Apoptosis (2010) 15:257–268
DOI 10.1007/s10495-009-0453-4

Necrosis in yeast

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Published online: 13 January 2010

Abstract Necrosis was long regarded as an accidental cell death process resulting from overwhelming cellular injury such as chemical or physical disruption of the plasma membrane. Such a definition, however, proved to be inapplicable to many necrotic scenarios. The discovery that genetic manipulation of several proteins either protected or enhanced necrotic cell death argued in favor of a regulated and hence programmed process, as it is the case for apoptosis. For more than a decade, yeast has served as a model for apoptosis research; recently, evidence accumulated that it also harbors a necrotic program. Here, we summarize the current knowledge about factors that control necrotic cell death in yeast. Mitochondria, aging and a low pH are positive regulators of this process while cellular polyamines (e.g. spermidine) and endonuclease G as well as homeostatic organelles like the vacuole or peroxisomes are potent inhibitors of necrosis. Physiological necrosis may stimulate intercellular signaling via the release of necrotic factors that promote viability of healthy cells and, thus, assure survival of the clone. Together, the data obtained in yeast argue for the existence of a necrotic program, which controls longevity and whose physiological function may thus be aging.

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Keywords Necrosis · Yeast programmed cell death · Yeast PCD · Programmed necrosis · Mitochondria · Spermidine · Aging

Introduction

Programmed cell death (PCD) defines all modes of death, whose execution is carried out in a regulated manner and is hence under molecular control. This requires, on the one hand, that the process follows a specific, orchestrated choreography, in which one event is triggered by another (like, for instance, during the caspase cascade). On the other hand, such a process should implicate an advantage for the particular organism or cell population. In this sense, particularly apoptosis has been classified as PCD. However, cell death, as it occurs during physiological development or during normal immune response, can manifest non-apoptotic features [1–3], thus illustrating that other types of death are, indeed, involved in a cell's programmed demise.

Necrosis was first described in detail by Walker et al. in 1988 [4] and has been regarded for a long time as a rather accidental, uncontrolled form of cell death. Necrotic cell death is morphologically characterized by an increase in cell volume (oncosis), swelling of organelles and plasma membrane rupture followed by loss of intracellular contents. Additionally, it shows bioenergetic impairment, random DNA degradation and leads to the release of factors involved in immune response stimulation or active wound repair [5]. In contrast, during apoptosis, which is an organized energy-dependent process, the plasma membrane integrity is maintained, intracellular content (like DNA) undergoes ordered degradation, and exposure or secretion of diverse factors promotes phagocytic cell

elimination or acts as an immuno-suppressant. However, mounting evidence suggests that a molecular regulation may underlie the execution of necrotic cell death through a series of signal transduction pathways and catabolic mechanisms [6–8]. For instance, calpains, cathepsins and cyclophilin D are often needed for necrotic cell death execution [6, 7, 9] and a crucial role for the serine/threonine kinases RIP1 and RIP3 has been demonstrated [10]. Moreover, using *C. elegans* as a model system, we demonstrated that the process of autophagy and proper lysosomal function can contribute to necrotic cell death in degenerating neurons [11, 12].

Necrosis is prominent in ischemia, trauma and possibly some forms of neurodegeneration [6, 9, 13, 14] as well as during various cardiovascular disorders [15]. In addition, some viruses, bacteria and protozoa can induce necrosis [16]. Thus, further biochemical understanding of the basal mechanisms that govern necrotic cell death in general is imperative to develop new strategies for therapeutic intervention of these disorders or pathological conditions [17, 18].

In recent years, yeast has been established as a powerful model to study the mechanisms and phylogenetically conserved pathways of PCD, in particular apoptosis. First reported in 1997, yeast exhibits typical morphological and biochemical hallmarks of metazoan apoptosis upon apoptotic stimuli, such as mutation of *CDC48* or treatment with hydrogen peroxide [19, 20]. A variety of other inducers of yeast apoptosis have since been identified and recently reviewed [21–23]. Among others, yeast homologs of the apoptosis-inducing factor [24], HtrA2/Omi [25], endonuclease G [26] or of mammalian caspases [27] have been functionally characterized as executors of yeast apoptosis. Combining the relatively easy genetics and low costs with powerful assays that determine the precise survival rates of a yeast culture (clonogenic survival) as well as distinct morphological and biochemical characteristics of dying cells on a single cell level, yeast has been successfully used to identify complex scenarios of apoptosis. These include detailed cascades of mitochondrial changes [28–30], epigenetic cell death regulation [31–33] or aging [34–36]. Aging belongs, in fact, to a series of other physiological scenarios (e.g. mating failure, colony differentiation), which culminate in yeast apoptosis, thus suggesting a teleological explanation for the existence of such a process in a unicellular organism [37].

Though most studies in the field of yeast PCD have addressed the process of apoptosis, it is increasingly recognized that necrosis as a primary cause of cell death also exists in yeast. Importantly, evidence is accumulating that necrotic cell death does not only follow after brutal chemical or physical torture but also occurs under normal physiological conditions and reasonable concentrations of cell death inducing substances. In this review we summarize, for the first time, the accumulating studies that recognize regulated necrotic cell death in yeast and give an overview about the available markers and means to discriminate necrotic from other types of cell death.

Scenarios of yeast necrosis

Agents capable of inducing a necrotic phenotype in yeast

A variety of agents capable of inducing yeast necrosis have been described. Several of them are well-known cell death inducers, like H_2O_2 or acetic acid, which trigger apoptosis when applied at low doses, but shift cell death to a rather necrotic phenotype when used at higher concentrations [20, 38]. This also applies to copper and manganese [39], the antifungal agent amphotericin B [40] and possibly also pheromones [41]. Hence, a concentration dependent switch between apoptosis and necrosis might be a common aspect of cell death inducing agents [42]. However, merely increasing the dosage of typical apoptotic triggers may, in most cases, stimulate an accidental, non-regulated type of necrotic death, likely resulting from radical damage of cellular structures and integrity. In the case of acetic acid, however, it is also feasible that increasing concentrations interfere with intracellular pH homeostasis in a regulated way that might resemble the detrimental effects of vacuolar dysfunction (see chapter below on vacuolar functions and necrosis).

Alongside conditions deriving in a possibly non-regulated type of necrosis, scenarios in which necrotic cell death rather follows a molecular program, may also exist in yeast (Fig. 1). Tunicamycin, a known ER-stress causing agent, was shown to induce cell death in *S. cerevisiae* with excessive reactive oxygen species (ROS) production at elevated temperature on synthetic complete medium [43] or when cultured in full (YPD) medium [44]. The sensitivity towards tunicamycin was strongly enhanced in calcineurin-deficient mutants or by the calcineurin inhibitor FK506. Under these "calcineurin-less" conditions tunicamycin caused massive cell death accompanied by the loss of membrane integrity, while at the same time lacking apoptotic DNA fragmentation (as assessed by the TUNEL assay) [44]. Therefore, it was concluded that tunicamycin induced a non-apoptotic, possibly necrotic death of yeast cells. The human heat shock protein Hsp90 is a cytosolic chaperone for many kinases including RIP1 [45], which serves a pro-necrotic signaling function in mammalian cells. Here, necrosis induction by Fas- and tumor-necrosis factor receptor 1 can be circumvented by the Hsp90 inhibitors geldanamycin and radiciol [46]. Interestingly,

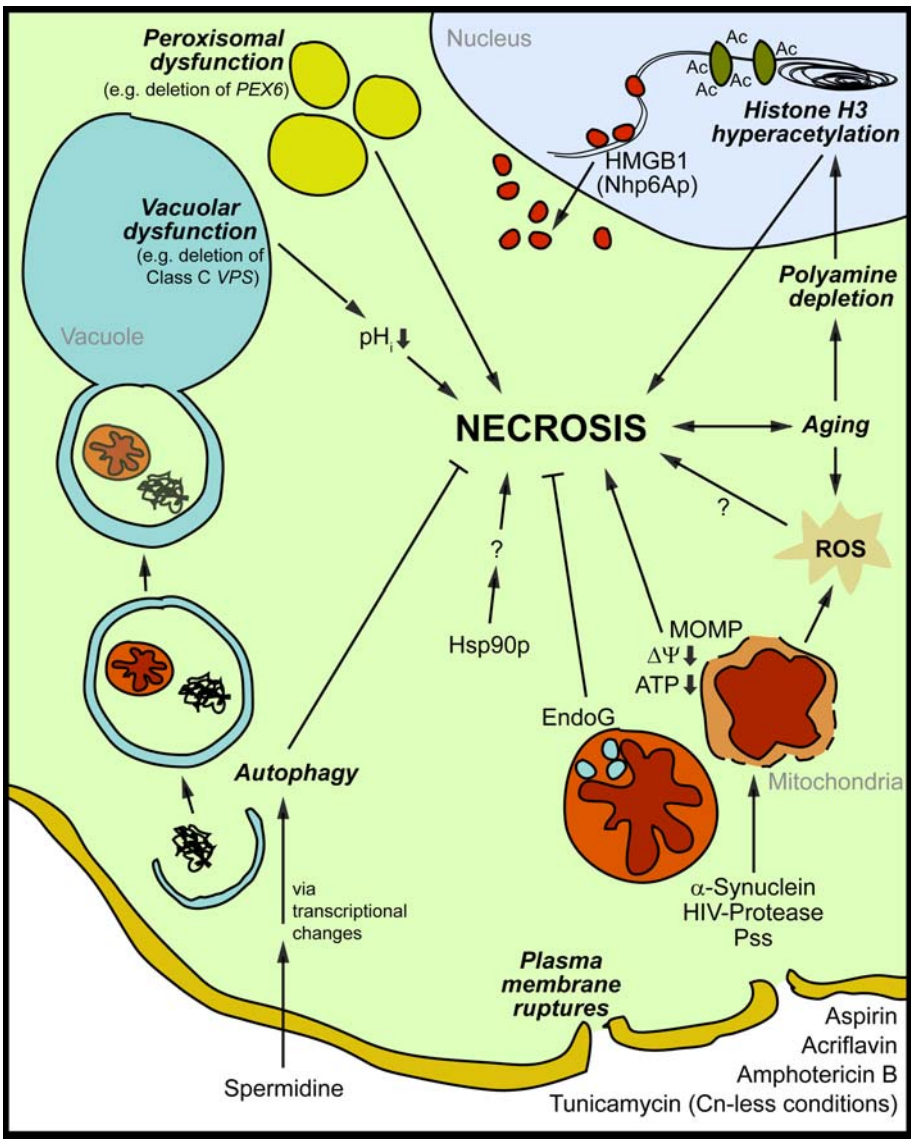

Fig. 1 Schematic view of stimuli and cellular processes that interfere with yeast necrosis. Autophagy as well as proper vacuolar and peroxisomal functions are homeostatic means that counteract necrosis in yeast. Dysfunction of these processes induces defined cellular events (e.g. cytosolic acidification) that can culminate into increased necrotic death accompanied by typical morphological and cell biological changes. These include plasma membrane ruptures, mitochondrial outer membrane permeabilization (MOMP), dissipation of mitochondrial potential ($\Delta\psi\downarrow$), ATP depletion (ATP$\downarrow$), overproduction of reactive oxygen species (ROS) and nuclear release of high mobility group box-1 (HMGB1) protein. Beside external stimuli (e.g. Acriflavin or Amphotericin B), endogenous triggers of yeast necrosis exist including EndoG depletion, heterologous expression of α-synuclein, HIV-protease and proteinaceous elicitor harpin (Pss). In addition, chronological aging represents a physiological scenario closely interrelated to necrotic cell death, which is regulated by a network of epigenetic histone modifications and intracellular levels of polyamines

yeast Hsp90p has been functionally associated with the regulation of necrosis as well [44]. Massive cell death caused by tunicamycin and simultaneous abrogation of calcineurin function (either via deletion of *CNB1*, the regulatory subunit of calcineurin or by calcineurin inhibition using FK506) could be prevented by additional disruption of Hsp90p function using the inhibitor radicicol [44]. Thus, it is plausible that calcineurin and Hsp90p act with opposing functions in the regulation of controlled necrotic cell death in yeast. However, the lack of a clear homolog of RIP1 in yeast raises the question of the exact molecular function of Hsp90p during yeast necrosis [44]. Other types of yeast serine/threonine kinases such as Slt2p (Mpk1p), having been suspected to be targets of Hsp90p [47, 48], might bear a comparable role to RIP1. It should be noted that controversy exists about the appearance of typical markers of apoptosis and thus about the exact type of cell death induced by tunicamycin. In accordance with earlier observations that ER-stress caused by *wbp1-1* mutation led to apoptotic cell death accompanied by DNA fragmentation (TUNEL positivity) and phosphatidylserine (PS) externalization we have suggested a rather apoptotic phenotype of tunicamycin treatment—at least when cells are cultured at elevated temperatures [43]. It is possible, for example, that cell death shifts to a necrotic phenotype when tunicamycin is applied under calcineurin-less conditions. Whether this discrepancy is attributed to technical problems or variations of critical experimental conditions remains to be elucidated. The use of additional markers such as Annexin V staining to detect PS externalization (possibly the most reliable apoptosis marker) and yeast HMGB1 translocation (see sections below Fig. 2 for

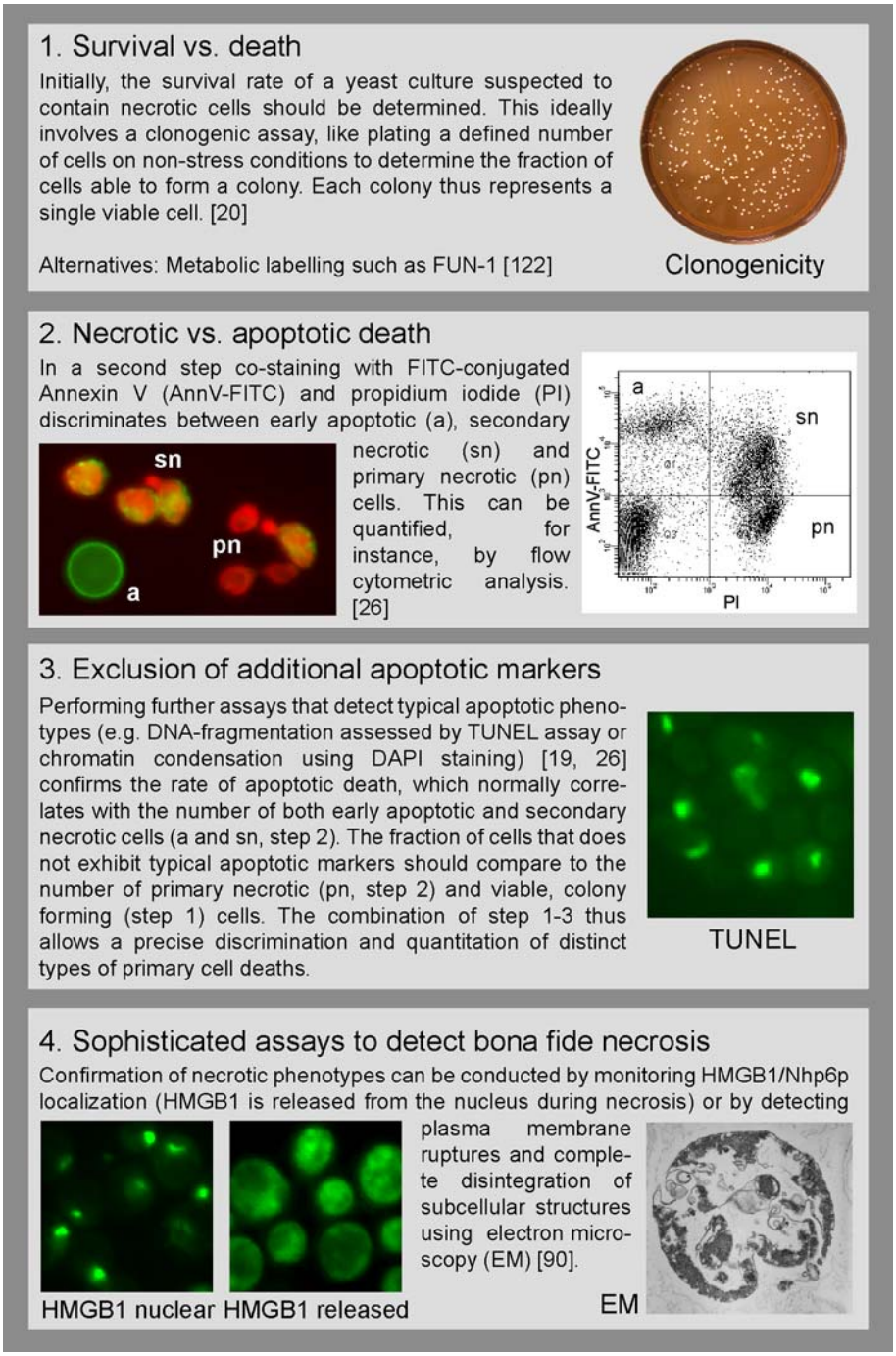

Fig. 2 A strategy to determine primary necrotic cell death in yeast

details) in order to confirm the necrotic phenotype is generally required and might help to resolve this discrepancy.

Furthermore, expression of the human immunodeficiency virus (HIV-1) protease in yeast provoked cell death that resembled a necrotic phenotype, similarly observable in mammalian COS7 cells [49]. HIV-1 protease-induced yeast cell death was accompanied by an increase in plasma membrane permeability with subsequent cell lysis and the release of intracellular content as a result of cell wall breakage. Consistently, also HIV-1 infection of cultured mammalian $CD4^+$ T lymphocytes culminated in necrotic cell death (in addition to apoptosis) [50, 51]. Importantly, the inhibition of HIV-1 protease by specific inhibitors has been discussed as pharmacological intervention in the context of AIDS-associated pathologies [52].

Mitochondria contribute to necrotic cell death in yeast

During the last decade it has become clear that mitochondria play a pivotal role during execution of PCD

pathways. This is particularly true for apoptosis both in mammals [53, 54] and in yeast [28–30] as well as during necrosis of mammalian cells [16, 55–57]. Among others mitochondria-derived ROS [7] and cyclophilin D, a regulatory component of the mitochondrial permeability transition pore (MPTP) [6], have been attributed to necrotic processes.

Several scenarios corroborate the mitochondrial connection to necrotic cell death also in yeast. For instance, acriflavin, an anti-septic, anti-parasitic and fungicide agent, caused both apoptosis and necrosis in the yeast *Candida utilis*, possibly by inducing mitochondrial outer membrane permeabilization and a subsequent collapse of the electrochemical proton gradient and ATP synthesis [58].

Necrotic cell death of *S. cerevisiae* induced by expression of a proteinaceous elicitor harpin (Pss) from *Pseudomonas syringae* [59] occurred with loss of mitochondrial membrane potential but a lack of cytochrome *c* release. Death was avoided in "petite" mutants, which lack functional mitochondrial DNA and thus are unable to respire. In addition, a clear loss of membrane integrity with the absence of nuclear fragmentation or chromosomal condensation was evident [59].

Heterologous expression of human α-synuclein, one of the principle triggers of neurodegeneration during Parkinson's disease (PD), induced both apoptosis and necrosis in an aging yeast model of PD. Cell death with excessive ROS production and loss of membrane integrity occurred in a mitochondria-dependent manner [60]. Aspirin, a known inducer of yeast apoptosis [61], can also trigger primarily necrotic death when cells are deleted in the mitochondrial superoxide dismutase *SOD2* and cultured on glucose as fermentative carbon source [62].

Mitochondria-produced ROS have been associated to yeast apoptosis under numerous conditions [28, 29, 63]. Some studies have addressed a causal role of ROS in the execution of cell death, including apoptotic death after treatment with low doses of hydrogen peroxide [20] or the superoxide-mediated altruistic program of aging [34]. To which extent ROS may also play an executory role during necrotic cell death in yeast remains unknown. Interestingly, many studies have suggested a crucial involvement of ROS during necrosis of mammalian cells [7]. Increased ROS production was shown to occur upon necrotic killing of L929 fibrosarcoma cells by TNFα [64] or during dsRNA-induced necrosis [65]. A possible downstream event of excessive ROS production has been discussed and attributed to the irreversible modification of proteins at lysine and arginine residues by formation of advanced glycation end products (AGEs). These covalent modifications of proteins are increased, for example, during the pathogenesis of diabetes as a result of hyperglycaemia [66]. AGEs themselves can cause additional oxidative stress and induce protein crosslinks within cells and tissues. Thus, they are thought to contribute to several pathophysiological conditions, including tissue damage after ischemia/reperfusion [67] and aging [68]. Future research will have to clarify if ROS can directly contribute, apart from apoptosis, also to necrotic cell death in yeast or if it is rather a bystander that accumulates as a result of cellular demise.

Remarkably, the pro-apoptotic mitochondrial endonuclease G (EndoG) also harbors an anti-necrotic function in yeast. While deletion of yeast EndoG (*NUC1*) diminished apoptotic death, especially when cells were cultured on non-fermentable carbon sources (enhancing mitochondrial mass and respiration), it increased rates of necrosis upon aging or peroxide treatment, which became most prominent when cells were grown on glucose media [26]. Thus, yeast EndoG encompasses a vital (anti-necrotic) as well as a lethal (pro-apoptotic) function. Interestingly, EndoG has also been demonstrated to be required for the survival of tetraploid mammalian and yeast cells, again underlining its cytoprotective function under specific conditions [69]. Dual—vital and lethal—functions are well described for several other crucial regulators of apoptosis, including caspases, cytochrome *c* or the apoptosis inducing factor AIF [70, 71]. The benefit of such dual capacity seems obvious: while activating a pro-death pathway (e.g. apoptosis), concomitant inhibition of crucial vital functions ensures efficient cell death execution. In this sense, it appears reasonable and advantageous that one factor mediates its vital and lethal function via two distinct death pathways (necrotic and apoptotic), as it seems to be the case for Nuc1p.

Vacuolar functions prevent necrotic cell death

Lysosomal functions have been closely connected to necrotic processes. In *C. elegans*, mutations in genes that result in altered lysosomal biogenesis and function markedly affect neuronal necrosis [11, 72]. Moreover, necrosis induction by TNFα or zVAD.fmk both requires the vacuolar V-ATPase Atp6v1g2 in mammalian L929 cells [73]. Importantly, lysosomal cell death pathways initiated by lysosomal membrane permeabilization have been recognized with necrotic morphology and relevance to human cancer [74] or ischemic neuronal death [75–77].

The vacuole (which is the yeast counterpart of lysosomes) plays a crucial role in maintaining cellular homeostasis including the regulation of intracellular pH. Deletion of class C VPS genes (involved in homotypic vacuole fusion, vacuolar protein sorting and thus critical for proper vacuolar function) resulted in a drastically enhanced sensitivity to cell death induced by acetic acid (applied at moderate concentrations that normally induce apoptosis) whereas the sensitivity to peroxide treatment

remained largely unchanged [78]. Phenotypic inspection of cell death markers revealed a shift from apoptotic to a necrotic type of cell death upon deletion of class C VPS genes and treatment with acetic acid. Importantly, the intracellular pH was strongly acidified in VPS mutant cells whereas wild type cells with functional vacuoles were able to sufficiently buffer the cytosolic pH [78]. Cell death induced by high concentrations of acetic acid [38] could similarly be the result of a collapse of intracellular pH homeostasis, again shifting the mode of death from apoptosis to necrosis, even when vacuolar functions are not disrupted. These data obtained from yeast go in line with findings in *C. elegans*, where acidification of the cytosol is reportedly required for necrotic cell death, whereas alkalinisation displays a cytoprotective effect [72]. Thus, disruption of normal vacuolar/lysosomal function derives in necrotic cell death, at least in part due to breakdown of intracellular pH control. Intriguingly, lysosomal proteins (i.e. cathepsins, which need a rather acidic pH to exhibit its proteolytic function) together with non-lysosomal proteases (i.e. calpains) have been directly linked to necrotic cell death execution [79–81]. This rises the possibility that dysfunction of vacuoles/lysosomes (and thus disruption of homeostatic pH control) may trigger necrosis by release of pro-necrotic proteases, which would find an optimal pH for their enzymatic activity in the acidified cytosol.

Peroxisomal function inhibits yeast necrosis

Necrosis is a typical feature of and contributes to inflammation [16, 82]. Interestingly, a significant reduction of peroxisomes and their enzymes is observed during inflammatory processes including infections, ischemia-reperfusion injury, and allograft rejection [83]. Additionally, peroxisomes comprise an important role in ROS homeostasis and oxidative stress [83, 84]. For example, in *PEX5*$^{-/-}$ knockout mice lacking functional peroxisomes severe alterations of mitochondria were observed in various organs accompanied by an increase in oxidative stress [85].

Recently, several studies have addressed the potential interrelation and mechanistic aspects of peroxisomal function and necrosis in yeast. Deletion of *S. cerevisiae PEX6*, encoding a protein crucial for peroxisomal protein import, increased the sensitivity towards both acetic acid stress and stress upon entry into early stationary phase. This was accompanied by excessive ROS production and markers of necrosis [86]. In a similar approach employing the yeast *Hansenula polymorpha*, which grows well on methanol media requiring a peroxide producing peroxisomal oxidase, deletion of the peroxisomal peroxiredoxin Pmp20 resulted in massive cell death by necrosis when cells were cultured on methanol as the sole carbon source [87]. This death was associated with enhanced ROS production and accumulation of lipid peroxidation products causing a marked difference in fatty-acid composition as compared to wild type cells. Cell death was paralleled by the release of peroxisomal matrix proteins into the cytosol [87]. The aforementioned studies suggest that—similar to mitochondrial players—peroxisomal factors might actively contribute to pro-death pathways of necrosis, when released into the cytosol.

Necrosis drives the physiological process of chronological aging in yeast

Convincing evidence for regulated necrosis in yeast came from studies using one of the most physiological scenarios of yeast cell death induction, namely chronological aging [37]. During chronological aging, which determines the viability of yeast during stationary phase modelling the aging of post-mitotic tissues in higher eukaryotes [88], cells eventually die exhibiting biochemical and morphological markers of apoptosis as well as necrosis [34, 35, 89]. Recently, we demonstrated that the fraction of cells dying by necrosis is prominent, suggesting a similar physiological relevance as apoptotic death [90]. At least 50% of dead cells showed a loss of membrane integrity (measured by PI staining) and complete disintegration of subcellular structures (determined by electron transmission microscopy) while at the same time lacking markers of apoptosis such as PS exposure or DNA fragmentation [90] (and our unpublished observations). Additional evidence for *bona fide* necrotic cell death was given by the nuclear release of Nhp6Ap, the yeast homolog of the mammalian high mobility group box-1 protein (HMGB1). Nuclear release of HMGB1 is a defining feature of necrosis in mammalian cells [91]. The nucleo-cytosolic translocation of Nhp6Ap appeared in a large fraction of yeast cells after 14 days of aging [90].

Among the numerous biochemical correlations of aging, a notable decline of natural polyamines (e.g. spermidine) can be observed during aging of various organisms including humans [92, 93] and yeast [90]. In addition, polyamines harbor anti-oxidant and anti-inflammatory activities [94, 95], both (oxidative stress and inflammation) suspected to accelerate the aging process. By externally administrating spermidine or by genetic modulation of polyamine biosynthesis, we demonstrated a causal role of intracellular polyamines on the regulation of necrosis and longevity. Depletion of endogenous polyamines led to early necrotic death, excessive ROS production and a decreased life span in chronologically aging yeast. Conversely, exogenous supply of spermidine counteracted

necrosis, inducing longevity in yeast and in various other organismal and cellular aging models [90].

Spermidine exerts its anti-necrotic function at least partly by induction of autophagy, in this case a clearly cytoprotective process. Consistently, deletion of crucial autophagy genes (e.g. *ATG6* or *ATG7*) delimitated the capacity of spermidine to promote longevity in yeast [90]. Of note, under physiological aging conditions, yeast cells were sensitized for early necrotic (and to some extent also apoptotic) death by deletion of *ATG7*, again demonstrating the cytoprotective function of autophagy under these conditions. Importantly, autophagy was further proven to be crucial for spermidine-mediated life span extension in worms and flies [90]. The mechanisms by which polyamines interrelate with autophagy and necrosis have been elucidated to some extent, providing strong indications for an epigenetic level of regulation, at least in yeast. Whereas polyamine depletion resulted in hyperacetylation of histone H3, spermidine treatment induced global histone H3 deacetylation at all N-terminal lysyl residues (Lys9, 14 and 18), possibly via inhibition of histone acetyltransferases. Intriguingly, the promoter region of *ATG7* was protected from strong deacetylation maintaining its transcriptional activity and suggesting a differential regulation of certain "pro-survival genes" on an epigenetic level [90]. Finally, deletion of two proteins involved in histone acetylation (Iki3p and Sas3p) increased survival during aging by mimicking to some extent the beneficial effects of spermidine application: induction of autophagy, inhibition of necrosis and reduction of oxidative stress [90].

Taken together, these data strongly argue for the existence of yeast necrosis in a highly regulated fashion, mainly for two reasons. First, necrotic cell death occurs during a highly physiological process, namely chronological aging. Second, necrosis can be inhibited (e.g by spermidine treatment or by genetic manipulation of histone acetyltransferase activity) and, hence, appears to be regulated by superior epigenetic processes. However, the detailed cellular events and the proteins that finally execute this type of necrotic cell death certainly still need to be characterized.

Cell biological and morphological markers for the detection of yeast necrosis

Studying necrosis is a young field and thus ill-defined in terms of nomenclature and precise markers. Though a common biochemical denominator of necrotic cell death is still missing [96] we summarize below different cellular features that currently serve as indicators for necrotic death in yeast.

Necrosis is often identified in negative terms by the absence of typical apoptotic markers, in particular when cells undergo early plasma membrane permeabilization [96]. In yeast, at least during chronological aging, the loss of plasma membrane integrity without simultaneous exposure of PS (as determined by co-staining with Annexin V and PI) appears, indeed, to be in good correlation with other markers of necrosis [90] and thus serves as an indicator for *bona fide* necrotic cell death. These additional markers include the nuclear release of yeast HMGB1 (Nhp6Ap) and complete disintegration of subcellular structures [90]. Figure 2 summarizes the available methodologies in more detail and outlines a strategy to determine the different modes of cell death in yeast. Importantly, (i) various ROS sensitive dyes (e.g. DHE, dihydrorhodamine, H2FDA, dihydrofluorescein diacetate, etc.) indicative of the level of oxidative stress and (ii) a clonogenic survival assay that determines the number of viable cells, i.e. which are able to form a colony (by plating a defined number of cells under non-stress conditions) should be used in combination with the purely necrotic assays, but can not serve solely to distinguish between apoptotic and necrotic death.

Necrotic cell death in yeast—secondary or primary?

It could be argued that the fate of an apoptotic yeast cell will inevitably lead into necrosis (loss of plasma membrane integrity) since it is unlikely to be phagocytosed by neighboring yeast cells. Of course, an apoptotic 'dead cell', will eventually suffer from a collapse of metabolism necessarily causing the breakdown of plasma membrane integrity and hence necrotic morphology [97]. This phenomenon is defined as "secondary necrosis" and, in principle, can be the result of various types of cell death including apoptosis. The exact time frame in which this occurs in yeast cells and its detailed characteristics has yet to be determined. First evidence obtained from studies using the pathogenic yeast *Candida albicans* implicate that secondary necrosis occurs very quickly, within 2–3 h after the onset of primary apoptotic death induced by acetic acid treatment [98]. The process of secondary necrosis might still be orchestrated by defined molecular events and thus distinguishable from un-regulated necrosis inflicted by brutal chemical or physical insults.

However, the fact that necrotic markers appear without apoptotic characteristics, strongly suggests an additional type of necrotic cell death, which can be distinguished from secondary necrosis and should be defined as "primary necrosis". In order to detect "primary necrosis" a combination of several markers is essential: as already pointed out above, more than one assay to detect typical necrotic features (for details see Fig. 2) needs to be employed, while the exclusion of markers that indicate apoptotic death (PS

Table 1 Yeast homologs of selected factors suspected to execute or regulate cascades of mammalian necrosis

Mammalian	Necrotic function	References	Yeast[a]	Known function in yeast	References
Cyclophilin D	Regulator of MPTP. Involved in necrosis during ischemia-reperfusion injury	[105, 106]	Cpr3p	Peptidyl-prolyl *cis–trans* isomerase involved in protein refolding after import into mitochondria	[107, 108]
				Suggested to be dispensable for yeast apoptosis induced by acetic acid or H_2O_2	
Calpains	Mediates lysosomal membrane permeabilization upon excessive Ca^{2+} overload leading to CathD release and PLA_2 activation	[76, 109, 110]	Cpl1p	Calpain-like cysteine protease involved in proteolytic activation of Rim101p in response to alkaline pH	[111]
Cathepsins	Lysosomal proteases released upon lysosomal membrane permeabilization and required for certain necrotic scenarios	[6, 76]	Pep4p	Cathepsin D homolog. Vacuolar aspartyl protease (proteinase A) important for turnover of oxidized proteins	[14, 112]
				Shown to be released from vacuole after H_2O_2-induced cell death and to degrade nucleoporins	
Hsp90	Involved in pro-necrotic signaling by stabilization of RIP1	[45, 46]	Hsp90	Cytoplasmic chaperone required for pheromone signaling and negative regulation of transcription factor Hsf1p	[44, 113]
				May regulate a pro-necrotic pathway in yeast opposing to calcineurin function	
RIP1 + RIP3	Serine/threonine kinases involved in pro-necrotic signaling. RIP1 may activate acid SMase	[7, 10]	(n.h.)	Yeast serine/threonine targets of Hsp90 might serve a comparable role to RIP1	[44]
PARP-1	Overactivation of PARP-1 (i.e. after extensive DNA damage) causes depletion of NAD+ and subsequent profound drop of ATP	[114]	(n.h.)	Enzymes with (ADPribosyl)ation activity may exist in yeast	[115]
PARP-2	Required for zVAD.fmk and TNFα-induced necrosis. May have similar function as PARP-1	[73]	(n.h.)	Enzymes with (ADPribosyl)ation activity may exist in yeast	[115]
PKA	Phosphorylates glyoxalase-1, which is required for TNFα induced necrosis in L929 cells	[116]	Tpk1p, Tpk2p, Tpk3p	Isoforms of c-AMP-dependent protein kinase (PKA), the effector kinase of the Ras-cAMP signaling pathway	[102, 117]
				Pro-aging signaling pathway	
Glyoxalase-1	Promotes AGE formation	[116]	Glo1p	Monomeric glyoxalase I, catalyzes the detoxification of methylglyoxal	[118]
Rab25	Ras-related protein involved in zVAD.fmk and TNFα-induced necrosis	[73]	Ypt31p, Ypt32p	GTPase of Rab family involved in exocytosis and intra-Golgi trafficking	[119]
				Rab25 shares considerable homology to other Ras-family yeast proteins including Ras2	
FOXI-1	Forkhead transcription factor required for zVAD.fmk and TNFα-induced necrosis	[73]	Fkh1p, Fkh2p, Hcm1p, Fhl1p	Forkhead transcription factor family proteins involved in cell cycle regulation	[120]
Atp6v1g2	Catalytic subunit of V-ATPase. Required for zVAD.fmk and TNFα-induced necrosis	[73]	Vma10p	G subunit of the yeast V-ATPase involved in vacuolar acidification	[121]

Yeast sequence homologs of selected factors that were shown to be required or to critically modulate necrosis in mammalian cells. Note that a putative involvement of the yeast proteins in necrotic cell death yet remains to be investigated

[a] (n.h.) indicates that no clear homolog based on sequence comparisons exists in yeast

exposure, DNA fragmentation, chromatin condensation, etc.) should simultaneously be controlled.

But what is the physiological sense of a necrotic program in yeast? In higher eukaryotes, necrosis has been postulated, beside its function as a 'backup system' for unsuccessful apoptosis [99, 100], to serve a role in initiating both inflammatory and reparative responses in the host [5, 16]. By activating adaptive immune responses programmed necrosis may therefore contribute to tissue homeostasis and host integrity. A recent study further suggests that programmed necrosis may also be important for proper function of innate immunity, regulating survival of activated primary macrophages [73]. Interestingly, genes required for regulated necrosis of mammalian L929 cells, which were identified in a large scale siRNA screen, are enriched in the nervous and immune system [73], arguing for a physiological function of necrosis in these two compartments. Molecules that are released from necrotic cells and govern the initiation of the immune response include the chromatin bound protein HMGB1 [91]. As discussed above, the nuclear release of HMGB1 also occurs during yeast necrosis, for example under physiological conditions of chronological aging [90]. Although the situation in mammals may not—at first sight—be directly applicable to the yeast system (certainly lacking an immune system), it indicates that the basic molecular characteristics of programmed necrosis are functional and conserved in yeast. In fact, it is tempting to speculate that yeast populations, which do not represent a group of non-communicating individuals but rather behave as an interacting multicellular community [21, 37], might use programmed necrosis as an intercellular signal. For instance, release of necrotic-specific factors (yet to be indentified) may be important for intraclonal signaling and overall cell death regulation to promote survival of the whole clone. This may be especially important during physiological processes that demand the cooperation of all cells within the population. Intriguingly, such an altruistic situation has been described by us for apoptotic death of yeast cells during the course of chronological aging [35]. As discussed above primary and secondary necrotic processes significantly contribute to the age-associated demise of yeast cells. Therefore, it will be interesting to investigate if and to which extent also necrosis is required for the release of pro-survival factors during chronological aging.

Conclusion

Yeast has proven to be a valuable model for understanding conserved pathways of apoptosis and has repeatedly contributed to the understanding of several aspects of mammalian apoptosis [19, 31–33, 69, 101]. Investigation of the basal necrotic machinery in yeast will likely be of similar value and help to understand necrotic processes in human cells and tissues, which are associated with various types of disease or aging as already demonstrated recently [90].

Nevertheless, yeast necrosis research (and in particular that on regulated necrosis) is at the very beginning and important questions need to be directly addressed in the near future. For example, what are the molecular players that execute cellular necrotic events in yeast? Table 1 summarizes yeast homologs of known mammalian mediators of necrosis. However, most of these proteins have not yet been investigated in the context of necrotic cell death in yeast and their possible role in this process needs to be clarified. Notably, protein kinase A (PKA) and the Ras-like Rab protein Rab25, both of which are required for TNF-induced necrosis of mammalian cells, are well conserved in yeast (Table 1). Ras2/PKA signaling belongs to one of the major pro-aging pathways that are conserved in various organisms [102, 103]. This includes the regulation of yeast chronological life span [104] where necrotic cell death is prominent as a physiological and primary cause of cell death [90]. Therefore, it is an intriguing possibility that the Ras2/PKA cascade comprises a conserved pro-necrotic function also in yeast cells under specific conditions. Furthermore, is there a teleological explanation for necrosis in yeast? What is the evolutionary benefit for the yeast clone (in this sense a multicellular population of genetically identical cells) to undergo necrosis compared to apoptosis? Can necrosis be characterized as altruistic death, similarly to what has been demonstrated for apoptosis [34, 35, 37]? Will it include quorum sensing mechanisms, since necrotic cell death leads to leakage of intracellular compounds? Can necrosis serve as a "backup" mechanism as described for mammalian cells? These questions need to be answered as research into this new field goes along. In general terms, understanding the mechanisms of necrosis and its interplay with apoptosis and autophagy in yeast will likely help to solve some important issues that relate to necrosis and the pathogenesis of various human diseases.

Acknowledgments We are grateful to the European Union for grant Apo-Sys (FP7) to F. M. and T. E. and to the Austrian Science Fund (FWF) for grant S-9304-B05 and "Lipotox" to F. M. and D. C.-G. and for grant T-414-B09 to S. B. (Hertha-Firnberg fellowship). N. T. acknowledges funding support by grants from EMBO, the European Research Council (ERC), and the European Commission Framework Programmes 6 and 7.

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