cellular LACE1 levels are based on direct protein–protein interactions, we usedmitochondria from LACE1-KD cells transfected with wt LACE1–FLAG construct to perform anti-FLAG affinity purification. To control for possible nonspecific results, we used mitochondria from LACE1-KD cells transfected with CLPP–FLAG (GenBank® accession number NM_006012)

construct. CLPP is a proteolytic subunit of matrix-localized CLPXP complex and thus unlikely to share common interacting partners with LACE1, which is predicted to be an inner membrane protein (Khalimonchuk, Bird et al. 2007). Mitochondria were solubilized using 0.5% Triton X-100, and co-purifying components were analysed using SDS/PAGE immunoblotting with a battery of validated antibodies against mitochondrial proteins. We obtained highly efficient FLAG pull-down with both LACE1 and CLPP proteins (Figure 4.14. B). Subsequent Western blot analysis revealed significant amounts of COX4 and COX5A subunits to specifically co-purify with LACE1–FLAG but not CLPP–FLAG protein (Figure 3.5. A).

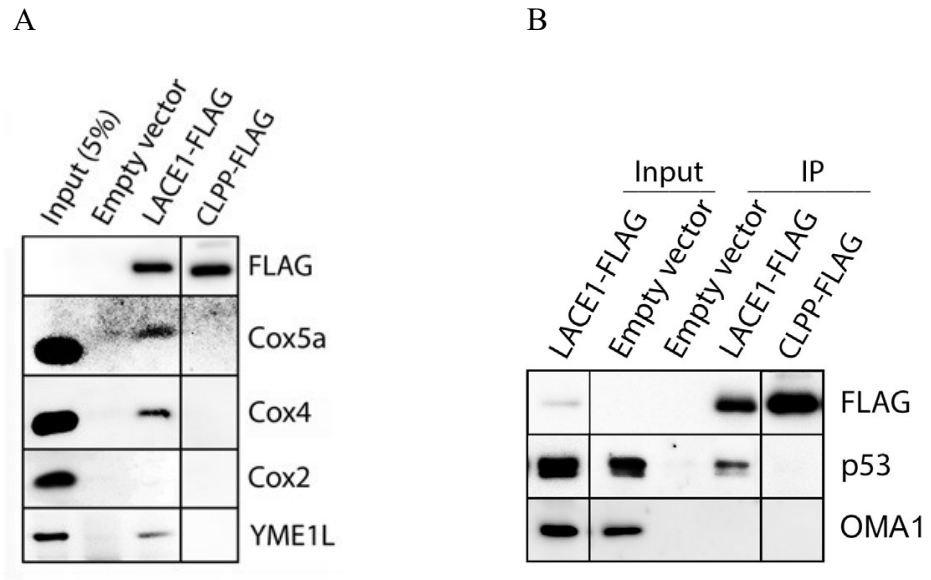

Figure 3.5. LACE1 co-immunoprecipitates with COX4, COX5A, YME1L protease and p53 tumor suppresor protein. (**A**) COX4, COX5A and YME1L and (**B**) p53 tumor supppresor protein co-immunoprecipitates with LACE1–FLAG expressed in LACE1-KD cells. LACE1-KD cells were transientlytransfected with empty expression vector, wt LACE1–FLAG construct and CLPP–FLAG construct. Isolated mitochondrial fractions were solubilized with 1% Triton X-100 and subjected to anti-FLAG affinity purification. The bound antigens were eluted with 3× FLAG peptide solution under native conditions and processed for SDS/PAGE immunoblotting. A 5% (v/v) amount of the purification input was loaded on the gel along with the purified samples and immunoblotted with antibodies against (**A**) FLAG, COX4, COX5A, YME1L and COX2 and against (**B**) FLAG, p53 and CLPP.

Importantly, additional Western blotting screening also identified a significant amount of YME1L protease (Figure 3.5. A) and the p53 tumor suppresor protein (3.5. B) in LACE1–FLAG purification samples. We were not able to identify any of the mitochondrially encoded complex IV subunits or additional nuclear-encoded complex IV components in LACE1–FLAG affinity preparations using Western blot analysis.

3.2.4. Loss of LACE1 leads to increased apoptotic resistance whereas its overexpression results in increased apoptotic sensitivity

Since LACE1 KD cells showed substantially diminished constitutive PARP cleavage (Figure 3.6. A), we next assessed staurosporine (STS)-induced apoptosis in these cells. Both control and LACE1 KD cells were treated with 0, 3 and 6 hours exposures to staurosporine (2 μM) and used either for preparation of whole cell lysates or for direct immunofluorescence staining. Whole cell lysates were subsequently processed for western blotting with antibody to cleaved PARP (Figure 3.6. B). For immunofluorescence, the cells were stained with antibody to cytochrome c and with DAPI (Figure 3.6. D). Consistently, western blotting of staurosporine treated cells showed reduced levels of cleaved PARP and cytochrome c-immunofluorescence microscopy revealed significantly milder apoptotic changes in STS-treated LACE1 KD cells, compared to identically treated controls (Figure 3.6. B and D). To confirm the cytochrome c immunofluorescence results, staurosporine treated cells were subjected to subcellular fractionation and the resulting cytoplasmic fractions were immunoblotted with antibody to cytochrome c. Western blotting detection of alfa-tubulin was used to control for equal cytoplasmic protein loading. The western blots revealed higher cytoplasmic cytochrome c content in control cells, when compared to identically treated LACE1 KD cells, confirming the immunofluorescence results (Figure 3.6. C).

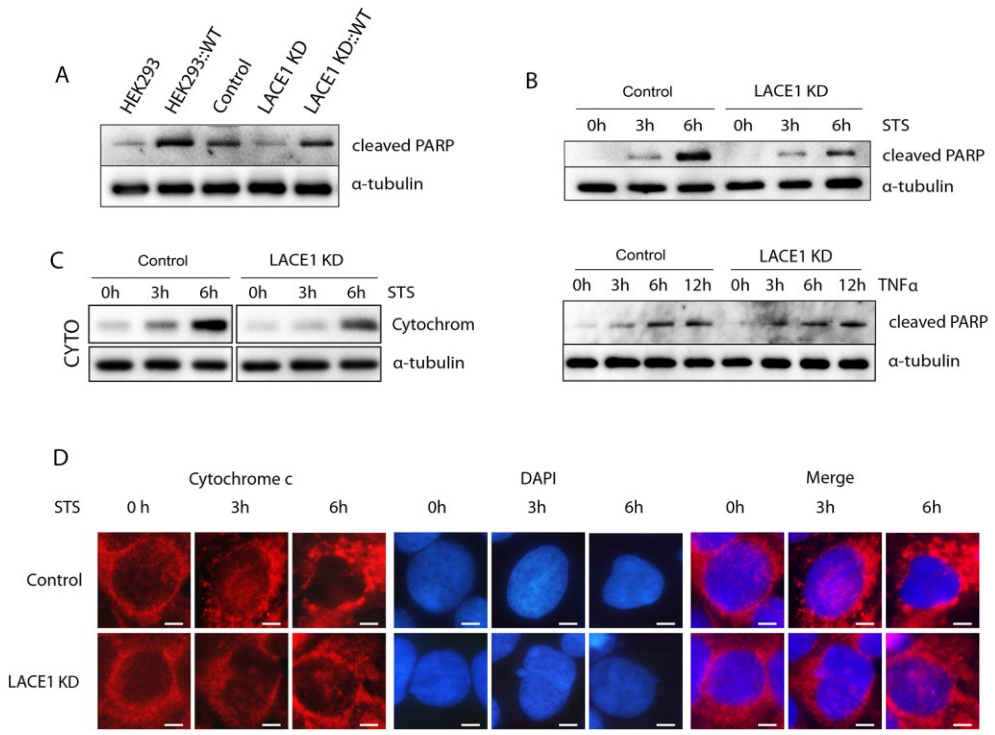

Figure 3.6. Loss of LACE1 leads to increased apoptotic resistance whereas its overexpression results in increased apoptotic sensitivity. (A) Overexpression of LACE1-FLAG in both wild-type HEK293 cells and LACE1 KD cells results in increased PARP cleavage. Wild-type HEK293 cells and LACE1 KD cells were transfected with LACE1-FLAG expression vector or with the empty vector. Whole-cell lysates were analyzed with western blotting using antibody to cleaved PARP. Antibody to α -tubulin was used to monitor equal protein loading. (**B)** LACE1 KD cells exhibit increased resistance to staurosporine-induced apoptosis but respond normally to TNFalpha-treatment. Control and LACE1 KD cells were treated with staurosporine (STS; 2 μM) or TNF alpha (10 ng/ml) and IFN-gamma (80 ng/ml) for 0, 3 and 6 hours. Whole cell lysates were prepared from treated cells and used for immunoblotting with antibody to cleaved PARP. Antibody to α-tubulin was used to control for equal protein loading. (**C)** Cells were treated with staurosporine (STS; 2 μM) for 0, 3 and 6 hours and then harvested and used for subcellular fractionation using dounce homogenization and differential centrifugation. The resulting cytoplasmic fractions were subjected to SDS-PAGE western blotting with antibody to cytochrome c. Western blotting of alpha-tubulin was used as loading control. (**D)** Cells grown on coverslips were treated with staurosporine (STS; 2 μM) for 0, 3 and 6 hours and then fixed with 4% paraformaldehyde at room temperature and permeabilized with 0.1% Triton X-100. Cells were blocked by 10% Fetal Bovine Serum and primary detection was performed with anti-cytochrome c antibody. After secondary fluorescent detection, cells were analyzed at 24°C using a Nikon Diaphot 200 inverted microscope equipped with a Plan-Apochromat 60×, numerical aperture 0.95, oil objective. The images were acquired with an Olympus DP50 CCD camera and Viewfinder Lite 1.0 software. Bar, 10 μM.

The main points related to aim B)

Human LACE1 protein mediates degradation of nuclear-encoded complex IV subunits, interact with p53 and mediates its mitochondrial translocation and apoptosis. Our results show that:

- LACE1 is a mitochondrial integral membrane protein which exist as part of three protein complexes of approximately 500, 400 and 140 kDa
- LACE1 variants with a T143V substitution in their Walker A motif fail to accumulate in mitochondria
- Loss of LACE1 leads to fragmented mitochondrial reticulum and aberrant mitochondrial ultrastructure
- Loss of LACE1 leads to increased accumulation of nuclear-encoded complex IV subunits, increased PARL, associated with increased amount of short OPA1 isoforms and K142A substitution in Walker A motif and E214Q substitution in Walker B motif of LACE1 abrogate LACE1-mediated clearance of complex IV subunits
- Knockdown of LACE1 by shRNA leads to diminished activity of mitochondrial respiratory chain and enzyme activity of complexes of electron transport chain
- Loss of LACE1 leads to increased PARL and reduced Omi/HTRA2, whereas its overexpression leads to accumulation of p53 in mitochondria and concomitant p53 reduction in nucleus
- COX4, COX5A, YME1L and p53 co-purify with wt LACE1 expressed in LACE1-KD cells
- Loss of LACE1 leads to increased apoptotic resistance whereas its overexpression results in increased apoptotic sensitivity
- LACE1 is required for mitomycin c-induced translocation of p53 to mitochondria and its concomitant reduction in nucleus
- The LACE1-mediated apoptosis is dependent on p53

4. DISCUSSION AND COCLUSIONS

Mitochondrial protein quality control is essential for the maintenance of the cell integrity. This is ensured by sequential use of a large system of mitochondrial proteases and proteolytic complexes located across the various mitochondrial subcompartments. Errors in this system may lead to mitochondrial damage and ending with apoptosis. Moreover, mitochondrial proteases were found altered in many serious diseases. The exact cause of their occurrence is not yet clear.

Based on our previous results, we have focused on characterization of functional overlap and cooperativity of proteolytic subunits AFG3L2 and YME1L of the IMM complexes m- and i-AAA in the maintenance of mitochondrial structure and respiratory chain activity. We demonstrate that loss of AFG3L2 and YME1L both, alone and in combination, results in significant growth retardation in HEK293 cells. Interestingly, both YME1L and YME1L/AFG3L2 knockdown mitochondria contained significantly increased levels of Drp1. In contrast, mitochondrial fraction from single AFG3L2 KD cells showed normal Drp1 levels.

Markedly diminished Spg7 levels were found in all three knockdown cell lines analyzed. Spg7 forms a hetero-oligomeric m-AAA protease isoenzyme together with AFG3L2 subunit. In the absence of AFG3L2 the stability of Spg7 might be compromised. However, the markedly diminished Spg7 levels found in YME1L KD cells are difficult to explain. In addition to YME1L, OMA1 protease mediates the processing of OPA1 (Baker, Lampe et al. 2014, Korwitz, Merkwirth et al. 2016). Whereas YME1L-dependent OPA1 processing promotes tubular mitochondrial morphology, OMA1-dependent processing induces mitochondrial fragmentation (Anand, Wai et al. 2014, Rainbolt, Lebeau et al. 2016). We found elevated OMA1 in all three knockdown cell lines with the most prominent change in AFG3L2 KD cells. Indeed, OMA1 was first identified in yeast as a peptidase with overlapping activities with the m-AAA protease (Khalimonchuk, Jeong et al. 2012).

LACE1 protein is human homologue of yeast Afg1 ATPase. Afg1 protein has an ATPase domain but lacks a proteolytic domain. The highly evolutionary conserved Afg1 ATPase probably acts as a chaperon in degradation of unassembled or misfolded complex IV subunits (Lee and Wickner 1992, Khalimonchuk, Bird et al. 2007). The human LACE1 ortholog consists of ATP/GTP binding Walker A motif and has predicted ATPase activity too. LACE1 protein is built up of a common five domain structure and shows high level of expression in

active mammary gland, myocardium, kidney and liver tissue. Likewise, physical interaction and co-operation of the LACE1 complex with YME1L may stimulate proteolysis of nuclear-encoded complex IV subunits by the i-AAA complex.

We show that mitochondrial integral membrane protein LACE1 exists as a part of three complexes of approximately 140, 400 and 500 kDa. The native molecular mass of approximately 140 kDa observed with dodecyl maltoside-treated mitochondria rather suggested a homodimeric complex or low-molecular-mass heteromeric LACE1 complex. However, using digitonin for protein solubilization instead of dodecyl maltoside, two high-molecular-mass LACE1 complexes of approximately 500 and 400 kDa were detected using two dimensional immunoblots in both mitochondrial and purified protein samples. It is likely that the higher delipidating properties of dodecylmaltoside, comparedwith digitonin, may have affected the stability of high-molecular-mass LACE1 complexes. Indeed, both of the observed high-molecular-mass complexes might contain the expected homohexameric LACE1 ATPase complex. The slightly higher apparent molecular mass of these complexes than expected for the pure LACE1 homohexamer, could stem from bound membrane lipids and/or interacting proteins (D'Aurelio, Gajewski et al. 2006, Stiburek, Cesnekova et al. 2012, Crichton, Harding et al. 2013).

The ectopic expression of the K142A LACE1 mutant shows the essential character of the Walker A motif for LACE1's involvement in degradation of excess nuclear-encoded complex IV subunits. Indeed, the essential lysine residue of Walker A motif is known to support binding of nucleotides. The role of the flanking threonine residue is much less understood (Nagy, Wu et al. 2009). Owing to failure of both T143V LACE1 mutants to accumulate in mitochondria, we were not able to assess their effects on clearance of excess nuclear-encoded complex IV subunits. However, a significantly increased amount of the unprocessed protein form found in all LACE1 Walker A motif variants suggests that ATP binding may be required for efficient mitochondrial import/processing of LACE1 protein. Despite high intramitochondrial protein levels, the Glu214Gln Walker B mutant also failed to rescue the increased accumulation of nuclearencoded complex IV subunits in the LACE1 RNAi background. The conserved glutamate residue of theWalker B motif is required for ATP hydrolysis (Hanson and Whiteheart 2005). Thus ATP binding and hydrolysis appear to be essential for LACE1-mediated degradation of nuclearencoded complex IV subunits.

We demonstrate that LACE1, similarly as its yeast ortholog, mediates degradation of nuclear-encoded complex IV subunits COX4, COX5A and COX6A, and moreover using affinity

purification we revealed direct interation with COX4 and COX5a. LACE1 protein is required for normal activity of complexes III and IV of OXPHOS. We demonstrate by ectopic expression of both Lys142Ala Walker A and Glu214Gln Walker B mutants that an intact ATPase domain is essential for LACE-mediated degradation of nuclear-encoded complex IV subunits. Yeast Afg1 was shown to facilitate degradation of mitochondrially encoded complex IV subunits COX1, COX2 and COX3, and a yeast Afg1 deletion strain showed respiratory growth impairment and diminished activities of respiratory complexes III and IV (Khalimonchuk, Bird et al. 2007). The fact that nuclear-encoded complex IV subunits COX4, COX5A and COX6A were markedly elevated in LACE1-KD cells, but the mitochondrially encoded subunits were completely unaffected, suggests changes in substrate specificity of LACE1 throughout evolution. A similar, but more profound, shift in substrate specificity between yeast and mammalian orthologues concerning complex IV subunits was found for the inner membrane translocase OXA1L (Stiburek, Fornuskova et al. 2007). LACE1 is shown to interact physically with COX4 and COX5A subunits of complex IV and with the inner membrane protease YME1L. In addition, loss of LACE1 is associated with marked up-regulation of YME1L. Indeed, COX4 subunit is one of the previously identified proteolytic substrates of YME1L protease. This fact provides a further functional link between LACE1 and YME1L (Stiburek, Cesnekova et al. 2012). On the other hand, the matrix-localized LON protease, which was reported to degrade COX4 and COX5A, exhibited a marked reduction in LACE1-KD cells (Lee and Suzuki 2008). In contrast with increased YME1L that could stem from a simple compensatory response to LACE1 deficiency, the observed downregulation of LON is more difficult to explain. On the basis of its structure–function characteristics, yeast Afg1 was suggested to resemble cytosolic Cdc48 (VCP/p97) (Khalimonchuk, Bird et al. 2007).

Amongst the most obvious features of LACE1 KD cells is the increased resistance to apoptosis. Since overexpression of LACE1 in both LACE1 KD cells as well as wild-type HEK293 cells led not only to suppression of the apoptotic defect but to major apoptotic phenotype point to significant pro-apoptotic character of human LACE1. Importantly, the LACE1 mediated apoptosis is shown to depend on p53 in normal human dermal fibroblasts. Furthermore, although increasingly resistant to intrinsic apoptosis, the LACE1 KD cells are fully susceptible to TNFalpha-induced cell death. The mitochondrial rhomboid protease PARL functions anti-apoptotic in mammalian cells by controlling cristae remodeling and cytochrome c release (Cipolat, Rudka et al. 2006). Hence, the markedly increased levels of PARL in LACE1 KD cells are consistent with their increased apoptotic resistance. The tumor

suppressor protein p53 is activated upon various stress stimuli to either support cell survival or promote apoptosis. Besides its well established nuclear, transcription-dependent function, p53 was shown to act also extranuclearly. Indeed, in response to acute cellular stress or even acute exercise, p53 can rapidly translocate into mitochondria and activate here apoptosis via fast, transcription-independent manner. Several lines of experimental evidence support the direct involvement of LACE1 in mitochondrial partition of p53 protein. First, overexpression of LACE1 leads to dramatic increase in mitochondria-associated p53 and to concomitant reduction in nuclear p53 content accompanied by reduced expression of p53 transcription target genes BAX and PUMA. Second, the DNA damage agent mitomycin c fails to induce mitochondrial translocation and concomitant nuclear reduction of p53 in LACE1 knockdown cells. Finally, LACE1-FLAG protein expressed in LACE1 knockdown cells physically interacts with p53. The role of LACE1 in mitochondrial partition of p53 is consistent with both the significant pro-apoptotic character of LACE1 protein and with the previously reported tumor-suppressor properties of LACE1 gene region (Karube, Nakagawa et al. 2011). The unaffected levels of SCO2 in LACE1 overexpressing cells further confirm the extranuclear, transcription-independent character of LACE1-induced p53 upregulation. Besides its function in complex IV assembly, the SCO2 metallochaperone was identified as a p53 transcription target gene required for shift of cellular metabolism from glycolysis to increased OXPHOS utilization (Bensaad, Tsuruta et al. 2006, Matoba, Kang et al. 2006, Madan, Gogna et al. 2013). On the other hand, positive genetic interaction between the mitochondrial metallopeptidase OMA1 and p53 was observed in OVCA cells, which is consistent with LACE1-induced OMA1 upregulation seen in our HEK293 knockdown model (Kong, Wang et al. 2014). Besides its specific role in mitochondrial homeostasis and apoptosis, the subcellular partitioning of p53 into mitochondria has the potential to substantially affect its nuclear activity (Zhuang, Wang et al. 2013). Indeed, the pattern of nuclear distribution of p53 protein is substantially affected in LACE1 KD cells. On the other hand, expression of p53 transcription target genes BAX and PUMA was found significantly diminished in LACE1 overexpressing cells. Several mechanisms have been proposed to drive the mitochondrial translocation of p53 (Ahn, Trinh et al. 2010, Trinh, Elwi et al. 2010, Zhuang, Wang et al. 2013). Similarly to our report, one of them utilizing the mitochondrial disulfide relay protein CHCHD4 was shown to be connected to mitochondrial respiratory capacity (Zhuang, Wang et al. 2013) . The yeast homologue of human LACE1 (Afg1) was shown to be required for normal activity of respiratory complexes III and IV (Khalimonchuk, Bird et al. 2007). Similar to the yeast Afg1 deletion strain, the rather moderate respiratory

defect of human LACE1 KD cells suggests possible redundant character of LACE1 role in respiratory chain maintenance. Given that ATP- or ADP-binding state may change biochemical property of LACE1, it is interesting to speculate that LACE1-p53 interaction could serve as an immediate apoptotic response to altered mitochondrial ATP levels. Further work is required to address the effects of LACE1-mediated subcellular p53 partitioning towards cell metabolism and proliferation.

Collectively, our results establish a crucial role for LACE1 in mitochondrial protein homeostasis and identify a possible novel accessory protein of the IMM i-AAA complex. Future studies will be focused on the identification of additional interacting partners of LACE1 protein.

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6. LIST OF ORIGINAL ARTICLES (in chronological order)

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