Programmed cell death: Superman meets Dr Death

Pascal Meier and John Silke

This year's Cold Spring Harbor meeting on programmed cell death (September 17–21, 2003), organised by Craig Thompson and Junying Yuan, was proof that the 'golden age' of research in this field is far from over. There was a flurry of fascinating insights into the regulation of diverse apoptotic pathways and unexpected non-apoptotic roles for some of the key apoptotic regulators and effectors. In addition to their role in cell death, components of the apoptotic molecular machinery are now known to also function in a variety of essential cellular processes, such as regulating glucose homeostasis, lipid metabolism, cell proliferation and differentiation.

Take the backdrop of Cold Spring Harbor, expose a bunch of scientists to the frantic pace of cell death research and out comes an intoxicating mixture of information and ideas. As it is impossible to report on all the excellent talks and posters, we have taken the approach of a tabloid journalist, primarily covering talks that generated lots of discussion. To look for an overall theme for the meeting, we need go no further than that other well-known journalist, Clark Kent, alias Superman. Many of the talks revealed the secret lives of proteins that until now had been considered to have humble existences and purposes, but which under the right circumstances exposed their alter egos and latent super powers.

Yoshihide Tsujimoto (Osaka University Graduate School of Medicine, Osaka, Japan) presented a striking example of this, showing that after γ-irradiation-induced release from the nucleus, the mild-mannered heterochromatin molecule, histone H1.2, acquired the extraordinary power of promoting cytochrome *c* release from mitochondria, thereby engaging the intrinsic cell death programme. Although all nuclear histone H1 variants were also released into the cytoplasm, only histone H1.2 translocation led to cytochrome *c* release. The high degree of sequence conservation between H1.2 and other H1 variants (70–80% identity) should allow rapid identification of the key residues required for its cytochrome *c*-releasing activity. Another unresolved question is the mechanism by which p53 mediates H1.2 translocation from the nucleus to the cytoplasm[1]. The pro-death Bcl-2 family member Bad was also shown to have a more beneficial, non-apoptotic, role in regulating intracellular glucose metabolism in liver and pancreatic islet cells. Nika Danial (Dana Farber Cancer Institute, Boston, MA) showed that Bad exists in a complex with Hexokinase IV (HK4). This complex was not formed in the Bad knockout mice and those mice displayed abnormal glucose homeostasis[2]. HK4–Bad complexes were exclusively localized to mitochondria, which is unexpected as both Bad and HK4 (~99% of total) predominantly localize to the cytoplasm in healthy cells. Thus, an outstanding question concerns the role of the uncomplexed cytoplasmic HK4 in glucose homeostasis[2]. Moreover, it will be important to establish the relative importance of cytoplasmic versus mitochondrial-bound Bad in different life and death processes.

Most pro- and anti-apoptotic members of the Bcl-2 family either normally localize to mitochondria or flock to this organelle after a death stimuli. François Vallette (INSERM, Nantes, France) showed that different subunits of the mitochondrial protein import complex TOM (translocase of the outer membrane) function as mitochondrial receptors for distinct Bcl-2-family members: TOM22 is required for the recruitment of Bax to the mitochndria and TOM20 for Bcl-2. This raises the question of whether there is an analogous receptor for Bcl-2 at the endoplasmic reticulum. One possible explanation for how healthy cells maintain the potentially lethal pro-apoptotic effector Bak in a monomeric, inactive, conformation at mitochondria was provided by Emily Cheng (Dana Farber Cancer Institute, Boston, MA), who suggested that Bak oligomerization is blocked by its association with VDAC2 (a low-abundance isoform of the mitochondrial voltage-dependent anion channel). In this model, cell death is induced when BH3 proteins such as Bim displace VDAC2 from Bak, allowing Bak aggregation and cytochrome *c* release.

Although a balance between pro- and anti-apoptotic Bcl-2 family members determines whether cells live or die, it remains unclear whether all pro-survival Bcl-2 family members have equal efficacy. Xiadong Wang (University of Texas, Dallas, TX) provided evidence that anti-apoptotic Bcl-2 family members are non-equivalent and function at specific steps to intercept the intrinsic mitochondrial death programme. Treatment with DNA-damaging agents or adenoviral infection, as described by Eileen White (Howard Hughes Medical Institute, Rutgers University, Piscataway, NJ), resulted

Pascal Meier is at The Breakthrough Toby Robins Breast Cancer Research Centre, Institute of Cancer Research, Mary-Jean Mitchell Green Building, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK. John Silke is at the Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia. e-mail: pmeier@icr.ac.uk or silke@wehi.edu.au

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in rapid proteasome-mediated degradation of the anti-apoptotic Bcl-2 family member Mcl-1. Loss of Mcl-1 was essential for several downstream mitochondrial events, such as translocation of Bax and cytochrome *c* release, placing Mcl-1 at the top of the pro-survival Bcl-2 family hierarchy[3]. However, Mcl-1 is not the sole arbiter of cell fate, as *mcl-1* small-interfering RNA (siRNA) was not cytotoxic, indicating that other events in addition to loss of Mcl-1 are required for induction of apoptosis. Xiadong Wang also presented new data on the identification of an E3 ubiquitin ligase, Mule, which is responsible for the ubiquitin-dependant degradation of Mcl-1 and hence may well function as a potent tumour suppressor. Loss of Mcl-1 results in the translocation of both pro-apoptotic Bim_{EL} and anti-apoptotic Bcl-x_L from the cytosol to the mitochondria. At first glance, it seems paradoxical that loss of Mcl-1 results in translocation of Bcl-x_L to mitochondria; however, Jerry Adams (Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia) showed that membrane insertion inactivates Bcl-w's pro-survival activity[4], potentially resolving the paradox.

Bcl-2 family members are not the only proteins that have double lives, Jürg Tschopp (University of Lausanne, Epalinges, Switzerland) showed how $FLIP_L$, long-considered an inhibitor of caspase 8/10, could also function as an activator of caspase-8. Activation of initiator caspases has been proposed to proceed through dimerization of their monomeric zymogenic forms[5]. For instance, dimerization of caspase-8 or -9 leads to their cleavage-independent activation. Importantly, dimerization causes a conformational change of the zymogen's catalytically active pocket, making it accessible to substrates[6]. Viewed in this way, it is not surprising that the catalytically inactive pseudo-caspase $FLIP_L$ induces caspase-8 activation after their dimerization. As $FLIP_L$ induces the activating structural changes in caspase-8, it does not matter that $FLIP_L$ is itself catalytically inactive. This finding presents a conundrum, if $FLIP_L$ is indeed an activator of caspases, how does $FLIP_L$ prevent caspase-8/10-mediated cell death? The simple answer is that not all caspase activation events result in death, a theme that was raised in several other talks (see below). In particular, the active caspase-8 in caspase-8–$FLIP_L$ heterodimers is not pro-apoptotic. At least in the case of the death receptor TNFR1, the answer to the question of why caspase-8–$FLIP_L$ complexes do not trigger cell death seems to be a complex issue, namely complex I and complex II. Whether or not caspase-8 is pro-apoptotic, at least in the case of the TNFR1 death receptor it is dependent on the complex in which it resides. Ligand-mediated activation of the cell-surface receptor TNFR1 results in the recruitment of complex I (TNFR1–TRADD–RIP1–TRAF2–cIAP1–NEMO) to TNFR1, triggering NF-κB activation. Shortly after receptor stimulation, complex I dissociates from TNFR1 and the liberated components (TRADD–RIP1–TRAF2–cIAP1) bind FADD–caspase-8–caspase-10 to form the 'cytoplasmic' complex II, and it is here that $FLIP_L$ regulates whether cells undergo apoptosis as a result of TNFR1 stimulation. If sufficient $FLIP_L$ is present, complex II contains TRADD–FADD–caspase-8–$FLIP_L$ and the cell lives, but if insufficient $FLIP_L$ is present then it is replaced by caspase-10 and the cell dies. As $FLIP_L$ is a transcriptional target of NF-κB, the activity of complex I can determine whether a cell lives or dies, and if NF-κB activity is impaired then the levels of $FLIP_L$ might not be sufficient to prevent cell death. Why active caspase-8 results in apoptosis when it is in the caspase-10-containing complex II, but not in the $FLIP_L$-containing complex II, remains unclear.

Activated caspases are the effectors of the apoptotic programme, but it seems that in certain developmental processes even these proteins are two-faced. Eli Arama (Howard Hughes Medical Institute, The Rockefeller University, New York, NY) showed that differentiating *Drosophila melanogaster* spermatids expel intracellular compartments containing superfluous cytoplasm through a caspase-mediated process. Intriguingly, cytochrome-*c*-d (cyt-*c*-d), one of the two *Drosophila* cytochrome *c* genes, was found to be essential for the activation of the effector caspase drICE during spermatogenesis. This is somewhat surprising given that cytochrome *c* is not required for the intrinsic cell death programme in the soma. It seems that an involvement of cyt-*c*-d in apoptosis and development is restricted to spermatogenesis, as male flies with a cyt-*c*-d loss-of-function mutation are sterile but otherwise normal. However, Hiroshi Kanda (University of Tokyo, Tokyo, Japan) demonstrated that such loss-of-function fly mutants are surprisingly resistant to TNF-induced apoptosis. How spermatids stay alive despite possessing activated caspases remains an open question. The developing *Drosophila* oocyte achieves a similar miracle. Here, dying nurse cells dump all their 'apoptotic' cytoplasm into the oocyte; however, the oocyte itself does not undergo apoptosis. Nurse cell death is a developmental prerequisite for proper oocyte maturation, as inhibition of this process results in infertile eggs. Kim McCall (Boston University, Boston, MA) showed that this nurse cell death does not require the activity of Reaper, Grim or Hid [11] (antagonists of the *Drosophila* inhibitor of apoptosis (DIAP1), or the effector caspase Dcp-1, as originally thought. Moreover, nurse cell death cannot be blocked by overexpression of DIAP1, a potent inhibitor of the initiator caspase Dronc and the effector caspases drICE and Dcp-1. However, nurse cell apoptosis does require Pita, a novel zinc-finger transcription factor that is present in the same locus as Dcp-1, and was simultaneously knocked out by the original P-element insertion [12]. How Pita promotes nurse cell death is unknown; however, given that most of the normal apoptotic molecules are not required, studies on this developmentally regulated death process should unearth some interesting genes.

As caspases have non-apoptotic roles, it follows that IAPs might regulate caspases with regard to these roles. Ezra Burstein (University of Michigan Medical School, Bethesda, MD) took this a step further by showing that the caspase inhibitor XIAP has a caspase-independent role as a regulator of copper homeostasis. XIAP was found to interact with MURR1, a factor that binds to the copper transporter ATP7B and is mutated in a hereditary form of copper toxicosis. Cells and tissues derived from XIAP knockout mice showed reduced intracellular copper levels, and XIAP seems to mediate this function through ubiquitination and degradation of MURR1. Hyung Don Ryoo (Howard Hughes Medical Institute, The Rockefeller University, New York, NY) showed that clones of cells mutant for the DIAP1 proliferated excessively and even switched developmental fates. Whether these effects are caused by aberrant caspase activity or other caspase-independent functions of DIAP1 remain unclear. One of the caspases inhibited by DIAP1 is the *Drosophila* initiator caspase Dronc. It has been known for some time that inhibition of Dronc requires the ability of DIAP1 to interact with the pro-domain of Dronc. Although Dronc is most homologous to caspase-9, it does not require proteolytic cleavage for IAP binding or contain the IAP-binding motif (IBM) found in caspase-9. The structural basis of the interaction between Dronc and DIAP1 was resolved by Yigong Shi (Princeton University, Princeton, NJ), who showed that 12 amino acids in the pro-domain of Dronc, with no apparent sequence homology to the canonical IBM, mediates DIAP1-binding. Despite the lack of sequence homology, the Dronc 12 amino-acid motif and the IBM of IAP antagonists bind to the same hydrophobic surface groove on DIAP1. The observation that two very different motifs bind to the same rigid

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IAP surface pocket may indicate that proteins with unrelated types of IAP-binding motifs can modulate IAP function. It remains to be seen whether the Dronc-type IBM is conserved in other IAP-binding proteins, such as MURR1 or ARTS (see below).

Although cell survival is ensured by members of the IAP family, apoptosis in *Drosophila* requires the activity of the IAP antagonists Reaper, Grim and Hid. Although in theory *Drosophila* IAP antagonists could induce cell death solely by liberating caspases from IAP complexes, it seems that some of these IAP antagonists also actively promote IAP-depletion. Daniel Colón-Ramos (Duke University Medical Centre, Durham, NC) showed that a small internal stretch within Reaper (the GH3 domain) was required for mitochondrial localization and IAP degradation. The *Drosophila* IAP antagonists Sickle, Reaper and Grim all contain a GH3 domain, but Hid does not. Lei Zhou (University of Florida, Gainesville, FL), however, identified another GH3-containing protein, Corp, that does not contain an IBM, but which can synergize with Hid to promote apoptosis. Thus, it is possible that Corp supplies the missing GH3 motif to make Hid functionally equivalent to Reaper and Grim. This observation is consistent with earlier genetic observations, indicating that *Drosophila* IAP antagonists are non-equivalent but function in synergy to effectively promote cell death[14]. Although none of the known mammalian IAP antagonists have been shown to trigger IAP degradation, Sarit Larisch (RamBam Medical Centre, Haifa, Israel) described how ARTS could promote apoptosis through binding and reducing XIAP levels. Similarly to the other mammalian IAP antagonists, ARTS is a mitochondrial protein; however, it does not contain an IBM. It will be interesting, therefore, to discover the mechanism of IAP binding and explain how binding promotes XIAP degradation. Emad Alnemri (Thomas Jefferson University, Philadelphia, PA) presented novel data about HtrA2/Omi, one of the known mammalian IAP antagonists. Htra2/Omi is normally resident in the mitochondrial intermembrane space but when released functions to antagonize IAPs and promote apoptosis. However, the *Escherichia coli* homologue of HtrA2/Omi is a protease/chaperone that protects *E. coli* against heat stress. Emad's data indicate that this protective function of Htra2/Omi is also required to maintain healthy mammalian mitochondria, because mice that are mutant for the serine protease activity of HtrA2/Omi suffer from a neurodegenerative disease typical of mitochondrialopathies.

A bewildering number of signals and events, such as genotoxic stress, nutrient deprivation, oncogene activation and some developmentally regulated death triggers, all seem to conspire on the mitochondria to induce release of cytochrome *c*. For instance, after genotoxic stress, p53 kills predominantly by the mitochondrial pathway, through transcriptional upregulation of pro-apoptotic genes such as the Bcl-2 family members Puma and Noxa. Surprisingly, however, Jerry Adams showed that thymocytes from Noxa knockout mice were as sensitive as wild-type thymocytes to γ-irradiation-induced apoptosis, although Noxa loss conveyed some protection to fibroblasts. However, thymocytes and fibroblasts from Puma knockout mice were highly refractory to such apoptotic signals, albeit such cells were not as resistant as p53-null counterparts. It remains unclear whether p53 signals for death entirely through BH3-only proteins (for example, Puma and Noxa) or whether other p53-induced gene products (for example, PIDD, PIG3, PIG8, PERP, p53AIP1 or p53DINP1) also contribute. One controversial hypothesis maintains that p53 has a transcription-independent cytochrome *c*-releasing activity that might synergize with Puma. Many people thought that this hypothesis had been dealt a mortal blow when transcriptionally defective p53 mutant knock-in mice displayed the same phenotype as p53 knockout mice[9]. However, Douglas Green (La Jolla Institute for Allergy and Immunology, San Diego, CA) resurrected the discussion by showing that an inducible p53 could cause exposure of phosphatidyl serine, indicative of apoptosis, even in the absence of a nucleus (that is, in cytoplasts) and that this cytoplastic apoptosis required Bax. He argued that studies using p53 transcription activation mutants[9] did not disprove the relevance of his observations because such mutants, unlike wild-type p53, did not accumulate in the cytoplasm after DNA damage. p53 mutants that cannot activate transcription of apoptotic genes but retain the ability to upregulate p53 may finally address this issue *in vivo*. Such a mutant is not such a far-fetched idea, because Xin Lu (Ludwig Institute for Cancer Research, London, UK) presented data that ASPP proteins selectively enhance the apoptotic function of p53, but not its ability to induce cell cycle arrest[10]. Xin Lu also reported on a novel member of the ASPP protein family, iASPP, which inhibits p53-induced apoptosis. RNA interference (RNAi) of iASPP in *Caenorhabditis elegans* actually induces apoptosis, arguing that iASPP constantly keeps p53's apoptotic activity in check. However, increased expression of mammalian iASPP inhibits p53-induced death and therefore, not surprisingly, can cooperate with Ras or E1A in cellular transformation. Strikingly, several human breast tumour samples with wild-type p53 express significantly higher levels of iASPP, providing a potential therapeutic target for tumours with wild-type p53.

Bruce Hay (California Institute of Technology, Pasadena, CA) elaborated on one of the recent surprises in biology, the microRNAs. Short (20–30mer) single-stranded non-coding RNAs (microRNAs) also seem to be widely used by *Drosophila* to regulate translation. In a genetic screen, Bruce Hay identified four microRNAs as efficient inhibitors of cell death. One of these microRNAs, *mir-14*, increases the threshold for apoptosis and lowers the overall protein level of the apoptotic effector caspase drICE. Surprisingly, the microRNA mir-14 not only suppresses apoptosis, but also seems to regulate fat metabolism, because mir-14 mutant flies are obese and have increased levels of diacylglycerol (DAG), a potent activator of protein kinase C (PKC). As neither drICE nor DAG-regulatory proteins are direct targets for mir-14, it will be interesting to see whether there is a direct molecular relationship between lipid biology and apoptosis. A link between lipid metabolism and apoptosis was also suggested by Roya Khosravi-Far (Harvard Medical School, Boston, MA), who reported that death receptor stimulation modifies the lipid composition of mitochondrial membranes, thereby facilitating the action of Bid in releasing apoptogenic factors.

The terms 'programmed cell death' and 'apoptosis' are frequently used as synonyms to describe physiological cell death. Clearly, not all physiological suicides occur through apoptosis. Cells can also die by an entirely different form of programmed cell death called autophagy[13]. Autophagy is a fascinating but poorly understood form of cell death in which cells die in a spectacular manner by chewing themselves up. Unlike apoptosis, cells that die by autophagy are degraded with little or no help from phagocytes and utilize lysosomes within the dying cell for their own degradation. Interestingly, most developmentally regulated cell deaths actually seem to occur through autophagy. For instance, cells in the interdigital webbing of mammals display autophagic morphological features during their death. Given the vast numbers of cells that die synchronously at specific developmental stages, it is not surprising that phagocytes cannot cope with such a work load on

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their own and that alternative strategies must be in place to clear the heap of accumulating dying cells. Eric Baehrecke (University of Maryland Biotechnology Institute, College Park, MD) showed that in *Drosophila* salivary glands, autophagic cell death requires Rpr, Hid, Dark and the caspases Dronc and Drice. Inhibition of caspases was, however, not sufficient to block morphological changes in these dying cells. Additional components, such as matrix metalloproteases (MMPs) and genes related to the yeast autophagy-ubiquitin-conjugation system (apg-related genes), are also required. Given the importance of autophagy in development and our limited knowledge on its regulation and execution, it is bound to be an exciting research topic for the future.

Taken together, many of the presentations revealed that apoptotic proteins are unlikely to function in isolation, but rather in huge molecular machines. These multiprotein complexes co-ordinate diverse aspects of cellular physiology to decide whether a cell dies or adopts a different cellular fate. These machines have been thrown together by evolution tacking on, or taking off, a little bit here or there as was expedient at the time. The processes of life and death have therefore been evolutionarily tangled, with the same proteins often present in different complexes. Viewed in this way, it is not surprising that individual proteins seem to have a perplexing variety of functions. To understand these puzzles we don't need Supermen, but rather supercomputers. John Albeck's (Massachusetts Institute of Technology, Boston, MA) talk on combining experimental and computational approaches to tease apart these interactions provided an interesting appetiser for what we predict will be an increasingly important part of research that is bound to appear more frequently in future meetings. We look forward to seeing you there. □

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ERRATUM

In the abstract of Meier and Silke (*Nature Cell Biol.* **5**, 1035–1038; 2003), the organizers for the Cold Spring Harbor meeting on programmed cell death should have read Hermann Steller, Craig Thompson and Junying Juan.

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