



# Oxeiptosis: a discreet way to respond to radicals

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One of the best-studied cellular responses to toxic signals and pathogens is programmed cell death. Over the past years, it became apparent that the specific mechanisms of cell death have tremendous influence at both cellular and organismal level, highlighting the importance of sensors and pathways involved in this decision-making process. Central signalling molecules involved in a variety of cell death pathways are reactive oxygen species (ROS). However, the molecular mechanisms regulating differential responses and cellular fates to distinct ROS levels remain incompletely understood. Recently, we uncovered a caspase-independent cell-death pathway named 'oxeiptosis', which links the ROS sensing capacity of KEAP1 to a cell death pathway involving PGAM5 and AIFM1. Alike apoptosis, oxeiptosis is anti-inflammatory when activated by increased intracellular ROS levels and upon pathogens encounter. Here we discuss the potential impact of oxeiptosis in pathogens clearance and teratogenic cells.

## Addresses

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## Introduction

A variety of different stimuli induces synthesis of intracellular reactive oxygen species (ROS). These stimuli include many activators of pathways that play an active role in immunity. For instance, it has been shown that ROS are generated and required for proper activation of pyroptosis, an inflammasome-dependent cell death pathway [1], in sterile inflammation caused by radiation [2], as well as in apoptosis and necroptosis [3–6]. ROS scavenging agents negatively regulate these pathways suggesting direct involvement of ROS, but until now relatively little is known about the source and the activity of ROS at the molecular level.

During the last two decades, several studies gathered experimental evidence linking viral infection or teratogenic transformation to the production of ROS. ROS accumulation can be caused by diverse virus families ranging from blood-borne hepaciviruses (i.e. HBV, HCV and HDV), to large DNA- (i.e. Epstein-Barr virus (EBV)) and small RNA viruses (i.e. respiratory syncytial virus (RSV) or Influenza)[7,8]. Functionally, accumulation of ROS during acute respiratory viral infections has been associated with epithelial barrier dysfunction and increased susceptibility to secondary infections. Similarly, in chronic viral hepatitis, the commonly observed liver cirrhosis, fibrosis and cancer, have been causally associated with a sharp increase in oxidative stress [9,10]. Likewise, bacterial infections can also induce oxidative stress, rapidly increasing intracellular ROS levels that are in turn critically required by macrophages to clear bacteria within phagosomes [11].

Despite the overwhelming evidence of the role of oxidative stress in acute and chronic infection and the associated diseases, the impact of the majority of infectious agents on the host-redox systems and the consequences of these perturbations for the outcome of viral infection and inflammatory processes are incompletely understood. In particular, it is still unclear which proteins are directly activated by ROS and how this activation leads to different functions.

## Intracellular ROS sensing

ROS commonly modify proteins within amino acid residues intrinsically susceptible to oxidation. Depending on the oxidized substrate, such modifications translate into a variety of effects influencing signalling pathways or cellular integrity. Intracellular ROS levels are tightly monitored by multiple scavenging systems, constantly monitoring the abundance of oxidative elements to ensure prompt and proportionate countermeasures. One of the best studied intracellular ROS sensors is KEAP1, which functions as a Cullin3 adaptor, leading to constant degradation of the transcription factor NRF2 [12–15]. Accumulation of ROS induces oxidation of a subset of C-terminal cysteine residues of KEAP1, leading to a conformational rearrangement of the protein. Depending on the oxidizing compound, the cysteines at positions 151, 273 and 288 are targeted and have been shown to impair Cullin3 and/or binding to Nrf2 and allow NRF2 translocation into the nucleus to mediate expression of cytoprotective genes with anti-oxidant function, which counterbalance intracellular ROS levels [16].

Increased intracellular ROS can additionally have dramatic effects on key cellular components including lipids and proteins. For instance, lipid peroxidation is an oxidative process linked to cell damage leading to lipids degradation and death by a process called ferroptosis [17]. Similarly, oxidation of proteins can lead to their fragmentation, aggregation, unfolding, altered conformation and interactions with other proteins. Although the notion that increased ROS levels lead to cell death is well accepted, the exact sensor proteins and downstream signalling pathways are not well studied.

Recently, we identified oxeiptosis as a novel cellular pathway, which utilizes the ROS-sensing capabilities of KEAP1 to execute a cell-death program, independently from any previously described canonical cell death pathway. Importantly, oxeiptosis appears to be functionally important for diverse biological processes ranging from viral infections to inflammation and cancer.

### **Oxeiptosis: a dedicated pathway that links ROS sensing to cell death**

Despite the reported cytoprotective function of KEAP1 in response to elevated levels of ROS, KEAP1-depleted cells exhibit increased endurance to high levels of ROS [18\*\*]. KEAP1 thus appears to have dual functions, regulating expression of cytoprotective genes at moderate levels of ROS, while triggering a cell death program in the presence of higher ROS levels. This program, named oxeiptosis, involves KEAP1 engagement of the mitochondria-tethered phosphatase PGAM5 and the mitochondrial apoptosis inducing factor 1 (AIFM1) [18\*\*].

The association between KEAP1 and PGAM5 appears to be directly modulated by the relative amount of intracellular ROS: while low or moderate ROS levels (sufficient to induce KEAP1-mediated stabilization of NRF2) do not affect this interaction, high levels of ROS displace KEAP1 from mitochondria, abrogating its interaction with PGAM5 (Figure 1) [18\*\*]. These data therefore support a model whereby KEAP1 has the ability to sense and modulate differential responses to different intracellular concentrations of ROS. The distinctive signal transduction ability of KEAP1 might be regulated by the numerous cysteine residues at its c-terminus, which may allow a fine and tight sensing of different ROS levels. In this respect, while the cysteine residues modulating the association of KEAP1 with CULLIN3 and/or NRF2 have been characterized in detail, the functional role of the additional cysteine residues is not yet known. One interesting possibility would encompass oxidation of an alternative set of residues in response to higher concentrations of ROS, conferring an individual protein the ability to serve as a molecular switch between cytoprotective countermeasures or execution of cell death.

PGAM5 appears as a central signalling hub within the oxeiptosis pathway. Although this protein has already been proposed as a crucial player in necroptotic cell death, recent reports have questioned the originally proposed direct involvement of PGAM5 in RIPK3-mediated responses [19]. Indeed, PGAM5 does not physically associate with components of the necroptosis pathway, but rather binds to Apoptosis inducing factor 1 (AIFM1), a multifunctional protein that, among other functions, was previously shown to mediate a caspase-independent type of cell death [20].

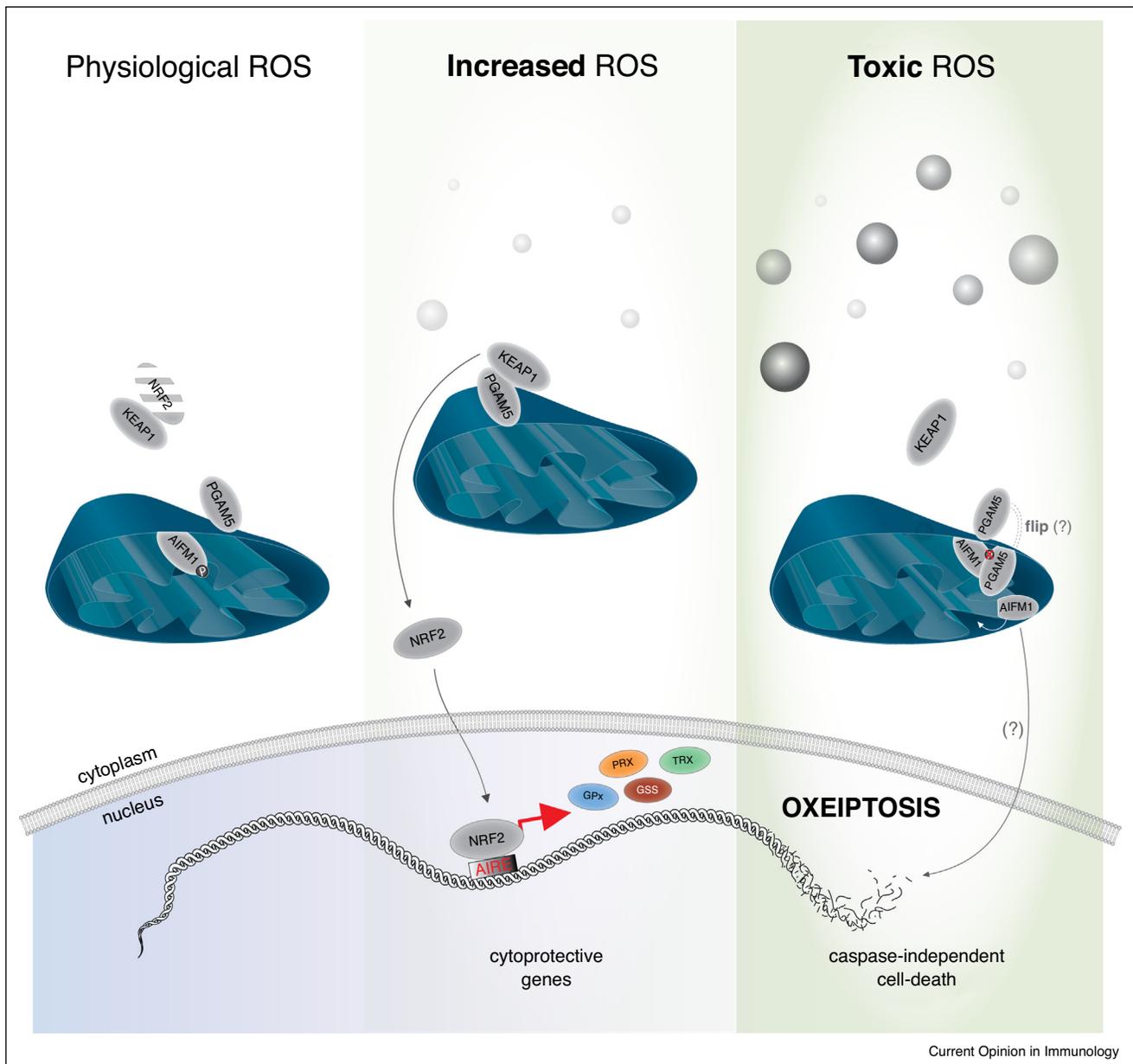
Under physiological ROS levels, PGAM5 and AIFM1 reside within physically segregated intracellular compartments (the outer and the inner mitochondrial membrane, respectively), and is therefore unclear in which subcellular compartment a direct protein-protein interaction could take place. However, in presence of high levels of ROS, KEAP1 and PGAM5 dissociate, potentially allowing PGAM5 to translocate into the mitochondrial lumen and to bind its substrate AIFM1 (Figure 1). Interestingly, PGAM5 localizes to the outer mitochondrial membrane and it associates with members of the TIM complex, which facilitates the transport of proteins to the inner mitochondrial membrane. In addition, PGAM5 bears a characteristic 'CX9C' motif, a motif used by the Mia40 complex to import proteins into mitochondria [21].

PGAM5 has the ability to dephosphorylate AIFM1 at a highly conserved Serine residue at position 116 and expression of an AIFM1 [S116A] mutant induced spontaneous cell death. However, the molecular consequences of AIFM1<sup>S116</sup> dephosphorylation have not yet been mechanistically clarified. AIFM1 has multiple functions including regulation of cell death, and through its NADH oxidoreductase activity modulates complex I, a multiprotein machinery that is essential for the energy-generating respiratory chain [22]. Cumulative data suggest that these cellular functions are not directly interconnected since mutations modulating NADPH oxidoreductase activity do not affect cell death, and ROS-inducing stimuli do not influence complex I functionality despite the concomitant AIFM1 dephosphorylation [18\*\*]. Earlier studies have shown that AIFM1 translocates into the nucleus and mediates fragmentation of chromatin [20]. However, in our hands, AIFM1 [S116A] does not leave mitochondria and relocalizes to circular structures within mitochondria (unpublished observations). Further work will be required to delineate the exact functions of AIFM1 in the context of oxeiptosis.

### **Oxeiptosis, apoptosis, necroptosis, ferroptosis & inflammation**

The relation of oxeiptosis to other cell death pathways is still unclear. Indeed, as central activator of oxeiptosis, ROS have also been implicated in necroptosis, ferroptosis and pyroptosis [1,3,6]. It is tempting to speculate that different threshold levels or intracellular sources of ROS

Figure 1



Activation of alternative pathways by KEAP1 in response to different intracellular ROS levels.

Under steady state conditions and physiological intracellular concentrations of ROS, the transcription factor NRF2 is retained within the cytosol by KEAP1, which targets it to the proteasome for degradation through an Ubiquitin E3 ligase-dependent mechanism. Intermediate levels of ROS, induce conformational changes within KEAP1 via oxidation of a subset of C-terminal cysteine residues, triggering its dissociation from NRF2, which translocates into the nucleus stimulating the transcription of several cytoprotective genes such as glutathione peroxidase (GPx), glutathione synthetase (GSS), peroxiredoxin (PRX) and thioredoxin (TRX). Conversely, high intracellular ROS levels (e.g. generated by persistent cellular stress upon chronic or fulminant virus infections), mediate the release of PGAM5 from KEAP1 and its translocation into the mitochondrion, where it dephosphorylates AIFM1 at S116 triggering a caspase independent cell-death.

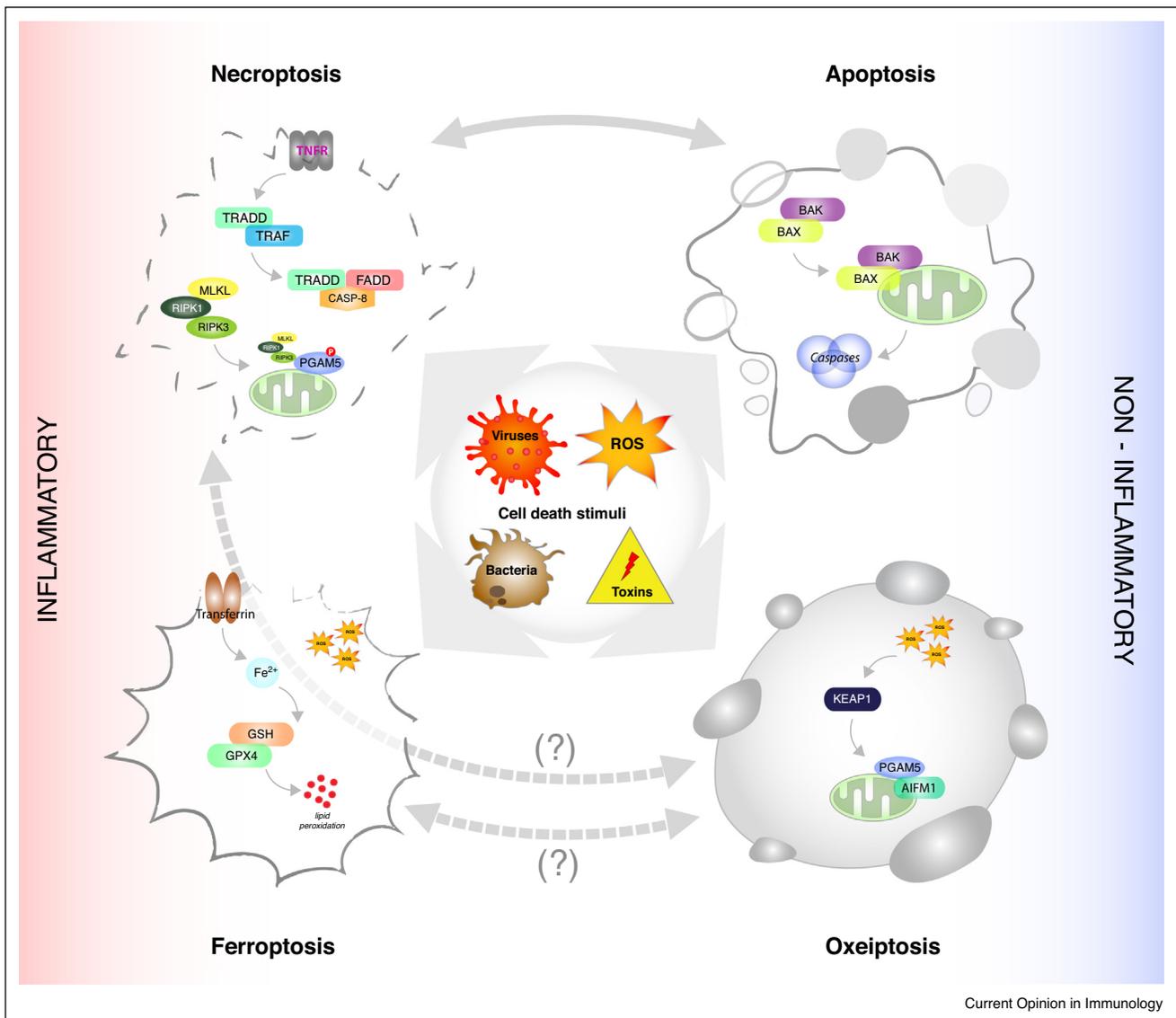
may lead to distinctive activation of cell death programs. Cells deficient in central components of the oxpeitosis pathway tolerate significantly higher levels of ROS *in vitro*, but are not protected against long-term or high doses of ROS, and will eventually die by means of

alternative pathways. It still needs to be established which pathways are activated in the absence of oxpeitosis and the immunological consequences thereof. It is likely that diverse cell death programs are differentially activated in diverse cell types.

The relative contribution of cell death pathways to inflammation is at the centre of research of many laboratories and appears to be highly-dependent on the exact experimental model used [23,24]. However, it is commonly accepted that necroptosis releases danger associated molecular patterns (DAMPs) that elicit pro-inflammatory signals in immune cells (Figure 2) [23]. Under most experimental conditions tested, necroptosis, mediated through RIPK3, results in a pro-inflammatory (i.e. lymphocyte recruiting and chemokine/cytokine producing) response *in vivo* [25,26]. For

instance, RIPK3-deficient mice show reduced signs of inflammation in a necroptosis prone knockout mouse model that lacks the apoptosis-factor FADD. In contrast, bronchoalveolar lavages of ozone-treated *Pgam5*<sup>-/-</sup> mice, show increased levels of pro-inflammatory cytokines and chemokines including IL-6, CXCL1 and CCL [18\*\*]. This indicates that PGAM5 prevents inflammatory responses in this ozone exposure model, suggesting that oxeiptosis is a cell death pathway that does not cause inflammatory reactions.

Figure 2



Oxeiptosis in the context of other cell death pathways.

Diverse intracellular and extracellular stimuli, such as virus or bacterial infections, toxic molecules or increased reactive oxygen species (ROS) trigger tailored and highly-specific cell death pathways of diverse nature. Necroptosis and ferroptosis are considered 'inflammatory'-like cell death pathways, while apoptosis and the recently identified oxeiptosis pathway cause 'non-inflammatory' cell death. Of note, late apoptosis eventually lead to secondary necrosis. Furthermore, besides triggering oxeiptosis, accumulating doses of ROS may activate inflammatory cell death through necroptosis or ferroptosis. Potential interconnections and compensatory mechanisms to the other cell death pathways have not yet been elucidated.

In influenza A virus infection models, genetic depletion of cIAP2 – a negative regulator of apoptosis – results in increased inflammation in the lungs, rendering mice more susceptible to lethal Influenza A virus challenges [27] and highlighting an important role for apoptotic pathways in dampening inflammatory responses. Importantly, in cIAP2 deficient and wild-type mice, depletion of RIPK3 or pharmacological inhibition of RIPK1 reduced FluAV-dependent necrosis, indicating that RIPK1/3 promotes a lethal phenotype in response to Influenza A virus infections [27,28\*\*]. Alike cIAP-deficient mice, *Pgam5*<sup>-/-</sup> animals exhibit a high mortality rate and an increased accumulation of cytokines and infiltrating lymphocytes upon Influenza A virus challenge [18\*\*]. Collectively, these observations suggest that PGAM5 exerts a non-inflammatory function and that in absence of a functional oxeiptosis pathway, alternative inflammatory types of cell death, such as necroptosis, are triggered in a compensatory manner. In this perspective, a non-inflammatory cell death pathway, such as oxeiptosis, may be beneficial to mitigate detrimental effects caused by immunopathologies on organismal scale. At this stage, we can only speculate on potential crosstalks between oxeiptosis and other cell death mechanisms (Figure 2). In addition, depending on the cell type the relative contribution of individual pathways may differ. For instance, while mouse embryonic fibroblasts and epithelial cells are highly sensitive to the damages induced by ROS, other cell types such as lymphocytes appear to be significantly more resistant (unpublished observations). This may explain the reported involvement of PGAM5 in pro-inflammatory functions of lymphocytes [29,30].

A central question is the initial source of the necroptotic signal in virally infected cells. Virus infection commonly leads to accumulation of toxic amounts of ROS in infected cells. However, it has recently been shown that the intracellular Z-DNA binding protein 1 (ZBP1, also known as DAI) directly binds FluAV RNA, recruits RIPK3 through its RHIM domain and thereby induces necroptosis [31\*\*,32\*\*]. ZBP1 therefore serves as a direct pathogen sensor to initiate cell death during FluAV infection (reviewed in [33]). In contrast to this pathway, which relies on direct sensor-pathogen contact, ROS-sensing oxeiptosis samples the cellular environment and therefore has the ability to respond to elevated intracellular ROS levels regardless of the specific endogenous or exogenous triggers. It is tempting to speculate that the choice of specific cell death pathways likely results from the integration of various direct and indirect signals, allowing the cell to balance/adapt responses and activate-specific pathways tailored to the surrounding environment.

### Oxeiptosis: pro-viral or anti-viral functions?

Regulation of cell death is a key cellular mechanism to limit virus spread and therefore many distinct virus

families evolved interfering mechanism to restrict cell death pathways. For instance, different viruses inhibit apoptosis (e.g. through CrmA, a cowpox encoded caspase inhibitor, or E1B-19K, a BCL-2 class of mitochondrial apoptosis blocker encoded by adenoviruses) to promote virus spread [34,35]. Similarly, RIPK1/3 was shown to induce necrosis thereby limiting vaccinia virus growth in mice [36], and to be directly targeted by the M45 protein of Murine cytomegalovirus [37]. Likewise, oxeiptosis is activated in FluAV infected cells, as inferred by dephosphorylation of AIFM1<sup>S116</sup> and increased viability of *Pgam5*<sup>-/-</sup> MEFs after FluAV infection [38]. Surprisingly, *Pgam5*-deficiency *in vitro* does not directly influence FluAV virus growth, suggesting that oxeiptosis is not involved in virus spread. Conversely, as discussed above, activation of oxeiptosis may be beneficial for viruses *in vivo*. Indeed, evolutionary divergent viruses bind proteins of the oxeiptosis pathway [38]. For example, the K3 protein of Kaposi Sarcoma Herpes virus (KSHV, HHV-8) directly binds PGAM5. In this case, K3 appears to impair PGAM5's phosphatase activity [18\*\*]. However, interactions between viral and host proteins can have diverse outcomes [39,40] and it will be important to characterize the ability of each individual viral protein in activating or inhibiting oxeiptosis in the context of pathogen encounter.

### KEAP1-PGAM5-AIFM1: oxeiptosis beyond virus infections

An additional line of future research should address the role of oxeiptosis in diverse biological functions. ROS can be generated in response to different challenges and developmental stages. Interestingly, both, depletion of PGAM5 and AIFM1, lead to neurological dysfunctions in mice [20,41], hinting towards a role of oxeiptosis during neuronal development.

Furthermore, this newly identified complex might play important roles in tumorigenic processes. A catalogue of somatic mutations in various cancers revealed 274 and 389 coding mutations within the KEAP1 and the NRF2 gene, respectively; accounting for approximately 0.9% of all cancer samples examined [42]. While cancer cells harbouring somatic mutations in NRF2 lose the KEAP1–NRF2 interaction, no information is currently available on the consequences of KEAP1 mutations on the oxeiptosis pathway and whether-specific modulation of the downstream AIFM1 effector function is also affected. For instance, mutations within a specific subset of KEAP1 C-terminal cysteines might specifically trigger or inhibit oxeiptosis, suggesting a potentially important role and therapeutic potential of this pathway for a variety of tumours.

### Conclusions

Oxeiptosis has recently emerged as a distinct cellular pathway, likely to operate in parallel to other cell-death

pathways and in a wide range of cell types (with the potential exception of lymphocytes), promoting a non-inflammatory, caspase-independent and apoptosis-like cell-death phenotype.

From an evolutionary perspective, it appears advantageous to unify cytoprotective and pro-apoptotic pathways into an integrated mechanism relying on a shared cellular sensor (e.g. KEAP1), to activate divergent downstream effectors (e.g. NRF2 and PGAM5) whose activity and regulation are tightly regulated by the entity of the stimulus (ROS levels). In case of oxeiptosis, this mechanism would constantly monitor the redox fitness of the cell, rapidly activating supporting survival mechanisms or inducing cell-death terminating inflammation. The integration of the mechanisms governing apoptosis and inflammation by the KEAP1-PGAM5-AIFM1 axis has far-reaching therapeutic implications. Notably, the finding that distantly related virus families actively target the oxeiptosis pathway provides an attractive therapeutic target to develop novel death regulating agents that could be beneficial for treatment against pathogens, cancer or neurological disorders. Future work should address the consequences of KEAP1 deregulation with respect to oxeiptosis activation, and its causal relation with the level of inflammatory cytokines and ROS-induced damage to the immune system.

### Conflict of interest statement

Nothing declared.

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