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Review

Selective induction of programmed cell death using synthetic biology tools

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ABSTRACT

Regulated cell death (RCD) controls the removal of dispensable, infected or malignant cells, and is thus essential for development, homeostasis and immunity of multicellular organisms. Over the last years different forms of RCD have been described (among them apoptosis, necroptosis, pyroptosis and ferroptosis), and the cellular signaling pathways that control their induction and execution have been characterized at the molecular level. It has also become apparent that different forms of RCD differ in their capacity to elicit inflammation or an immune response, and that RCD pathways show a remarkable plasticity. Biochemical and genetic studies revealed that inhibition of a given pathway often results in the activation of back-up cell death mechanisms, highlighting close interconnectivity based on shared signaling components and the assembly of multivalent signaling platforms that can initiate different forms of RCD. Due to this interconnectivity and the pleiotropic effects of 'classical' cell death inducers, it is challenging to study RCD pathways in isolation. This has led to the development of tools based on synthetic biology that allow the targeted induction of RCD using chemogenetic or optogenetic methods. Here we discuss recent advances in the development of such toolset, highlighting their advantages and limitations, and their application for the study of RCD in cells and animals.

1. Introduction

Programmed or Regulated cell death (PCD, RCD respectively) is a fundamental biological process and a ubiquitous feature of cellular systems. While it is best studied in animals, regulated forms of cell death can be observed across all kingdoms of life, including plants, fungi, archea and bacteria [1]. In contrast to non-regulated cell death or necrosis, which results from extreme physical or chemical damage and manifests as a rapid non-selective loss of cellular integrity, RCD is initiated by specific extra- or intracellular signals and typically involves a tightly coordinated sequence of molecular and cellular events, which vary between different RCD modalities. In multi-cellular organisms, RCD contributes to multiple developmental programs, homeostatic cell turnover and tissue adaptation [2]. Moreover, RCD is crucial for the timely elimination of damaged, infected or otherwise compromised cells and is thus a key component of innate and adaptive immunity[3]. Dysregulation of cell death programs is implicated in many human diseases, including developmental disorders [4], cancer [5], chronic inflammation [6], multiple forms of neurodegeneration [7], and sepsis [8]. Given its importance, it is not surprising that in the past decades a tremendous effort has been devoted to understanding its molecular and physiological mechanisms and biological relevance across the various biological scales and model systems. Nonetheless, despite many advancements in understanding RCD, multiple cellular and mechanistic aspects of cell death and their immunological consequences are still incompletely understood. Currently, the main challenges are, I. the complexity and interconnectivity between different RCD pathways, II. the pleiotropic and variable effects of RCD on the adjacent tissues, III. the limitations of current methods for inducing selected forms of RCD, and IV. the limited methodology to distinguish different forms of RCD from each other. To overcome these challenges, it is necessary to develop on one hand new tools that enable highly controlled, spatio-temporal resolved and specific induction of different types of RCD both in cell culture and in live organisms. On the other hand, it requires novel methods to detect specific forms RCD with high sensitivity. In this review, we summarize and discuss recent developments in the selective induction of RCD using synthetic biology tools, and the advantages and limitations of their application.

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2. Major forms of RCD in mammals

While first observations of cell death in animals were already documented in the first half of the 19th century, it was not until mid-20th century that cell death began to be experimentally explored. Apoptosis, the first type of cell death to be recognized as regulated, or "programmed", was described first in 1972 [9] in the context of mammalian malignant neoplasms. Five decades later, we distinguish over twelve different RCD programs based on their morphological and mechanistic features, as well as specific circumstances of their occurrence [10]. For the detailed mechanistic characterization of all the different RCD modalities, we refer the reader to excellent reviews of this topic [10]. Below, we provide a brief description of apoptosis, pyroptosis, necroptosis and ferroptosis as the most frequently studied forms of RCD.

2.1. Apoptosis

Apoptosis is the best-studied RCD pathway and presumed to account for most of the developmental and homeostatic cell death in animals [11]. Morphologically, apoptotic cells are characterized by shrinkage and the formation of apoptotic blebs and apoptotic bodies, the disassembly and degradation of intracellular structures and organelles and the shutdown of cellular functions [12]. A hallmark of apoptosis is the maintenance of plasma membrane integrity, which distinguishes it from many other forms of RCD, and facilitates a "silent" and non-inflammatory removal of apoptotic corpses via expulsion [13] or efferocytosis (effective clearance of apoptotic cells by professional and non-professional phagocytes) [14]. It is important though to note that if apoptotic cells are not cleared by phagocytes, they will eventually loose plasma membrane integrity, a process often referred to as secondary necrosis. Two main pathways can induce apoptosis (Fig. 1**A**): the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway [15]. These two signaling axes control the activation of apoptotic caspases, a group of cysteine proteases that control apoptosis execution.

Intrinsic apoptosis is induced in response to various cellular stressors, among them nutrient depletion, loss of pro-survival factors and irreparable DNA damage. These signals activate pro-apoptotic members of the BCL-2 protein family that can inhibit pro-survival BCL-2 family members. This results in the activation of the BCL-2 proteins BAX, BAK and BOK, which form ring-shaped oligomeric pores in the mitochondrial outer membrane, leading to mitochondrial outer membrane permeabilization (MOMP) as a key signaling checkpoint [16–18]. MOMP releases several mitochondrial intermembrane proteins into the cytosol, among them cytochrome c, second mitochondrial activator of caspase (SMAC, or DIABLO) and OMI [19]. Cytochrome c is essential for inducing intrinsic apoptosis, as it binds APAF1 (Apoptotic protease activating factor 1) and thus initiates the formation of a multimeric complex (apoptosome) [20,21], which acts as a platform for the recruitment and activation of the initiator caspase-9 [22]. SMAC and OMI promote apoptosis induction by antagonizing cellular inhibitors of apoptosis (cIAP1/2, XIAP), which inhibit caspase activity [23,24]. Extrinsic apoptosis on the other hand, is triggered by the ligand-induced oligomerization of a subset of membrane bound death receptor family members, such as TNFR1, FAS, and TRAIL. Death receptors feature a cytosolic death domain (DD), that allows the recruitment of the adaptor FADD and the apoptotic initiator caspase-8, forming the caspase-activating DISC (death inducing signaling complex) [25]. Signaling via TNF results initially in the formation of the pro-survival complex-I consisting of TNFR1, TRADD, TRAF2, RIPK1 and the E3 Ubiquitin ligases cIAP1 and cIAP2. Within this complex, RIPK1 and other proteins are conjugated with Ubiquitin linkages, which allows the recruitment of the kinase complex TAK1/TAB2/TAB3 and the LUBAC (linear Ub chain assembly complex) complex. Further linear ubiquitination stabilizes the complex and allows an NF-κB-dependent upregulation of the anti-apoptotic genes [26]. Disrupting ubiquitination of complex I results in its dissociation and the formation of the cytosolic complex-IIa/b that include RIPK1, TRADD, FADD and caspase-8. Once activated, caspase-8 and − 9 converge on the activation of the effector caspases-3, − 6 and − 7 that cleave multiple downstream substrates [27], together responsible for apoptosis execution [28], corpse disassembly [29] and apoptosis-induced anti-inflammatory signaling [30]. Besides effector caspases, activated caspase-8 can also cleave Bid, additionally engaging a mitochondrial pro-apoptotic signaling [31].

2.2. Necroptosis

Necroptosis was originally discovered as a necrotic cell death that is induced by TNF-α if caspase-8 is absent or caspase activity is inhibited. Under these conditions, RIPK1 recruits RIPK3 to form a signaling complex known as necrosome (Fig. 1**B**). Other receptors, such as TLR4, TLR3 and ZBP1 (DAI), can trigger necroptosis as well, as they also contain a RHIM-domain that allows RIPK3 recruitment. Activation of RIPK3 allows the phosphorylation and activation of the necroptosis executioner, the pseudokinase MLKL [32,33]. MLKL consists of two domains, with the C-terminal domain acting as autoinhibitory relative to the N-terminal 4 helix bundle (4HB) and two brace helices [34]. MLKL phosphorylation results in confirmational switch and exposure of the 4HB, that allows the protein to translocate to the plasma membrane where the 4HB inserts and oligomerizes [35–37]. The mechanism of MLKL-mediated plasma membrane rupture is still incompletely understood, as it is hypothesized to either do it directly [38,39] or via interaction with the downstream partners, such as TRPM7 [35]. Intriguingly, in contrast to the other forms of regulated necrosis, MLKL-induced membrane rupture is only partially dependent on ninjurin-1 (NINJ1), a protein that executes plasma membrane rupture during pyroptosis (see below) [40], and often occurs at the MLKL "hotspots" [41] suggesting that MLKL can act itself as a membrane lysing protein.

2.3. Pyroptosis

Pyroptosis is best known as a form of cell death activated by inflammasomes, signaling complexes formed by pattern recognition receptor upon the detection of invading pathogens or endogenous danger signals (Fig. 1**C**) [42,43]. Canonical inflammasomes activate caspase-1 and are assembled by the proteins Pyrin, AIM2, CARD8 or members of the NOD like receptor family (NLR). Activation of these receptors leads to their oligomerization and subsequent recruitment of adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD), creating a multimeric signal-amplifying caspase-1-activating platform [44] like the apoptosome. A non-canonical inflammasome pathway exists as well and it comprises human caspase-4 (−5) or mouse caspase-11 that are activated by bacterial LPS [45,46]. Caspase-1, − 4, − 5, or − 11 induce pyroptosis by cleaving gasdermin-D (GSDMD) within its interdomain linker leading to the separation of the cytotoxic N-terminus from the regulatory C-terminal domain. The gasdermin N-terminus targets the plasma membrane and forms large β-barrel pores with an internal diameter of approximately 21.5 nm [47,48]. These pores are large enough to leak cytosolic content, among them IL-1 family cytokines. A hallmark of pyroptosis is plasma membrane rupture and cell lysis, which requires additionally NINJ1 [40]. NINJ1 oligomerizes to form amphipathic filaments that rupture the membrane [49], yet the exact signal that triggers NINJ1 activation remains unknown. GSDMD shares the pore-forming ability with other gasdermin family members, which have been shown to be activated by a variety of different proteases such as such as caspase-8 [50,51], granzymes [52,53], neutrophil elastase [54] and cathepsin G [55], and even bacterial proteases [56], expanding the scope of mechanisms and circumstances under which the pyroptosis can occur. The recent discovery of bacterial and fungal gasdermin-like proteins [57] even suggests that the use of pore-forming proteins for induction of cell death is evolutionarily conserved and that pyroptosis might be one of the most ancient forms of RCD.

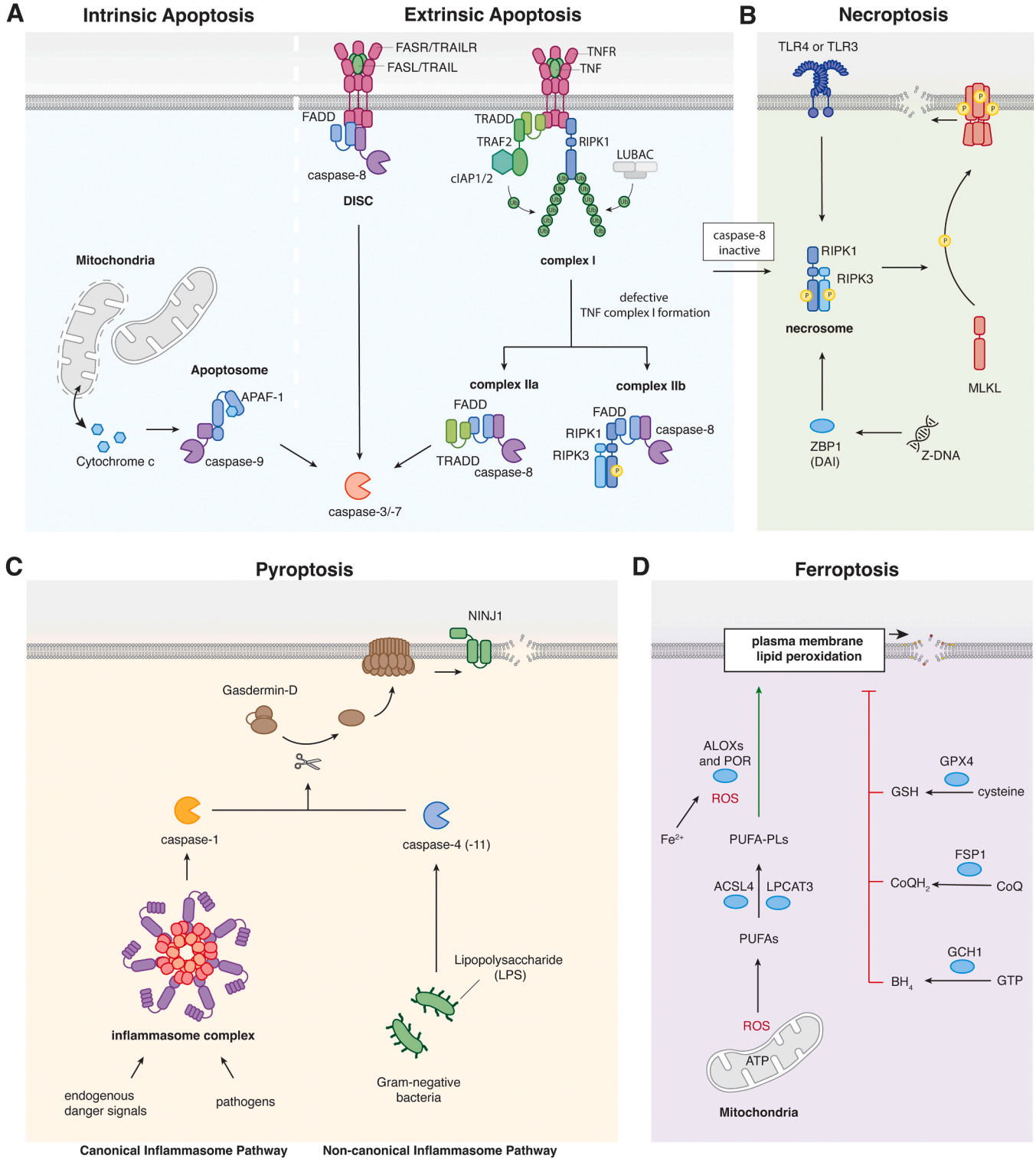

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Fig. 1. Major cell death pathways in mammals. A. Extrinsic apoptosis can be induced by the death-inducing signaling complex (DISC) formed after FAS-TRAIL receptor ligation, or by TNF complex IIa/b if complex I formation is defective. Intrinsic apoptosis is triggered after the release of cytochrome c from mitochondria and the assembly of an apoptosome complex. Initiator caspases-8 and − 9 cleave caspase-3/− 7 to drive apoptosis. **B.** Necroptosis is induced under conditions when caspase-8 in inactive and involves the formation of the necrosome complex downstream of TNF treatment, TLR3 or TLR4 activation, or upon detection of Z-DNA by the sensor ZBP1. Phosphorylation and activation of RIPK3 in the necrosome allows the phosphorylation of MLKL to induce plasma membrane rupture. **C.** Sensing of endogenous or pathogen-derived stimuli by canonical or non-canonical inflammasomes controls the activation of caspase-1 or − 4 (−11). These caspases process GSDMD to yield an N-terminal fragment that forms plasma membrane pores to induce pyroptosis. Plasma membrane rupture requires in addition NINJ1 oligomerization. **D.** Ferroptosis is induced by iron-dependent peroxidation of PUFA (Polyunsaturated fatty acids)-containing phospholipids (PUFA-PLs) and leads to plasma membrane lesions and rupture. Lipid peroxidation is counteracted by several detoxifying systems that can protect against ferroptosis. FASR, FAS-Receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor; FADD, Fas Associated Via Death Domain; TRADD, TNFRSF1A Associated Via Death Domain; TNF, Tumor Necrosis Factor; TNFR, TNF receptor; TRAF2, TNF Receptor Associated Factor 2; RIPK1, Receptor-Interacting Protein Kinase; LUBAC, linear ubiquitin chain assembly complex; APAF-1, Apoptotic protease activating factor 1; MLKL, mixed lineage kinase like; TLR, Toll-like receptor; ZBP1, Z-DNA-binding protein 1; DAI, DNA-dependent activator of IFN-regulatory factors; NINJ1, ninjurin-1; ROS, reactive oxygen species; PUFA, Polyunsaturated fatty acids; ALOX, arachidonate lipoxygenase; POR, Cytochrome P450 reductase; GPX4, Glutathione peroxidase 4; GSH, Glutathione; FSP1, ferroptosis suppressor protein 1; GCH1, GTP cyclohydrolase I; BH4, tetrahydrobiopterin; CoQ, coenzyme Q10; ACSL4, cyl-CoA Synthetase Long Chain Family Member 4; LPCAT3, Lysophosphatidylcholine Acyltransferase 3.

2.4. Ferroptosis

Ferroptosis is another recently characterized type of regulated necrosis and is characterized by the accumulation of lipid peroxides and iron-dependent reactive oxygen species (ROS), which ultimately lead to oxidative membrane damage and cell death (Fig. 1**D**). In contrast to other forms of RCD, ferroptosis still lacks a well-defined molecular mechanism of execution and is thought to rather occur because of the loss of activity of the cellular anti-oxidation systems, such as Glutathione and Glutathione Peroxidase 4 (GPX4), Ferroportin and Iron Regulatory Protein 2, leading to the accumulation of peroxidized lipid, which eventually leads to cell lysis [58]. Similarly to other forms of necrotic cell death, ferroptotic cells are characterized by membrane permeabilization and membrane scrambling, a loss of metabolic activity and release DAMPs, such as ATP, HMGB1 and others, as well as oxidized lipids [59]. The pathophysiological role and tissue-level consequences of ferroptosis remain controversial, as different studies found it to exhibit both pro-inflammatory and pro-immunogenic [60] or anti-immunogenic [61] effects.

2.5. Other forms of cell death

Besides the four major forms of cell death described above, there are other types of death defined by either their cell type specificity (NETosis, eryptosis, cornification)[62], their distinctive molecular or cellular features (methuosis, parthanatos, lysosomal cell death, autophagy-mediated cell death) or circumstances of occurrence (entosis, phagoptosis, corneoptosis). Despite their unique features, these RCD forms still often converge on one or several of the major molecular pathways described above. For a recent review on these types of cell death we refer the reader to [10].

3. Plasticity and interconnectivity in cell death signaling

While a clear distinction between different RCD programs has historically been useful for experimental and clinical studies, it does not reflect the mechanistic crosstalk and interconnectivity between different RCD pathways that has been shown to exist in tissues or sometimes even within individual cells. The examples of such cross-talk include the engagement of the NLRP3 inflammasome in apoptotic [50] or necroptotic cells [63]; cleavage of gasdermin family members (such as GSDMD, GSDME [64] and GSDMC [65]) by apoptotic caspases; the activation of the apoptotic caspases − 3 and − 7 downstream of caspase-1 in cells treated with inflammasome activators; recruitment and activation of caspase-8 at the ASC speck [66,67], and caspase-3/7-mediated inactivation of GSDMD during apoptosis [68]. Even more intriguingly, catalytically inactive caspase-8 can also serve as a scaffold to directly promote ASC nucleation and inflammasome assembly [69,70], and more examples involving also other forms of RCD are likely to be found in the future. While it is not clear why such plasticity and interconnectivity exist, it is plausible to speculate that it could have evolved as a backup mechanism to ensure that a cell is eliminated even if one of the primary pathways is blocked, as it can for example occur during infections with viral pathogens, many of which encode inhibitors of RCD [71]. Alternatively, it could also represent a means to fine-tune cell death-associated proinflammatory signaling based on the threat level [72].

Our understanding of cell death is further complicated by the growing evidence demonstrating a non-lethal engagement of RCD pathways in different pathological or physiological circumstances, and observations showing that cells can progress surprisingly far into cell death pathways before reaching a “point of no return”. For example, while MOMP and apoptotic caspase activation was originally thought to proceed in a one-or-none manner [73,74], it is becoming increasingly clear that cells can survive a substantial degree of caspase activation and associated damage and revert to normal morphology after the withdrawal of the apoptotic stimulus - a process termed anastasis [75]. This survival is not specific to apoptosis, as necrotic cells can also rescue themselves by engaging various membrane repair systems [76–80]. Additionally, it was reported that cell death pathways can be activated without any apparent loss of cell viability in some cell types. Neutrophils, for example, engage canonical inflammasome and gasdermins for IL-1β secretion without lysing [81,82]. Moreover, apoptotic caspases have been shown to regulate immune and inflammatory responses, proliferation, cell fate determination and differentiation, and cellular and cytoskeletal remodeling even without inducing cell death [83]. In viable cells, caspase-8 activity is essential for putting a brake on necroptosis [84], and both its enzymatic and non-enzymatic activity contributes to the regulation of NF-kB signaling and gene expression independently of apoptosis [85,86]. Increasing evidence also suggests that necrotic cell death effectors ca have non-lethal functions. Examples include gasdermin pores as mediators of unconventional protein secretion, mucus secretion and in modulating actin cytoskeleton functions [87] and MLKL in regulating vesicular trafficking and secretion [88]. How the balance between the death-related and physiological functions of cell death machinery is achieved and what mechanisms define the switch between cell death and cell survival remains to be determined.

4. Tools for cell specific manipulation of different cell death pathways

Experimental cell death studies often require the targeted induction of a selected form of cell death and use genetic or pharmacological interference to reveal its molecular mechanisms or role in a specific experimental model. In a clinical context, induction of cell death is used for targeted cell elimination (for example killing cancer cells or HIV-infected cells) or introduced as safety switch for cell therapies. This creates a need for novel methods to specifically induce the RCD of choice

Table 1
Summary of chemogenetic tools for cell death induction.

Category	Tool	Target or effector protein	Type of cell death	References
PROTAC	N/A	Bcl-XL	Apoptosis	[90]
		PARP1	Apoptosis	[91,92]
		GPX4	Ferroptosis	[93]
Inducible exporession	Tet-ON	revCaspase-3	Apoptosis	[96,97]
		tBID	Apoptosis	[98]
		BIM	Apoptosis	[99]
		BAX	Apoptosis	[100]
		Gasdermin N-termini	Pyroptosis	[48]
		ASC	Pyroptosis	[103]
		MLKL N-terminus	Necroptosis	[34]
Chemically inducible dimerization	FKBP*	FAS	Apoptosis	[105-108]
		BAX	Apoptosis	[109]
		FADD	Apoptosis, necroptosis	[99,110,111]
		caspase-2	Apoptosis	[112]
		caspase-3	Apoptosis	[106,113]
		caspase-8	Apoptosis	[102,103,114,115-119]
		caspase-9	Apoptosis	[106]
		caspase-10	Apoptosis	[106,120,121]
		caspase-1	Pyroptosis	[77,117,106,122]
		caspase-11	Pyroptosis	[77,123,124]
		RIPK1	Necroptosis	[125]
		RIPK3	Necroptosis	[126,127,125,110]
		MLKL	Necroptosis	[39,76]
	Gyrase	FADD	Apoptosis	[99]
		caspase-1	Pyroptosis	[128]
		RIPK1	Necroptosis	[129]
		RIPK3	Necroptosis	[129]
		MLKL	Necroptosis	[130]
	Cyclosphilin	FAS	Apoptosis	[105]

in selected cells, ideally without affecting bystander cells. The earliest attempts to induce targeted cell death often involved rather harsh physical or chemical treatments [89], however later many much more selective triggers have been identified. The commonly used agonists include 1) pharmacological compounds, which either directly trigger some form of cellular damage or inhibit a process crucial for the maintenance of cell viability (for example, BCL2 inhibitor venetoclax for apoptosis or GPX4 inhibitors for ferroptosis induction) 2) biologicals, such as antibodies and monobodies used to cross-link and activate death receptor or to block pro-survival signals; cytokines and signaling factors (such as TNF and FasL that trigger extrinsic apoptosis) that induce endogenous cell death pathways; 3) purified bacterial toxins and virulence factors or live pathogens, often used in studies of pathogen-induced cell death and cell-autonomous innate immunity; 4) physical factors, such as UV irradiation, temperature and mechanical stress, and 5) depletion of growth factors or nutrients. A separate category of tools includes targeted proteosomal degradation of the anti-apoptotic proteins or proteins crucial for cell survival, such as Bcl-Xl [90], PARP1 [91,92] and GPX4 [93] that has been used to induce cancer cell apoptosis and is currently explored for therapeutic applications.

Since the abovementioned triggers generally lack specificity, several strategies for more selective cell elimination have been introduced, such as antibodies that bind to the specific surface antigens on the target cells, or diphtheria toxin which can selectively deplete the cells expressing the diphtheria-toxin receptor under tissue-specific promoters [94]. Nevertheless, commonly used inducers of cell death lack the desired spatio-temporal precision, display pleiotropic or off-target effects, or trigger several forms of death simultaneously, which makes it difficult to study a given cell death modality in isolation [95]. These limitations thus prompted the development of many more controlled “clean” cell death induction methods based on genetic, chemogenetic and optogenetic approaches.

4.1. Chemogenetic approaches for cell death induction

4.1.1. Inducible expression of cell death effectors

Chemogenetic approaches usually involve the expression of so-called a RASSL (receptor activated solely by a synthetic ligand) or a DREADD (designer receptor exclusively activated by designer drugs) (summarized in Table 1), i.e. artificially engineered protein receptors whose activity can be modulated by a specific small molecule compounds. In their most basic form, chemogenetic tools enable chemically induced expression of an active cell death effector, using doxycycline- or tamoxifen-inducible promoters, as done for the active form of caspase-3 [96,97], tBID [98], BIM [99] and BAX [100], or the N-termini of gasdermins [48] and MLKL [34] (Fig. 2**A**). This group of tools has been extensively utilized to reveal the roles and regulation of these proteins and downstream events during the respective forms of cell death both in vitro and in vivo. For example, inducible expression of the uninhibited N-terminal domains of different gasdermins [48] was instrumental for demonstrating their pore-forming activity, and later for uncovering their complex post-translational regulation [101,102]. Inducible overexpression of ASC that results in its spontaneous oligomerization, has revealed insights into inflammasome assembly and its consequences in vivo [103]. The major disadvantages of these systems include limited temporal control, since cell death induction depends on a gradual accumulation of the expressed protein, and the absence of an “off switch” that would allow a rapid inactivation of the expressed protein. The prolonged accumulation of the active effectors and gradually increasing cellular damage may lead to experimental artifacts, as it can induce cellular stresses that are not present if the pathway is engaged by classical activators. It may thus significantly affect the cellular state, leading to the altered repertoire of signaling factors released during cell death. Additionally, engagement of the various repair and adaptation systems could lead to inefficient or incomplete cell death execution, potentiating caspase-mediated DNA damage and oncogenicity [104].

4.1.2. Chemically induced dimerization

More advanced chemogenetic approaches enable post-translational manipulation of target proteins, offering substantial advantages both in terms of improved dose dependency and kinetics, as it is now defined almost exclusively by the rate of ligand diffusion (seconds to minutes) rather than protein expression and accumulation (hours). In addition, such systems offer reversibility that can be achieved by the ligand

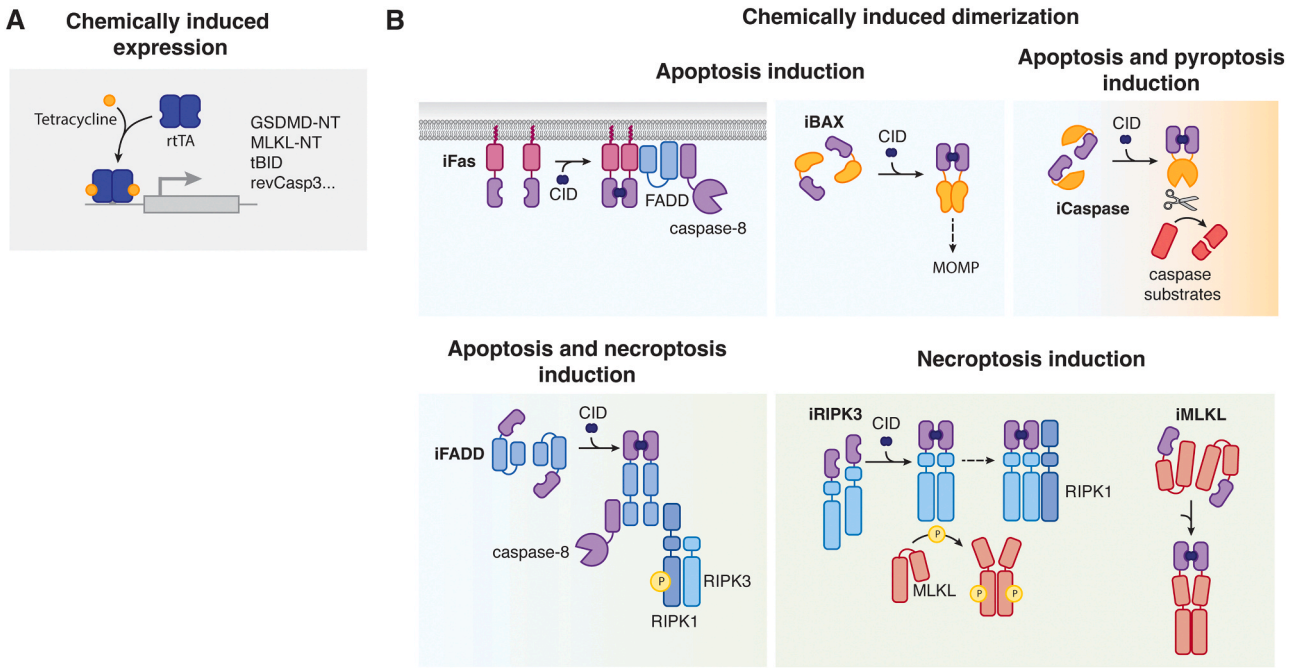

Fig. 2. Chemogenetic approaches for the induction of cell death. A. Inducible expression of active cell death effectors, such as $GSDMD^{NT}$, MLKL-NT, tBID etc., can be used to induce specific forms of cell death. **B.** Chemically induced dimerization as a tool for cell death induction. Fusion of chemically induced dimerization (CID) modules to specific components of cell death pathways (iFas, iBAX, iCaspases, iFADD, iRIPK3 and iMLKL) enables their oligomerization with small chemical compounds (CIDs) leading to their activation and the initiation of downstream signaling events.

removal or competitive autoinhibition. Such protein manipulation strategies include chemically induced proximity, protein re-localization and trapping, or the release from an inactive precursor. Given the central role of higher order multimerization in cell death and innate immune signaling, the former strategy is particularly useful when it comes to the initiation of cell death as forced oligomerization can induce the activation of cell death effectors. Most often this is achieved by fusing the effector part of the protein to a chemically induced dimerization (CID) module that can be then forcibly homo- or heterodimerized using small cell-permeable molecules (dimerizers) (Fig. 2**B**). The most widely used CIDs are based on mutated FKBP or FRB domains dimerized by rapamycin or its biologically inert synthetic derivates, with latter enabling CIDs to function orthogonally to endogenous signaling pathways [131]. Other, although less commonly used, CID systems include abscisic [114], gibberellic [115] and vanillic acid [132] and synthetic agrochemical mandipropamid [133] for plant or bacterial-derived domain dimerization, coumermycin-induced homodimerization of bacterial Gyrase-B [134], cyclophilin dimerization by cyclosporin A [105], ABT-737-driven dimerization of Bcl-Xl with a recombinantly expressed antibody [135], and SNAP-tag and HALO-tag based systems [136]. The current efforts to expand the CID repertoire rely on directed evolution and random mutagenesis [137], computational design of novel ligand-binding interfaces [138], or combining several CID modules [139] to enhance the system's robustness and biorthogonality. Additionally, recent advancements in photochemistry resulted in the development of photoactivatable CIDs [140–142], providing an additional level of control via patterned illumination. Due to their highly modular design, CID systems can be easily adapted to control a variety of biological targets, with applications ranging from split protein assembly to regulation of transcription and translation, cell signaling, vesicular trafficking and cell migration [143]. In cell death studies, both FKBP-based and gyrase-based CIDs have been first employed to decipher the role of induced proximity in apoptotic and inflammatory caspase activation [116,117,106,123,121]] and, later, to provide further mechanistic insights into caspase biology and downstream events [118, 119,122,124,120,128]. This approach was also applied to demonstrate the role of homotypic interactions in the activation of necroptotic effectors RIPK3 and MLKL [39,126,129,130,127,125], and the role of BAX dimerization in its mitochondrial translocation and in bypassing of BCL-XL inhibition [109]. Finally, on a more upstream level, a similar approach has been applied to dimerize the cytosolic Fas domains for inducible apoptosis [105,117,107,108], and chemically forced dimerization of FADD was also shown to be sufficient to initiate recruitment of RIPK1 and RIPK3, necrosome assembly and necroptosis [99,110]. Interestingly, selective incorporation of FADD death (DD) or death effector (DED) domains in such system enable to convert it into more selective apoptosis or necroptosis-initiating platform [111].

While most of the CIDs enable homo- or heterodimerization of their targets, this is insufficient to achieve the activation of some proteins, such as inflammasome sensors, which require a higher-order multimerization to initiate downstream signaling. An alternative is the use of trimerization systems, such as foldon (the natural trimerization domain of T4 fibritin), which was for example shown to be sufficient to oligomerize the $NLRP3^{PYD}$ for the induction of downstream IL-1β processing and pyroptosis [144]. An alternative approach, which is yet to be explored, could involve the combination of several dimerization modules into a single polypeptide, that could also facilitate higher-order protein-protein interactions of their targets. CID systems that utilize biorthogonal ligands offer several significant advantages, including minimal background cytotoxicity compared to cell ablation systems based on transcriptional induction [113], rapid kinetics of cell death following ligand administration and a reduced collateral damage to bystander cells [145,146].

4.2. *Using light to induce cell death*

4.2.1. *Laser ablation and photosensitizers*

Despite the versatility of chemogenetic approaches, they offer only limited spatiotemporal resolution and reversibility, and are limited by dimerizer administration and its diffusion, in particular in vivo. In contrast, light can be easily and rapidly delivered to biological samples using widely available microscope setups and inexpensive custom-made

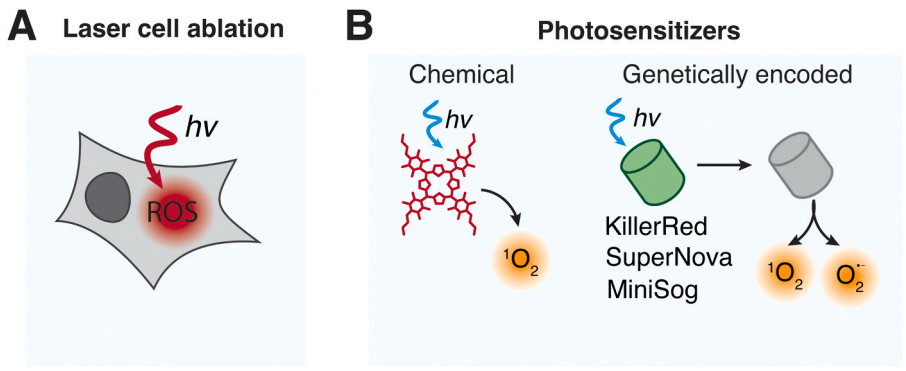

Fig. 3. Using light to induce cell death. A. Short pulses of high-intensity UV or near-infrared (NIR) lasers can be used to specifically kill cells (also known as laser ablation). **B.** Laser ablation can be enhanced by chemical or genetically encoded photosensitizers.

hardware. Unlike chemical approaches, it can be patterned or targeted to the specific regions of interest, enabling targeting specific cells or subcellular locations. Early studies took advantage of the well-known property of the ultraviolet (UV) light to induce apoptosis to study biological processes such as cell extrusion [147]. However, these early attempts were characterized by the random pattern of apoptosis induction within the population, prompting the development of the more selective tools which enable targeting of specific cells together with the simultaneous time-resolved monitoring of their environment. Some attempts to overcome these initial limitations relied on patterned illumination using specially adapted hardware [148], while other involved targeted killing of selected cells or cell populations using short pulses of high-intensity UV [149] or near-infrared (NIR) [150,151] lasers (also known as laser ablation) (Fig. 3**A**). The mechanisms of laser-induced cell death are unclear and potentially involve ROS production [151,152] and mitochondrial and nuclear DNA damage [153], or direct membrane permeabilization [154]. While being often referred to as "apoptosis--like", the dying cells typically display a mix of both apoptotic and necrotic features, such as membrane blebbing, mitochondrial fragmentation and caspase activation [149,152,155] coupled to the rapid calcium influx and loss of membrane integrity [151,152], making it challenging to pinpoint the exact cell death pathway that is activated and delineate its consequences for the neighbors. High-intensity laser beams can also induce collateral damage in adjacent cells, further complicating the data interpretation and limiting their application in the deeper tissues or non-transparent samples.

While due to its relative technical simplicity and spatiotemporal flexibility these laser-assisted techniques are still sometimes employed in cell death and wound healing studies, their limitations prompted the development of more specific methods which limit the damage to the neighboring cells. Chemical photosensitizers, such as organic and organometallic compounds and nanoparticles, produce cytotoxic ROS species in response to illumination, enabling a more selective killing of the cells with the less intense light [156] – a property which makes them highly attractive targets for the development of photodynamic therapies [157]. Moreover, advances in fluorescent protein engineering resulted in the development of genetically encoded photosensitizers (Fig. 3**B**), such as Killer Red [158], miniSOG [159] and SuperNova Green [160]. These proteins are structurally close to other fluorescent proteins or flavoproteins and, similarly to chemical photosensitizers, are not cytotoxic on their own but bear an enhanced capacity to generate ROS upon illumination - a property that can be harnessed for ROS-mediated inactivation of specific intracellular targets [161], or, at the higher dose, for inducing ROS-dependent cytotoxicity and cell death. As with other genetically encoded tools, these photosensitizers can be expressed under cell-type specific promoters and targeted to different intracellular compartments using specific localization sequences, which makes them suitable both for single-cell biology and targeted cell ablation in live animals. However, as for laser ablation and chemical photosensitizers, the mechanisms of such ROS-induced cell death are still insufficiently characterized and highly pleiotropic [162], therefore not suitable for studying specific types of cell death.

4.2.2. Optogenetics

Optogenetics is an umbrella term encompassing a fraction of synthetic biology tools which include a variety of plant, bacterial and animal photoreceptors that have been repurposed or re-engineered to control specific protein or signaling functions. The modern optogenetic toolbox contains tools that can be activated by various excitation wavelengths, and that have distinct modes of actions (summarized in Table 2), thus enabling a light-mediated control of a wide variety of cellular processes. Some of these tools combine sensory and effector function within a single photosensitive domain, enabling interference with or direct activation of endogenous signaling networks (Fig. 4**A**). Among these are type I rhodopsins (bacteriorhodopsins, halorhodopsins and channelrhodopsins), transmembrane light-gated ion channels, that derive from algae and prokaryotes and were subjected to extensive protein engineering to alter their wavelength sensitivity and ion selectivity (the recent advances in rhodopsin-based optogenetics are reviewed in [163]). While they are most used in neurophysiology and behavioral studies to locally activate or inhibit neuronal circuits [164–166], they are increasingly applied in other areas of biology to manipulate transmembrane ionic fluxes both in excitable and non-excitable cells. Type II rhodopsins are G protein-coupled receptors (GPCRs) that derive from animals, where they mediate visual perception by activating G protein-mediated signaling – a function that can be transferred to other cell types or species (reviewed in [163]). Chimeric optoXRs combine photosensitive part of rhodopsins with the intracellular loops of endogenous GPCRs, enabling de novo light-induced activation of the respective signaling pathways ([167,168], the further advances reviewed in [169]). Finally, light-activated cytoplasmic enzymes, such as adenylyl cyclases [170–174], guanylyl cyclases [171, 175] and phosphodiesterases [176–178], can be used to directly manipulate intracellular levels of second messengers.

Other optogenetic tools do not have a signaling function on their own but enable to control a protein of interest (POI) by manipulating its intracellular localization, protein-protein interactions, or activity. Optogenetic control of protein-protein interactions is achieved via light-induced di- or oligomerization of Cryptochromes (including the original Cry2-CIB1 [195,196] heterooligomer and Cry2 versions Cry2olig [193] and Cry2clust [194] with enhanced homooligomerization), various Light-Oxygen-Voltage-sensing (LOV) domain proteins [189,200] including Vivid (VVD) [181], Magnets [186], TULIPs [188] and EL222 [179,180], UV-sensitive receptors such as UVR8 [185,210,211], and far-red sensitive plant and bacterial Phytochromes [212,190,191,183, 192,184] (Fig. 4**B**). Localizing one of the heterodimer partners to the specific intracellular location also provides the possibility to light-dependently alter POI localization [195,212,187]. Moreover,

Table 2
Summary of optogenetic tools and their modes of action.

Category	Mode of action	Name	Origin	λ on (nm)	λ off (nm)	Cofactor	References
Light-activated ion channels and pumps	**Type I rhodopsins**	Channelrhodopsins	algae, procaryotes	various	dark	retinal	reviewed in [163]
Light-activated GPCR	**Type II rhodopsins**	Mammalian Opsins	animal origin	various	dark	retinal	reviewed in [163]
		OptoXRs	synthetic	various	dark	retinal	[167–169]
Light-activated enzymes	**Light-activated adenylate cyclases**	cPAC	*Microcoleus sp. PCC 7113*	410	520	PCB	[173]
		BlgC	*Beggiatoa sp.*	450	dark	FAD or FMN	[171]
		bPAC	*Beggiatoa sp.*	450	dark	FAD or FMN	[170]
		NgPAC	*Naegleria gruberi*	450	dark	FAD	[174]
		euPAC	*Euglena gracilis*	450	dark	FAD or FMN	[172]
	Light-activated guanylate cyclases	BlcG	*Beggiatoa sp.*	450	dark	FAD or FMN	[171]
		BphG	*Rhodobacter sphaeroides*	660	760	Biliverdin	[175]
	Light-activated phosphodiesterases	EB1	*Magnetococcus marinus*	450	dark	FAD	[176]
		BlrP1	*Klebsiella pneumoniae*	450	dark	FAD or FMN	[177]
		LAPD	*Deinococcus radiodurans**	650	750	Biliverdin	[178]
Light-activated protein-protein interactions and protein recruitment	**Homodimerization**	EL222	*Erythrobacter litoralis*	450	dark	FMN	[179,180]
		Vivid (VVD)	*Neurospora crassa*	450	dark	FMN or FAD	[181]
		YtvA	*Bacillus subtilis*	450	dark	FMN, FAD or RF	[182]
		iLight (IsPadC–PCM)	*Idiomarina sp. A28L*	660	760	Biliverdin	[183]
		Cph1	*Synechocystis sp. PCC 6803*	660	740	PCB	[184]
	Heterodimerization	UVR8/COP1	*Arabidopsis thaliana*	300	dark	n.a.	[90,185]
		Magnets (pMag/nMag)	*Neurospora crassa*	450	dark	FAD	[186]
		iLID (AsLOV2–SsrA/SspB)	*Avena sativa*	450	dark	FMN	[187]
		TULIP (LOVpep/ePDZ)	*Avena sativa*	450	dark	FMN	[188]
		FKF1/GI	*Arabidopsis thaliana*	450	dark	FMN	[189]
		PhyB/PIF3/6	*Arabidopsis thaliana*	660	740	PCB	[190]
		BphP1/PpsR2	*Rhodopseudomonas palustris*	760	dark / 640	Biliverdin	[191]
		BphP1/Q-PAS1	*Rhodopseudomonas palustris*	760	dark / 640	Biliverdin	[192]
	Homooligomerization	Cry2olig, Cry2clust	*Arabidopsis thaliana*	450	dark	FAD	[193,194]
	Heterooligomerization	Cry2/CIB1	*Arabidopsis thaliana*	450	dark	FAD	[195,196]
	Multimer dissociation	PixD/PixE	*Synechocystis sp. PCC6803*	450	dark	FAD or FMN	[197,198]
		UVR8/UVR8	*Arabidopsis thaliana*	300	dark	n.a.	[185]
		LOVTRAP (AsLOV2/Zdk)	*Avena sativa*	450	dark	FMN	[199]
		RsLOV	*Rhodobacter sphaeroides*	450	dark	FMN	[200]
		Dronpa*	*Pectiniidae*	500	400	n.a.	[201,202]
		ttCBD	*Thermus thermophilus*	545	dark	cobalamin derivates	[203]
		MxCBD	*Myxococcus xanthus*	545	dark	cobalamin derivates	[203]
Allosteric protein regulation	**Uncaging by multimer dissociation**	Dronpa	*Pectiniidae*	500	400	n.a.	[202]
	Uncaging by intramolecular conformational change	AsLOV2	*Avena sativa*	450	dark	FMN	[204,205,182,206]
		cpLOV2	*Avena sativa*	450	dark	FMN	[207]
		PYP	*Halorhodospira halophila*	450	dark	p-coumaric acid	[208]
Light-induced proteolysis	**Photocleavable protein**	PhoCl	*Clavularia sp.*	380	n.a.	n.a.	[209]

UVR8 [185], BLUF proteins [197,198], Cobalamin-binding domains [203] and some LOV2-based systems, such as LOVTRAP [199] and RsLOV [200] provides control over POI activity via light-induced protein complex dissociation (Fig. 4**C**). Another level of optogenetic control over POI can be gained via light-induced uncaging of effector POI domains (Fig. 4**D-E**). These approaches usually utilize LOV domain proteins [204,205,182,206,207], photoactive yellow protein (PYP) [208] or Dronpa [201,202], which allosterically inhibit POI it in the dark but rapidly dissociating following illumination, exposing POI effector domain and allowing its activity. Finally, the last group of tools encompasses photocleavable protein PhoCl [209], which undergoes autoproteolysis and dissociation following violet light exposure, enabling light-dependent release or proteolysis-dependent activation of some targets (Fig. 4**F**). These tools and the molecular mechanisms of

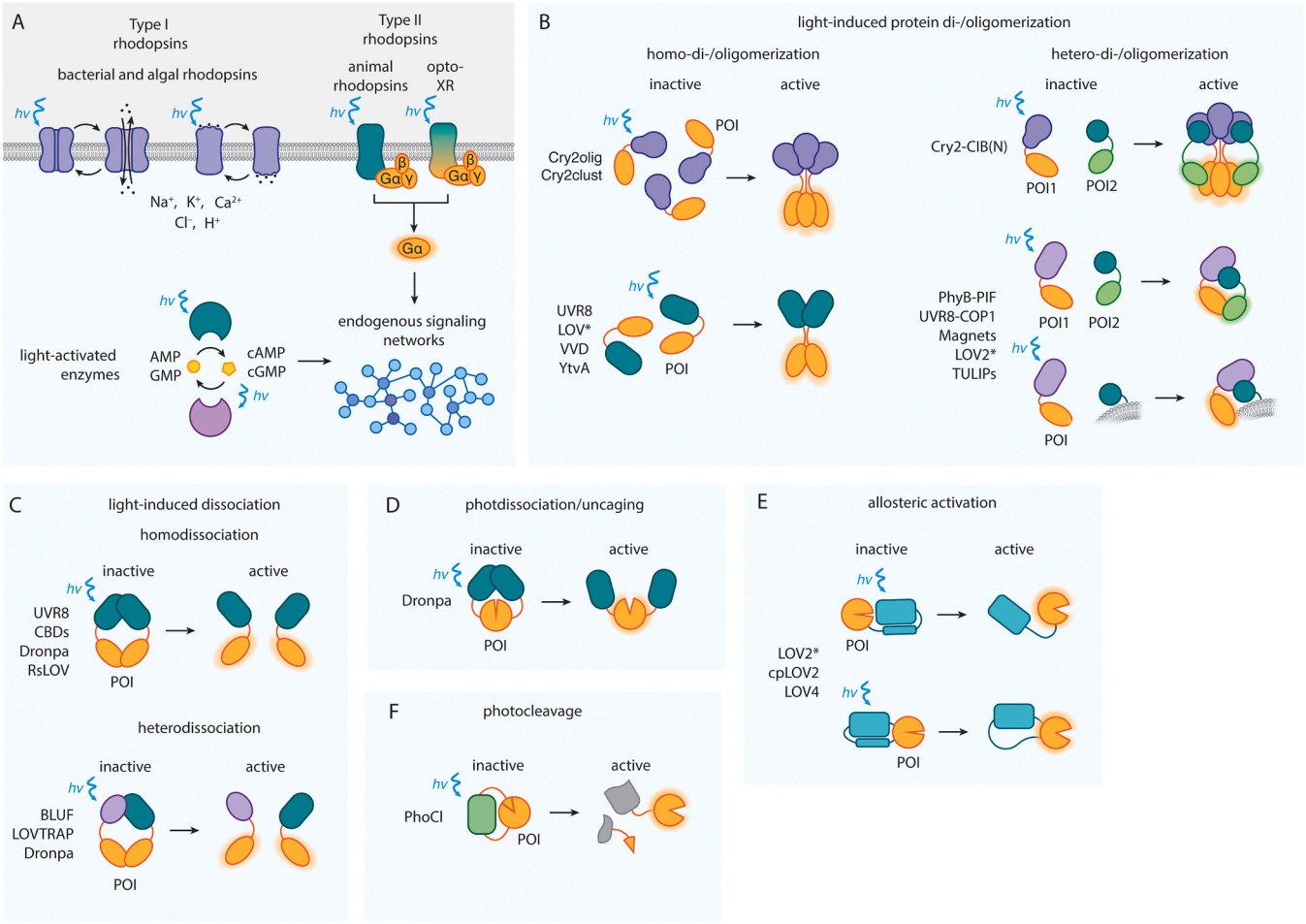

Fig. 4. Overview of commonly used optogenetic tools. A. Optogenetic systems, where light response and cellular function are combined in a single protein domain, include bacterial and algal type II rhodopsins (light-gated ionic channels and pumps), animal type II rhodopsins, and light-activated enzymes. **B.** Optogenetic tools which enable light-induced control over protein-protein interactions. When one of the interacting partners is tethered to specific cellular structure, these systems also enable light-induced protein recruitment or sequestration. **C.** Tools used for light-induced dissociation of multimeric protein complexes. **D.** Fluorescent protein Dronpa can be used for light-induced effector protein uncaging. **E.** LOV domain proteins can be re-engineered for allosteric regulation of target proteins. **F.** Proteolysis-dependent activation of the target protein using a photocleavable protein PhoCl. Curved blue arrows indicate light-sensitive photoactuator domains.

their function are reviewed in more detail elsewhere [213,214], and for the most recent information about the currently available photoswitches we refer the reader to OptoBase (https://www.optobase.org/) [215]. While optogenetic tools have many advantages over chemogenetic tools, they are not without limitations. One of these is their low activation threshold, which necessitates to for example culture the cells in the dark or in red-light conditions, or to express optogenetic tools under the control of inducible promoters.

4.2.3. *Optogenetic induction of transcription of cell death-inducing effectors*

Similarly to chemogenetics, optogenetically activated transcription can be used for the expression of cytotoxic proteins, such as the cytotoxic ion channel variant M2(H37A) (Fig. 5**A**) or when using a joint genetic-pharmacological approach exploiting the nitroreductase/nitrofuran system, which is suitable for the ablation of neuronal or other cells in zebrafish embryos [216]; and similar expression systems could be utilized in the future to induce expression of active caspases or pore-forming proteins using illumination. An opposite approach could also involve spatially resolved conditional knock-out of critical pro-survival factors using recently developed light-induced Cre recombinases [217] or CRISPR strategies [218], although no such studies have been reported yet.

4.2.4. *Cell death induction by prolonged membrane depolarization*

Several groups reported the use of long-term activation of ChR2 for the induction of cell death. In one study, low-intensity chronic activation of a plasma membrane-localized calcium channel ChR2 variant with prolonged activation kinetics was used to trigger melanoma cell death in vitro and in mouse xenograft model [219], while another group demonstrated the lethal effect of sustained activation of another ChR2 mutant that localizes to the inner mitochondrial membrane [220]. A more recent study also employed the light-activated proton pump Archerhodopsin-3 (AR3) to trigger cell death through intracellular alkalization [221]. The exact mechanisms and modality of induced cell death in all these cases remain unclear, as the dying cells displaying both apoptotic and necrotic features. The authors propose that they converge on the mitochondrial apoptosis pathway, and at least one of these studies also reports small but significant background cytotoxicity of the channel, which is likely due to the spontaneous dark-state activation of ChR2 mutant [219].

4.2.5. *Pathway specific activation*

A more selective category of optogenetic tools provide pathway-specific activation of the death receptors, adaptors or effector proteins (Table 3). Optogenetic tools designed to activate extrinsic apoptosis (Fig. 5**B**) include optogenetically activated Fas and its adaptor partner TRADD. In one of these studies, membrane-anchored cytoplasmic Fas

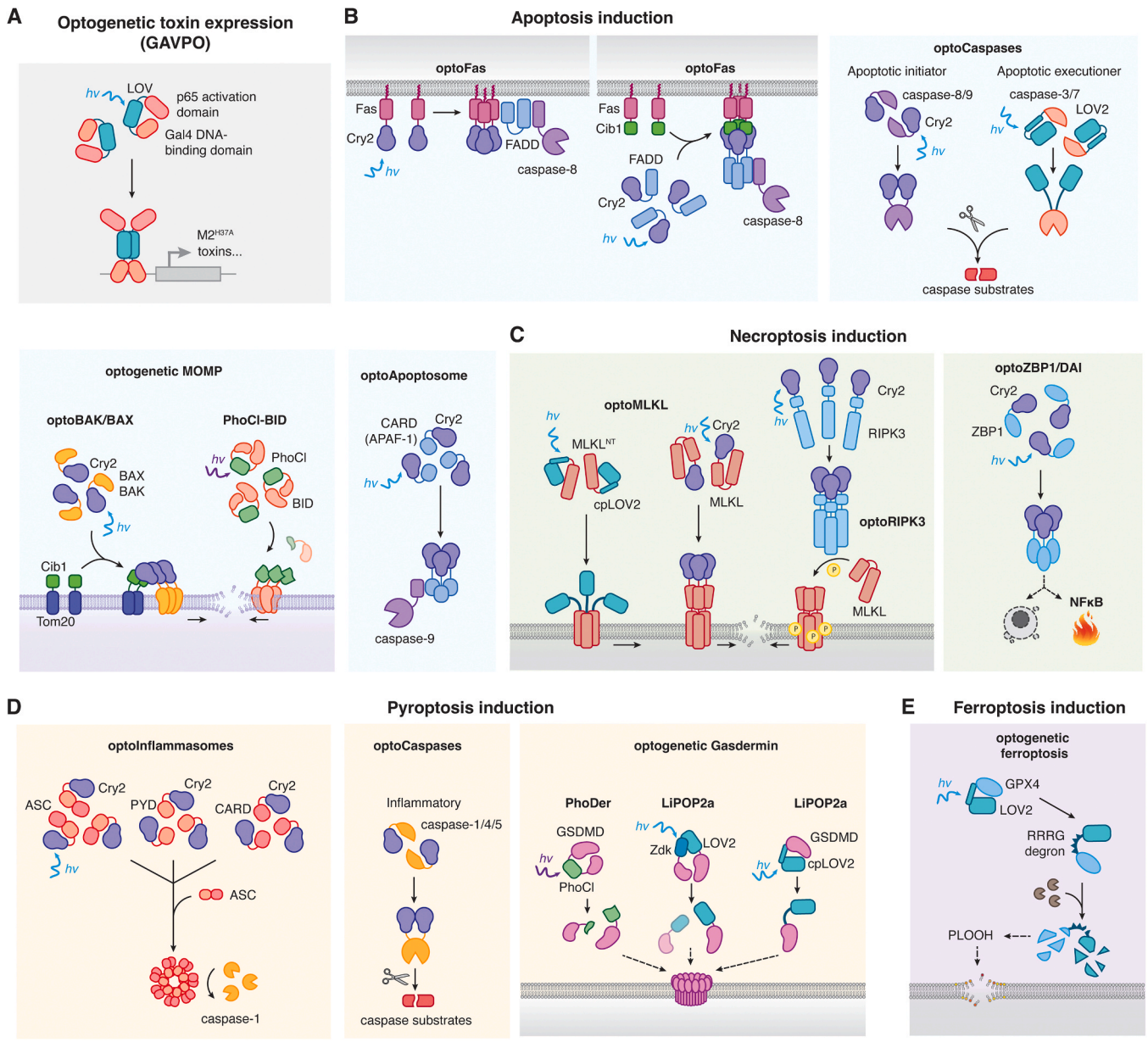

Fig. 5. Pathway-specific activation of cell death using optogenetic tools. A. Optogenetic control of gene expression using for example LOV domain-controlled transcription factors can be used to specifically induce expression of target genes, such as toxins or constitutively active cell death proteins. **B.** Apoptosis induction can be achieved by Cry-driven oligomerization of Fas, FADD or APAF-1. Apoptotic caspases can be activated using Cry2-driven oligomerization of initiator caspase-8/−9 or by using the LOV2 domain insertion in caspase-3/-7 precursors. Upstream, MOMP can also be induced using Cry2 fusions, or photocleavable BID (PhoCl-BID). **C.** Necroptosis induction has been achieved by oligomerizing MLKL, RIPK3 or ZBP1 via Cry2, or via LOV2 dependent activation of MLKL. **D.** Optogenetic pyroptosis induction can be achieved by Cry2-driven oligomerization of inflammatory caspases, ASC, or the PYD or CARD domains of inflammasome forming receptors. Several approaches have been developed to photoactivate gasdermins using either LOV2 domains or PhoCl. **E.** Ferroptosis can be induced using a LOV-based photodegradation tool encompassing an RRRG degron sequence and a human GPX4 into a single fusion construct. Curvy blue arrows indicate photoactuator domains of fusion proteins.

domain was fused to Cry2, yielding an optoFAS construct. Due to the lack of ligand-binding sites, this optoFas is insensitive to Fas ligand, thus functioning orthogonally to endogenous extrinsic apoptosis pathway, but can be rapidly oligomerized with the blue light, providing efficient apoptosis induction both in vitro and in vivo [222]. Two other groups also reported a Cry2-CIB1-based system for light-induced Fas-CIB1 and Cry2-FADD di(oligo)merization [223,224]. A similar principle was also applied to generate optogenetically activated versions of intracellular innate immune receptors, such as ZBP1/DAI [225] and IFI16 [226] and adaptors MAVS and MyD88 [227], all of which have known dual functions both in inflammatory responses and cell death. This could further be expanded to the activation of other membrane-localized death receptors, such as TNF receptor and DR4/5, as well as cytoplasmic inflammasome sensors, such as NLRs and AIM2, and other innate immune proteins. We have developed a system that mimics inflammasome sensor activation by fusing inflammasome-nucleating domains of several human and mouse NLRs and AIM2 to Cry2olig, a mutated version of the original Cry2 protein which undergoes rapid blue-light induced homo-oligomerization. In response to blue light, these optoInflammasomes rapidly assemble nucleation "seeds" that trigger rapid recruitment of adaptor ASC and caspase-1 and induce pyroptosis (Shkarina and Broz, unpublished results). A similar approach was later proposed by other group [228], and zerrafish opto-ASC was also recently tested for in vivo zebrafish inflammasome nucleation [229].

Table 3
Summary of light-base or optogenetic approaches for cell death induction.

Category	Tool	Target or effector protein	Type of cell death	References
Laser ablation	NA	NA	Apoptosis / non-specific	[149–152,155]
Chemical photosensitizers	NA	NA	Apoptosis / non-specific	[156]
Optogenetics	GAVPO (inducible expression)	M2(H37A)	Non-sepcific	[216]
	KillerRed	NA	Apoptosis / non-specific	[158,161]
	MiniSOG	NA	Apoptosis / non-specific	[159]
	SuperNova	NA	Apoptosis / non-specific	[160]
	Channelrhodopsin 2 (ChR2)	NA	Apoptosis / non-specific	[219,220]
	Archerhodopsin 3 (AR3)	NA	Apoptosis / non-specific	[221]
	Cry2	Fas	Apoptosis	[222]
		Caspase-8	Apoptosis	[80]
		Caspase-9	Apoptosis	[80]
		APAF-1 CARD	Apoptosis	[215]
		NLR or ASC PYD/CARD	Pyroptosis	[229,231,228]
		caspase-1/4/5/11	Pyroptosis	[80]
		RIPK3	Necroptosis	[80]
		MLKL	Necroptosis	[80,232,233]
		ZBP-1/DAI	Necroptosis (?)	[225]
		IFI16	Pyroptosis (?)	[226]
	Cry2/CIB1	Fas	Apoptosis	[223,224]
		BAX	Apoptosis	[234,235]
		BAK	Apoptosis	[232]
	LOV2	caspase-3	Apoptosis	[236]
		caspase-7	Apoptosis	[237]
		GSDMD	Pyroptosis	[233]
		MLKL	Necroptosis	[238]
		GPX4	Ferroptosis	[239]
	LOV2/Zdk	GSDMD	Pyroptosis	[233]
	PhoCl	BID	Apoptosis	[139,140]
		GSDMD	Pyroptosis	[240]

Interestingly, direct oligomerization of adaptor protein ASC turned out to be an equally or even more efficient way to trigger inflammasome assembly than oligomerizing NLRs, potentially due to the additional post-translational regulatory mechanisms safeguarding NLR activation. Besides cell death induction, such optogenetic constructs can also be used to gain further mechanistic insights into receptor/adaptor activation or to test known and unknown protein-protein interactions. For example, NLRP6-Cry2 fusions were recently used to reveal the role of intrinsically disordered regions and phase separation in NLRP6 inflammasome activation [230]. In another study, a forced death fold domain clustering was highly useful to probe the novel protein-protein interactions, such as those between inflammasome sensor AIM2 and apoptosis adaptor TRADD, and to provide evidence for the role of protein supersaturation, i.e., a soluble state that persist despite a thermodynamic drive towards a solid phase, and complex stability in innate immune signaling and apoptosis [231].

The largest group of optogenetic tools provides direct activation of different cell death effectors. Among others, these include light-activated BCL-2 and BH3 proteins which trigger MOMP to unleash the mitochondrial apoptosis pathway (Fig. 5A). These include blue light-induced mitochondrial recruitment of BAX [234,235] or dimerization of BAK [232] using a Cry2-CIB1 heterodimerization system consisting of mitochondrially localized CIB1 and cytoplasmic Cry2-mCherry-BAX fusion protein (optoBAX). Importantly, optoBAX contains serine 184 (S184E) mutation in BAX C-terminus, maintaining it in its predominantly cytoplasmic state in the absence of illumination, while blue light exposure triggers Cry2-CIB1 interaction and rapid enrichment of optoBAX at the outer mitochondrial membrane, resulting in MOMP. An alternative approach uses photocleavage of BID, which is achieved by inserting a photocleavable protein (PhoCl) between its C- and N-termini [241,242]. The violet light illumination triggers a photocleavage of PhoCl and subsequent dissociation of the C- and N-terminal parts, permitting the activated Bid N-terminus to trigger MOMP. In all the above cases, the onset of this optogenetically induced apoptosis is remarkably fast, with the first signs of MOMP and morphological changes observed within minutes post-illumination. Release of mitochondrial proteins, including XIAP inhibitors SMAC and OMI, results in a more complete downstream caspase activation, and is particularly relevant in Type II cells, e.g., cell in which engagement of the mitochondrial pathway is required for successful execution of extrinsic apoptosis [243].

We also recently reported the generation of optogenetic apoptosis, pyroptosis and necroptosis toolbox consisting of apoptotic initiator caspases-8 and − 9, inflammatory caspases-1, − 4 and − 5 and necroptotic effectors RIPK3 and MLKL, using a Cry2olig-mediated induced proximity activation approach [80] (Fig. 5**B-D**). All these tools can be expressed in cultured human and mouse cells or in vivo, and induce corresponding types of cell death upon illumination, with the first signs of cell death being detectable within minutes. Similar tools have been also independently developed by several other groups [233,244]. This approach offers several major advantages, in particular the remarkably fast activation of cell death given the direct activation at the effector level, which allows bypassing upstream pathway steps that are typically subjected to more stringent endogenous post-translational regulation, and the possibility to directly compare these types of cell death and their effects on the bystander cells under similar experimental conditions.

Unlike initiator caspases, executor caspases are activated via an interdomain linker cleavage rather than dimerization alone and thus require an alternative engineering approach for their optogenetic activation [113]. This was first solved by integrating the *Avena sativa*-derived LOV2 into the interdomain linkers of caspases-3 [236]. In the dark, LOV2 acts as an alternative pro-domain by preventing the correct folding of the caspase domain into its active conformation. The blue light exposure triggers the unfolding of LOV2 Jα helix and an extension of the inter-subunit linker, thereby releasing the block and allowing the caspase-3 to fold into the active conformation and gain catalytic activity. Later, a similar approach was used for photoactivatable caspase-7, where the LOV2 domain was fused to the catalytic domain of human caspase-7, in this case acting as an artificial pro-domain and restricting the caspase-7 activity in the dark and similarly releasing this inhibition upon illumination [237]. Additionally, considering direct CID-based activation of caspase-3 achieved in the earlier studies [113,117], its optogenetic multimerization could also be explored in the future as an alternative, although it is not clear how it would compare to the two

abovementioned strategies for the release of the pro-domain in terms of efficiency.

The most terminal effectors of the pyroptosis pathway, gasdermins, can also be optogenetically controlled (Fig. 5**C**). One group reported the development of optogenetically activated GSDMD (PhoDer), where PhoCl was inserted in the interdomain linker region between N- and C-termini. Similarly to previously described optoBID, the violet light stimulation and PhoCl cleavage are sufficient to release the C-terminus-mediated autoinhibition, allowing the N-terminus to translocate to the membrane and assemble pores [240]. Besides this, two alternative strategies for GSDMD activation were also introduced. LIPOP2a involves a light-induced dissociation of co-expressed C- and N-termini of GSDMD using a bipartite LOV2 Trap and Release of Protein (LOVTRAP) module. In this construct, N- and C-termini of GSDMD are fused to LOV2 and Zdk, which form a stable complex in the dark, maintaining GSDMD C- and N-termini interaction, but rapidly dissociate upon illumination, releasing active pore-forming N-terminus. In an alternative LIPOP2b construct the GSDMD C-terminus is replaced by LOV2, the latter acting as a synthetic autoinhibitory domain relative to N-terminus in the non-illuminated state [233] (of note, a very similar approach was also applied in another study by the same authors to engineer a photo-uncaged MLKL N-terminus [207], in which MLKL is sterically inhibited by LOV2 at the basal state but exposed upon illumination). These constructs enable to uncouple the GSDMD-mediated membrane damage from caspase activation and thus allow to address to what degree caspases contribute to pyroptotic cell death and downstream inflammatory responses beyond gasdermin activation. This approach could also be expanded to other gasdermins, for example to study which subcellular organelles are targeted by different gasdermin family members.

The lack of clearly defined molecular mechanism and execution pathway presents a major hurdle for the induction of ferroptosis using "clean" cell death tools. Recently, an attempt has been made to overcome this hurdle by developing a photodegradable GPX4 (Opto-GPX4Deg), which combines a previously developed LOV-based photodegradation tool [238] encompassing an RRRG degron sequence and a human GPX4 into a single fusion construct [239] (Fig. 5**E**). Upon illumination, the degron sequence exposure leads to rapid degradation of the GPX4 protein, sufficient to induce lipid peroxidation and ferroptotic cell death. In line with the previous studies, this tool could be applied to visualize the previously observed ferroptosis propagation to the neighboring bystander cells not expressing Opto-GPX4Deg, potentially providing new means to study ferroptosis mechanisms and consequences in a more controlled and molecularly defined system.

5. Applications of the artificial cell death systems to address biological questions

The major advantage that optogenetic and chemogenetic tools provide is the possibility to selectively control specific steps or the molecular players in the cell death pathways, creating the means to disentangle different signaling networks, reduce their complexity and minimize the inter-pathways crosstalk. Such simplified networks can also be reconstituted step-by-step in minimal cell systems that lack some or most endogenously expressed pathway components, providing valuable insights into the exact molecular mechanisms of cell death execution and regulation and revealing what is sufficient as opposed to what is required for cell demise or survival. The artificial cell death systems that we describe in this review could also aid pharmacological or genetic screening, as reduced pathway complexity could result in a reduced number of off-targets hits.

In heterogenous cell populations, cell death is usually stochastic and difficult to anticipate on a single-cell level, making it challenging to monitor highly dynamic molecular and cellular events, such as vesicular trafficking or membrane repair. Synthetic cell death tools facilitate the observations of the dying cells with the unmatched spatiotemporal resolution and might reveal new insights into cell death mechanisms and consequences [235]., as they allow to induce the process of interest in a selected cell at a specific timepoint and, in most cases, are compatible with live cell imaging. Moreover, transient activation of such tools, which can be achieved via pulsed illumination [80] or, in the case of chemogenetic constructs, by competitive ligand removal with so-called "washout" compounds [76,77], could be used to track the membrane repair and anastasis events on a single-cell level [80,245]. This would be important to gain further mechanistic understanding of these processes and to reveal the factors that determine the balance between the cell death and survival or that enable some cell types to engage cell death machinery without any apparent loss of cell viability.

Another research area where chemo- and optogenetics are becoming increasingly useful is the understanding of the tissue-level and organism-level consequences of RCD. Not all forms of cell death have been shown to be equally immunogenic or inflammatory, and our understanding of these differences and underlying biological mechanisms is still incomplete.

Complex biological and pharmacological triggers, particularly when used in vivo, often induce a mix of various forms of cell death and result in convoluted and difficult-to-interpret phenotypes, as it is often difficult to separate the immunogenic or inflammatory effects of cell death itself from the impact of these triggers on the surviving tissue. By contrast, optogenetic and chemogenetic tools enable highly controlled and selective engagement of specific cell death pathways in chosen cells, providing the means to study the bystander cell responses in a "clean" ligand-independent system. Coupled with live imaging, they can be used to monitor dynamic signaling events in cell populations, such as apoptosis- and pyroptosis-induced waves of calcium and ERK activation [246–248] and bystander motility [80,249]. "Clean" cell death systems proved to be extremely useful in illuminating the differences between several forms of cell death in immunogenicity and antitumor immunity in vitro and in mouse models [98,110,250–252], where they also provided the means to segregate different forms of cell death from each other or even uncouple cell death execution and signaling functions of cell death effectors, such as RIPK3 and MLKL [250,253]. Finally, such tools could also find their utility in context of the rapidly developing gene and cellular therapies, where they could either be delivered to the pre-existing cancer [219,254,255] or infected [256,257] cells using various types of gene delivery vectors, or introduced as "safety switches" during adaptive or regenerative cell therapies [258–260] to provide the possibility of their timely elimination without impacting healthy tissues [261], with some of such tools already being tested in humans [261].

6. Summary and outlook

Over the last years, a wide variety of methods have been developed that allow the specific induction of RCD modalities in cultured cells or even in animals. Nevertheless, despite these advances, further development and application of these toolsets is still necessary to better investigate and understand the intricate network of RCD pathways. Besides the development of novel tools for the induction of cell death, one of the areas where technological improvement is necessary is the development of tools that allow to specifically identify what RCD pathway a dying cell is undergoing, particularly in the complex in vivo settings and in clinical samples. The recent advances in multiplexed [262], label-free microscopy [263,264] and Raman spectroscopy [265], combined with the computer vision and neuronal network-aided detection of cell death events in high-throughput microscopy data [266] have the potential to greatly accelerate studies of cell death and even reveal previously unrecognized features associated with the different cell death modalities. Alternatively, new and specific caspase activity reporters could be generated by encompassing extended caspase recognition motifs and exosites, or new sensors for non-caspase-dependent cell death types, such as necroptosis and ferroptosis could be developed based on specific features of these RCD

modalities. These reporters could be combined with intravital imaging or recently developed tissue clearing techniques to identify where in the organism a certain type of cell death or a non-lethal pathway activation occurs during homeostasis or disease – a question which was previously predominantly addressed using reverse genetic tools such as tissue-specific knockouts.

While we know a lot about the morphophysiological changes that occur during apoptosis, we know comparably less about other types of (non-apoptotic) cell death. Advanced imaging techniques, such as expansion microscopy and multiplexed imaging or CRISPaint [267], and gene editing approaches like optical CRISPR screening [268], could provide novel insights into how and why cells die and how their corpses are dismantled or utilized by their neighbors. The ability to precisely control different cell death pathways, aided by newly developed tissue biology approaches such as spatial multi-omics or in vivo spectral imaging of multiplexed biosensors will be critical for understanding how different types of cell death impact tissues and organisms in a spatially and temporarily resolved manner, or how certain types of cancers or infectious pathogens evade or block this signaling to aid their survival and dissemination.

The recent advances in synthetic biology field also include generation of the more effective and bioorthogonal chemical and chemogenetic tools and expansion of the photoreceptor repertoire, particularly proteins sensing red and near-infrared illumination with different modes of action. Combined with the further improvements in multiphoton microscopy and alternative light delivery strategies using bioluminescence and nanoparticles [214], these technologies would enable their simpler activation in the deeper and more intact tissues, expanding the scope of biological questions which can be addressed with these approaches. The rapid growth of synthetic biology will also enable more targeted manipulation or repurposing of existing cell death pathways or their separate elements, reprogramming them to sense novel user-defined inputs, or even creating completely new cell death pathways which could be used for targeted cell elimination with experimental or therapeutic purposes. These synthetic effectors can be derived from other organisms or even other kingdoms of life (given our rapidly increasing knowledge of the plant and microorganismal cell death pathways) or designed from scratch using de novo protein engineering and can be programmed to sense user-defined synthetic or endogenous inputs [269]. Given the role of apoptosis in morphogenesis during embryonic development, it is also plausible to imagine that in future, such tools could be even utilized for creating complex biological shapes, sculpturing synthetic tissues or even building synthetic embryos [270]. We thus expect that in the coming years synthetic biology tools for cell death induction will not only continue to revolutionize the way we investigate existing cell death signaling pathways, but also allow the building of novel signaling networks and the application of targeted cell death induction beyond its traditional field of research.

Declaration of Competing Interest

The authors declare no competing interests.

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