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Guidelines and recommendations on yeast cell death nomenclature

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ABSTRACT Elucidating the biology of yeast in its full complexity has major implications for science, medicine and industry. One of the most critical processes determining yeast life and physiology is cellular demise. However, the investigation of yeast cell death is a relatively young field, and a widely accepted set of concepts and terms is still missing. Here, we propose unified criteria for the definition of accidental, regulated, and programmed forms of cell death in yeast based on a series of morphological and biochemical criteria. Specifically, we provide consensus guidelines on the differential definition of terms including apoptosis, regulated necrosis, and autophagic cell death, as we refer to additional cell death routines that are relevant for the biology of (at least some species of) yeast. As this area of investigation advances rapidly, changes and extensions to this set of recommendations will be implemented in the years to come. Nonetheless, we strongly encourage the authors, reviewers and editors of scientific articles to adopt these collective standards in order to establish an accurate framework for yeast cell death research and, ultimately, to accelerate the progress of this vibrant field of research.

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Abbreviations:

ACD - Accidental cell death, **ADCD** - Autophagy-dependent cell death, **ALP** - Alkaline phosphatase, **ATCD** - Autophagic cell death, **ATG** - Autophagy-related, **CFU** - Colony-forming unit, **DAMP** - Damage-associated molecular pattern, **DAPI** - 4',6-diamidino-2-phenylindole, **DHE** - Dihydroethidium, **DHR123** - Dihydrorhodamine 123, **EM** - electron microscopy, **H2-DCF-DA** - 2,7-dichlorodihydrofluorescein diacetate, **HMGB1** - High mobility group box 1, **IDAI** - Indwelling device-associated infection, **IMS** - Intermembrane mitochondrial space, **KO** - Knockout, **MC** - Mitotic catastrophe, **MOMP** - Mitochondrial outer membrane permeabilization, **MPT** - Mitochondrial permeability transition, **NCCD** - Nomenclature Committee on Cell Death, **OD** - Optical density, **PCD** - Programmed cell death, **PI** - Propidium iodide, **PS** - Phosphatidylserine, **RCD** - Regulated cell death, **ROS** - Reactive oxygen species, **TUNEL** - Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, **$\Delta\psi_m$** - Mitochondrial transmembrane potential.

INTRODUCTION

Yeast, a fungus that predominantly lives as a unicellular organism, has had an extraordinary influence on humanity throughout millennia, from its usage for baking and brewing to the potential of some species to cause life-threatening human diseases. The cultural, industrial, biotechnological, and medical impact of this organism remains unparalleled. The use of yeast fermentation to produce alcoholic beverages and to leaven bread coincided with the rise of ancient civilizations and has persisted until our days. Importantly, the continued development of yeast strains as vehicles for the development of new technology, for example in bioethanol, drug, and enzyme production, as well as the implementation of unconventional yeast species in industrial processes, highlights the ever increasing importance of yeast now and in the future [1, 2]. This is exemplified by the fact that the global market for yeast products is in the multibillion dollar range and is expected to grow further [3]. Beyond the mentioned applications, yeast has a direct impact on human health and disease. Many fungi, including some yeasts, can exist as commensals, i.e., they are part of our natural microbiota, forming the mycobiome [4]. In fact, it is being increasingly recognized that fungi are a major determinant in establishing commensal microbial communities and are thus vital for healthy individuals [5]. However, under certain circumstances, e.g., compromised immunity, commensal fungi may become opportunistic pathogens and as such are a potential cause for infectious diseases [6]. These include superficial infections of the skin and nails (especially by dermatophytes) that affect billions worldwide, biofilm colonisations of mucosal surfaces and more serious invasive infections, which can have very high mortality rates and are estimated to lead to 1.5 million deaths per year [7]. A significant number of these deaths arise from infections caused by the yeasts *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans* in immunocompromised individuals. This socio-economic burden is further amplified by the unprecedented rise in fungal diseases that are affecting plants and animals [8]. These examples highlight the importance of a full understanding of fungal biology, and the study of yeast cell biological processes has been crucial in this respect.

Yeasts have served as a successful research tool for the last century, *Saccharomyces cerevisiae* (the budding yeast) being one of the most thoroughly studied eukaryotes at the cellular and molecular levels. Indeed, yeast continues to be one of the preferred model organisms to explore eukaryotic cell biology, both due to its technical advantages in devising/sophisticating molecular tool kits to study cellular biology, and to a high degree of functional conservation [9]. Also, yeast offers rapid growth and inexpensive accessibility paired with a high amenability to biochemical and genetic manipulation. This enables the establishment of various experimental setups, ranging from single experiments to high-throughput, genome-scale, unbiased screenings in a short time frame. Notably, many insights obtained in yeast have proven to be transferable to higher eukaryotes. Indeed, over the past decades, yeast

studies have unveiled individual gene functions as well as gene and protein interactions, and have instrumentally contributed to the understanding of fundamental cellular processes such as eukaryotic cell cycle control [10–15], autophagy [16–19], mitochondrial function [20, 21], including mitochondrial import [22–25], protein degradation [26], vesicle fusion [27, 28], genetic instability [29, 30], epigenetic control [31, 32], metabolic regulation [33–35], or cellular nutrient sensing [36].

In addition, studies on yeast have shed light on human diseases, providing a cellular platform to examine, for instance, prion biology, virus-host interactions, metabolic diseases, neurodegenerative disorders, cancer, or aging [37–61]. Among the pathophysiologically relevant pathways that can readily be explored in yeast are those governing cellular demise. Indeed, cell death regulation is structurally and functionally conserved in yeast [21, 62–66], and yeast has even served to uncover and establish factors and pathways involved in apoptosis and other controlled cell death subroutines, which have later been corroborated in metazoan or other multicellular systems, e.g., the AAA-ATPase Cdc48/VCP [63, 67], the BAX inhibitor-1 [68], the implication of metacaspases as cell death regulators [69–71], the role of cathepsin D in non-autophagic mitochondrial degradation [72, 73], or the lethal impact of ER-Golgi transport blockage as one of the mechanisms explaining the demise of dopaminergic neurons during Parkinson's disease [74]. To sum up, on the one hand, cell death represents a key process that can be feasibly modeled in yeast. On the other hand, the understanding of yeast cell death and its putative modulation may improve industrial and biotechnological applications, provide insights into mycobiome dynamics, and help develop the fight against fungal and other diseases.

In multicellular organisms, the controlled suicide of single cells is crucial for development and homeostasis, providing a system that eliminates superfluous cells. The presence of such a mechanism also allows for the removal of damaged cells that might compromise organismal fitness. In a single-celled organism like yeast, this paradigm does not seem to apply at first sight, since – in this case – cellular suicide entails the death of the whole organism. However, in a way, a population of yeast cells *de facto* behave as a multicellular entity of communicating individuals rather than a group of isolated cells that do not interact with each other. In fact, a given yeast population originates from a single clone, and the ultimate biological goal of that population is the survival of the genetic information representing that very clone. Thus, under certain circumstances, the death of unfit or damaged yeast cells promotes the survival of the population as a whole. A number of physiological scenarios have been described that corroborate this teleological explanation for a cellular suicide program in yeast, including antagonistic interactions between yeasts, aging, mating, or colony formation [54, 61, 75–85]. Of note, also other unicellular organisms, including bacteria and protozoan parasites, incorporate regulatory processes that are at least partly reminiscent of higher eukaryotic cell death programs [86–91].

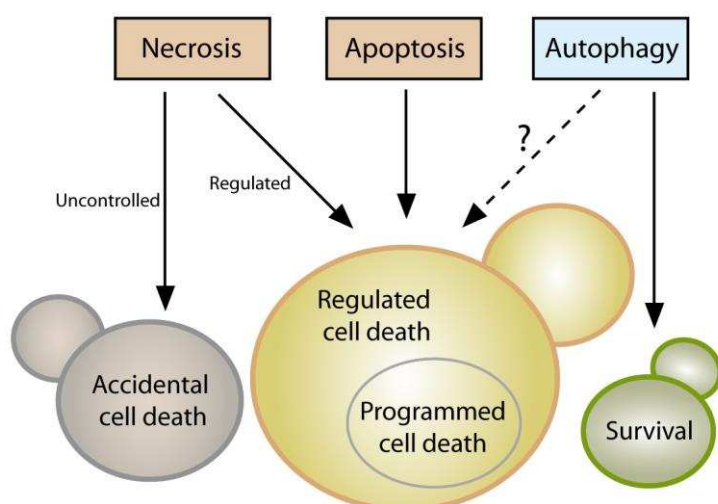


FIGURE 1: Yeast cell death. Yeast cells can die either upon exposure to very harsh microenvironmental conditions via accidental cell death (ACD) or in the context of a failing response to mild stress via regulated cell death (RCD). While ACD invariably manifests with a necrotic morphology (disintegration of cell structure, plasma membrane rupture), RCD can exhibit a spectrum of morphologies and can result from multiple signaling pathways, including regulated necrosis or apoptosis. Programmed cell death (PCD), which occurs in strictly physiological scenarios (e.g., development), represents a specific type of RCD. The possible role of autophagy as a cell death pathway in yeast remains elusive, while its cytoprotective function is well established.

Even though it is now clear that yeast can indeed undergo cellular suicide, the corresponding terminology to describe this multifaceted process remains heterogenous and potentially misleading. Thus, we believe that there is timely need for a more precise and consistent nomenclature that clearly defines the concept of “yeast cell death”, considering morphological, enzymological, and functional aspects. Such standardization seems of importance, given that the field of yeast cell death is continuously expanding with significant progress being made at the phenotypical and mechanistic levels, including the finding that, akin to higher eukaryotes, yeast can also engage in distinct cell death modalities (**Figure 1**). In this paper we thus attempt for the first time to formulate a series of recommendations and caveats with respect to cell death-related results obtained in yeast. To this aim, we have followed the directions of the Nomenclature Committee on Cell Death (NCCD) [92–95] and adapted them to the particularities of *Saccharomyces cerevisiae*, which we think can be extended to other yeast species and to multicellular filamentous fungi. Our goal is to frame a uniform set of guidelines that facilitate the communication among yeast cell death researchers, ultimately supporting and accelerating scientific advance (**Box 1**). In that respect, the nomenclature presented herein will likely need to be revised and updated as the field of yeast cell death moves forward and even more precise definitions are required.

YEAST CELL DEATH AND SURVIVAL

A crucial issue that demands a clear definition is the question of cell death itself. When is a cell dead? According to the NCCD guidelines this is only the case upon irreversible plasma membrane breakdown or complete cellular fragmentation, because only then the cell is factually disintegrated, irrespectively of which upstream pathway or routine has been engaged [93]. In fact, no earlier marker can be defined that reliably determines death in all settings.

Thereby, this lethal irreversibility might start with the collapse of the electrochemical membrane potential across the plasma membrane through formation of a leak. In yeast, the most common method to monitor cell membrane integrity *in vivo* is to use propidium iodide (PI). PI is a fluorescent nucleic acid intercalator that can only enter cells with a ruptured cell membrane, and can be routinely employed in both low and high throughput formats [96–98]. Along similar lines, colorimetric dyes like trypan blue may be used, but are less common [99–101]. Further potential alternatives exist (e.g., 7-aminoactinomycin D), but will need to be thoroughly tested with respect to their suitability for yeast cell death applications in future studies. As mentioned, assessing cell membrane disintegrity is the only technique to quantify actual cell death and must be performed irrespectively of the lethal setting being analyzed. This is imperative, since lethal signaling does not imply that the final stage (cell death) is reached or even that it will be reached at a later stage (see below). In fact, specific subpopulations engaged in lethal pathways that maintain plasma membrane integrity (e.g., early apoptotic cells, see below) are by definition not (yet) dead. In that respect, timecourse experiments are important to monitor both the lethal subroutine-specific phenotypes and the actual occurrence of cell death over time. Of note, indications exist that upon specific stress insults, a small subpopulation of yeast retains the ability to repair cell membrane damage even after having stained positive for PI [102]. Given the lack of other comparably well established dyes in this context and the large body of data supporting PI staining as a valid method to quantify loss of survival, we conclude that determining PI positivity is – at this point – the best technique to quantitatively approach yeast cell death. Still, for the sake of accuracy and waiting for further evidence supporting the above-mentioned indications, we suggest expressing a corresponding quantification as “% PI-positivity” or “% cell death (PI positive)” instead of

BOX 1: DEFINITIONS OF KEY CONCEPTS IN YEAST CELL DEATH

Accidental cell death describes cellular death following exposure to very harsh microenvironmental conditions.

Apoptosis represents a regulated cell death subroutine characterized by specific morphologic and biochemical features and executed via different pro-apoptotic factors; eventually, it culminates in secondary necrosis.

Autophagy defines a predominantly cytoprotective process that orchestrates the digestion of intracellular material (e.g. proteins, organelles) in the vacuole.

Autophagy-dependent cell death describes a lethal subroutine, in which the molecular machinery of autophagy (or parts thereof) causally contributes to cellular demise.

Cell death defines a status of irreversible plasma membrane breakdown (only then, the cell is factually disintegrated, irrespectively of which upstream pathway or routine has been engaged).

Cell viability reflects the ability of a cell to divide and thus to proliferate.

Cell vitality reflects the physiological capabilities of a cell and thus its metabolic activity.

Necrosis is a cell death instance mainly characterized by plasma membrane permeabilization; primary necrosis (cellular necrosis occurring *ab initio*) may take place in an accidental or regulated manner; secondary necrosis (combined necrotic and apoptotic features) is the final stage of the apoptotic process.

Programmed cell death designates a specific type of regulated cell death, which occurs in strictly physiological scenarios (e.g., development, aging).

Regulated cell death describes cellular death occurring in the context of a failing response to internal or external mild stress.

Regulated necrosis is a regulated cell death modality with characteristic features of necrosis that can be inhibited by specific pharmacological or genetic interventions.

“% death” or “% survival” upon using this method. In the long term, the development and establishment of alternative dyes should be explored in order to validate data obtained with PI. A number of approaches allow to experimentally assess (i) cell viability, which reflects the ability of a cell to divide, and (ii) cell vitality, defined as the physiological capabilities of a cell [100]. Nonetheless, an impaired/compromised (i) proliferation or (ii) metabolic capacity does not necessarily result in cellular demise. Thus, these techniques alone cannot be used to demonstrate cell death. Still, they are very useful to complement and corroborate data obtained with PI or alternative dyes.

Assessing clonogenicity with plating assays is the most commonly used method to quantify cell viability [62, 103]. Here, a defined number of cells from a given culture are plated on rich medium agar plates that are further incubated to allow colony formation. The ratio between the resulting colony-forming units (CFUs) and the originally

plated number of cells reflects the viability state in the culture. Theoretically, however, it is possible that under specific conditions (of genetic nature, for instance), colony formation may be blocked in cells that *per se* are still alive (a condition usually referred to as senescence). Additional caveats include the possibility that live cells at the point of plating might die before forming a colony and/or that the plating procedure itself might drive (a fraction of) cells into death, which would be indistinguishable from cell senescence. Nonetheless, the literature suggests clonogenic capacity as a very good correlate to cell death in a plethora of different settings [69, 96, 104, 105] and thus represents a valid approximation to quantify survival in yeast populations. Of note, clonogenicity can also be measured by monitoring CFU formation at the microcolony level (time-lapse photomicroscopy) [106, 107]. Even though cell and colony counting can be automated, clonogenicity assays are rather time-consuming and used for low- to medium-throughput analyses.

A further technique to assess yeast viability follows the growth rate of a given culture, which may decrease as a consequence of increased cell death. For this purpose, an aliquot is inoculated into fresh liquid medium and the growth is monitored, for instance, via photometric measurement of optical densities over a specific period of time [108, 109]. Optionally, spot dilution assays can be performed, where cultures are spotted in serial dilutions on agar plates [110]. Here, the growth ability is compared between cultures at the various dilution steps in a semi-quantitative manner, although automated readout of microcolonies can be used to yield a quantitative result [111]. Monitoring growth can be scaled up and performed either manually or using robotics support, which makes it an attractive technique, especially for screen-based analyses. As with other viability assays, an important disadvantage is that a decreased growth rate can also result from a non-lethal event such as modulation of cell cycle progression or a reduced metabolism due to an alteration in the use of media components.

One possibility to evaluate yeast cell vitality is to directly assess the activity of specific enzymes directly. Although this is not widely employed in yeast cell death research, it represents an avenue to assay the physiological state of a metabolic pathway within the cell [100, 112, 113]. As pointed out below, a caveat of this approach is the possible distortion of results by residual activity in dead cells. A further option is to use vital dyes, like the two-color fluorescent probe FUN-1, which diffuses into cells, irrespectively of their viability status, and results in green fluorescence of the cytoplasm. Dead cells fluoresce green while (live) cells that have both plasma membrane integrity and metabolic capability, can further process the probe, resulting in red vacuolar fluorescence [114, 115]. Similarly, several tetrazolium salts are reduced into colored formazan crystals [116]. Methylene blue is converted to the colorless leucomethylene blue only in metabolically active cells [117], while the red dye phloxine B is only retained in metabolically inactive cells that are unable to actively export it [100, 118]. Other methods aim at assessing further aspects of

cellular physiology, including the cellular ATP content (e.g., based on the luciferin-luciferase reaction) [119] or mitochondrial transmembrane potential (e.g., upon staining with rhodamine 123, JC-1, TMRM/E, DiOC₆(3)) [120, 121]. It should be noted that the readout of metabolic signatures has considerably improved with new generation extracellular flux analyzers, offering the possibility to simultaneously measure mitochondrial respiration and glycolysis (and thus mitochondrial function). A drawback of metabolic assays resides in the fact that cells may be able to maintain some metabolic activities until cell membrane rupture occurs, and that some rely on specific metabolic processes such as oxidative phosphorylation that are not mandatory for cell survival. Thus, such techniques may fail to detect subpopulations of dead (or alive) cells, reflecting the notion that a decrease in growth or metabolic activity (i.e., viability or

vitality) cannot be placed on a par with an increase in cell death. In conclusion, as mentioned above, the term cell death should be used only upon observing breakdown of the plasma membrane and thus loss of cell integrity (e.g., upon PI staining). In addition, we suggest to strengthen this observation by simultaneously assessing clonogenic capacity (**Figure 2**), since (i) it represents the best-established output to accurately monitor overall cellular viability and (ii) it empirically correlates very strongly with actual cell death markers. Importantly, both methods are easy, quick and relatively inexpensive. The use of additional dyes/stainings/assays provides valuable complementary information, but cannot be used alone to unequivocally define a cell as dead.

Yeast cell death is often accompanied by oxidative damage and thus, a widely employed method in the field is

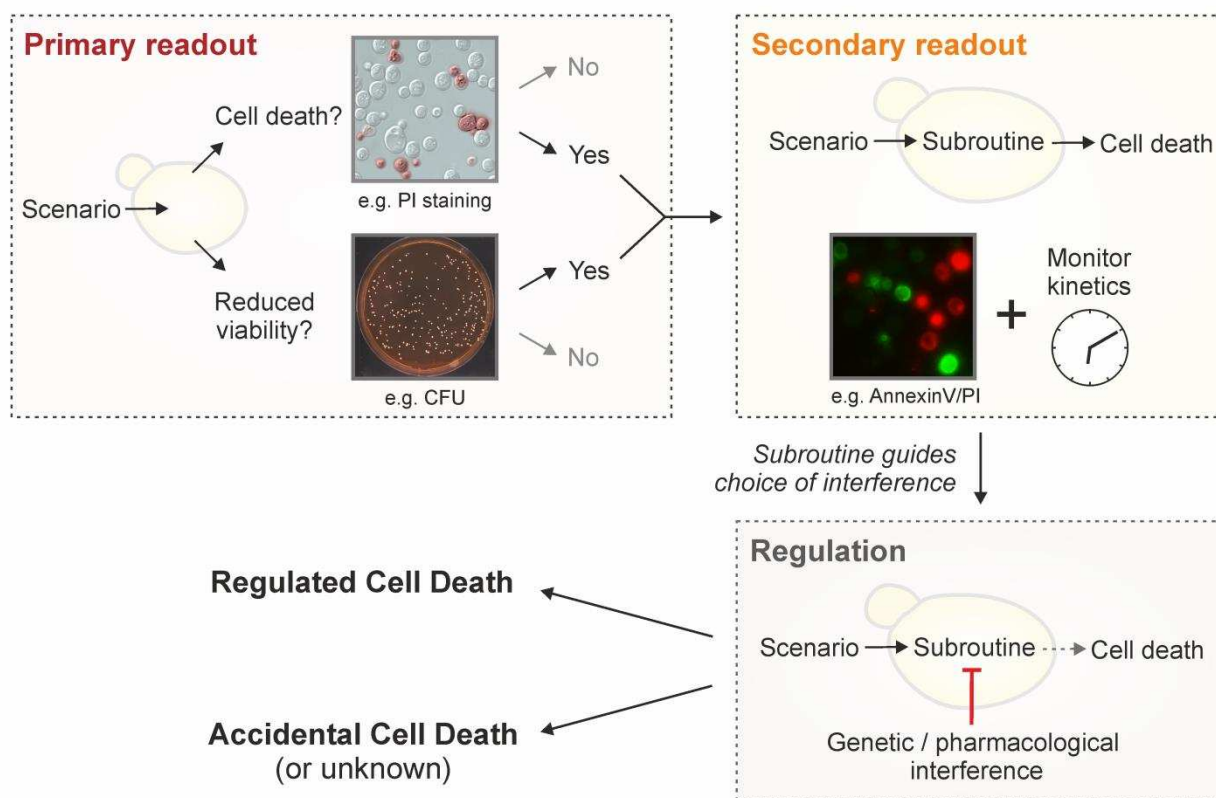


FIGURE 2: Strategy to characterize yeast cell death. To define a lethal scenario in yeast, we recommend to sequentially evaluate the following three levels. (i) The occurrence of cell death should be assessed by monitoring loss of plasma membrane integrity (e.g., by staining with exclusion dyes such as propidium iodide, PI). We suggest to complement this assessment by determining viability with clonogenic tests, knowing that, in many scenarios, clonogenic capacity correlates exceptionally well with cell survival. Other viability and vitality assays may be performed to corroborate the results obtained, but do not replace these two assays. (ii) If cell death is demonstrated, the possible RCD subroutine(s) should be examined via morphological and biochemical observations. While necrotic and autophagic phenotypes demand a further clarification (inhibition studies) to conclude whether the observations correspond to an RCD modality (regulated necrosis, autophagic cell death), ACD (accidental necrosis), or a cell death correlate (protective autophagy), an apoptotic phenotype directly indicates RCD (via apoptosis). Irrespectively, it is imperative to follow the scenario over time (kinetics). (iii) Regulation *per se* and/or assessment of the regulatory network should be tackled by means of genetic and/or pharmacological interventions. Importantly, these interventions should inhibit or shift cell death and the observed subroutine-specific phenotypes to conclude on the involvement of an RCD modality (for regulated necrosis, autophagic cell death) and/or to provide mechanistic insight (all RCD types).

the detection of reactive oxygen species (ROS) [122]. Indeed, a number of different ROS, like the superoxide anion, hydroxyl radical, and hydrogen peroxide, can accumulate upon mitochondrial disturbances, ER stress or other cellular derangements [96, 122–125]. ROS can generally be detected using membrane-permeable dyes that are oxidized to fluorescent products in a ROS-dependent manner. Importantly, these stains do not measure ROS as a group, but rather react with specific species. For instance, dihydroethidium (DHE) preferentially reacts with superoxides, while dihydrorhodamine 123 (DHR123) and 2,7-dichlorodihydrofluorescein diacetate (H₂-DCF-DA) are converted by a broad range of other ROS, but only poorly by superoxides [126]. Such specificities should be taken into account when measuring ROS with a particular stain, since distinct lethal triggers might result in the production of a differential ROS subset [123]. Thus, we recommend to specifically indicate the ROS subtype that is being monitored instead of generally referring to ROS production. Of note, to a certain degree, DHE may also be oxidized unspecifically (independently of superoxide). In order to exploit the full potential of DHE as a superoxide-specific dye, a range of methodological possibilities (e.g., the use of optimized spectra) exist [127, 128]. The standardization of such refinements for DHE assays, which are a preferred tool in yeast cell death research, should be addressed in the future. While ROS measurements allow for high-throughput approaches due to their simplicity and relatively low cost, it is imperative to realize that this method does not discriminate between living and dead cells, although ROS usually precede and are often causative for cell death in yeast [125]. In fact, ROS play a crucial role in intracellular signaling [129–132], functioning, for instance, as direct and indirect regulators of diverse physiologically relevant targets [133–135]. In addition, limited ROS generation might be beneficial under certain conditions, since the resulting adaptive responses can promote stress resistance as a form of preconditioning (hormesis) [131, 136–139]. Thus, an increase in ROS should be regarded as a cell death-correlated phenotype only in connection with assays that directly determine increased plasma membrane disintegration and loss of clonogenicity (see above). Similarly, a decrease in ROS production by incubation with anti-oxidants might support the mechanistic involvement of ROS in the lethal process, but only when cell death is adequately monitored.

ACCIDENTAL VERSUS REGULATED CELL DEATH

Cellular demise in yeast may occur in two mutually exclusive variants: either as an accidental event or through a regulated pathway. Accidental cell death (ACD) occurs upon exposure to severe conditions, resulting in a rapid, uncontrollable and unavoidable form of death. ACD may follow a series of extreme stimuli, including physical conditions, such as very high temperatures or pressures, severe chemical insults like strong detergents and high concentrations of acids or bases as well as mechanical challenges, for instance, vigorous shearing or ultrasonic treatment. The

immediate nature of ACD, which is characterized by a virtually immediate structural breakdown of cells, allows no form of pharmacologic or genetic inhibition. Thus, this form of cell death does not constitute a direct target for modulation or prevention. However, it remains unclear whether yeast cells undergoing ACD may release endogenous, bioactive molecules to the extracellular space [75, 79]. If so, such molecules could interact with local cells that have survived the primary insult and ignite a response within the whole yeast population. Such a consequence of ACD may resemble the release of damage-associated molecular patterns (DAMPs) by dying human cells. DAMPs can stimulate a direct or indirect (via innate immune effectors) cytotoxic response in surrounding bystander cells that have survived ACD [140–144]. In such a case, interfering with the effects of ACD on the rest of the population remains possible.

ACD is often equated with necrosis, which in yeast is usually identified as a cellular condition of early plasma membrane permeabilization in the absence of typical apoptotic markers and of complete disintegration of sub-cellular structures [103]. Indeed, ACD usually exhibits morphological features of necrosis, but mounting evidence suggests that – as it is the case in human cells – a physiologically relevant, regulated type of necrosis does also exist in yeast. Thus, we recommend to avoid using the term “necrosis” to define an accidental and uncontrollable type of death, and to favor the term “ACD”. We believe that this will avoid any potential misunderstandings regarding the two fundamentally distinct (accidental *versus* regulated) modalities of yeast cell death manifesting with a necrotic morphology (see below).

That said, many lethal stimuli result in a form of yeast cell death that – at odds with ACD – is executed by a genetically encoded, dedicated molecular machinery. In higher eukaryotes, a distinction is made between such a controlled form of cell death when it occurs (i) in the framework of a purely physiological program, e.g., during (post-) embryonic development or tissue homeostasis, or (ii) as a response to either a perturbation of intracellular or extracellular homeostasis, e.g., upon exposure to mild stress or as a consequence of mutations. Cell death occurring in the former scenario is termed “programmed cell death” (PCD), while the expression “regulated cell death” (RCD) encompasses both PCD as well as all other instances of cell death that depend on a molecular machinery [145–148].

For yeast cell death, many authors have used the term PCD to interchangeably refer to all types of cellular demise that are not accidental (i.e., to all instances of RCD). However, emerging evidence is confirming that a yeast population, be it a liquid culture or a solid colony, bears a degree of complexity reminiscent of multicellular organisms that demands a revision of this terminology. For instance, during yeast gametogenesis (or sporulation), immature meiotic products as well as the mother cell itself succumb via activation of vacuolar rupture [149, 150]. Interestingly, the mother cell’s demise is delayed until spores have reached a threshold degree of differentiation. Thus, in this scenario, RCD occurs in the frame of a developmentally coordinated

program, *de facto* representing an instance of PCD. During yeast chronological aging, the cellular community maintains homeostasis thanks to the programmed death of dysfunctional or old cells, which spares and provides nutrients to the fitter individuals [75, 76]. In yeast colonies, stationary-phase or slow-growing cells differentiate into specific subpopulations with unique metabolic properties and particular functions within the colony [151, 152]. These examples show that, indeed, yeast populations can harness cell death to control coordinated development, homeostasis and differentiation. Hence, we propose to define PCD in yeast as a specific instance of RCD that is executed in the frame of such physiologic programs. All other forms of regulated demise (e.g., cell death induction upon stress, or as a consequence of specific genetic alterations) should be referred to with the superordinate term of RCD.

Importantly, since RCD depends on a defined molecular machinery, it can be modulated with pharmacologic or genetic means. The extent of such modulation depends on the progression of the process across a hitherto poorly defined point-of-no-return. According to the NCCD, the processes preceding such point are part of cellular stress responses, while those following it belong to actual cell death signaling [93]. Adopting this rationale, RCD can be accelerated or delayed (but not avoided) if the point-of-no-return has been trespassed. Instead, prior to that point, modulating stress responses or avoiding stress can prevent RCD. However, the definition of this point-of-no-return has not been established yet, implying that the exact boundary between the reversibility of a stress stimulus and the irrevocable engagement in a lethal cascade remains to be specified.

Yeast RCD may follow different subroutines (see below) that can be differentiated from each other by a series of morphological and biochemical features. To precisely characterize the lethal phenotype, we recommend (i) to first determine if cell death actually occurs (as opposed to only reduced viability/vitality), (ii) if it does, to then examine the subroutine(s) involved via morphological and biochemical observations, using at least two different detection methods [155], and (iii) finally, to corroborate the implicated mechanism(s) via genetic and pharmacological interventions (**Figure 2**). Finally, it should be noted that in cell death research, it is generally advisable to determine the kinetics of the parameters under scrutiny [156]. In order to detect the differential appearance of apoptotic or necrotic characteristics, we recommend assessing such features at different time points to yield a better resolution of cell death events. Importantly, subroutine-specific markers should precede cell death. In the following sections, we will describe yeast RCD subroutines and the techniques to precisely discriminate amongst them (**Table 1**). Beyond the specificities outlined below, a number of general issues and notes of caution also need to be considered (**Box 2**).

APOPTOSIS

Most studies on RCD in yeast have been conducted in the budding yeast *Saccharomyces cerevisiae*. This includes the first observation of an apoptotic phenotype in yeast, specifically in a strain with a point mutation in the gene coding for the cell cycle protein Cdc48 [63]. One of the early indications for an active cellular participation in the yeast apoptotic process was that RCD in this setting can be prevented by inhibiting *de novo* protein synthesis, e.g. by cycloheximide [125]. Ever since these discoveries, a set of methods has been established, validated and refined that allows to specifically determine whether a yeast cell has engaged in an apoptotic pathway [62]. These techniques are mainly based on the key morphologic and biochemical features of an apoptotic cell. We suggest employing at least two of these apoptosis-specific methods (one of them should be Annexin V staining, see below) and include at least one viability assay (preferably clonogenic capacity) to describe a corresponding phenotype.

One of the events most commonly associated with apoptosis is the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane [182]. However, PS externalization might be context-dependent to a certain degree, at least within the complexity of the human cellular network [93, 183, 184]. It remains unclear whether this is also the case in yeast, although the current evidence suggests that PS externalization is a universal feature of yeast cells undergoing apoptosis. PS externalization can be assessed via monitoring PS-binding to Annexin V, which is usually fluorescently labeled for quantitative (e.g., fluorescence reader-based or flow cytometric analyses) and qualitative (microscopic) evaluation. To this aim, the cell wall needs to be (partially) digested in order to make the externalized PS accessible to Annexin V and permit binding. Usually, the Annexin V assay is performed as a co-staining with a marker for plasma membrane rupture like PI [63, 96, 104, 157]. This allows for the discrimination between several subpopulations as they occur in yeast: (i) Annexin V/PI double negative, (ii) Annexin V positive, (iii) PI positive, and (iv) Annexin V/PI double positive cells.

We believe that the second (Annexin V positive) and third (PI positive) subpopulations can be readily interpreted as apoptotic and primary necrotic, respectively, provided that at least one more assay is performed to validate this assumption. For the fourth subpopulation (Annexin V/PI double positive cells), we favor the following interpretation: unlike multicellular animals, a yeast population presumably does not eliminate apoptotic cells via the phagocytic activity of other yeast cells. In the absence of such clearance by scavengers, an apoptotic cell eventually undergoes a metabolic collapse that results in breakdown of the plasma membrane integrity and thus a necrotic phenotype. This phenomenon is termed “secondary necrosis” to discriminate it from “primary necrosis”, which describes the phenotype of “cellular necrosis occurring *ab initio*” [185, 186]. We thus view the above-mentioned fourth subgroup (Annexin V/PI double positive cells) as a late apoptotic population that has undergone secondary necrosis.

Table 1. Methods commonly used for the assessment of cell death, viability and vitality as well as for the identification of different cell death subroutines in yeast.

	Feature	Method	Limitations	References
Death	Loss of cell membrane integrity	PI, trypan blue	Does not distinguish between primary and secondary necrotic cells (unless coupled with Annexin V assay and kinetics study)	[96, 97, 99]
Viability	Colony formation	Clonogenic capacity (quantification of colony-forming units)	Slow, work-intensive, does not differentiate between cell death and senescence	[69, 96, 104]
	Growth capacity	Spot dilution	Possible non-lethal modification in cell cycle or metabolism; semi-quantitative (except for microcolony count)	[110, 111]
	Growth capacity	OD ₆₀₀	Possible non-lethal modification in cell cycle or metabolism; concurrent growing and dying subpopulations	[108, 109]
Vitality	Metabolic activity	Specific enzymatic activity	Possible non-lethal modification in cell cycle or metabolism	[100, 112, 113]
	Metabolic activity	Physiological parameters	Possible residual activity in factually dead cells	[119, 120]
	Metabolic activity	General activity (e.g., FUN-1, methylene blue, phloxine B)	Possible residual activity in factually dead cells, or living cells without assayed metabolic activity	[114–118]
	Oxidative stress	ROS (e.g. DHE, DHR123, H2-DCF-DA)	ROS specificity, only correlative and non-exclusive feature	[96, 122–125]
Apoptosis	PS externalization Chromatin condensation	AnnexinV/PI DAPI	Need of spheroplasts Not fully specific, can occur in some form of necrosis	[63, 96, 104, 157] [63, 125]
	Chromatin condensation DNA fragmentation	EM TUNEL	Slow, non compatible with quantification Possible staining of vital processes, can occur in some forms of necrosis	[63, 125] [63, 96, 104, 157, 158]
	MOMP (release of pro-apoptotic proteins from the IMS)	Western blot, immunofluorescence microscopy, enzymatic activities of IMS proteins	Also occurs during necrosis	[96, 159, 160, 161]
	Loss of $\Delta\psi_m$	Potentiometric fluorescent probe imaging or flow cytometry	Also occurs during necrosis	[121, 162, 163, 164]
	Dependence on known regulators Dependence on <i>de novo</i> protein synthesis	Knockout analysis Protein synthesis inhibitors (e.g., cycloheximide)	May affect other signal cascades Interferes with the synthesis of anti-apoptotic proteins; may apply to other RCD instances	[62] [125]
Necrosis^a	Disruption of plasma membrane	EM	Slow, quantification	[103, 157]
	Disruption of plasma membrane	Unpermeable fluorochromes (e.g. , PI)	Does not discriminate between primary and secondary necrosis	[103, 157]
	Nucleo-cytosolic translocation of Nhp6A	Fluorescence microscopy (GFP-tag)	Does not discriminate between primary and secondary necrosis	[104, 157, 165]
	Disintegration of subcellular structures	EM	Slow, quantification	[103, 157]
	MOMP (release of pro-apoptotic proteins from the IMS)	Western blot, immunofluorescence microscopy, enzymatic activities of IMS proteins	Also occurs during apoptosis	[149]
	Loss of $\Delta\psi_m$	Potentiometric fluorescent probe imaging or flow cytometry	Also occurs during apoptosis	[121, 149]
Regulated necrosis (additional features to discriminate from accidental necrosis)	Pharmacological inhibition	Spermidine	May affect other signal cascades	[157]
	Genetic dependence	Inhibition by spermidine biosynthesis	May affect other signal cascades	[157]
	Genetic dependence	Inhibition by Pep4 propeptide	Propeptide-free Pep4 is not expressed (limited to propeptide overexpression studies)	[104]
Autophagy	GFP-Atg8 processing	Western blot	May occur with some degree of non-specificity	[166, 167]
	GFP-Atg8 processing	Fluorescence microscopy	May occur with some degree of non-specificity	[168, 169, 170]
	Modified ALP activity	ALP assay	May result from other signaling pathways, results could be influenced by changes in Pho8 expression levels	[171, 172]
	pH-change upon delivery of cellular compartments to the vacuole	Rosella fluorescent pH biosensor	May result from other signaling pathways	[173]
	Others	Biochemical, microscopic	-	[175]
ADCD (additional dependency assessment)^b	Pharmacological inhibition (dependence on autophagic degradation)	Inhibition of vacuolar proteolysis	May affect other signal cascades	[175, 176]
	Pharmacological inhibition (dependence on autophagic degradation)	Inhibition of autophagic process	May affect other signal cascades, insufficient specificity	[175, 177]
	Genetic dependence (dependence on autophagic machinery)	KO of ATG genes	May affect other signal cascades	[174, 175]
	Genetic dependence (dependence on autophagic machinery)	KO of vacuolar proteolysis	May affect other signal cascades	[175, 176]
	Genetic dependence (dependence on autophagic machinery)	Constitutive activation of TOR or RAS/PKA	May affect other signal cascades	[178–181]

^a Please note that necrotic features are common to different subpopulations and phenotypes (primary necrosis, secondary necrosis, accidental necrosis, regulated necrosis).^b Please note that autophagy is often cytoprotective and that the additional experiments are necessary to establish the occurrence of ADCD.ADCD, autophagy-dependent cell death; ALP, alkaline phosphatase; $\Delta\psi_m$, mitochondrial transmembrane potential; EM, electron microscopy; IMS, intermembrane mitochondrial space; KO, knockout; MOMP, mitochondrial outer membrane permeabilization; OD, optical density; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Still, these cells might also have undergone secondary necrosis following other cell death subroutines, but at this point there is no evidence for this possibility, which should be evaluated earlier in the cascade of events leading to cellular demise. Importantly, the phenotypical shift from apoptosis to secondary necrosis might reflect defined molecular events and thus be experimentally distinguishable from ACD with necrotic features and primary necrosis also at the functional level [187, 188]. It could be argued that, in turn, primary necrotic cells might eventually stain for apoptotic markers like Annexin V, thus also yielding AnnexinV/PI double-stained cells. However, necrotic markers do appear without apoptotic characteristics and such primary necrotic populations are stably maintained during long-term physiological conditions like chronological aging. This strongly suggests that primary necrosis can be distinguished from secondary necrosis by the absence or presence of apoptotic markers. Still, no study has yet systematically evaluated this distinction at the cellular level, for instance, via cell sorting analysis. Until such further analysis, this interpretation remains a valid approximation. In any case, we suggest determining the kinetics of the cell death process (see above) to accurately resolve the appearance of these subpopulations. In general, any approaches that facilitate monitoring death scenarios time-dependently represent a helpful improvement, for instance replicative age-associated changes using microfluidic platforms [189–193].

In multicellular animals, clearance of apoptotic cells is a central physiological feature for maintenance of organismal homeostasis. Still, secondary necrosis does occur under certain circumstances [186]. *In vitro*, cultured metazoan cells that are left to finalize the apoptotic process without interruption (e.g., without interference of phagocytic scavenging) eventually succumb with features of secondary necrosis [186, 194, 195]. *In vivo*, secondary necrosis may occur in multicellular animals, for example, when apoptotic cells are shed into the lumina of hollow organs with low probability to encounter scavengers or when apoptotic cell death occurs at a pace that surpasses the local scavenging capacity [186, 196, 197]. These observations suggest that secondary necrosis following apoptosis is a conserved outcome upon exposure to pro-apoptogenic stimuli if clearance mechanisms are absent or insufficient.

Besides PS externalization, apoptotic cells exhibit chromatin condensation, which can be readily assessed by nuclear staining with dyes such as 4',6-diamidino-2-phenylindole (DAPI) followed by microscopic inspection [63, 125]. Another characteristic that accompanies yeast apoptosis – especially at late steps of the process – is DNA fragmentation. It is often assessed via the “terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling” (TUNEL) test, which allows for the fluorescent labelling of free 3'-hydroxyl ends that can be easily monitored via microscopy analysis and quantified using a fluorescent plate reader or a flow cytometer [63, 96, 104, 157]. In many yeast cell death scenarios, TUNEL positivity matches apoptotic markers determined by other assays [96, 104, 157, 198]. However, TUNEL staining detects free 3'-hydroxyl

ends regardless of the molecular mechanism involved in generating them. In fact, in some conditions, necrosis, DNA repair, or active gene transcription have all been shown to yield TUNEL positivity, at least in human cells [199–204]. In yeast, the nature and the kinetics of DNA fragmentation detected by the TUNEL test need further investigation, even though previous studies have partly addressed these issues [79, 205]. In summary, we recommend using the TUNEL test as a method to determine the occurrence of DNA fragmentation associated with yeast apoptosis rather than a technique for quantifying apoptosis on its own. In addition, the TUNEL test may provide an assay to screen for cellular demise in high-throughput assays. In this setting, hits must be confirmed by testing cellular membrane integrity and clonogenic capacity. Furthermore, apoptotic DNA damage may be tested using the so-called “comet assay”, or single cell gel electrophoresis, whereby physiologic DNA strand breaks are distinguished from apoptotic DNA dissolution in individual cells (the latter forms a distinct cluster of fragmented DNA at the ‘tail’ of the comet) [206]. In addition, the flow cytometric detection of a subpopulation with hypoploid DNA content (sub- G_0/G_1) has been previously employed as an alternative to assess apoptotic DNA degradation [207]. However, such results should be interpreted carefully, since apparent hypoploidy may also reflect an artefact from the debris associated with necrotic cells, unless discarded by cell sorting analyses [208].

Apoptotic cell death often follows mitochondrial outer membrane permeabilization (MOMP), which culminates with the release of pro-apoptotic proteins from the intermembrane space and irreversible loss of mitochondrial transmembrane potential ($\Delta\psi_m$) [96, 159, 160, 162–164, 209,210]. A detailed analysis of these mitochondrial sub-events requires precise kinetic determinations. For instance, in acetic-acid induced RCD, pro-apoptotic cytochrome *c* release, which depends on the ADP/ATP carrier [211], occurs before mitochondrial integrity is lost [212]. All of these biochemical features might be evaluated to determine an apoptotic phenotype, though it should be kept in mind that mitochondria have also been associated with at least one other RCD subroutine (regulated necrosis) [149]. Thus, we recommend the involvement of mitochondria in apoptosis to be validated by at least two specific methods (one of them should be assessing PS externalization) and at least one viability assay (preferentially clonogenic capacity).

A large number of apoptotic regulators and executors have been identified in yeast so far [62]. This enables testing whether RCD occurring upon a given stimulus is at least partly dependent on one of these factors based on genetic manipulations, pending confirmatory experiments with morphological and biochemical assays. We advise to interpret results from genetic disruption or inhibition studies with caution, as it is difficult to estimate whether other or how many signaling cascades have been affected by a manipulation *a priori* specific. Indeed, many yeast cell death regulators, e.g., cytochrome *c*, apoptosis-inducing factor (Aif1), endonuclease G (Nuc1) and the yeast metacaspase (Yca1), exert both lethal and vital functions [62,

69, 96, 159, 160, 213-216]. Importantly, the molecular network underlying apoptosis regulation in yeast is starting to be uncovered and additional regulators and subroutines that are yet unknown are expected to emerge. Thus, if a given cell death phenotype is not dependent on any of the known apoptotic regulators this does not exclude apoptosis as a possible cell death modality.

For exploring a putative apoptotic mechanism in a given cell death scenario, the deletion strains of known apoptotic regulators should be harnessed, since distinct apoptotic subroutines exist that rely on different factors that may act independently from each other to orchestrate cellular demise. For instance, the yeast metacaspase Yca1 is involved in many apoptotic RCD and PCD settings [62, 69, 75]. Thus, cell death inhibition in *yca1* knockout strains may point towards an apoptotic mechanism. However, under certain conditions, apoptosis is not executed via Yca1, but instead relies on other factors, including Aif1, Nuc1, the human cyclophilin D ortholog Cpr3, the BH3-only protein Ybh3 or ceramides [96, 160, 217-223]. Importantly, while yeast harbors a single metacaspase-encoding gene (*YCA1*), it is possible that other proteases might functionally substitute for metacaspases [224-226]. Thus, in cases where Yca1 is not involved in cell death regulation, we favor the expression “Yca1-independent” instead of “metacaspase-” or “caspase-independent” cell death. For cell death stimuli that are dependent on Yca1, we consider that the terms “Yca1-“, “metacaspase-“, and “caspase-dependent” are all appropriate. In fact, though

much controversy has accompanied the denomination of metacaspases as true homologs of caspases, recent advances strongly indicate that this is the case [71]. Indeed, caspases and metacaspases seem to be evolutionary distinct variants with a functional commonality that do fulfill the criteria of homology, since they both share (i) a common cellular program (RCD) and (ii) common or at least overlapping substrates [70, 227, 228].

In human cells, extrinsic apoptosis defines a caspase-dependent cell death subroutine that is induced by extracellular lethal ligands. These ligands are sensed and transmitted either via specific transmembrane death receptors or through so-called ‘dependence receptors’. Dependence receptors can trigger two opposite signaling pathways: in the presence of ligand, they elicit signals involved in cell survival, migration and differentiation, but in the absence of ligand, they promote apoptotic RCD. Thus, dependence receptors only exert lethal functions when the concentration of their specific ligands falls below a critical threshold level [229]. While in yeast no such dedicated receptors are known, cases of metacaspase-dependent apoptosis induction by molecules that may operate from the extracellular microenvironment have been described. For instance, toxins secreted by virus-infected killer strains and a number of drugs have been shown to trigger apoptosis executed by Yca1 [82, 230-233]. Yet, it remains unknown whether these factors act on intracellular targets, or whether they may also bind to plasma membrane-localized receptors. Given the complexity and interactivity of a yeast population, it is

BOX 2: GENERAL NOTES OF CAUTION

Besides the specific points to be addressed for appropriately classifying an observed cell death phenotype, various general issues need to be considered, as well.

(i) In general, we recommend to sequentially test the following: first, whether cell death occurs (defined as loss of plasma membrane integrity, which may be accompanied by decreased proliferation and/or diminished metabolic activity), second, the hallmarks of cell death subroutines as implied at the descriptive level (morphology, biochemistry), and third, the mechanisms of cell death as determined at the interventional level (genetic, pharmacological). Thus, it is imperative to combine **multiple and complementary approaches**, also with respect to **kinetics** (markers should precede cell death), to characterize a specific cell death type. We recommend performing at least two independent and subroutine-specific assays, preferably not just restricted to the assessment of morphological features.

(ii) While mainly qualitative or arduously quantifiable methods (e.g., electron microscopy) offer the possibility to accurately define specific cell death phenotypes, they may be poorly representative of the general sample conditions. Thus, we encourage using these methods for exploratory purposes, but strongly suggest accompanying them with **quantitative assays**.

(iii) In many cell death settings, the dependence on specific factors may be tested by inhibiting their function. Where possible, we recommend employing **genetic tools** (i.e., knockout, temperature-sensitive mutants) instead of pharmacological inhibitors. Indeed, the specificity of such compounds might not be sufficient to precisely block the activity of a single pathway/factor that characterizes a cell death subroutine [153].

(iv) If knockout strains are used to inquire the involvement of the corresponding gene/protein in a given cell death scenario, we recommend employing **self-generated deletion strains** and control results by **complementation analysis** (i.e., ectopic re-expression of deleted genes etc.). Those available at public strain collections constitute useful starting tools for experimentation, but may have accumulated secondary mutations that might lead to misinterpretations [154].

(v) For the quantification of fluorescence-based detection methods, we recommend using **flow cytometry** rather than a fluorescent plate reader. Data obtained with a plate reader may indeed be influenced by the fluorescence of the entire culture, which may vary with several parameters including strain-specific cell size. Few highly fluorescent cells may yield the same signals compared to a substantial fraction of moderately or low fluorescent but still positively stained cells. Thus, even upon normalization to the OD₆₀₀, bulk results are less accurate than results obtained with flow cytometry, which is based on actual single-cell fluorescence. Plate readers may be convenient for high-throughput studies, but positive hits should be validated using flow cytometry.

conceivable that a yet-to-be-determined extrinsic apoptotic pathway may co-regulate cell death within a yeast community [79, 234]. However, and meeting the definition of extrinsic apoptosis put forward by the NCCD, we suggest not to use this term until dedicated death receptors or dependence receptors are discovered. Similarly, another specific type of apoptosis in human cells, anoikis, which defines a form of intrinsic apoptosis restricted to adherent cells that detach from the matrix [235], is theoretically possible in yeast. Indeed, adhesion mediated by cell-wall-bound adhesins is crucial for colony and biofilm formation as well as for host-pathogen interactions [236-238]. While it remains conceivable that normally adherent yeast cells, which detach in a specific scenario where adhesion is important, might undergo a form of anoikis, this form of RCD has not (yet) been described in yeast.

REGULATED NECROSIS

In dying yeast, necrotic characteristics may appear in the frame of a primary or secondary necrotic process. While secondary necrosis is probably a consequence of apoptosis in most if not all cases (see above), a primary necrotic phenotype (which occurs without any preceding apoptotic traits) may result from two cell death modalities: ACD or RCD. Indeed, yeast primary necrosis can not only be the outcome of severe insults (accidental necrosis), but also develop as an event orchestrated by a genetically controlled machinery (regulated necrosis) [103]. In both cases, cell death is characterized by a set of distinct morphological and biochemical features that defines it as necrotic.

Necrosis first leads to a gain in cell volume and organelle swelling (oncosis), which may be observed, for instance, using fluorescent microscopy of GFP-fused proteins that mark organellar membranes [149]. Eventually, necrotic cells also show the complete breakdown and disintegration of subcellular structures, which can be assessed using electron microscopy [157]. Similarly, the rupture of the plasma membrane that accompanies necrosis can easily be assayed via electron microscopy or fluorochromes like PI that only enter cells with a disintegrated cell membrane, but are excluded by healthy or early apoptotic cells [103, 157]. In yeast, the release of intracellular material has not yet been systematically employed as an assay to characterize necrotic cell death. However, the nucleo-cytosolic translocation of Nhp6A may be used to assess necrosis in yeast [104, 157, 165, 239]. Nhp6A is the yeast homolog of the mammalian protein high mobility group box 1 (HMGB1), whose release accompanies immunogenic cell death mammalian cells [144, 240]. We suggest assessing at least two of these markers in order to define *bona fide* primary necrosis in yeast. In addition, viability should be measured with at least one assay (preferably by assessing clonogenic capacity) to corroborate cellular demise. Finally, we strongly recommend to exclude the presence of apoptotic death indicators, and most importantly to differentiate the observed phenotype from secondary necrosis.

As in higher eukaryotic cells, in yeast, ACD may be triggered upon the challenge to extremely detrimental condi-

tions. Thus, agents like hydrogen peroxide, acetic acid, amphotericin B, or several metals that are pro-apoptotic at low doses may induce necrosis at high concentrations [125, 218, 241, 242]. We assume that necrosis is the consequence of radical cellular damage in most of these cases, and hence a *bona fide* instance of ACD. This is in line with the concept that not only the type but also the intensity of a given perturbation determines the form of death [91, 243].

As mentioned above, yeast can undergo regulated necrosis, reminiscent of the RCD instances detected in human cells [244]. Indeed, genetic and chemical manipulations demonstrate that yeast necrosis can be inhibited, at least in some settings, indicating that it results from the activation of a molecular mechanism. In order to differentiate regulated from accidental necrosis, it is necessary to test whether a pharmacological or genetic intervention is capable of inhibiting necrosis in the scenario that is being studied. Known necrosis-modulatory approaches include the exogenous administration of the naturally occurring polyamine spermidine, which can specifically reduce primary necrotic cell death in the context of chronological aging [157]. A similar outcome can be obtained by genetic modulation of polyamine biosynthesis [157]. In addition, the proteolytically inactive propeptide of the vacuolar endoprotease Pep4, the homolog of human cathepsin D, has been shown to mediate antinecrotic effects. Accordingly, prolonged overexpression of Pep4 (or its propeptide) can extend chronological lifespan via specific inhibition of necrosis [104, 245]. Intriguingly, the antinecrotic function of Pep4 depends on polyamine biosynthesis [104]. In fact, further vacuolar factors as well as other organelles, e.g., peroxisomes, might be connected to regulated necrosis, but this requires further investigation [246-250].

Under certain circumstances, regulated necrosis in mammalian cells may be mechanistically linked to primary $\Delta\psi_m$ dissipation [251, 252], and such a mitochondrial permeability transition (MPT)-driven necrosis is connected to a series of pathological conditions [253]. In yeast, necrotic cell death also seems to depend on mitochondria in several settings [149, 165, 221]. In addition, recent reports show that necrotic cell death upon a lipotoxic insult requires a functional Rim101 signaling cascade that involves the calpain-like protease Rim13/Cpl1 for lethal execution [254, 255]. To interrogate a possible case of regulated necrosis, it is thus advisable to evaluate a possible mitochondrial involvement. For that purpose, it would be indicated to examine whether necrosis is diminished upon abrogation of mitochondrial function, e.g., in a ρ^0 strain (which lacks mitochondrial DNA). However, as previously mentioned, mitochondria are the main executors of apoptotic cell death. Thus, mitochondrial dependence cannot be used as a sole determinant to characterize regulated necrosis and must be accompanied by a set of other assays that demonstrate the primary necrotic nature of cell death. Of note, several known mammalian mediators of regulated necrosis have homologs in yeast, including cathepsins, cyclophilin D, calpains, Hsp90, or protein kinase A, among others [244], but only a few of them have been examined in this context

[103, 256]. It will be interesting to see whether these factors possess a conserved necrotic function in yeast, which would expand the possibilities to determine *bona fide* regulated necrosis. Similarly, it remains to be seen whether known inhibitors of regulated necrosis in mammals also interfere with some cell death scenarios in yeast as well [257].

A number of questions remain to be answered with regard to the actual existence of a necrotic RCD subroutine in yeast. In mammals, regulated necrosis plays a number of key roles, most prominently due to its immunogenic nature, for instance upon pathogen infection [244]. Such interaction with the immune system, however, is a feature of complex multicellular organisms. Nonetheless, several physiological scenarios in which regulated necrosis seems to be instrumental for yeast, provide a teleological explanation for its existence in a unicellular organism. During chronological aging, for instance, yeast cells die exhibiting markers of both early/late apoptosis and primary necrosis [61, 75, 157]. Interestingly, the fraction of cells dying by primary necrosis actually represents the majority of the dying population that is reduced upon a cytoprotective intervention, at least via polyamine-mediated lifespan extension [104, 157]. Another example is the necrotic death of the meiotic mother cell during the terminal stages of gametogenesis (sporulation) [149]. In this setting, necrosis occurs after the spores have reached the final phases of development, suggesting a controlled coordination that allows for gamete differentiation prior to the elimination of the mother cell. This might well constitute an instance of necrotic PCD, reinforcing the notion that yeast populations must be seen as a multicellular community of genetically identical cells that responds to selective pressures by ensuring the long-term survival of at least one clonal individual. Therefore, it is conceivable that regulated necrosis might participate in cell-to-cell communication via the inevitable release of intracellular contents, as this is the case in higher eukaryotes [244]. Such hypothetical necrosis-related quorum-sensing molecules, however, are yet to be identified in yeast.

In human cells, different types of regulated necrosis have been defined, with MPT-driven regulated necrosis and necroptosis among the most extensively studied forms [258, 259]. In yeast, mechanistic insights into the control of necrosis are still very limited at this point. Thus, we strongly discourage the use of neologisms to avoid confusion. Instead, we propose to employ the term “regulated necrosis” to describe any genetically controlled form of necrosis in yeast (or “programmed necrosis” if it is a form of PCD). Further research into the molecular activators, transducers and executioners of regulated necrosis in yeast will reveal whether potentially different subroutines of the process exist.

OTHER RCD TYPES

In mammalian cells, a series of other RCD modalities have been defined. Macroautophagy (hereafter referred to as autophagy) is a conserved catabolic process that orches-

trates the digestion of intracellular material (e.g., protein aggregates, organelles) in the vacuole. During autophagy, double-membraned vesicles (so-called autophagosomes) form and engulf cytoplasmic components, followed by the fusion of autophagosomes with the vacuole, where the cargo is degraded and the resulting macromolecules are released into the cytoplasm for reuse [16, 260]. Thus, autophagy is predominantly a cell survival mechanism (see below). Historically, though, “autophagic cell death” (ATCD) was one of the three distinct cell death manifestations (besides apoptosis and necrosis) that were described for human cells based on morphological criteria [261]. Although this original description did not indicate any functional connection, it became a widespread belief that ATCD would point to cell death as a mechanistic outcome of autophagy. The term ATCD has indeed been extensively misused to describe cell death instances that occur in the presence of autophagic markers, instead of testing an actual dependency on the process and/or its molecular machinery, i.e., assessing the retardation of cell death via pharmacological or genetic inhibition of autophagy [260]. In fact, the NCCD has recently agreed to identify such forms of cell death as “autophagy-dependent cell death” (ADCD) [95]. ADCD can in principle describe (i) cell death dependent on the autophagic machinery (in its whole, or parts thereof) and (ii) cell death dependent on actual autophagic degradation. Indeed, (i) components of the autophagic machinery have been etiologically implicated in specific settings of RCD in *Drosophila melanogaster* and human cells [243, 262-266]. In these contexts, the molecular apparatus for autophagy contributes to cellular demise. To our knowledge, however, there is no study in which cell death has been directly linked to (ii) a functional autophagic flux. Thus, we surmise that most cases of ADCD rather depend on components of the autophagic machinery than on autophagic responses. In fact, the molecular machinery of ADCD and adaptive autophagy partially differ (at least in *D. melanogaster*) [267, 268].

In yeast, the term ATCD has been used to describe cellular demise occurring under specific external stress conditions like zinc-induced cell death [269], heterologous expression of human α -synuclein [174] or human p53 [270] as well as internal deficiencies like defects in inorganic pyrophosphatases [271]. Following the recent proposition by the NCCD, we favor the use of the term ADCD (instead of ATCD) in yeast, as well. Again, ADCD should be used to describe cell death only when autophagy (or at least two proteins from the autophagic machinery, see below) has been experimentally given an etiological implication in the process. As a note of caution, it is important to underscore that the term ADCD should be avoided if the autophagic machinery (or components thereof) is activated parallel to (rather than triggering) RCD or if it promotes other RCD subroutines [95]. In fact, in most known cases from yeast to human, autophagy acts as a cytoprotective response to detrimental stress conditions, in which it disposes damaged cellular material [37, 272, 273]. Accordingly, cell death is rather accelerated than repressed upon inhibition of autophagy in both human cells and yeast [274-276]. In

fact, and despite the evidence for autophagy activation in the course of cell death (see above), the very existence of ADCD as an actual cell death type has been questioned [277, 278]. In any case, cell death may often be preceded or accompanied by autophagy markers, probably mirroring the final effort of dying cells to counteract a lethal stress. Thus, in most cases, cells showing biomarkers of autophagy might be dying with, and not by, autophagy. We thus consider that the use of the term ADCD should be used with utmost care, taking into account the aforementioned NCCD recommendations [95].

A number of microscopic, biochemical and enzymatic assays are available and established [175, 279, 280] to determine autophagic flux, i.e., the progression through the pathway and thus its degradation activity [175, 176]. One of the most common methods to measure autophagic flux in yeast is to evaluate the vacuolar processing (or GFP liberation) of N-terminally GFP-tagged Atg8, a central modulator of autophagosome formation, and its delivery to the vacuole, via fluorescence or immunoblot analysis [166-170]. Other widely used assays include assessing the autophagy-dependent activity of a modified version of the vacuolar alkaline phosphatase Pho8 via a specific enzymatic assay [118, 171] or monitoring the pH-change of cellular compartments upon delivery of pH-sensitive fluorescent proteins to the vacuole (such as Rosella) [173]. However, such quantitative assessments – while necessary – are not sufficient to characterize ADCD: for that purpose, a functional dependency on the autophagic machinery (or components thereof) must be concluded, as mentioned above. Thus, all cases of cell death that are accompanied by autophagic markers, but cannot be suppressed or retarded by inhibiting the (at least parts of) the molecular apparatus of autophagy should not be considered as ADCD.

The causative implication of autophagy in cell death may be explored by deletion of autophagy-related (ATG) genes, which are the key orchestrators of the process [175]. However, ATGs may have autophagy-unrelated functions as well [281]. Thus, akin to the recommendations for higher eukaryotes [260], we suggest testing at least two (and better more) distinct ATG deletions to assess dependency on the autophagic machinery. Inhibitory components of the autophagic apparatus can also be targeted, e.g., by constitutively activating the TOR complex 1 or the RAS/PKA signaling pathway, resulting in autophagy suppression [178-181]. As mentioned above, dependence of cell death on the molecular machinery of autophagy (in its whole, or parts thereof) does not imply cell death to be dependent on autophagic degradation. To evaluate if the autophagic response is implicated in the lethal execution, one may take advantage of chemical inhibition [175, 177]. Vacuolar proteolysis can be blocked through direct inhibition of proteases either genetically (e.g., by deleting *PEP4* or *PRB1*) or pharmacologically (e.g., by addition of pepstatin A, E-64D, leupeptin alone or in combination) as well as by neutralizing the vacuolar pH (e.g., by means of chloroquine) [175, 176]. In yeast, chemical inhibition of autophagosome formation (as it is commonly applied in mammals using specific suppressors of phosphatidylinositol 3-kinase) is not typi-

cally employed, since substantially higher concentrations of these drugs are often needed [262, 265]. In fact, genetic approaches are generally favored in the ADCD field due to insufficient specificity of most pharmacological autophagy inhibitors [260].

The expression “mitotic catastrophe” (MC) was first employed to illustrate the lethal phenotype of a temperature-sensitive fission yeast mutant strain that enters mitosis prematurely without effectively completing it [11]. The term MC has since been most frequently used to define cell death that occurs upon aberrant mitosis [94], which is frequently accompanied by gross nuclear alterations. In yeast, as in mammals, it may result from genome instability, microtubule destabilization, DNA damage, or alterations in cell cycle checkpoints [282-285]. Intriguingly, yeast RCD has been connected to most of these features [63, 286-288]. It will be interesting to follow whether known MC scenarios culminate in specific RCD subroutines.

In mammalian cells, death following mitotic aberrations can, indeed, be either apoptotic or necrotic [289]. Since mitotic defects may contribute to malignant transformation in the mammalian system, MC can be viewed as an oncosuppressive mechanism that operates via cell death or senescence [94, 289]. In fact, suppression of MC provokes tumorigenesis and cancer progression in mammals [290]. By analogy, MC in yeast might be a mechanism to eliminate mitosis-incompetent and thus unfit cells from the population. Adhering to the recommendations by the NCCD [94], we thus propose to use the term MC as an independent molecular avenue that precedes RCD, but does not constitute a *bona fide* cell death executioner mechanism by itself [290].

A series of other cell death subroutines have been defined in human cells that, however, are restricted to specific cell types and thus do not apply to yeast.

RCD IN OTHER YEASTS AND FILAMENTOUS FUNGI

As previously mentioned, yeast cell death has been most extensively studied in *S. cerevisiae*. However, other yeast species have been shown to share similar cell death characteristics and also bear a set of comparable cell death subroutines. Thus, we propose to extend the above-described recommendations formulated above to all yeast species.

Schizosaccharomyces pombe (fission yeast) has been shown to express an RCD machinery that responds to various stimuli. These include physiological triggers such as aging, defects like the abnormal metabolism of intracellular lipids [291-294], and a number of insults, including ER stress [295], inositol starvation [292, 293] or the heterologous expression of several metazoan apoptotic effectors, e.g., BAX and BAK [296]. All of these stimuli converge on the activation of apoptosis. Of note, according to our definitions, neither regulated necrosis nor ADCD have been demonstrated in *S. pombe* (yet). Among the described *S. pombe* apoptosis executors are the chaperone Cnx1 (calnexin) and the metacaspase Pca1 [295, 297]. Pca1 is involved in the apoptotic response to inositol starvation [295, 297] and lipid-induced, non-apoptotic cell death in

minimal medium. Conversely, Pca1 does not seem to play any role during apoptosis induced by ER stress [295], valproic acid treatment [298], or lipotoxic stress in minimal medium [292]. *S. pombe* apoptosis is expected to involve additional players, as there is evidence for the presence of different factors in fission yeast that are homologous to effectors of *S. cerevisiae* apoptosis, including the protease Nma111 [299], Aif1 [300] or endonuclease G [301].

The major opportunistic human pathogen *Candida albicans*, which has become a molecular genetics model to study pathogenicity, virulence and fungal development [302, 303], can also undergo apoptosis following the exposure to a plethora of different agents [242, 304–306]. To date, no RCD subroutines other than apoptosis have been described. Interestingly, apoptosis can occur in both the blastospore and the hyphal form of this organism [305] as well as in *Candida* biofilms, which are highly tolerant to standard antimycotics and hence difficult to eradicate. Exploiting the apoptosis machinery in cells constituting biofilms may pave the way to their effective eradication, and hence limit the incidence of indwelling device-associated infections (IDAls) [307–309]. *C. albicans* also harbors a gene encoding a metacaspase (*CaMCA1*) [310], which mediates apoptosis, for instance, upon treatment with farnesol [311], caspofungin [312], and micafungin [232] or upon interaction with murine macrophages [311, 313]. Conversely, *CaMca1* is not involved in other apoptotic settings like exposure to the plant defensin RsAFP2 [314]. The Ras–cAMP–PKA signaling pathway [315] and the bZip transcription factor Cap1 [316, 317] have also been implicated in distinct apoptotic scenarios. Finally, other closely related *Candida* species, e.g., *Candida glabrata* [318], *Candida krusei* [319], *Candida dubliniensis* [320], *Candida tropicalis* [321], or *Candida parapsilosis* [232, 322], have been reported to exhibit apoptotic markers upon lethal challenge.

Cryptococcus neoformans, an important pathogen of immunocompromised and immunocompetent patients, also undergoes apoptosis [323, 324], with apoptosis-inducing factor and two metacaspases independently orchestrating this lethal subroutine [324]. At least one other *Cryptococcus* genus member, *Cryptococcus laurentii*, has also been shown to respond to some stimuli with apoptotic RCD [325]. Furthermore, a number of other yeast species, e.g., *Kluyveromyces lactis* [326, 327], *Pichia pastoris* [328], *Rhodotorula glutinis* [329], or *Zygosaccharomyces bailii* [330, 331], may develop signs of apoptosis under certain conditions. We surmise that similar lethal programs are to be discovered in other yeast species. In fact, such discoveries and further characterization of both identified and yet uncovered RCD programs are expected to follow in the near future, given that antifungal therapeutics for medical and industrial purposes may increasingly rely on targeting the yeast RCD machinery [332, 333]. We thus suggest adopting the recommendations formulated above for the description of cell death in all types of yeasts.

It should be noted that a growing body of work is addressing RCD in multicellular fungi. A major human patho-

genic fungus that causes life-threatening disease is *Aspergillus fumigatus*, which has also been demonstrated to undergo apoptosis under certain conditions [334, 335]. The genome of *A. fumigatus* codes for two metacaspases (CasA and CasB), whose relative contribution to cell death seems to depend on the scenario [335–337]. In fact, other fungal proteases might also exert metacaspase activities that are relevant for cell viability and/or survival [337]. *Aspergillus nidulans* is another member of the *Aspergillus spp.* that has been demonstrated to undergo RCD [338, 339]. The genome of *A. nidulans* appears to code for an apoptotic machinery with relevant players like apoptosis-inducing factor and two putative metacaspases [339, 340]. Another filamentous fungus, *Podospira anserina*, is used as an aging model that incorporates crucial apoptotic factors, including two metacaspases (PaMCA1 and PaMCA2) and at least five Aif members, of which only mitochondrial (but not cytosolic) isoforms seem to be relevant for aging-driven RCD [341, 342]. A role for the *P. anserina* cyclophilin D ortholog in RCD [343–345] as well as for autophagy in aging and lifespan control of *P. anserina* [346, 347] have been reported. Further instances of fungal RCD [306, 348] have been documented in *Paracoccidioides brasiliensis* [349], *Colletotrichum gloeosporioides* [350], *Fusarium oxysporum* [351], *Fusarium graminearum* [352], *Mucor racemosus* [353], *Botrytis cinerea* [354], *Penicillium expansum* [355], *Rhizopus oryzae* [356], *Scedosporium prolificans* [357] and *Neurospora crassa* [358]. As multicellular organisms, filamentous fungi have developed programs that are reminiscent of organismal RCD. For instance, several putative homologs of factors relevant for animal apoptotic control that are not found in unicellular yeast are present in the genomes of filamentous species [359]. Thus, multicellular fungi may have complex traits not present in yeasts that may add to the criteria and definitions presented herein.

CONCLUDING REMARKS

The impact of yeast (and other fungi, including filamentous species) on our lives at multiple socioeconomic, scientific and medical levels emphasizes the importance of decoding the mechanisms that determine its survival and control its demise. Therefore, the molecular comprehension and potential manipulation of yeast cell death hold major promise for biotechnological and biomedical applications. We anticipate that numerous fields might benefit from the possibility to modulate yeast cell death. For instance, the productivity of yeast during large-scale processes in the pharmaceutical and industrial arenas largely depends on its viability and ultimately on its tolerance to stress and its demise in stationary cultures. Also, novel pharmacological approaches that specifically target the RCD machinery of yeast pathogens may bypass the ever-increasing resistance to classical antimycotics, which is an emerging public health problem. Other medical manipulations of yeast RCD are also conceivable, e.g., strategies to intervene on pathogenic deviations of the mycobiome. Finally, yeast will continue to help the community in deciphering eukaryotic cell death pathways as it serves as an important model for human disease. Given its power to study the relationship

between genotype and phenotype, we expect to gain further insights from yeast to identify actionable targets that may be subjected to pharmacological (drug discovery) or genetical manipulation.

For all these reasons, it is now imperative to set the standards for defining and studying cell death in yeast. That said, we want to emphasize that the present set of recommendations should be taken – as any scientific overview – as a snapshot of the current knowledge, rather than as a definitive compilation. Indeed, as research continues, we surmise that the present guidelines will have to be extended and revised. For instance, other nuanced changes to - or even novel types of - RCD may emerge from continued efforts to characterize the multicellular character of yeast populations, including but not restricted to uncovering intercellular communication, interaction between populations or cellular differentiation within colonies and biofilms. Still, neologisms should be introduced with care and only when the characterization of a lethal process that bears new functional and biochemical aspects requires it. Otherwise, new expressions should be avoided to limit confusion.

Another crucial point is to acknowledge the inherent complexity and dynamic nature of RCD in general and its different subroutines in particular. In fact, it is the crosstalk between pro-life and pro-death signals that determines cellular fate, and the activation of pro-survival pathways (such as autophagy) may often accompany lethal signals. Also, stress conditions may activate different RCD subroutines that can be interconnected or may occur independently, sequentially, or in parallel. Indeed, the inhibition of one specific RCD modality might trigger backup mechanisms that still ensure cell death execution. It is thus important to keep these points in mind when classifying a lethal phenotype.

Altogether, the present guidelines attempt to unify the nomenclature and definition of yeast cell death modalities

and - in our opinion – will help other fields of unicellular research (e.g., bacteriology, parasitology, etc.) to establish their set of recommendations using the present one as a basis. We are convinced that some degree of linguistic and experimental standardization is necessary for facilitating communication among researchers, especially at a point where the existence of yeast RCD is scientifically accepted and its socioeconomical impact is ever growing.

AUTHOR CONTRIBUTIONS

DCG, MAB, LG, GK and FM conceived and wrote the manuscript. All authors participated in the critical reading of the manuscript, completed or approved the cited literature, approved the conclusions, and endorse the consensus of the herein defined terminology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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