Doughnuts, daisy chains and crescent moons: the quest for the elusive apoptotic pore

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How the two killer proteins Bax and Bak form the putative “apoptotic pore” that is responsible for irrevocably damaging mitochondria leading to cell death during apoptosis is considered the “holy grail” of apoptosis research. Indeed, even whether Bax and Bak form a pore remains contentious largely due to the failure to detect such structures in cells or mitochondria. Two new super-resolution microscopy studies in this issue of *The EMBO Journal* now provide tantalising evidence of ring-like “apoptotic pores” on mitochondria of dying cells and provide new insight into how Bax and Bak bring about a cell’s demise.

See also: L Große *et al* (February 2016) and R Salvador-Gallego *et al* (February 2016)

Bax and Bak are members of the Bcl-2 family of proteins and the critical executioners of the intrinsic pathway of apoptosis (Wei *et al*, 2001). Following reception of an apoptotic stress, interaction with another subclass of the Bcl-2 family, the BH3-only proteins, induces a drastic change in conformation of Bax and Bak. These activated conformations are then compatible for self-association on the mitochondrial outer membrane. A long-held notion is that these oligomeric proteins form pores that damage the outer membrane of mitochondria. The “apoptotic pore” then acts as a conduit for a variety of intermembrane space proteins including cytochrome *c* and Smac/DIABLO that trigger the final proteolytic destruction and packaging of the cell during apoptosis. Recent biochemical and structural advances have provided

much needed insight into how these important proteins morph into their deadly form and undergo the initial steps in oligomerisation (Dewson *et al*, 2008, 2012; Bleicken *et al*, 2010; Oh *et al*, 2010; Zhang *et al*, 2010; Czabotar *et al*, 2013). However, the very nature of the apoptotic pore remains enigmatic. Although elegant studies have observed large pore openings in model membranes, inability to detect anything that resembles a protein pore in cells or mitochondria has fuelled argument that such structures may not contribute to membrane damage during apoptosis. Rather, large and heterogeneous clusters (Zhou & Chang, 2008), or perhaps even monomers (Kushnareva *et al*, 2012), mediate mitochondrial outer membrane damage.

Two studies using powerful super-resolution microscopy approaches, single-molecule localisation microscopy (SMLM) and stimulated emission depletion (STED) microscopy now report ring-like Bax structures (*doughnuts*) in mitochondria of apoptotic cells (Große *et al*, 2016; Salvador-Gallego *et al*, 2016). These structures varied in size from 100 to 400 nm in diameter, broadly consistent with holes reported in vesicles derived from mitochondrial outer membrane (Gillies *et al*, 2015), and appear to precede formation of, or are distinct from, the large Bax and Bak clusters that have been reported in dying cells (Nechushtan *et al*, 2001; Zhou & Chang, 2008). Remarkably, both studies also observed distinct structures including lines (*daisy chains*) and arcs (*crescent moons*). Whether the line and arc structures represent qualitatively different oligomers or whether they are intermediaries on the path to forming a

closed pore is currently unclear, although 3D reconstructions may suggest the latter (Große *et al*, 2016). However, both studies reported the somewhat surprising finding that arcs were sufficient to permeabilise the mitochondrial membrane. As proposed by the authors, this may suggest that Bax molecules only need to partially line the pore surface, throwing weight behind previous biophysical studies that Bax and Bak form a lipidic pore rather than a barrel-stave proteinaceous pore (Terrones *et al*, 2004; Qian *et al*, 2008). However, a recognised limitation of both approaches is that the presence of other mitochondrial proteins that may contribute to the pore cannot be ruled out. For example, as Bax can also associate with Bak as well as self-associate during apoptosis, the arc structures may in fact be hetero-oligomers of Bax and Bak. As Salvador-Gallego *et al* (2016) observed similar arc-like structures in *Bax^{-/-}Bak^{-/-}* cells expressing Bax tagged with green fluorescent protein, any influence of endogenous Bax or Bak can be excluded in this study. Although the possibility remains that other mitochondrial proteins contribute to the pore, this may be unlikely given that activated Bax alone is sufficient to at least permeabilise model membranes. Preventing Bax from oligomerising with an engineered disulphide tether reduced the occurrence of ring-like pores and membrane damage supporting that they are important for Bax apoptotic function (Salvador-Gallego *et al*, 2016). Additionally, as the “lumen” of the pore was devoid of abundant mitochondrial outer membrane proteins such as TOM20 and SAM50 suggests that these structures indeed punched holes in the membrane

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Figure 1. Forming the Bax apoptotic pore.

Bax is normally cytosolic but accumulates on the mitochondrial outer membrane (MOM) during apoptosis. Interaction with BH3-only proteins downstream of an apoptotic stress leads to drastic conformation change in Bax including exposure of its BH3 domain, exposure of N-terminal epitopes and dissociation of $\alpha 1-5$ from $\alpha 6-9$. Conformation changes facilitate the self-association of Bax to initially form symmetrical homodimers involving reciprocal insertion of the exposed BH3 domain into a surface groove on the partner protein. Super-resolution microscopy has now revealed that these dimers multimerise to form distinct structures in the MOM including lines (daisy chains), arcs (crescent moons) and rings (doughnuts) that puncture the MOM and promote cell death. Whether these structures are distinct or are snapshots of different stages of a forming circular pore is not known. Also whether other mitochondrial proteins (purple) contribute to the pore is unclear. That arcs appear sufficient to permeabilise the MOM and that the pore appears heterogeneous in size support the notion that the pores are lipidic with intercalated lipid headgroups playing an important role. The molecular events that allow dimers to multimerise and the topology of Bax dimers (depicted here in a “side by side” arrangement) in these higher order structures remain unclear.

(Große *et al*, 2016), which was supported by atomic force microscopy in lipid bilayers (Salvador-Gallego *et al*, 2016).

So why have such pores in dying cells eluded detection until now? The diffraction limit of conventional light microscopy, which precludes resolution of structures smaller than 200 nm, has certainly confounded detection of the apoptotic pore. Advances in super-resolution microscopy approaches such as those employed in these current studies make resolution of such structures tractable. Also, Große *et al* observed that these discrete ring-like pores often occurred adjacent to very large clusters that have been previously reported. These large (and so very bright) clusters tended to dominate the imaging and steps had to be taken to mitigate their presence (Große *et al*,

2016). Intriguingly, such clusters were not prevalent in the Salvador-Gallego *et al* (2016) study, suggesting that they are cell type-specific or dependent on how Bax is labelled and imaged.

These current studies have provided us with the first glimpse of the pore structures that are present on mitochondria during apoptosis and so are important steps to understanding how Bax and Bak (for we presume that given their functional redundancy that Bak is likely to work in a similar manner) kill cells. However, we still have some way to go on our quest for our “holy grail”. Crucial questions remain as to how these structures actually form. What are the molecular interactions that mediate oligomerisation of these *daisy chains* and *doughnuts*? What is the minimal oligomer

required for membrane damage? Is membrane damage by the Bax/Bak pore sufficient for cell death or are other downstream events required? That we now have evidence that ordered pore complexes actually exist in dying cells raises confidence that we shall answer these questions. However, given certain implications of the current studies including heterogeneity in size and the inherent instability of proteo-lipidic pores, structural characterisation of these complexes is likely to continue to prove extremely challenging, with success likely depending on combinations of biochemical, biophysical and structural approaches. Upon completing our quest, we will finally resolve how Bax and Bak form the apoptotic pore to kill cells, but perhaps even more importantly, we may identify potential avenues to block it and so inhibit excessive or inappropriate apoptotic cell death, for example following traumatic brain injury or ischaemic stroke.

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