Apoptotic Factors Are Evolutionarily Conserved Since Mitochondrial Domestication

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Abstract

The mechanisms initiating apoptotic programmed cell death in diverse eukaryotes are very similar. Basically, the mitochondrial permeability transition activates apoptotic proteases, DNases, and flavoproteins such as apoptosis-inducing factors (AIFs). According to the hypothesis of the endosymbiotic origin of apoptosis, these mechanisms evolved during mitochondrial domestication. Various phylogenetic analyses, including ours, have suggested that apoptotic factors were eubacterial proto-mitochondrial toxins used for killing protoeukaryotic hosts. Here, we tested whether the function of yeast *Saccharomyces cerevisiae* apoptotic proteases (metacaspases Mca1 and Nma111), DNase Nuc1, and flavoprotein Ndi1 can be substituted with orthologs from remotely related eukaryotes such as plants, protists, and eubacteria. We found that orthologs of remotely related eukaryotic and even eubacterial proteins can initiate apoptosis in yeast when triggered by chemical stresses. This observation suggests that apoptotic mechanisms have been maintained since mitochondrial domestication, which occurred approximately 1,800 Mya. Additionally, it supports the hypothesis that some of these apoptotic factors could be modified eubacterial toxins.

Key words: apoptosis, apoptotic factors, mitochondrial domestication, ortholog complementation, yeast.

Significance

The origin of eukaryotic apoptosis is an open question in biology. We postulate that yeast apoptotic factors have been functionally maintained since mitochondrial domestication (i.e., 1,800 Mya). Performing complementation studies, we showed that the apoptotic functions of these factors can be substituted not only by orthologs from remotely related eukaryotes but, in some cases, even by bacterial orthologs. The presented results support our hypothesis that apoptosis evolved due to antagonistic interactions between the protoeukaryotic host and protomitochondrial prey. These observations shed new light on the evolution of apoptosis, further emphasizing its endosymbiotic origin.

Introduction

Apoptotic cell death is a mechanism that regulates the development of multicellular organisms. However, apoptosis-like cell death, a form of primordial apoptosis, also occurs in unicellular organisms such as yeast or unicellular protists. The origin of the mitochondrial pathway of apoptosis is an open question in biology, although the "endosymbiotic hypothesis" is generally accepted. It was proposed for the first time by Guido Kroemer (Kroemer 1997), who observed that the release of apoptotic factors from the mitochondria initiated apoptosis in the so-called permeability transition. He hypothesized that apoptosis originated from the ancestral mechanism of killing eukaryotic cells and that this ancient mechanism evolved into current apoptotic mechanisms. Various phylogenetic studies, including ours

(Aravind et al. 2001; Klim et al. 2018), have confirmed that apoptosis-initiating factors are usually of bacterial origin, akin to mitochondria. Although this conclusion is generally accepted, it still contains some controversial elements. It is not clear why organisms use different apoptotic factors and pathways. For example, although the animal apoptotic caspase–based pathway is described exclusively in animals, we previously identified caspase homologs in the unicellular protist *Reticulomyxa* (Klim et al. 2018). The apoptotic programed cell death of nonanimal eukaryotes, such as fungi, protists, or slime molds, occurs without the caspase pathway. These organisms use other proteases called metacaspases, which are distantly related to caspases (Madeo et al. 2002). Caspases, metacaspases, and other proteases (e.g., paracaspases) contain the PFAM PF00656 caspase/metacaspase domain. The traditional classification of these proteases is based on the described biochemical specificity. Caspases are known as "cysteine-dependent aspartate-specific protease;" as they cleave after aspartate residues, paracaspases are arginine specific, whereas metacaspases can cleave after either arginine or lysine (Minina et al. 2020). Although caspases and metacaspases have different specificities, both plant metacaspases and animal caspases cut and inactivate TSN (Tudor staphylococcal nuclease, a phylogenetically conserved protein), which has been shown to participate in apoptotic programed cell death (Sundström et al. 2009). It is worth mentioning that the apoptosis-inducing factor (AIF)–based pathway is described both in animals (Xie et al. 2005) and different nonanimal eukaryotes (Wissing et al. 2004).

Yeast apoptosis was discovered in 1997 (Madeo et al. 1997), and that of *Saccharomyces cerevisiae* is currently a classical model of apoptosis in unicellular organisms (Carmona-Gutierrez et al. 2010). As in other organisms, yeast apoptosis is initiated by the release of proapoptotic factors, such as AIFs, Nuc1 nuclease, and cytochrome c, followed by the degradation of DNA. It is induced by various chemical compounds, such as hydrogen peroxide, acetic acid, metals, drugs, UV irradiation, and heat stress (Carmona-Gutierrez et al. 2010). Apoptosis is also activated during the yeast life cycle by both chronological and replicative aging (Rockenfeller and Madeo 2008; Kaczanowski 2016).

Various apoptotic factors derived from other organisms and heterologously expressed in yeast can induce apoptosis. Although the yeast genome does not encode the ortholog of the main human proapoptotic factor BAX, its expression induces yeast apoptosis (Greenhalf et al. 1996). This phenomenon was used for the discovery of BAX suppressors. Conversely, heterologous expression of human suppressors of apoptosis Bcl-2 and Bcl-xL prevents Bax-induced lethality (Xu and Reed 1998). Later studies on the Bcl-2 family revealed that proteins from this family govern the permeabilization of the outer mitochondrial membrane, which induces apoptosis. It turns out that the mechanisms of induction of cell death by this permeabilization slightly differ in animals and yeast. In animals, permeabilization leads to the release of cytochrome c and several other apoptogenic proteins from mitochondria. In yeast, the release of cytochrome c is not required for the induction of cell death (see a review (Polčic et al. 2015)). Additionally, the heterologous expression of human α-synuclein was used to discover the molecular mechanism of pathological apoptosis that occurs in Parkinson's disease (Mochizuki et al. 1996; Outeiro and Lindquist 2003). Also *Trypanosoma* and *Arabidopsis* metacaspases have been shown to induce apoptosis upon their expression in yeast (Szallies et al. 2002; Watanabe and Lam 2005).

In this study, we tested the hypothesis that the function of apoptotic factors has been evolutionarily conserved since mitochondrial domestication. Accordingly, the mechanism of apoptosis of all eukaryotes would have evolved together during mitochondrial domestication. This implies that apoptotic factors from remotely related organisms should be able to replace the original yeast ones. We tested the above hypothesis using *S. cerevisiae* as a model. We selected yeast genes encoding apoptotic factors, that is, *NDI1* (Li et al. 2006; Cui et al. 2012), *MCA1* (Madeo et al. 2002), *NMA111* (Fahrenkrog et al. 2004), and *NUC1* (Büttner et al. 2007), and replaced them with orthologs identified through our phylogenetic analysis. Then, we tested the constructed strains in terms of cellular response to proapoptotic factors. Moreover, we tested the range of apoptosis in response to acetic acid stress using an annexin V–based flow cytometry assay.

We found that in most cases, selected original yeast proteins could be functionally replaced with phylogenetically distant orthologs. This strongly suggests that the proapoptotic functions of the analyzed factors have been evolutionarily conserved since the origin of eukaryotes.

Results

Identification of Yeast–Ortholog Complementation Gene Pairs

In our previous studies, we identified the core of apoptosis machinery in *S. cerevisiae*, which included proteins; the lack of which decreases apoptosis, whereas overproduction of these proteins induces it (Klim et al. 2018). Using a phylogenetic approach, that is, calculating phylogenetic trees, we indicated putative orthologs of these proteins (e.g., proteins AtMC [1–9] of *Arabidopsis thaliana* and Pca1 of *Schizosaccharomyces pombe* as orthologs of yeast Mca1) thus creating yeast gene ortholog complementation pairs (shown in table 1). In this study, we tested these pairs, in which selected orthologs represent the taxonomic diversity of eukaryotes, including plants, animals (humans), slime

Table 1
Orthologs of *S. cerevisiae* Core Apoptotic Genes Selected for the Study (Orthology Prediction Based on Phylogenetic Study by Klim et al. 2018)

Yeast gene (systematic name)	Organism	Ortholog gene name	UniProtKB ID	Identity (%) (Smith–Waterman algorithm)
MCA1 (*YOR197W*)	*Arabidopsis thaliana*	*MCA1*	Q8L7E9	37.0
	Bacillus cereus	*bcere0018_55570*	C2UNI0	30.4
NMA111 (*YNL123W*)	*Desulfatibacillum alkenivorans*	*Dalk_4440*	B8FNF0	29.6
	Rickettsia australis	*MC5_06825*	H8K8L0	23.0
	Arabidopsis thaliana	*DEGP7*	Q8RY22	31.7
NDI1 (*YML120C*)	*Dictyostelium discoideum*	*DDB0168392*	Q86AE2	34.8
	Arabidopsis thaliana	*NDB2*	F4JGL5	31.2
	Actinomyces urogenitalis	*HMPREF0058_0119*	C0W2M5	25.8
NUC1 (*YJL208C*)	*Homo sapiens*	*NUCG_HUMAN*	Q14249	42.9
	Leishmania major	*LMJF_10_0610*	Q4QHF4	23.3

molds, and protists. We also tested bacterial orthologs because their functionality in yeast may contribute to the "endosymbiotic" origin of apoptosis, which claims that eubacterial ancestors of extant mitochondria used toxin–primordial apoptotic factors to kill protoeukaryotic cells. The apoptotic activity of eubacterial factors may show that "eubacterial" biochemical machinery can induce apoptosis.

Ortholog-Bearing Yeast Strains

To check whether heterologous expression of apoptotic factors from remotely related organisms complements orthologous gene deletion in yeast, we constructed strains in which selected genes were replaced by chosen orthologs from various organisms. The individual orthologs for each of the four genes under study were introduced to the genome of the BY4743 diploid strain by homologous recombination. After sporulation of the resulting heterozygous diploid, tetrads were dissected and offspring strains were analyzed to select haploid strains with the ortholog allele. The presence of orthologs did not cause lethality, and in all tetrads, the four spores were viable. The presence of the orthologous gene in the proper gene locus was verified by PCR analysis followed by DNA sequencing, and the correct strains were used for further studies. These strains were designated as follows: yeast gene name followed by abbreviations of genus and species names of the organism from which orthologous genes were selected, for example NMA111_Da: ***D****esulfatibacillum* ***a****lkenivorans* ortholog of yeast *NMA111* gene (listed in supplementary table S1, Supplementary Material online).

Expression and Cellular Localization of Orthologous Proteins in Recombinant Yeast Strains

To check the integrity, size, level of expression, and cellular localization of the orthologous proteins in yeast cells, strains bearing ortholog–GFP (green fluorescent protein) hybrid genes were constructed (supplementary table S1, Supplementary Material online). All fusion genes were prepared in the same manner (see SI MM) with the sequence encoding the GFP tag attached at the C-terminus of the gene. The fusion genes were introduced into the genome of the diploid BY4743 strain, and the final strains were obtained following the strategy mentioned above. Positively verified haploid strains bearing ortholog–GFP gene fusions were tested by western blotting and fluorescence microscopy. In agreement with the reported abundance of the wild-type (WT) yeast proteins under study in the Saccharomyces Genome Database (SGD), we observed variability in the intensity of the western blot bands as well as in the fluorescence signals. Using anti-GFP antibodies, we demonstrated that all analyzed hybrid proteins were of the expected size (fig. 1; table 2). The level of expression of fusion ortholog proteins of Ndi1 and Nuc1 was similar to that of the WT. However, in the case of Nma111 and Mca1, we observed differences in the orthologous protein level, that is, the Nma111_Da-GFP band intensity was significantly stronger, while that of Mca1_Bc-GFP was much weaker in comparison with the respective GFP-tagged WT protein. Notably, despite multiple attempts, we were unable to generate the strain bearing the GFP-tagged *NMA111_At* ortholog (in contrast to the untagged *NMA111_At*).

Next, we tracked the cellular localization of orthologous proteins by fluorescence microscopy. As a reference, the respective yeast strains expressing the corresponding WT protein tagged with GFP were used (supplementary table S1, Supplementary Material online). In accordance with previous studies (Li et al. 2001; Fahrenkrog et al. 2004; Büttner et al. 2007; Lee et al. 2010; Cui et al. 2012) on WT proteins, our data show the localization of Ndi1 and Nuc1 and their orthologs in the mitochondria, while Mca1 and Nma111 and their corresponding orthologs stayed predominantly in the cytoplasm and nucleus, respectively (fig. 2).

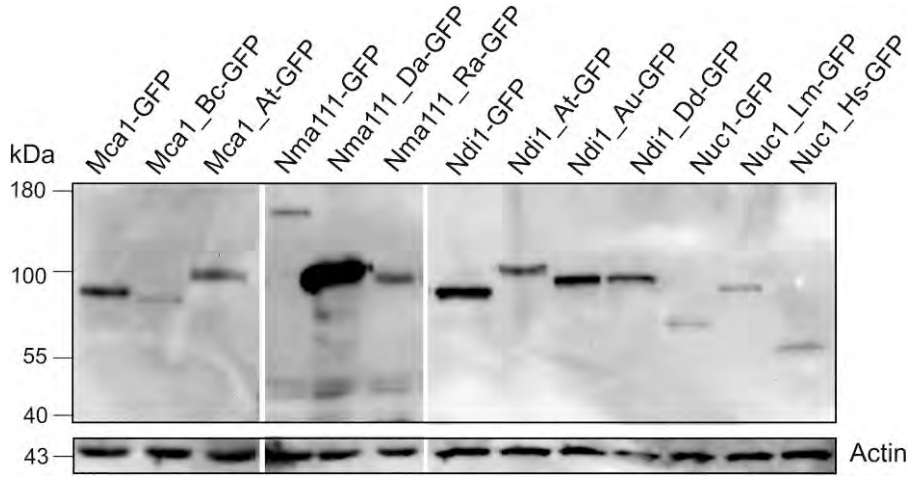

Fig. 1.—Orthologous proteins of various origins expressed in *S. cerevisiae* have the expected molecular weight. Western blot analysis of SDS–PAGE–separated proteins probed with antibodies recognizing GFP (anti-GFP antibodies) or actin (anti–actin antibodies), used as the loading control. WT, BY4741; At, *Arabidopsis thaliana*; Bc, *Bacillus cereus*; Da, *Desulfatibacillum alkenivorans*; Ra, *Rickettsia australis*; Dd, *Dictyostelium discoideum*; Au, *Actinomyces urogenitalis*; Hs, *Homo sapiens*; Lm, *Leishmania major*.

Table 2

Size and Abundance of Tested Proteins. Protein Molecular Weights Were Estimated Based on Amino Acid Composition as a Sum of the Given Protein and GFP

Gene	Cellular localization	Median abundance (molecules/cell)	Protein name	MW of a GFP fusion protein (kDa)
MCA1	Cytoplasm	5,025 ± 2,001	Mca1	74.9
			Mca1_At	90.0
			Mca1_Bc	71.5
NMA111	Nucleus	4,177 ± 1,547	Nma111	137.8
			Nma111_Da	84.7
			Nma111_At	151.7
			Nma111_Ra	86.2
NDI1	Mitochondria	7,696 ± 2,456	Ndi1	84.3
			Ndi1_Dd	88.2
			Ndi1_At	95.0
			Ndi1_Au	81.7
NUC1	Mitochondria	3,900 ± 1,714	Nuc1	64.2
			Nuc1_Hs	56.4
			Nuc1_Lm	74.7

In conclusion, western blotting and microscopy observations confirmed the expression and correct localization of orthologous proteins in recombinant yeast strains.

Phenotypic Analysis of Yeast Expressing Orthologous Genes

Many orthologous genes have been shown to successfully complement the defects of their yeast counterparts; however, this is not a general rule (Kachroo et al. 2015, 2017). To test whether the selected yeast gene orthologs can functionally replace the yeast *NUC1*, *MCA1*, *NDI1*, and *NMA111*, the yeast strains were subjected to a series of phenotypic tests. Initially, we tested their viability under apoptosis-inducing agents, that is, acetic acid and hydrogen peroxide using a semiquantitative drop assay followed by CFU quantification assay. Both agents induce mitochondria-dependent apoptotic cell death in *S. cerevisiae* displaying characteristic apoptotic markers, including chromatin condensation, phosphatidylserine exposure, and accumulation of DNA strand breaks (Madeo et al. 1999; Ludovico et al. 2001). We checked the effect of these oxidative stressors under continuous stress conditions on solid YPD medium as well as under short-term but sublethal exposure in liquid YPD medium (for details, see SI MM). In accordance with the SGD database, we found that except for *nuc1Δ*, the deletion strains showed elevated resistance to acetic acid and H_2O_2-induced cell death (fig. 3; supplementary figs. S2 and S3, Supplementary Material online). As reported by Ludovico et al. (Ludovico et al. 2001), *NUC1* deletion enhances the sensitivity toward these oxidative stresses in the presence of glucose while protecting against the abovementioned stresses in glycerol-containing medium. In contrast, we observed no phenotypic differences for the *nuc1Δ* strain on glycerol- and glucose-containing media (supplementary fig. S3, Supplementary Material online). *nuc1Δ* was more sensitive to acetic acid as well as to H_2O_2 stress than the WT was (fig. 3; supplementary fig. S2, Supplementary Material online). According to the spot assay (fig. 3*A*) and survival assay (fig. 3*B*), not all but some of the engineered strains showed sensitivity to the stresses aligning with the phenotype showcased by the WT. The strains bearing orthologs

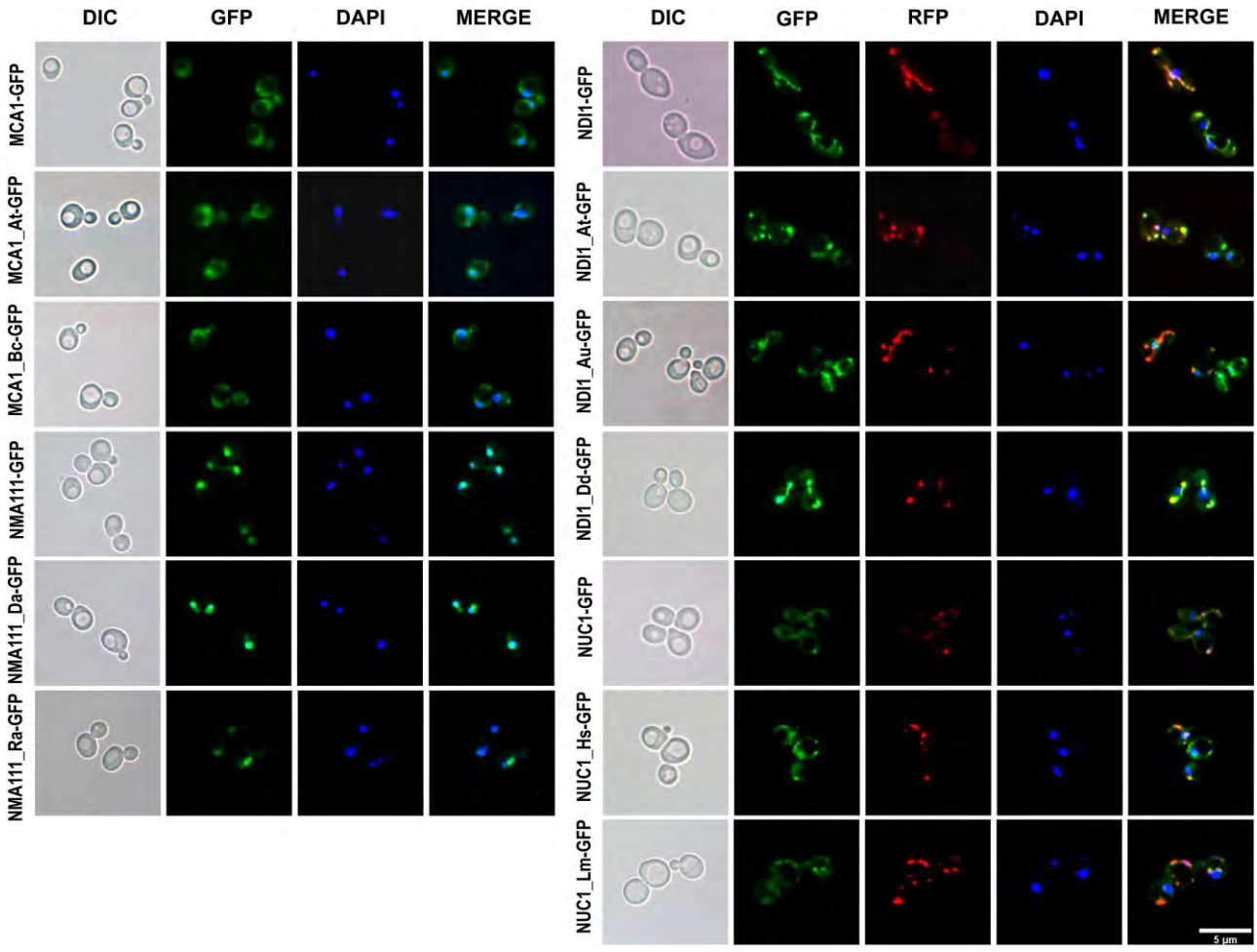

Fig. 2.—Orthologs of apoptotic factors expressed in yeast have the same localization as native proteins. Fluorescence microscopy of yeast cells expressing native or orthologous GFP-tagged proteins. Yeast cells were grown in synthetic complete medium up to the exponential phase. Mitochondria were visualized with mtRFP protein. To visualize nuclei, cells were stained with DAPI. Abbreviations as in Figure 1.

MCA1_At, *NMA111_Ra*, and *NMA111_At* were significantly more sensitive to both stresses as compared with their respective deletion mutant. In the case of *NDI1*, while drop assay (fig. 3*A*) showed that NDI1_Au and NDI1_Dd were sensitive to both stresses as opposed to *ndi1Δ*, survival assay (fig. 3*B*) revealed that all three *NDI1* ortholog-bearing strains were relatively more sensitive to acetic acid stress while only NDI1_Dd was more tolerant to H_2O_2 stress in comparison with the *ndi1Δ*. Among *NUC1* orthologs, yeast cells bearing the human counterpart were most sensitive to both stresses while Leishmanian orthologs were resistant to acetic acid stress but sensitive to H_2O_2 stress in comparison with the *nuc1Δ* as well as WT strain. Additionally, strains carrying plasmid-borne native *NUC1* did show a reversal of phenotypes of the *nuc1Δ* strain (supplementary figs. S2, S3, and S4, Supplementary Material online). The above assays indicated that several orthologs of respective yeast genes complement, at least in part, the native gene deletion.

Annexin V Assay for Apoptosis Detection

To further assess the functional complementation of yeast apoptotic genes by their orthologs, we performed an annexin V/propidium iodide (PI) costaining assay. This technique detects the extent of apoptosis- and necrosis-displaying cells in the analyzed population after treatment with proapoptotic compounds, for example, acetic acid. The addition of annexin V to normal healthy cells generates almost no intrinsic fluorescent signal. The exposition of phosphatidylserine, characteristic of apoptosis, allows its binding with annexin V linked to the fluorescent dye, yielding a fluorescent signal detectable by flow cytometry. Staining cells with PI in parallel labels the necrotic cells in the population. We adapted the annexin V/PI protocol for yeast cells by removing the thick cell wall first to allow phosphatidylserine detection. We observed that the proper preparation of spheroplasts was of crucial importance (see SI MM). Acetic acid was chosen as a proapoptotic stressor due to its stability and greater

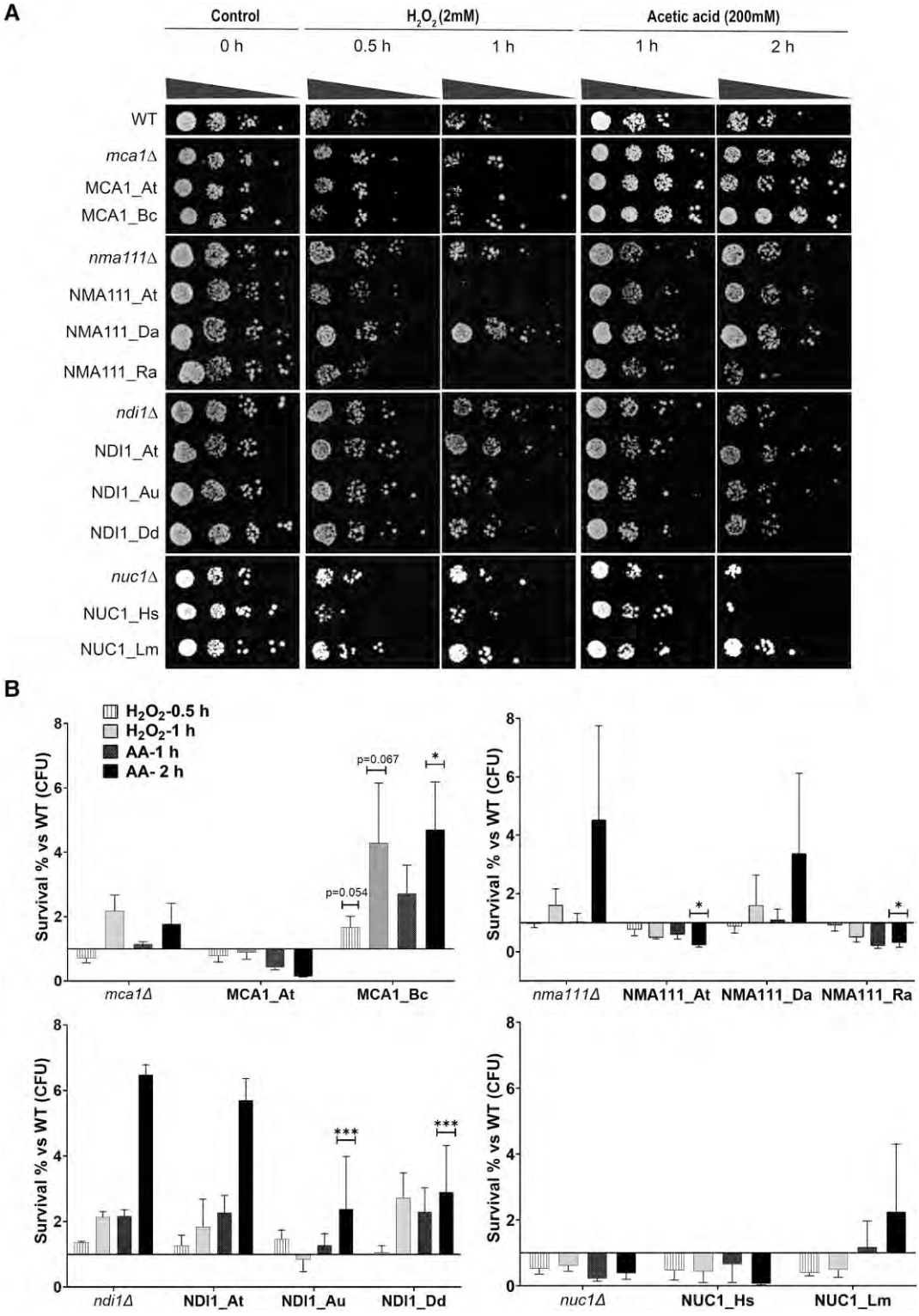

Fig. 3.—Yeast strains bearing orthologs of apoptotic factors exhibit diverse sensitivity to oxidative stressors. Indicated strains were grown in YPD medium to the exponential phase and subjected to hydrogen peroxide– and acetic acid–induced stress conditions. (*A*) Successive 10-fold dilutions of cell suspensions were spotted on solid YPD medium for drop assay. The results of all panels of the figure are representative of at least three independent growth experiments. (*B*) Yeast samples at indicated time points were plated on YPD medium, followed by incubation and counting the number of colonies to calculate cell viability. Data from at least three independent experiments are presented using the means ± SEMs. Abbreviations as in Figure 1.

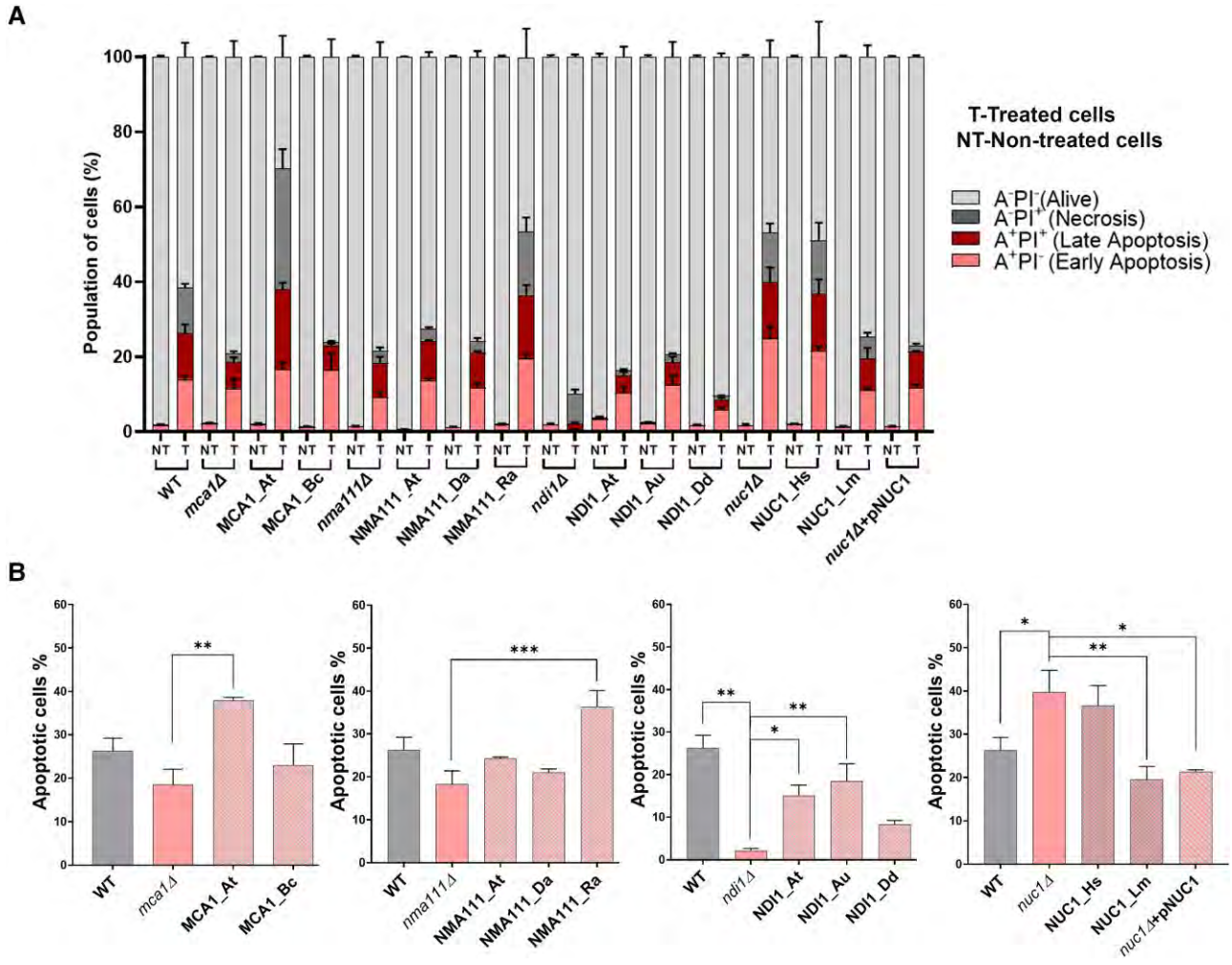

Fig. 4.—Annexin V/PI apoptosis assay on studied yeast strains. (*A*) Percentage of early apoptotic, late apoptotic, necrotic, and viable cells after exposure to 200 mM acetic acid for 2 h (T, treated cells) as quantified by flow cytometry. Nontreated cells (NT) were used as negative controls. (*B*) Comparison of the percentage of early and late (total) apoptotic cells after acetic acid treatment among the strains. $*P < 0.05$, $**P < 0.005$, and $***P < 0.001$. Data from at least three independent experiments are presented using the means ± T as quantified by flow cytometry.

reproducibility compared with H_2O_2. We performed annexin V/PI costaining on the WT, selected yeast deletion mutants, and ortholog-bearing yeast strains before and after apoptosis induction with acetic acid. The results (shown in fig. 4; supplementary fig. S5, Supplementary Material online) revealed that the percentage of apoptotic cells was approximately 26% for the WT, 18% for *mca1Δ*, 13% for *nma111Δ*, 1.7% for *ndi1Δ*, and 46% for *nuc1Δ* strains. Hence, the number of apoptotic cells in the strains lacking *NDI1*, *MCA1*, and *NMA111* was lower than that in the WT strain, which is in line with their suggested role in apoptosis. Notably, the *ndi1Δ* strain showed almost exclusively necrosis and a very small fraction of apoptotic cells.

Our data confirmed the ability of *MCA1*, *NMA111*, and *NDI1* orthologs to trigger apoptosis in yeast strains, which lost this ability due to the given deletion. In all these cases, cells exhibited elevated levels of apoptosis in comparison with the respective deletion mutants, albeit at different levels. However, in some cases, the difference was not statistically significant (fig. 4*B*). For instance, MCA1_At had significantly higher levels, while MCA1_Bc showed a slightly higher percentage of apoptotic cells compared with the *mca1Δ* strain. In comparison with the *nma111Δ* strain, only the bacterial *NMA111_Ra* ortholog showed a statistically significant increase in the number of apoptotic cells, while the other two strains had somewhat higher levels of apoptosis. Similarly, in the case of *NDI1* deletion, we observed statistically significant apoptosis complementation in the case of two orthologs (*NDI1_Au*, *NDI1_At*) and no significant complementation in the case of *NDI1_Dd*. In contrast to other deletion strains, *nuc1Δ* displayed a higher number of apoptotic cells than the control WT strain upon induction of apoptosis. This was in accordance with the drop assay indications. In a previous study, Büttner et al.

(Büttner et al. 2007) reported that disruption of *NUC1* did not provide protection against cell death in glucose-containing medium but instead sensitized them to necrotic death. In contrast, when grown in glycerol-containing medium and exposed to acetic acid, the cells showed improved survival compared with the WT. However, our experiments, conducted under similar conditions as the study by Büttner et al., yielded different results regarding the protective effect on the glycerol medium (supplementary figs. S3 and S4, Supplementary Material online). To further investigate this discrepancy, we performed Annexin V/PI assay using WT, *nuc1Δ*, and *nuc1Δ*+pNUC1 cells grown in both glycerol- and glucose-containing media and subsequently treated with acetic acid. Our results (supplementary fig. S6, Supplementary Material online) indicated that removing the *NUC1* from the yeast genome had little impact on the cell response to acetic acid when compared with WT in glycerol-containing medium, that is, the lack of *NUC1* does not protect against apoptosis under these conditions. In contrast, in a medium supplemented with glucose, it appeared to promote apoptosis. Notably, even slight overproduction of Nuc1, assured by expression from centromeric plasmid, provided protection to cells grown in both types of medium. Furthermore, when the *NUC1_Lm*-expressing strain was grown in a glucose-containing medium, it exhibited a similar number of apoptotic cells to the WT strain and significantly fewer apoptotic cells than that of the *nuc1Δ* strain. These observations are consistent with the results of the drop assay (shown in supplementary figs. S2 and S3, Supplementary Material online). These findings suggest that Nuc1 might have an antiapoptotic role and expression of the Leishmanian ortholog was able to reverse it successfully.

Taken together, these results suggested that the remotely related orthologs of studied yeast apoptotic genes complemented the apoptotic function in yeast.

Mitochondrial ROS Production Assay

It has been shown that apoptotic programmed cell death occurring in yeast cells exposed to acetic acid may also involve mitochondrial reactive oxygen species (ROS) accumulation associated with a decrease in mitochondrial transmembrane potential and oxygen consumption, underscoring the participation of mitochondria in acetic acid-induced programed cell death (Madeo et al. 1999). Thus, we checked the level of mitochondrial superoxide radical generation in the studied strains treated with acetic acid by employing MitoSOX™ Red fluorescent dye. An increased apoptosis level should be accompanied by an increased level of mitochondria-derived ROS, such as mitochondrial superoxide, a primary oxygen free radical. Different ROS production levels were detected in the analyzed strains after acetic acid treatment. The assay results showed two different types of cellular reactions to the acetic acid stress regarding the mitochondrial superoxide level: (a) the change in the ROS levels per cell visible as a histogram shift (toward the right side of the graph for the cells with elevated ROS or left side for the cells with decreased ROS levels) and (b) the change in the frequency of cells among strains with the same ROS levels as the strain to compare, visible as higher/lower peak on the graph. Consistent with previous studies (Yi et al. 2018), control strains *rho0* and *prx (5Δ)* showed the expected histogram shift as well as an increase or decrease, in the P2 percentage, that is, percentage of ROS^+ cells (fig. 5*A, B*). Our data exhibited a decrease in ROS^+ cells in the *mca1Δ* ($P = 0.006$), *nma111Δ* ($P = 0.02$), *ndi1Δ* ($P = 0.008$), and *nuc1Δ* strains, albeit at different levels. The low level of mitochondrial ROS was rescued by MCA1_At compared with *mca1Δ* ($P = 0.001$) as well as with the WT, while MCA1_Bc showed a higher ROS^+ cell percentage compared with *mca1Δ* but lower ROS^+ cell percentage compared with the WT. Similarly, NMA111_Ra showed a similar P2 percentage compared with the WT but an increased P2 percentage compared with *nma111Δ*. In contrast, both NMA111_At and NMA111_Da showed a low ROS^+ cell percentage compared with *nma111Δ* and WT. Notably, the low ROS levels resulting from the deletion of *NDI1* were increased but not significantly by its three orthologs. We observed a lower P2 percentage for both NUC1_Hs ($P = 0.003$) and NUC1_Lm strains than for *nuc1Δ* and WT (fig. 5*A-V*). The rescue of ROS levels by the abovementioned orthologs further confirms the functional complementation of the yeast apoptotic genes under study.

Discussion

There are different hypotheses of apoptosis origin and evolution. In this study, we tested the endosymbiotic hypothesis of apoptosis origin.

In a pioneering paper, Kroemer suggested that the interaction between the protomitochondrion and its initial host was analogous to the observed interaction between plasmids and bacteria (Kroemer 1997). According to this model, protomitochondria produce both toxins (apoptotic factors) and antitoxins (antiapoptotic factors), as seen in extant plasmids. The antitoxins were unstable and played the role of "addiction molecules," much like the addiction molecules of extant phages. As a result, a protoeukaryotic ancestral host colonized by a protomitochondrion could not survive after losing it. Later in the course of evolution, these toxins were transformed into extant apoptotic factors. Green and Fitzgerald (Green and Fitzgerald 2016) proposed that animal caspase–based apoptosis evolved from ancient primordial immune mechanisms. Their hypothesis is supported by the observation that proteins from the caspase family can elicit an immune response. According to this

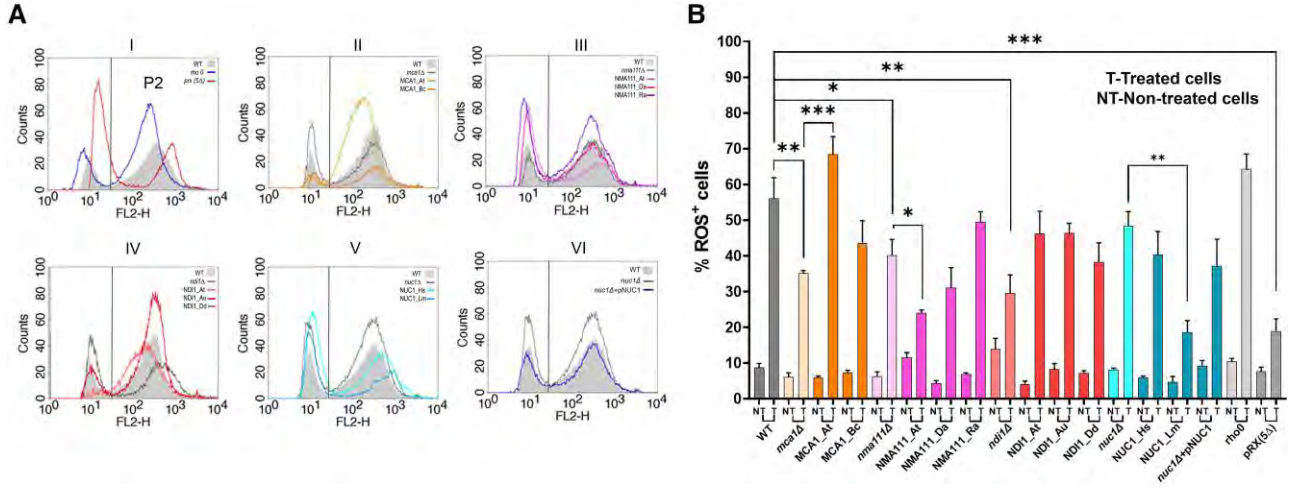

Fig. 5.—Mitochondrial ROS levels for studied yeast strains. The mitochondrial ROS levels were measured by flow cytometry using MitoSOX™ Red after acetic acid treatment. Indicated strain cells were exposed to 200 mM acetic acid for 2 h and incubated with MitoSOX™ Red to assess relative levels of mitochondrial superoxide. The *rho0* strain serves as a negative control, and the *prx (5Δ)* strain serves as a positive control. (*A*) Histograms averaged using CellQuestPro (Becton Dickinson) from at least three biological repetitions are shown. (I) Control assay. (II–V) Mitochondrial superoxide levels in the indicated strains. (VI) Complementation assay for *nuc1Δ* using pNUC1 plasmid. (*B*) Percentage of ROS$^+$ cells in the tested strains before and after acetic acid stress (mean ± SEM, $n = 3$–7). $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ compared with WT or deletion mutant cells (one-way ANOVA).

scenario, apoptotic cell death is one of the immunological mechanisms induced by caspases. A cell dies upon infection by bacteria before the invader can replicate, thereby sparing its clone mates from the subsequent infection. Indeed, caspases (via APAF-1) also bound and recognized bacterial protomitochondrial cytochrome c and interactions between "host" caspases and proteobacterial cytochrome c were transformed into extant apoptosomes (protein complexes that activate programed cell death in animals) during evolution. Another plausible scenario proposed by Gao et al. (Gao et al. 2019) is based on the assumption that some forms of programed cell death (PCD) might have evolved from antivirus defense mechanisms. Their findings shed some light on transitional forms of PCD by suggesting that the primary function of mitochondrial release of Nuc1 (ortholog of human endonuclease G [EndoG]) was protection against viral attenuation and thus perhaps was the function of apoptosis itself. We previously suggested another putative scenario for the evolution of apoptosis (Klim et al. 2018), and we theorized that protoeukaryotes were predators. They did not possess an oxidative respiration system and for this relied on bacterial prey which, in response, produced toxins against hosts. Later, bacteria were domesticated and served as mitochondria for protoeukaryotic cells and various eubacterial toxins were transformed into extant apoptotic factors (e.g., AIFs, caspases, metacaspases, and cytochrome c). We found that apoptotic systems of protoeukaryotes contained several apoptotic factors/protomitochondrial toxins: apoptotic DNases (plant ZEN1, animal NUC1/DNase II, and EndoG), caspase-type proteases, various HTRA/OMI proteases (both fungal and mammalian types), and diverse AIFs. Despite the differences, some of the factors have identical functions, for example, unrelated apoptotic nucleases that degrade DNA due to putative convergent evolution. Such richness of redundant toxins present in the protomitochondrion suggests that "red queen" coevolution may have shaped the protomitochondrion to contain as many toxins as possible (Van Valen 1973). We also noticed that redundant factors were subsequently lost in different lineages, for example, metacaspases in animals. It is worth mentioning that the evolution of the complexity of apoptotic mechanisms has not been restricted to early stages of eukaryotic evolution (Krasovec et al. 2021).

There is a controversy over why apoptotic mechanisms have been maintained after mitochondrial domestication. In various unicellular organisms, apoptosis is a kind of altruistic suicide and its presence could be explained by kin selection (Fabrizio et al. 2004; Herker et al. 2004; Duszenko et al. 2006; Kaczanowski 2016; Durand et al. 2019). However, as such mechanisms are ancient, it is surprising that kin selection could have preserved them for so long. One of the putative mechanisms is an already mentioned "addiction molecule" hypothesis (Kroemer 1997). Another possibility is antagonistic pleiotropy (Williams 2001; Kaczanowski 2016). Apoptotic factors usually have additional, nonapoptotic functions (Kaczanowski 2016). Therefore, cell death in unicellular organisms is often accidental and caused by the nonapoptotic activity of apoptotic factors. In summary, although it has been agreed that apoptosis likely evolved from mitochondrial mechanisms, it is unclear how this process has taken place. It is arguable whether the

mitochondrial apoptotic mechanisms of animals and other eukaryotes evolved independently. Furthermore, it is not clear why mechanisms of programed cell death are maintained in unicellular organisms.

According to our model based on previous phylogenetic studies, eukaryotic apoptosis evolved during mitochondrial domestication, which took place approximately 1,800 Mya (Knoll et al. 2006; Roger and Hug 2006; Parfrey et al. 2011). We concluded that in primordial apoptosis, eubacterial protomitochondrial toxins were likely killing the eukaryotic host. Later, they evolved into extant apoptotic factors: apoptotic proteases, DNases, AIFs, and other factors involved in respiration, such as cytochrome c. In our previous study describing the ancestral state reconstruction of the apoptosis machinery (Klim et al. 2018), we postulated that during the evolution of different systematic groups, some of these ancient apoptotic factors were lost. For example, our analysis suggests that caspases (apoptotic proteases) occur in animals, eubacteria, and some other eukaryotes like foraminifera (*Reticulomyxa filosa*) but not in plants or fungi. According to this analysis, it is likely that caspase-type proteases were present in the last common ancestor of eukaryotes. Animal and foraminifera caspase-type branches are separated, suggesting that a common ancestor (which is the last common ancestor of eukaryotes) contains caspases. Additionally, caspases are absent in plants, fungi, and the majority of protists, suggesting that these proteins were subsequently lost. These analyses were based on phylogenetic analysis of thousands of sequences and manually curated alignment of the small set of sequences, blast homology searches, and analysis of the presence of the InterPro domain (Peptidase C14A, caspase catalytic domain) (Klim et al. 2018).

We asked whether there is functional conservation of apoptotic factors. If that was the case, it would connote that yeast apoptotic factors have been functionally maintained since mitochondrial domestication. To answer the above question, we applied a complementation test using orthologs of yeast apoptotic genes. A similar approach has been successfully used to test the hypothesis that animal apoptotic mechanisms appeared at the beginning of the evolutionary history of this clade (Huang et al. 2000). We selected remotely related orthologs of proteins belonging to the main types of ancient apoptotic factors: AIF factors involved in respiration, proteases, and DNases, predicted by our phylogenetic study mentioned above. The family of AIFs included NDI1. According to our phylogenetic analysis, NDI1 has eubacterial and remotely related eukaryotic orthologs from such systematic groups as "slime molds" (*Dictyostelium*) or green plants (*Viridiplantae*) (Klim et al. 2018). However, it has no animal orthologs, although some older predictions suggested its existence (Park et al. 2007). We found that both eubacterial and eukaryotic orthologs from very diverse systematic groups of flowering plants and slime molds can substitute for the function of yeast Ndi1 during apoptosis induced by acetic acid. The progress made in phylogenetics has revealed that the likely branches of extant fungi and flowering plants diverged very early in the process of eukaryotic evolution, just after mitochondrial domestication (Roger and Hug 2006; Adl et al. 2012). Therefore, the apoptotic function of this factor is evolutionarily conserved, at least from that time. Interestingly, orthologs of Ndi1 could not restore the ability of *ndi1Δ* yeast to grow on glycerol (supplementary fig. S7, Supplementary Material online). This observation indicates that the evolutionarily conserved function of NDI1 in apoptosis is likely independent of its "respiratory" function.

We tested strains bearing orthologs of two apoptotic proteases: metacaspase Mca1 and serine protease Nma111. Metacaspase is the main apoptotic protease of nonanimal eukaryotes. It is a counterpart but not an ortholog of caspase. Previous studies revealed that orthologs from flowering plants and *Trypanosoma* can substitute for the apoptotic function of this protein in yeast (Szallies et al. 2002; Watanabe and Lam 2005). In the case of *A. thaliana* metacaspase, we reproduced this result. In contrast, bacterial orthologs from *B. cereus* only partially substitute for yeast metacaspase. It is tempting to speculate that *Bacillus* orthologs have different proteolytic specificities. Nma111 is a nuclear apoptotic protease that has mammalian counterpart proteases OMI/HTRA and whose apoptotic function is well described (Suzuki et al. 2001). Our phylogenetic analysis revealed that animal and fungal branches of NMA111 proteases most likely diverged before mitochondrial domestication, and therefore, HTRA proteases are not true orthologs of Nma111 (Klim et al. 2018). Additionally, both Nma111 and HTRA have eubacterial and plant orthologs and there are no archaeal orthologs. Eubacterial orthologs from *Desulfatibacillum* and *Rickettsia* are able to substitute for the yeast Nma111 function in apoptosis. This finding is particularly significant, as it includes the complementation by *R. australis*, which belongs to the diverse and ancient group of bacteria known as Alphaproteobacteria that includes the mitochondrial lineage (Emelyanov 2001). This finding further supports our hypothesis that eubacterial factors acquired new apoptotic functions during the process of mitochondrial domestication, contributing to the evolution of apoptotic machinery.

As a representative DNase, we chose Nuc1 (mitochondrial nuclease, ortholog of human EndoG). The apoptotic function of endonuclease G has been demonstrated in organisms belonging to two ancient eukaryotic phylogenetic branches, the branch of Opisthokonta comprising yeast and animals and the parasitic protist *Trypanosomes* belonging to the group Excavata (Kaczanowski 2016). In humans, endonuclease G is involved in the pathological induction of apoptosis in Parkinson's disease. This mechanism was discovered using yeast as a model for the process (Büttner

et al. 2013). Under different conditions, Nuc1 stimulates or suppresses cell death (Büttner et al. 2007). In our experiments, the deletion of *NUC1* has only a very little impact and it may promote apoptosis in glucose-containing medium but does not provide protection against apoptosis in glycerol-containing medium. Expression of human and Leishmanian orthologs to some extent substitutes for the antiapoptotic activity of Nuc1.

According to the literature, ROS accumulation and the involvement of mitochondria, where this usually occurs, are considered to be the most unifying features shared by apoptotic yeast cells exposed to various exo- and endogenous proapoptotic stimuli (Farrugia and Balzan 2012). Thus, we included this phenotype in our study. Oxidative stress can trigger a range of responses in yeast cells that lead to either their survival or their death. We followed the accumulation of mitochondrial ROS in response to acetic acid–induced oxidative stress. A lack of Mca1, Nma111, and Ndi1 proteins leads to a decrease in the number of ROS^+ cells, and complementation by orthologs (except *NMA111_At* and *NMA111_Da*) leads to an increase. Defects in respiratory complex I (orthologous to yeast Ndi1) have been linked to ROS overproduction, and expression of the yeast Ndi1 enzyme has been shown to be able to suppress the rate of ROS formation (Seo et al. 2006). This observation shows that the impact of mitochondrial ROS accumulation is not always proapoptotic, but in fact, ROS accumulation in living cells is a complex and multifactorial phenomenon (Leadsham et al. 2013; Yi et al. 2018).

In summary, our results indicate that the apoptotic function of different apoptotic factors has been evolutionarily conserved since the divergence of studied systematic groups which probably took place before or during the origin of eukaryotes. The extent of yeast apoptosis–associated gene replaceability suggests that the ancestral functions of selected orthologs have remained nearly intact, at least in terms of their primordial functionality. Even some eubacterial orthologs of apoptotic factors can substitute for the original yeast proteins, which suggests that orthologous apoptotic factors may confer functions across deep evolutionary distances. This observation supports the hypothesis that apoptotic machinery evolved due to the cooption of eubacterial factors, which gained new apoptotic functions during mitochondrial domestication. According to this hypothesis, protomitochondria used to kill their eukaryotic host and apoptotic factors are protomitochondrial proteins that gained a new apoptotic function. Later, apoptotic factors evolved from such toxins. It is worth mentioning that there are known cases of cooption of bacterial factors to the activities that lead to cell death by inducing apoptosis (e.g., bacterial proteases (Rust et al. 2016) and DNases (Faïs et al. 2016)). In conclusion, our data support the hypothesis that apoptotic factors first played the role of toxins used by the prey (protomitochondrion) against predators (protoeukaryotes) and later evolved into extant apoptotic factors.

Materials and Methods

Below, we provide a brief description of the methods applied in the study. Detailed Materials and Methods of the experiments and analyses are described in SI Appendix. This includes tables with listed strains and plasmids, description of growth conditions, strain generation, cloning procedures, western blots, microscopy, semiquantitative drop assay, Annexin V apoptosis assay, and determination of ROS production.

Since the present study considers proteins from diverse clades, the short-form abbreviations of protein nomenclature following the Pfam database style are used throughout the text, except for the yeast experiment section. Standard genetic nomenclature is used to designate *S. cerevisiae* strains.

Yeast Strains

All *S. cerevisiae* strains used for the study were derived from BY4741, and their genotypes are listed in supplementary table S1, Supplementary Material online.

In Silico Designing of Yeast Apoptotic Gene Orthologs

The putative promoter and terminator sequences and nuclear/mitochondrial localization signals of proteins encoded by yeast genes under study were annotated. The DNA sequences encoding orthologs of yeast genes (ORFs) were taken from either NCBI or their respective databases. The amino acid sequences of orthologs were obtained from UniProt. The annotated signal sequences of the ortholog protein sequence were identified and substituted by respective sequences of yeast homologs. Then, amino acids were codon optimized for yeast translation and the gene sequence thus obtained was used for the synthesis of DNA encoding orthologs of respective yeast ORF. Next, the terminator and *kanMX6* selection cassette were introduced before the native terminator region of the respective yeast gene.

Plasmid Construction

The details and general schematics of the construction of plasmids bearing orthologs are illustrated in supplementary figure S1, Supplementary Material online. Plasmids derived and constructed by us or from other sources using standard methods are listed in supplementary tables S2 and S3, Supplementary Material online.

Yeast Strain Construction

Strains with orthologs were constructed by gene replacement in diploid strain BY4743. Yeast cells were transformed

by the standard lithium acetate method, and transformants were selected on SD uracil dropout or YPD_{G418} media. Primers used in this study are listed in supplementary table S4, Supplementary Material online.

Microscopy

Cells were viewed under 1000× magnification with a fluorescence microscope and appropriate filter set. Images were captured, processed, and enhanced identically with Fiji software.

Western Blot Analysis

To visualize chimeric GFP-tagged proteins on western blots, protein extracts were prepared. Samples were separated and transferred onto PVDF membranes. The primary antibodies used were anti-GFP (1:5,000) and anti-actin monoclonal antibody (1:10,000). Secondary goat anti-mouse IgG horseradish peroxidase (HRP)–conjugated antibodies (1:10,000) were used to recognize the bound primary antibodies. The signal was detected by enhanced chemiluminescence.

Semiquantitative Drop Assay

The cultures were grown to an exponential phase and subjected to acetic acid (200 mM) or hydrogen peroxide (2 mM) stress for 120 or 60 min at 28 °C. Samples were taken at different time points, and 10-fold serially diluted cell suspensions were spotted on YPD plates. The plates were photographed after 48 h incubation.

Survival Assay

The cultures were grown to an exponential phase and adjusted to the same density. Cells were then treated with 2 mM H_2O_2 or 200 mM acetic acid and incubated at 28 °C, 150 rpm. Samples were taken at different time points, and 10-fold serially diluted cell suspensions were plated on YPD agar. After 72 h at 28 °C, the number of colony forming units (CFU) on each plate was counted and analyzed.

Annexin V Apoptosis Assay

Yeast cell cultures grown to an early exponential phase were divided into two portions, from which the first served as control and to the second acetic acid which was added to the final concentration of 200 mM. Cells were cultivated for an additional 120 min to induce apoptosis. The 10^8 cells from each culture were centrifuged, rinsed with water, centrifuged again, and suspended in the appropriate buffer. The pellet was suspended and treated with Zymolyase 100T to achieve spheroplasts. The 50-µl aliquots of the spheroplast suspension were taken for staining. Analysis was performed using the FL2 channel for propidium iodide and FL1 for Annexin V labeled with FITC. For each tested strain, at least three biological repetitions were made.

ROS Level Measurements

The mitochondrial superoxide radical (ROS) level was assessed by flow cytometry by employing a mitochondria-selective probe, MitoSOX™. Both cells from nontreated as well as acetic acid–treated (200 mM, 2 h) samples, were processed and preloaded with 2 µM dye for 25 min at room temperature in the dark. Afterwards, cells (20,000 per sample) were subjected to analysis using FACS Calibur (Becton Dickinson) at the FL2 channel. The level of mitochondrial ROS before and after acetic acid treatment was calculated by subtraction of ROS levels in nonstained cells. The rate of the ROS level change in analyzed strains versus WT was calculated for averages from all biological repetitions.

Statistical Analysis

Data are expressed as means ± SEM of at least three independent experiments. Statistical analysis between samples was performed by one-way ANOVA test in GraphPad Prism 8.0. Prior to analysis, the data were assessed for normal distribution using the Shapiro–Wilk test.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Author Contributions

U.Z. and S.K. designed the research; V.K. and J.K. performed research experiments; V.K., J.K., A.S., and T.E. performed Annexin V/PI experiments; V.K., A.S., J.K., U.Z., and S.K. analyzed the data; V.K., J.K., A.S., A.K., U.Z., and S.K. wrote the paper.

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

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