Biological Chemistry ‘Just Accepted’ Papers

Biological Chemistry ‘Just Accepted’ Papers are papers published online, in advance of appearing in the print journal. They have been peer-reviewed, accepted and are online published in manuscript form, but have not been copy edited, typeset, or proofread. Copy editing may lead to small differences between the Just Accepted version and the final version. There may also be differences in the quality of the graphics. When papers do appear in print, they will be removed from this feature and grouped with other papers in an issue.

Biol Chem ‘Just Accepted’ Papers are citable; the online publication date is indicated on the Table of Contents page, and the article’s Digital Object Identifier (DOI), a unique identifier for intellectual property in the digital environment (e.g., 10.1515/hsz-2011-xxxx), is shown at the top margin of the title page. Once an article is published as **Biol Chem ‘Just Accepted’ Paper** (and before it is published in its final form), it should be cited in other articles by indicating author list, title and DOI.

After a paper is published in **Biol Chem ‘Just Accepted’ Paper** form, it proceeds through the normal production process, which includes copy editing, typesetting and proofreading. The edited paper is then published in its final form in a regular print and online issue of **Biol Chem**. At this time, the **Biol Chem ‘Just Accepted’ Paper** version is replaced on the journal Web site by the final version of the paper with the same DOI as the **Biol Chem ‘Just Accepted’ Paper version**.

Disclaimer

Biol Chem ‘Just Accepted’ Papers have undergone the complete peer-review process. However, none of the additional editorial preparation, which includes copy editing, typesetting and proofreading, has been performed. Therefore, there may be errors in articles published as **Biol Chem ‘Just Accepted’ Papers** that will be corrected in the final print and online version of the Journal. Any use of these articles is subject to the explicit understanding that the papers have not yet gone through the full quality control process prior to advanced publication.

Review

**Coordination of stress, Ca²⁺ and immunogenic
signaling pathways by PERK at the
endoplasmic reticulum**

Alexander R. van Vliet, Abhishek D. Garg and Patrizia Agostinis*

Laboratory of Cell Death Research and Therapy, Department of Cellular and Molecular
Medicine, University of Leuven, Campus Gasthuisberg OandN1, Herestraat 49, Box 802, B-
3000 Leuven, Belgium

*Corresponding author

e-mail: Patrizia.Agostinis@med.kuleuven.be

Abstract

The endoplasmic reticulum (ER) is the main coordinator of intracellular Ca^{2+} signaling, protein synthesis and folding. The ER is also implicated in the formation of contact sites with other organelles and structures, including mitochondria, plasma membrane (PM) and endosomes, thereby orchestrating through inter-organelle signaling pathways, a variety of cellular responses including Ca^{2+} homeostasis, metabolism and cell death signaling. Upon loss of its folding capacity, incited by a number of stress signals including those elicited by various anticancer therapies, the unfolded protein response (UPR) is launched to restore ER homeostasis. The ER stress sensor PERK is a key mediator of the UPR and its role during ER stress has been largely recognized. However growing evidence suggests that PERK may govern signaling pathways through UPR-independent functions. Here we discuss emerging non-canonical roles of PERK with particular relevance for the induction of danger or immunogenic signaling and inter-organelle communication.

Keywords: endoplasmic reticulum; PERK; immunogenic cell death; mitochondria; MAMs; plasma membrane; SOCE; calcium signaling.

Introduction: features of the ER stress-associated pathways

The endoplasmic reticulum (ER) is a key organelle coordinating a myriad of biosynthetic and signaling functions, ranging from synthesis, folding and posttranslational modification (PTM) of proteins destined for the cell membrane or extracellular space (i.e. proteostasis), synthesis of lipids as well as spatiotemporal regulation of Ca^{2+} signaling (Garg *et al.*, 2015; Grolach *et al.*, 2006; Stevens and Argon, 1999).

Proper protein folding is one of the foundations of life and the ER environment has evolved to ideally perform this very demanding task. The ER lumen is equipped with chaperones and proteins specialized in assisting protein folding and key post-translational modifications, such as the glucose regulated protein 78 (Grp78, also known as BiP), the lectins - calnexin (CNX) and calreticulin (CRT), and various protein disulfide isomerase (PDIs) and oxidoreductases (Anelli *et al.*, 2012; Gilady *et al.*, 2010; Malhotra and Kaufman, 2007; Pollard *et al.*, 1998; Stevens and Argon, 1999). ER proteostasis is an energetically demanding process, with many ER associated proteins requiring ATP for their function. Typical examples are the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) that pumps Ca^{2+} from the cytosol into the lumen of the ER, thereby maintaining steady-state ER- Ca^{2+} levels, and the ER chaperone BiP, which hydrolyses ATP to allow binding to unfolded proteins and assist in their folding (Vishnu *et al.*, 2014). However when the ER folding capacity is challenged, as occurs in cancer cells in which the biosynthetic need outweighs ER capacity, or when ER homeostasis is perturbed, as triggered by tumor environmental and metabolic stress, this pressurizes the ER thereby creating ER stress (Garg *et al.*, 2015).

ER stress is sensed by three major transmembrane proteins: the S/T protein kinase RNA-like ER kinase (PERK), the endo-ribonuclease and protein kinase Inositol-requiring protein 1 α (IRE1 α) and the transcription factor activating transcription factor 6 (ATF6). In resting/homeostatic conditions these ER stress proteins bind, by virtue of their luminal domain, the chaperone BiP, which prevents their activation. When ER homeostasis is compromised, the consequent accumulation of unfolded proteins in the ER lumen titrates away BiP from these transmembrane proteins. This triggers the dimerization/oligomerization of PERK and IRE1 α , as well as the migration of ATF6 to the Golgi (Harding *et al.*, 1999; Hetz *et al.*, 2015; Shen *et al.*, 2002; Wang *et al.*, 1998). The concerted action of these ER stress sensors engages a highly conserved cytosol-to-nucleus signaling cascade, called the

unfolded protein response or UPR, which has as a primary goal of restoring ER homeostasis (Ron and Walter, 2007; Schroder and Kaufman, 2005). The UPR initially regulates various adaptive responses including ER expansion transcriptional expression of various chaperones or, folding enzymes and a temporary translational shut-down (Ron and Walter, 2007; Schroder and Kaufman, 2005). However, when the ER folding capacity is permanently compromised, this adaptive stress response turns into a lethal signal, inducing a cell death program that is executed mainly –albeit not uniquely- through caspase activation and apoptosis (Oyadomari and Mori, 2004; Ron and Walter, 2007).

Extensive reviews on the UPR and its signaling function in physiological and pathological conditions have been published recently (Garg *et al.*, 2015; Hetz *et al.*, 2015; Ron and Walter, 2007; Schroder and Kaufman, 2005) and will not be discussed further here. This essay is focused on emerging and non-canonical roles of PERK in cancer therapy and as a master regulator of inter-organellar contact sites.

PERKking the ER stress

Dimerization-induced activation of PERK, upon loss of ER homeostasis, results in its autophosphorylation and the phosphorylation of its main cytosolic substrate, the α subunit of the eukaryotic initiation factor 2 (eIF2 α) on Ser51. PERK-mediated eIF2 α phosphorylation causes a temporal translational block, which largely prevents protein translation, while causing the activation of transcription factor 4 (ATF4). ATF4 is the main mediator of the transcriptional program governed by PERK (Ron and Walter, 2007). PERK also phosphorylates nuclear factor 2 (NRF2), entailing its dissociation from Kelch-Like ECH-Associated Protein 1 (KEAP1), nuclear translocation and activation of a NRF2-driven transcriptional program (Cullinan *et al.*, 2003). These PERK-mediated signaling pathways tend to restore ER homeostasis by enabling the transcription of ER chaperones, folding enzymes and anti-oxidant proteins. However, when this protective signal fails to restore ER homeostasis and PERK activation remains sustained, the PERK-ATF4 axis is switched towards the induction of the pro-apoptotic transcription factor CHOP, inciting apoptosis (Ron and Walter, 2007; Schroder and Kaufman, 2005; Verfaillie *et al.*, 2013). The relevance of this PERK-mediated signal is highlighted by several findings showing that deletion of CHOP protects against the induction of apoptosis after persistent ER stress (Oyadomari and

Mori, 2004). However, *in vivo*, the overall role of CHOP is further influenced by the tissue context and environmental stress as well as inflammatory conditions (Malhi *et al.*, 2013).

PERK in ROS and ER stress-regulated immunogenic signaling

While the ability of ER stressors to induce apoptosis is a well-established phenomenon, it is only in the last decade that this process has been shown to be an essential prerequisite for the induction of immunogenic cell death (ICD) (Garg *et al.*, 2015; Kepp *et al.*, 2014). ICD is a cell death subroutine triggered by a restricted subset of anti-cancer modalities inducing the combined occurrence of reactive oxygen species (ROS) and ER stress (Dudek-Peric *et al.*, 2015; Panaretakis *et al.*, 2009). This form of cancer cell death is highly immunogenic, since it is coupled to the spatiotemporally-defined emission of danger signals or damage-associated molecular patterns (DAMPs) and other immunostimulatory molecules by the dying cancer cells (Bettigole and Glimcher, 2015; Garg *et al.*, 2015; Kroemer *et al.*, 2013). ICD-inducers elicit a ROS-based proteotoxicity at the ER, which eventually results in activation of danger signaling pathways that traffic DAMPs toward the extracellular space (Garg *et al.*, 2012). Indeed, any process that blunts this proximal ER-associated proteotoxicity also tends to suppress danger signaling e.g. macroautophagy and anti-oxidative signaling (Garg *et al.*, 2012, 2013). In fact, cancer phenotypes that suppress danger signaling are frequently associated with ablation of immunogenic phagocytosis, inhibition of immunotherapeutic or ICD efficacy and poor prognosis in cancer patients (Garg *et al.*, 2015; Vacchelli *et al.*, 2015; Yamazaki *et al.*, 2014). Interestingly, suppression of therapy induced ROS-modifications in general can reduce immunogenic potential of dying cancer cells (Vandenberg *et al.*, 2015).

In contrast to cancer cells dying through tolerogenic apoptosis, cancer cells dying through ICD establish a proficient interface with the immune system culminating in efficient T-cell mediated anti-tumor immunity (Garg *et al.*, 2015). According to their ability to target the ER and induce ER stress as a secondary/collateral or primary/on-target effect, ICD inducers – which share no chemical or structural features- can be conveniently classified into Type I (e.g. most chemotherapeutics) or Type II (hypericin-mediated photodynamic therapy or Hyp-PDT and certain oncolytic viruses), respectively (Garg *et al.*, 2015). Systematic investigation of the molecular pathways underlying chemotherapy based (Type I) ICD-induced CRT exposure showed that it was reliant on the sequential activation of three main modules: (i) a ROS-modulated ER-stress arm regulated by intracellular Ca^{2+} elevations

and dependent on PERK-mediated phosphorylation of eIF2 α , (ii) a caspase-module requiring Bax/Bak and BAP31 cleavage by ER-associated caspase-8 and (iii) an ER-to-Golgi anterograde transport-module ultimately eliciting SNARE-dependent exocytosis (Panaretakis *et al.*, 2009). On the other hand, CRT-exposure mechanisms elicited by the prototypical Type II ICD inducer, Hyp-PDT, relied on a more simplified danger signaling pathway consisting of: (i) a focused ROS-ER-stress module, (ii) a PERK-mediated proximal secretory pathway independent of eIF2 α phosphorylation, (iii) a similar ER-to-Golgi anterograde transport and, (iv) phosphoinositide 3-kinase (PI3K)-regulated exocytosis (Garg, *et al.*, 2012; Garg *et al.*, 2012). Remarkably, following Hyp-PDT, the PERK-regulated danger signaling pathway co-ordinated the concomitant pre-apoptotic export of both surface-CRT and secreted ATP (Garg *et al.*, 2012). Although danger signaling during ICD exhibits a certain level of plasticity depending on the type of ICD inducer under consideration (Garg *et al.*, 2016), ablation of PERK in cancer cells compromised DAMPs exposure and suppressed the tumor-rejecting anticancer vaccination effect of ICD *in vivo* for both Type I and Type II ICD inducers (Garg *et al.*, 2015; Panaretakis *et al.*, 2009). Thus while these studies show the vital relevance of PERK for ICD, they also disclose the capability of PERK to modulate trafficking of danger signals through UPR-independent secretory functions, whose relevance need to be further elucidated within the context of ICD (Garg *et al.*, 2012). The PERK-autophagy connection also needs to be revisited in an ICD context considering that ROS-based ER stress elicited macroautophagy has been shown to suppress ICD (Garg *et al.*, 2013). However, in spite of the still limited molecular knowledge, the concept of ICD is rapidly disclosing its clinical relevance, both on the level of anticancer immunotherapy as well as for prognostic/predictive cancer patient stratification (Fucikova *et al.*, 2015; Galluzzi *et al.*, 2014; Garg *et al.*, 2015; Garg *et al.*, 2015; Garg *et al.*, 2015). We recently challenged the relevance of the PERK-modulated danger signaling and anti-tumor immunity for the development of a next-generation Th1/cytotoxic T cells/Th17-biased dendritic cell (DC) immunotherapy for treatment of high grade glioma (HGG), a cancer associated with profound immunosuppression (Garg *et al.*, 2016). In an orthotopic-HGG mouse model we found that clinically-relevant Hyp-PDT-based DC vaccines showed remarkable HGG rejection capability (Garg *et al.*, 2016). In fact this anti-HGG immunity was reliant on cancer cell-associated danger signals eliciting a proficient interface with DCs and on CD8⁺ T cell responses (Garg *et al.*, 2016).

These studies highlight the potential of PERK-mediated signaling for the development of clinically relevant ICD-based vaccines.

PERK beyond the UPR: inter-organellar contact sites and Ca^{2+} signaling

The initial, adaptive phase of ER stress is accompanied by changes in ER morphology and contact sites between the ER and the juxtaposed mitochondria (Bravo *et al.*, 2011). For example, ER stress elicited by ER- Ca^{2+} depletion induced through ROS-based ER stress (i.e. Hyp-PDT; Verfaillie *et al.*, 2012) or tunicamycin, an inhibitor of N-linked glycosylation, is associated with a relocation of mitochondria towards the perinuclear ER and a strengthening of ER-mitochondria juxtaposition (Bravo *et al.*, 2011). Functionally, increased ER-mitochondria apposition may favor localized Ca^{2+} transfer between these organelles in order to increase Ca^{2+} uptake by the mitochondria, leading to a stimulation of ATP production and oxygen consumption (Bravo *et al.*, 2011). Increasing ATP production may be required to support the transcriptional machinery during the early adaptive phases of ER stress. Thus regulation of the ER-mitochondria contact sites during ER stress assumes a relevant biological role and is reflected by a growing body of evidence indicating that a dynamic link between ER and mitochondria may fine-tune the UPR response, the amplitude and consequences of ER stress (reviewed in van Vliet *et al.*, 2014).

Recently, the physiological role of PERK has been expanded beyond the regulation of the UPR transcriptional program, by the finding that this ER stress sensor is enriched at the mitochondria-associated membranes or MAMs (Verfaillie *et al.*, 2012). MAMs are ER membrane-derived proteinaceous structures at the interface between the ER and the mitochondria, which form a physical connection between these organelles (Raturi and Simmen, 2013). Although the molecular nature of the MAMs has not been completely solved, likely due to their dynamic nature, a growing number of proteins implicated in Ca^{2+} signaling, mitochondria metabolism, fission/fusion and movement mechanisms as well as autophagy have been shown to be recruited to the MAMs (for a complete discussion on MAMs readers are referred to recent reviews (Raturi and Simmen, 2013; Rowland and Voeltz, 2012; van Vliet *et al.*, 2014). PERK deficient cells display a less tight ER-mitochondria juxtaposition under resting conditions and after ROS-mediated ER stress, resulting in a reduced transfer of ROS-mediated lethal signals from the stressed ER to the closely apposed mitochondria

thereby decreasing cell death susceptibility (Verfaillie *et al.*, 2012). Reconstitution of the full-length wild type PERK or a PERK kinase-dead mutant in PERK^{-/-} cells tends to rescue ER-mitochondria appositions and the effects on mitochondria depolarization following ROS-induced ER- Ca^{2+} depletion (Verfaillie *et al.*, 2012). This indicates a major tethering role of PERK in the maintenance of ER-mitochondria juxtapositions.

A PERK-MAMs link is further emphasized, by the finding that PERK is able to interact with the mitochondrial dynamin-related protein mitofusin 2 (MFN2), a vital mediator of ER-mitochondria juxtapositions (de Brito and Scorrano, 2008). The interaction between PERK and MFN2 was reported to modulate fragmentation of the mitochondrial network, mitochondrial Ca^{2+} content and UPR progression as well as apoptosis. Knockdown of PERK in MFN2 deficient cells rescued their fragmented mitochondrial network and restored mitochondrial Ca^{2+} levels. (Munoz *et al.*, 2013).

Although the exact mechanism through which MFN2 regulates PERK function is still elusive, absence of MFN2 caused sustained PERK activation, but not enhanced UPR-related apoptosis (Munoz *et al.*, 2013). This suggests that altering MAMs composition affects PERK functionalities that are not directly superimposed to the UPR. Moreover, while (regulated) mitochondrial Ca^{2+} uptake boosts mitochondrial ATP production, Ca^{2+} overload promotes mitochondrial dysfunction and cell death. This indicates that PERK's function at the MAMs may be of a sensor and effector of organelle homeostasis and cell death.

However, while there is no doubt that MFN2 is a prime modulator of ER-mitochondria juxtapositions, few recent studies have raised some concerns about its exact tethering role (Filadi *et al.*, 2015). Thus whether MFN2 is a positive player and mediates ER-mitochondria tethers (de Brito and Scorrano, 2008) or –on the contrary- acts by weakening the contact sites between these key organelles (Filadi *et al.*, 2015), is currently a matter of debate. Future work will be required to reconcile these alternative outcomes and understand the functional effects of the PERK-MFN2 link on mitochondrial bioenergetics.

Whether PERK activity, or just its tethering function, is required to exert its effects at the ER-mitochondria contact sites, including the recently described role of these subdomains in mitochondria fission (Friedman *et al.*, 2011) and autophagy regulation (Hamasaki *et al.*, 2013), should also be further addressed.

Additionally, considering these novel functions of PERK, and that MAMs have been shown to influence immune responses *via* inflammasome regulation (Zhou *et al.*, 2011), it will be crucial to reach a better understanding of the role played by PERK as a modulator of ER-organellar contact sites, in danger signaling and ICD.

Interestingly, PERK has been shown to harbor an intrinsic lipid kinase activity enabling the phosphorylation of diacylglycerol (DAG) to generate phosphatidic acid (PA), a signaling lipid that plays a critical role in a variety of biological processes, including membrane and actin remodeling, as well as cytokine secretion and the regulation of pro-survival and anabolic pathways such as mTOR (Liu *et al.*, 2013). However, the broad functional role of PERK's lipid kinase activity has not been fully explored, and future studies are required to assess the role of this PERK-regulated lipid signal in cancer cells and ER stress-based anticancer therapy. However, considering the role of DAG and PA in the regulation of exocytosis, at secretory vesicle recruitment and late membrane fusion steps (Ammar *et al.*, 2013), the possibility that PERK coordinates danger signaling through its lipid kinase activity is intriguing. This possibility is further highlighted by the general involvement of exocytosis mediated by the lipid kinase PI3K in the tracking of DAMPs during ICD (Garg *et al.*, 2012).

Last but not least, PERK is regulated by autologous and heterologous tyrosine phosphorylation at different Y residues. PERK has been shown to exhibit a dual specificity protein kinase activity and to be able to autophosphorylate the Y615 residue found within the kinase domain (Su *et al.*, 2008). PERK mediated Y615 phosphorylation was reported to occur prior to ER stress and to sustain both PERK autocatalytic activation and eIF2 α phosphorylation. However, whether PERK may regulate other signaling pathways through its tyrosine kinase activity requires further studies. Moreover, PERK activity is regulated through the Y561 residue, found in the juxtamembrane domain. The phosphorylation of PERK at Y561, which is rapidly lost upon ER stress, is protected by the binding of Nck1, a Src-homology (SH) domain-containing adaptor protein (Yamani *et al.*, 2014), to this PERK phospho-tyrosine residue, thereby keeping PERK activity in check. Indeed, depletion of Nck1 in MIN6 cells enhanced physiological PERK activity that was accompanied by increased proinsulin synthesis (Yamani *et al.*, 2014). Given that a primary role of PERK during the UPR is to inhibit protein translation, this finding is intriguing and suggests that various regulatory mechanisms control the full spectrum of PERK's physiological roles.

Emerging studies also assign to PERK a crucial role in calcium signaling through mechanisms that are partially independent of eIF2 α phosphorylation (Bollo *et al.*, 2010; Liu *et al.*, 2014; Wang *et al.*, 2013). Although the PERK- Ca^{2+} link has been initially described in the context of β -cells in the exocrine pancreas, our recent data (van Vliet *et al.*, unpublished data) confirms that this might be a more general phenomenon.

Besides shaping Ca^{2+} signaling at ER-mitochondria contact sites, during ER stress induced by loss of ER- Ca^{2+} homeostasis, the interface between the ER and the plasma membrane (PM) becomes essential to regulate Ca^{2+} fluxes and the refilling of the ER- Ca^{2+} store through the evolutionary conserved process of SOCE (Varnai *et al.*, 2009). SOCE is stimulated in response to depletion of the ER Ca^{2+} store and is the main pathway regenerating ER Ca^{2+} levels and maintaining Ca^{2+} signaling. The molecular entities mediating SOCE are the ER Ca^{2+} sensors Stromal interacting protein 1 (STIM1) and the PM Ca^{2+} release-activated calcium channel protein 1 (Orai1) (reviewed in Varnai *et al.*, 2009). Upon ER- Ca^{2+} depletion STIM1 senses a decrease in the luminal Ca^{2+} levels through its ER luminal EF hand domain, which triggers STIM1 oligomers and their translocation to the PM (Luik *et al.*, 2006; Wu *et al.*, 2006), where they bind Orai1. Binding of STIM1 to Orai1 allows its clustering and generation of the PM channel that allows extracellular Ca^{2+} influx into the cytosol. This process is accompanied by a large remodeling of the ER (Orci *et al.*, 2009), which enables the formation of ER specialized compartments termed pre-cortical and cortical ER, consisting of thin subsections of the ER closely juxtaposed to the PM which are highly enriched in STIM1 but free of chaperones and ribosomes (Orci *et al.*, 2009; Shen *et al.*, 2011).

The molecular mechanism through which PERK regulates SOCE is still elusive but may involve the concerted action of calcineurin (CN), a Ca^{2+} -dependent phosphatase, and PERK activation after an elevation in cytosolic Ca^{2+} (Wang *et al.*, 2013). Interestingly, we reported that in PERK knock out (KO) mouse embryonic fibroblasts, agonist-mediated Ca^{2+} efflux through the IP₃R was compromised due to a disturbed contact between the ER and the PM (Verfaillie *et al.*, 2012). This further suggests that absence of PERK may contribute to exacerbate ER stress by hampering proper ER- Ca^{2+} refilling (through ER-PM contacts) and mitochondrial Ca^{2+} buffering (through the MAMs).

In conclusions these findings advocate a role for PERK as a vital enabler and regulator of the communication between the ER and different organelles/vesicular trafficking machinery in resting and ER stress conditions.

Conclusions

Emerging studies support the concept that the ER stress sensor PERK is endowed with novel functions beyond the UPR, in particular as a master orchestrator of inter-organellar contact sites and an inducer of danger/immunogenic signaling during ROS and ER stress-mediated immunogenic cell death. However, how this ER stress sensor is capable of coordinating such key physiological functions remains to be solved (Garg *et al.*, 2015). Future studies interrogating the PERK-interactome and its dynamics under physiological and therapy-induced ER stress conditions, will be required to reach a better understanding of the biological role of PERK and to devise therapeutic opportunities to modulate its pleiotropic functions in pathophysiological conditions and during anticancer therapy eliciting immunogenic cell death.

Acknowledgments

This work was supported by C16/15/073 grant of the KU Leuven, Federal Grant under the IAP7/32 of the Belgian Science Policy Office and FWO grant G0584.12 to Patrizia Agostinis. A.D.G. is a post-doctoral fellow of the FWO-Flanders.

References

- Ammar, M. R., Kassas, N., Chasserot-Golaz, S., Bader, M. F., and Vitale, N. (2013). Lipids in Regulated Exocytosis: What are They Doing? *Front Endocrinol (Lausanne)* 4, 125.
- Anelli, T., Bergamelli, L., Margittai, E., Rimessi, A., Fagioli, C., Malgaroli, A., Pinton, P., Ripamonti, M., Rizzuto, R., and Sitia, R. (2012). Ero1 α regulates Ca²⁺ fluxes at the endoplasmic reticulum-mitochondria interface (MAM). *Antioxid Redox Signal* 16, 1077-1087.
- Bettigole, S. E., and Glimcher, L. H. (2015). Endoplasmic reticulum stress in immunity. *Annu Rev Immunol* 33, 107-138.
- Bollo, M., Paredes, R. M., Holstein, D., Zheleznova, N., Camacho, P., and Lechleiter, J. D. (2010). Calcineurin interacts with PERK and dephosphorylates calnexin to relieve ER stress in mammals and frogs. *PLoS One* 5, e11925.
- Bravo, R., Vicencio, J. M., Parra, V., Troncoso, R., Munoz, J. P., Bui, M., Quiroga, C., Rodriguez, A. E., Verdejo, H. E., Ferreira, J., Iglewski, M., Chiong, M., Simmen, T., Zorzano, A., Hill, J. A., Rothermel, B. A., Szabadkai, G., and Lavandro, S. (2011). Increased ER-mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress. *J Cell Sci* 124, 2143-2152.
- Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J., and Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* 23, 7198-7209.
- de Brito, O. M., and Scorrano, L. (2008). Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* 456, 605-610.
- Dudek-Peric, A. M., Ferreira, G. B., Muchowicz, A., Wouters, J., Prada, N., Martin, S., Kiviluoto, S., Winiarska, M., Boon, L., Mathieu, C., van den Oord, J., Stas, M., Gougeon, M. L., Golab, J., Garg, A. D., and Agostinis, P. (2015). Antitumor immunity triggered by melphalan is potentiated by melanoma cell surface-associated calreticulin. *Cancer Res* 75, 1603-1614.
- Filadi, R., Greotti, E., Turacchio, G., Luini, A., Pozzan, T., and Pizzo, P. (2015). Mitofusin 2 ablation increases endoplasmic reticulum-mitochondria coupling. *Proc Natl Acad Sci USA* 112, E2174-2181.
- Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J., and Voeltz, G. K. (2011). ER tubules mark sites of mitochondrial division. *Science* 334, 358-362.
- Fucikova, J., Moserova, I., Urbanova, L., Bezu, L., Kepp, O., Cremer, I., Salek, C., Strnad, P., Kroemer, G., Galluzzi, L., and Spisek, R. (2015). Prognostic and predictive value of DAMPs and DAMP-Associated processes in cancer. *Front Immunol* 6, 402.
- Galluzzi, L., Vacchelli, E., Bravo-San Pedro, J. M., Buque, A., Senovilla, L., Baracco, E. E., Bloy, N., Castoldi, F., Abastado, J. P., Agostinis, P., Apte, R. N., Aranda, F., Ayyoub, M., Beckhove, P., Blay, J. Y., Bracci, L., Caignard, A., Castelli, C., Cavallo, F., Celis, E., Cerundolo, V., Clayton, A., Colombo, M. P., Coussens, L., Dhodapkar, M. V., Eggermont, A. M., Fearon, D. T., Fridman, W. H., Fucikova, J., Gabrilovich, D. I., Galon, J., Garg, A., Ghiringhelli, F., Giaccone, G., Gilboa, E., Gnjjatic, S., Hoos, A., Hosmalin, A., Jager, D., Kalinski, P., Karre, K., Kepp, O., Kiessling, R., Kirkwood, J. M., Klein, E., Knuth, A., Lewis, C. E., Liblau, R., Lotze, M. T., Lugli, E., Mach, J. P.,

- Mattei, F., Mavilio, D., Melero, I., Melief, C. J., Mittendorf, E. A., Moretta, L., Odunsi, A., Okada, H., Palucka, A. K., Peter, M. E., Pienta, K. J., Porgador, A., Prendergast, G. C., Rabinovich, G. A., Restifo, N. P., Rizvi, N., Sautes-Fridman, C., Schreiber, H., Seliger, B., Shiku, H., Silva-Santos, B., Smyth, M. J., Speiser, D. E., Spisek, R., Srivastava, P. K., Talmadge, J. E., Tartour, E., Van Der Burg, S. H., Van Den Eynde, B. J., Vile, R., Wagner, H., Weber, J. S., Whiteside, T. L., Wolchok, J. D., Zitvogel, L., Zou, W., and Kroemer, G. (2014). Classification of current anticancer immunotherapies. *Oncotarget* 5, 12472-12508.
- Garg, A. D., De Ruyscher, D., and Agostinis, P. (2015). Immunological metagene signatures derived from immunogenic cancer cell death associate with improved survival of patients with lung, breast or ovarian malignancies: a large-scale meta-analysis. *Oncoimmunology*, DOI: 10.1080/2162402X.2015.1069938.
- Garg, A. D., Dudek, A. M., Ferreira, G. B., Verfaillie, T., Vandenabeele, P., Krysko, D. V., Mathieu, C., and Agostinis, P. (2013). ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy* 9, 1292-1307.
- Garg, A. D., Elsen, S., Krysko, D. V., Vandenabeele, P., de Witte, P., and Agostinis, P. (2015). Resistance to anticancer vaccination effect is controlled by a cancer cell-autonomous phenotype that disrupts immunogenic phagocytic removal. *Oncotarget* 6, 26841-26860.
- Garg, A. D., Galluzzi, L., Apetoh, L., Baert, T., Birge, R. B., Bravo-San Pedro, J. M., Breckpot, K., Brough, D., Chaurio, R., Cirone, M., Coosemans, A., Coulie, P. G., De Ruyscher, D., Dini, L., de Witte, P., Dudek-Peric, A. M., Faggioni, A., Fucikova, J., Gaip, U. S., Golab, J., Gougeon, M. L., Hamblin, M. R., Hemminki, A., Herrmann, M., Hodge, J. W., Kepp, O., Kroemer, G., Krysko, D. V., Land, W. G., Madeo, F., Manfredi, A. A., Mattarollo, S. R., Maueroder, C., Merendino, N., Multhoff, G., Pabst, T., Ricci, J. E., Riganti, C., Romano, E., Rufo, N., Smyth, M. J., Sonnemann, J., Spisek, R., Stagg, J., Vacchelli, E., Vandenabeele, P., Vandenberk, L., Van den Eynde, B. J., Van Gool, S., Velotti, F., Zitvogel, L., and Agostinis, P. (2015). Molecular and translational classifications of DAMPs in immunogenic cell death. *Front Immunol* 6, 588.
- Garg, A. D., Krysko, D. V., Vandenabeele, P., and Agostinis, P. (2012). Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. *Cancer Immunol Immunother* 61, 215-221.
- Garg, A. D., Krysko, D. V., Vandenabeele, P., and Agostinis, P. (2016). Extracellular ATP and P2X7 receptor exert context-specific immunogenic effects after immunogenic cancer cell death. *Cell Death Dis* 7, DOI:10.1038/cddis.2015.1411.
- Garg, A. D., Krysko, D. V., Verfaillie, T., Kaczmarek, A., Ferreira, G. B., Marysael, T., Rubio, N., Firczuk, M., Mathieu, C., Roebroek, A. J., Annaert, W., Golab, J., de Witte, P., Vandenabeele, P., and Agostinis, P. (2012). A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *EMBO J* 31, 1062-1079.
- Garg, A. D., Maes, H., van Vliet, A. R., and Agostinis, P. (2015). Targeting the hallmarks of cancer with therapy-induced endoplasmic reticulum (ER) stress. *Mol Cell Oncol* 2, e975089.
- Garg, A. D., Vandenberk, L., Koks, C., Verschuere, T., Boon, L., Van Gool, S., and Agostinis, P. (2016). Dendritic cell vaccines based on immunogenic cell death elicit

- danger signals and T cell-driven rejection of high-grade glioma. *Sci Translat Med*, in Press.
- Gilady, S. Y., Bui, M., Lynes, E. M., Benson, M. D., Watts, R., Vance, J. E., and Simmen, T. (2010). Ero1 α requires oxidizing and normoxic conditions to localize to the mitochondria-associated membrane (MAM). *Cell Stress Chaperones* 15, 619-629.
- Gorlach, A., Klappa, P., and Kietzmann, T. (2006). The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. *Antioxid Redox Signal* 8, 1391-1418.
- Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., Oomori, H., Noda, T., Haraguchi, T., Hiraoka, Y., Amano, A., and Yoshimori, T. (2013). Autophagosomes form at ER-mitochondria contact sites. *Nature* 495, 389-393.
- Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397, 271-274.
- Hetz, C., Chevet, E., and Oakes, S. A. (2015). Proteostasis control by the unfolded protein response. *Nat Cell Biol* 17, 829-838.
- Kepp, O., Senovilla, L., Vitale, I., Vacchelli, E., Adjemian, S., Agostinis, P., Apetoh, L., Aranda, F., Barnaba, V., Bloy, N., Bracci, L., Breckpot, K., Brough, D., BuquÃ©, A., Castro, M. G., Cirone, M., Colombo, M. I., Cremer, I., Demaria, S., Dini, L., Eliopoulos, A. G., Faggioni, A., Formenti, S. C., Fucikova, J., Gabriele, L., Gaip, U. S., Galon, J. r. m., Garg, A., Ghiringhelli, F. o., Giese, N. A., Guo, Z. S., Hemminki, A., Herrmann, M., Hodge, J. W., Holdenrieder, S., Honeychurch, J., Hu, H.-M., Huang, X., Illidge, T. M., Kono, K., Korbelik, M., Krysko, D. V., Loi, S., Lowenstein, P. R., Lugli, E., Ma, Y., Madeo, F., Manfredi, A. A., Martins, I., Mavilio, D., Menger, L., Merendino, N., Michaud, M., Mignot, G., Mossman, K. L., Multhoff, G., Oehler, R., Palombo, F., Panaretakis, T., Pol, J., Proietti, E., Ricci, J.-E., Riganti, C., Rovere-Querini, P., Rubartelli, A., Sistigu, A., Smyth, M. J., Sonnemann, J., Spisek, R., Stagg, J., Sukkurwala, A. Q., Tartour, E., Thorburn, A., Thorne, S. H., Vandenabeele, P., Velotti, F., Workenhe, S. T., Yang, H., Zong, W.-X., Zitvogel, L., Kroemer, G., and Galluzzi, L. (2014). Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunology* 3, e955691.
- Kroemer, G., Galluzzi, L., Kepp, O., and Zitvogel, L. (2013). Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 31, 51-72.
- Liu, Y., Su, Y., and Wang, X. (2013). Phosphatidic acid-mediated signaling. *Adv Exp Med Biol* 991, 159-176.
- Liu, Z., Cai, H., Zhu, H., Toque, H., Zhao, N., Qiu, C., Guan, G., Dang, Y., and Wang, J. (2014). Protein kinase RNA-like endoplasmic reticulum kinase (PERK)/calcineurin signaling is a novel pathway regulating intracellular calcium accumulation which might be involved in ventricular arrhythmias in diabetic cardiomyopathy. *Cell Signal* 26, 2591-2600.
- Luik, R. M., Wu, M. M., Buchanan, J., and Lewis, R. S. (2006). The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. *J Cell Biol* 174, 815-825.
- Malhi, H., Kropp, E. M., Clavo, V. F., Kobrossi, C. R., Han, J., Mauer, A. S., Yong, J., and Kaufman, R. J. (2013). C/EBP homologous protein-induced macrophage apoptosis protects mice from steatohepatitis. *J Biol Chem* 288, 18624-18642.

- Malhotra, J. D., and Kaufman, R. J. (2007). The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* 18, 716-731.
- Munoz, J. P., Ivanova, S., Sanchez-Wandelmer, J., Martinez-Cristobal, P., Noguera, E., Sancho, A., Diaz-Ramos, A., Hernandez-Alvarez, M. I., Sebastian, D., Mauvezin, C., Palacin, M., and Zorzano, A. (2013). Mfn2 modulates the UPR and mitochondrial function via repression of PERK. *EMBO J* 32, 2348-2361.
- Orci, L., Ravazzola, M., Le Coadic, M., Shen, W. W., Demareux, N., and Cosson, P. (2009). From the Cover: STIM1-induced precortical and cortical subdomains of the endoplasmic reticulum. *Proc Natl Acad Sci USA* 106, 19358-19362.
- Oyadomari, S., and Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 11, 381-389.
- Panaretakis, T., Kepp, O., Brockmeier, U., Tesniere, A., Bjorklund, A. C., Chapman, D. C., Durchschlag, M., Joza, N., Pierron, G., van Endert, P., Yuan, J., Zitvogel, L., Madeo, F., Williams, D. B., and Kroemer, G. (2009). Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J* 28, 578-590.
- Pollard, M. G., Travers, K. J., and Weissman, J. S. (1998). Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol Cell* 1, 171-182.
- Raturi, A., and Simmen, T. (2013). Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* 1833, 213-224.
- Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8, 519-529.
- Rowland, A. A., and Voeltz, G. K. (2012). Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat Rev Mol Cell Biol* 13, 607-625.
- Schroder, M., and Kaufman, R. J. (2005). The mammalian unfolded protein response. *Annu Rev Biochem* 74, 739-789.
- Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* 3, 99-111.
- Shen, W. W., Frieden, M., and Demareux, N. (2011). Remodelling of the endoplasmic reticulum during store-operated calcium entry. *Biol Cell* 103, 365-380.
- Stevens, F. J., and Argon, Y. (1999). Protein folding in the ER. *Semin Cell Dev Biol* 10, 443-454.
- Su, Q., Wang, S., Gao, H. Q., Kazemi, S., Harding, H. P., Ron, D., and Koromilas, A. E. (2008). Modulation of the eukaryotic initiation factor 2 -subunit kinase PERK by tyrosine phosphorylation. *J Biol Chem* 283, 469-475.
- Vacchelli, E., Ma, Y., Baracco, E. E., Sistigu, A., Enot, D. P., Pietrocola, F., Yang, H., Adjemian, S., Chaba, K., Semeraro, M., Signore, M., De Ninno, A., Lucarini, V., Peschiaroli, F., Businaro, L., Gerardino, A., Manic, G., Ulas, T., Gunther, P., Schultze, J. L., Kepp, O., Stoll, G., Lefebvre, C., Mulot, C., Castoldi, F., Rusakiewicz, S., Ladoire, S., Apetoh, L., Bravo-San Pedro, J. M., Lucattelli, M., Delarasse, C., Boige, V., Ducreux, M., Delaloge, S., Borg, C., Andre, F., Schiavoni, G., Vitale, I., Laurent-

- Puig, P., Mattei, F., Zitvogel, L., and Kroemer, G. (2015). Chemotherapy-induced antitumor immunity requires formyl peptide receptor 1. *Science* 350, 972-978.
- van Vliet, A. R., Verfaillie, T., and Agostinis, P. (2014). New functions of mitochondria associated membranes in cellular signaling. *Biochim Biophys Acta* 1843, 2253-2262.
- Vandenberk, L., Garg, A. D., Verschuere, T., Koks, C., Belmans, J., Beullens, M., Agostinis, P., De Vleeschouwer, S., and Van Gool, S. (2015). Irradiation of necrotic cancer cells employed for pulsing dendritic cells (DCs), potentiates DC vaccine-induced antitumor immunity against high-grade glioma. *Oncoimmunology*, DOI:10.1080/2162402X.2015.1083669.
- Varnai, P., Hunyady, L., and Balla, T. (2009). STIM and Orai: the long-awaited constituents of store-operated calcium entry. *Trends Pharmacol Sci* 30, 118-128.
- Verfaillie, T., Garg, A. D., and Agostinis, P. (2013). Targeting ER stress induced apoptosis and inflammation in cancer. *Cancer Lett* 332, 249-264.
- Verfaillie, T., Rubio, N., Garg, A. D., Bultynck, G., Rizzuto, R., Decuypere, J. P., Piette, J., Linehan, C., Gupta, S., Samali, A., and Agostinis, P. (2012). PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. *Cell Death Differ* 19, 1880-1891.
- Vishnu, N., Jadoon Khan, M., Karsten, F., Groschner, L. N., Waldeck-Weiermair, M., Rost, R., Hallstrom, S., Imamura, H., Graier, W. F., and Malli, R. (2014). ATP increases within the lumen of the endoplasmic reticulum upon intracellular Ca²⁺ release. *Mol Biol Cell* 25, 368-379.
- Wang, R., McGrath, B. C., Kopp, R. F., Roe, M. W., Tang, X., Chen, G., and Cavener, D. R. (2013). Insulin secretion and Ca²⁺ dynamics in β -cells are regulated by PERK (EIF2AK3) in concert with calcineurin. *J Biol Chem* 288, 33824-33836.
- Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J* 17, 5708-5717.
- Wu, M. M., Buchanan, J., Luik, R. M., and Lewis, R. S. (2006). Ca²⁺ store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *J Cell Biol* 174, 803-813.
- Yamani, L., Latreille, M., and Larose, L. (2014). Interaction of Nck1 and PERK phosphorylated at Y(5)(6)(1) negatively modulates PERK activity and PERK regulation of pancreatic β -cell proinsulin content. *Mol Biol Cell* 25, 702-711.
- Yamazaki, T., Hannani, D., Poirier-Colame, V., Ladoire, S., Locher, C., Sistigu, A., Prada, N., Adjemian, S., Catani, J. P., Freudenberg, M., Galanos, C., Andre, F., Kroemer, G., and Zitvogel, L. (2014). Defective immunogenic cell death of HMGB1-deficient tumors: compensatory therapy with TLR4 agonists. *Cell Death Differ* 21, 69-78.
- Zhou, R., Yazdi, A. S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469, 221-225.

Tables and figures

Figure 1 The many faces of PERK at the endoplasmic reticulum (ER).

Various unfolded protein response (UPR) dependent and independent functions of PERK at the ER have emerged. Beyond the classical UPR-dependent role in cell death/survival regulation, PERK has been found to function in secretory trafficking of danger signals and help in ER-mitochondria tethering, in a UPR-independent manner. In fact many new functions of PERK continue to emerge and are currently subject of intense investigations. We envisage that this ER-sessile kinase may “hold in store” many more unknown functions that can come to light in coming years.