Scientists contemplate unexplained death in Austrian Alps

Charles Miller¹*, Christopher Dillon¹, Jennifer Martinez¹, Melissa Parsons¹, Ricardo Weinlich¹, Gerry Melino²,³

The recent EMBO Molecular Medicine Workshop on Cell Death and Disease was held this past March in the picturesque Alpen ski-town of Obergurgl, Austria. Scientists working on diverse mechanisms and pathways of cell death convened to present and discuss their current research. Topics included not only cell death signalling pathways, their etiology in human disease, and potential avenues for therapeutic intervention, but also new approaches and perspectives for understanding the subtle mechanisms regulating cell fate.

Cell death is a critical component of numerous cellular processes and must be executed with extreme precision to ensure proper embryonic development, normal functioning of immune system and termination of incipient tumours. As a consequence, deregulated cell death presents as a contributing mechanism in many disease pathologies including autoimmune disorders, cardiovascular disease and human cancer. For this reason, the therapeutic potential in modulating cell death in human disease remains an enormously plausible yet unrealized opportunity. Although many of the critical proteins involved in classical apoptosis have been described, additional research into cell death signalling pathways and protein networks continues at a fevered pace, elucidating the intricacies regulating established pathways and uncovering poorly understood mechanisms of cell death.

Recognizing the benefit of discussing these new findings, Andreas Villunger, Andreas Strasser and Gerry Melino invited approximately 100 scientific experts to the majestic Tyrolean Alps, to present their current research and discuss recent advances with colleagues. The result was the EMBO Cell Death and Disease workshop, appropriately nicknamed ‘Death in the Alps 3.0’, which presciently augured both the vigorous scientific program and inadequate schussing prowess of many conference attendees. Participants were treated to detailed scientific discussions, stimulating poster sessions and the tireless hospitality of the conference centre staff, all of which contributed to the good-spirited collegial atmosphere and by all accounts resounding success of the conference. In this meeting report, we will highlight the new discoveries presented in established cell death signalling pathways, how these pathways contribute to normal immune function and diseases, including cancer, and novel perspectives on how these myriad pathways ultimately regulate cellular decisions to die.

**p53 Family involvement in cell death and disease**

p53 is arguably the most important human tumour suppressor, but despite enormous research efforts, questions persist about how it executes this function. p53 knockout mice predominately develop thymic lymphomas, which are believed to result from failure of p53-induced apoptosis or cell cycle arrest, for which Puma and p21 are thought to be the most important transcriptional targets, respectively (Lane & Levine, 2010). In their presentations, Andreas Strasser (Walter and Eliza Hall Institute, Australia) and Alexander Egle (Medical University Salzburg, Austria) both addressed the pressing question of how p53 performs its tumour-suppressor function. Somewhat surprisingly, Egle and Strasser independently presented that Puma/p21 double-knockout as well as Puma/Noxa double-knockout mice do not develop thymic lymphomas or any other cancers for that matter, which would be predicted if these are the most relevant targets for p53-mediated tumour suppression. However, since neither mouse phenocopies the p53 knockout, the elusive mechanism of p53 tumour suppression remains obscure. Similarly, the mechanism by which stabilized p53 regulates cell fate, through transcriptional regulation of either cell cycle arrest or apoptotic targets, remains incomplete. Addressing this question, Ulrich Maurer (Institute of Molecular Medicine and Cell Research, Germany) showed that Glycogen Synthase Kinase-3 (GSK-3) inhibition prevents the transcriptional induction of Puma, and consequently cell death, following gamma-irradiation. His data suggested that inhibition of GSK-3-dependent phosphorylation of the histone acetyltransferase Tip60 and subsequent

(1) Department of Immunology, St. Jude Children’s Research Hospital, Memphis, TN, USA
(2) IDI-IRCCS & University of Rome, Tor Vergata, Rome, Italy
(3) MRC Toxicology Unit, Leicester, UK
*Corresponding author: Tel: +1 901 595 5850, Fax: +1 901 595 5766;
E-mail: charles.miller@stjude.org
DOI 10.1002/emmm.201100148
absence of p53. While caspase-2 has protease in promoting cell death in the ing interest in the function of this p53 family (Levine et al, 2011). evolutionarily ancestral function of the p53 function.

Ascribing specific functions to p53 and other family members (p63 and p73) is further complicated by partially overlapping functions between proteins. Xin Lu (Oxford, UK) reported that ASPP2 (apoptosis-stimulating protein of p53 2) not only regulates the cancer-related function of p53, but also the polarity and proliferation of neural progenitors. This function resembles that of p73 and is mediated by the physical interaction of ASPP2 with Par3’s apical/junctional localization, without affecting its expression or Par3’s apical/localization binding. Andreas Strasser described potential insights into p63 function using a model of radiation-induced death of primordial follicle oocytes, a cell type in which only p63 but not p53 or p73 are expressed. In these primordial follicle oocytes, loss of p63 (Suh et al, 2006) or loss of Puma (and even more so combined loss of Puma and Noxa) protects from DNA damage-induced apoptosis. This demonstrates that under physiological conditions p63 triggers apoptosis through transcriptional upregulation of Puma and Noxa. Importantly, this oocyte cell death leads to infertility that was rescued by loss of Puma (or combined loss of Puma and Noxa), suggesting that this pathway might be a potential therapeutic target for certain types of infertility. Significant data, in fact, strongly suggests that maternal reproduction was the primary evolutionarily ancestral function of the p53 family (Levine et al, 2011).

Caspase-2 is the most evolutionarily conserved caspase and there is continuing interest in the function of this protease in promoting cell death in the absence of p53. While caspase-2 has previously been described as a tumour suppressor in the context ofEr-Myc-driven lymphomas (Mo, 2009). Andreas Villunger (Innsbruck Medical University, Austria) presented that the onset of tumorigenesis in p53 knockout animals was unaffected by the additional loss of caspase-2. Furthermore, in efforts to define the mechanism of the checkpoint kinase 1 (Chk1)-suppressed, caspase-2-dependent apoptotic pathway in the absence of p53 (Sidi et al, 2008), Villunger discovered that, while conserved in zebrafish and humans, this pathway appears absent in mice. These unexpected results may bring into question the potential therapeutic value of targeting caspase-2 in tumours with compromised p53 function.

**Bcl-2 family proteins in apoptosis and autophagy**

The Bcl-2 family of proteins plays a critical role in regulating mitochondrial-dependent apoptosis in cells; consequently several insightful presentations were dedicated to discussion of Bcl-2 proteins and their mechanistic role in promoting cell death. Douglas Green (St. Jude Children’s Research Hospital, USA) described two ‘modes’ by which anti-apoptotic Bcl-2 proteins block activation of the cell death effectors Bak and Bax. In Mode 1, anti-apoptotic Bcl-2 proteins block MOMP by sequestering BH3-only proteins that directly promote effector activation, while in Mode 2, which appears more robust at blocking MOMP, anti-apoptotic Bcl-2 proteins block effector activity by binding to and inhibiting effectors directly.

The function of a new Bcl-2 family member (Bcl-2-like protein 12), which was described as a novel cell death protein over a decade ago, has remained enigmatic and was highlighted in a talk by Martin Brandenburg (Institute of Molecular Medicine and Cell Research, Germany). Interestingly, Brandenburg described a potential role for Bcl2L12 in cell cycle control and regulation by phosphorylation, perhaps by cyclin-dependent kinase 1 in M phase. Importantly, knockdown of Bcl2L12 protected against Taxol-induced death and cells continued to replicate in the presence of the drug. From this data, it would stand to reason that Bcl2L12 activity could be an important factor affecting cellular proliferation in tumours, particularly those where Taxol treatment is clinically relevant.

Beyond discussing new Bcl-2 proteins, Seamus Martin (Trinity College, Ireland) described a novel role for the protein Noxa in regulating autophagic cell death in tumour cells with oncogenic Ras. Martin showed that Ras-induced death was not associated with typical apoptotic markers, but with characteristics of autophagy. The Martin lab provided evidence that oncogenic Ras upregulates Noxa levels and displaces anti-apoptotic Bcl-2 family proteins from Beclin, allowing Beclin to induce autophagic cell death. These findings have obvious implications for Ras-induced tumorigenesis and suggest this pathway could be important in preventing tumour formation in organisms.

**TNF signaling in cell death and inflammation**

The role of inhibitor of apoptosis proteins (IAPs) as antagonists of cell death pathways is well described and IAP mutations are associated with chemoresistance and poor prognosis in human cancers. This discovery has prompted researchers to investigate the therapeutic potential of small pharmacological inhibitors of IAPs. Termed Smac mimetics, these IAP inhibitors are known to facilitate tumour necrosis factor (TNF)-induced death – both apoptosis and necroptosis – by inducing degradation of cIAP1 and cIAP2. A presentation by Pascal Meier (Institute of Cancer Research, England) provided insight into the role of IAPs in regulating etoposide-induced cell death. XIAP and cIAP1 normally ubiquitinate the receptor interacting protein kinase 1 (RIPK1), targeting it for proteasomal degradation and inhibiting caspase-8 mediated cell death. Meier indicated that treatment with etoposide induces cell death via loss of IAP-dependent suppression of the pro-death RIPK1-caspase-8 containing Ripoptosome. This suggests that etoposide, like second mitochondria-derived activator of caspases (SMAC)
mimetics, stimulates autoubiquitylation and degradation of IAPs. Further delineating the role of IAPs in RIPK1 function, John Silke (Walter and Eliza Hall Institute, Australia) outlined findings from his lab showing that cIAPs are important regulators of RIPK1 at the tumour necrosis factor receptor 1 (TNFR1) and regulate cellular sensitivity to TNF treatment. Interestingly, their work showed that genetic ablation of cIAP1/cIAP2 or cIAP1/XIAP, but not cIAP2/XIAP, results in an early embryonic lethal genotype, which was partially rescued by simultaneous loss of TNFR1 or RIPK1, at least until (soon after) birth. By demonstrating that the IAP family modulates RIPK1 recruitment to the TNFR1 platform, and that this mechanism can profoundly alter the outcome of TNF signalling, these findings have important implications for our understanding of IAP-regulated cell death.

Investigating the role of IAPs in regulating cell death during inflammation, a presentation by Martin Leverkus (University Heidelberg, Germany) demonstrated that Toll-like receptor 3 (TLR3)-induced apoptosis was inhibited by cIAP, the loss of which promoted the assembly of the ripoptosome, and sensitized cells to poly (I/C)-induced death. The ability of cIAP to dictate the mode of TLR3-mediated, and possibly other signalling-mediated, cell death pathways may have critical consequences during the inflammatory response.

RIPK2-dependent XIAP association with the nucleotide-binding oligomerization domain 2 (NOD2) signalosome is required for nuclear factor kappa B (NF-κB) activation in response to NOD2 ligands (Krieg et al, 2009), however, the mechanism for XIAP regulation of NF-κB signalling still awaits elucidation. In an intriguing presentation by Mads Gyrd-Hansen (NNF Center for Protein Research, Denmark), data from their lab demonstrated that XIAP coordinates the assembly of the NOD2 signalosome, via ubiquitination of RIPK2 and consequently the recruitment of additional factors. Loss of XIAP ligase activity resulted in decreased NF-κB activation.

Henning Walczak (Imperial College London, England) introduced us to a new member to the LUBAC complex named Sharpin. The ubiquitin ligases HOIL-1 and HOIP and Sharpin are present as a cytoplasmic, pre-formed high-molecular weight complex. Sharpin deficiency impairs TNF-induced NF-κB activation, similar to the HOIL-1/HOIP knockdown. Walczak presented evidence that RIPK1 can be linearly ubiquitinated, but importantly, NF-κB essential modulator (NEMO) receives only linear ubiquitin chains. Walczak’s group also showed that Sharpin-deficient mice develop severe chronic proliferative dermatitis with increased keratinocyte death and this phenotype is completely rescued by TNF deletion as shown in collaboration with John Silke’s group. Altogether, these data suggest that different types of ubiquitination modify the composition of the TNF signalling complex, altering the inflammatory outcome of TNFRI ligation.

Caspase-8 has distinct roles in promoting cell death and survival and understanding the mechanisms that regulate these opposing functions is still of considerable interest. Along these lines, Andrew Oberst (St. Jude Children’s Research Hospital, USA) presented evidence that caspase-8 functions in a catalytically active complex with FLICE-like inhibitory protein long (FLIPL) to prevent RIPK3-dependent necrosis, without inducing activation of apoptosis. This observation was further supported by the ability of RIPK3 deletion to rescue the development of Caspase-8 deficient mice, suggesting that caspase-8 is required to suppress run-away necrosis in the developing embryo. David Wallach (Weizmann Institute of Science, Israel) presented a novel mechanism for regulation of inflammation: the cleavage of RIPK1 by caspase-8. As observed in a variety of cell types in vitro, as well as in the in the liver of mice with caspase-8-deleted hepatocytes, activation of the interferon regulatory factor 3 (IRF-3) transcription factor by RNA viruses is enhanced by caspase-8 deficiency. Mechanistically, both caspase-8 and RIPK1 are recruited to the RNA helicase RIG-I signalling complex, and infection with Sendai virus induces linkage of poly-ubiquitin chains (that are in part K63-linked and thus do not prompt proteosomal degradation) to RIPK1, which marked it for caspase-8-mediated cleavage. Importantly, the cleavage of RIPK1 by caspase-8 not only arrested the IRF-3 activation by an activated complex via breakdown of functional RIPK1 in it, but it also generated a RIPK1 fragment that could displace full-length RIPK1 molecules from other (neighbouring) complexes. Therefore, caspase-8-mediated RIPK1 cleavage was proposed to be a two-pronged mechanism of limiting the induction of IRF-3 through RIG-I activation.

Elucidation of the myriad mechanisms that regulate cell fate is a formidable challenge. It is clear, however, from the quality of the discoveries, passionate discussions and technical advances presented at this meeting that scientists continue to embrace this challenge. Further understanding of the complex signalling pathways that regulate cell death are expected to yield important insights into human health and development and our ability to treat its associated pathologies. Not long after our meeting in the Austrian Alps we heard of the sudden and premature loss of Ju¨rg Tschopp, a primary scientist in the field of TNF signalling in cell death and inflammation and a leading figure in our community. We wish to pay tribute to him here and his contributions to our field; he will be deeply missed.

Acknowledgements

The authors wish to specifically thank the organizers and all the conference presenters and participants for their insightful presentations and stimulating discussions. We apologize to those whose work was unable to be discussed due to space limitations.

The authors declare that they have no conflict of interest.

References

Ho LH, Taylor R, Dorstyn L, Cakouros D, Bouillet P, Kumar S (2009) A tumor suppressor function for caspase-2. Proc Natl Acad Sci USA 13: 5336-5341
Krieg A, Correa RG, Garrison JB, Le Negrade G, Welsh K, Huang Z, Knoefel WT, Reed JC (2009) XIAP mediates NOD2 signalling via interaction with RIP2. Proc Natl Acad Sci USA 106: 14524-14529
Lane D, Levine A (2010) p53 Research: the past thirty years and the next thirty years. Cold Spring Harb Perspect Biol 12: a000893
Levine AJ, Tomasini R, McKeon FD, Mak TW, Melino G (2011) The p53 family: guardians of maternal reproduction. Nat Rev Mol Cell Biol 12: 259-265
Sidi S, Sanda T, Kennedy RD, Hagen AT, Jette CA, Hoffmans R, Pascual J, Imamura S, Kishi S, Amatruda JF, et al (2008) Chk1 suppresses a caspase-2 response to DNA damage that bypasses p53, Bcl-2, and caspase-3. Cell 133: 864-877
Suh EK, Yang A, Kettenbach A, Bamberger C, Michaelis AH, Zhu Z, Elvin JA, Bronson RT, Crum CP, McKeon F (2006) p63 protects the female germ line during meiotic arrest. Nature 444: 624-628