Cell

Shedding Light on Apoptosis at Subcellular Membranes

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http://dx.doi.org/10.1016/j.cell.2012.11.013

Regulation of apoptosis by Bcl-2 family proteins is a paradigm for complex protein-protein and protein-membrane systems. Elucidating the molecular mechanisms of these interactions in vitro in live cells and in animal studies has been significantly enhanced by using fluorescence techniques.

Apoptosis and Membranes: The Problem Posed

Ironically, the organelle that helps to provide cells with energy to live also serves as a platform to actively initiate cell death. Unlike forms of cell death triggered by mitochondrial dysfunction, during apoptosis, the signals that regulate the fate of the cell are integrated at functioning mitochondria and govern the irreversible step of mitochondrial outer membrane permeabilization (MOMP). Upon induction of apoptosis, the integrity of the mitochondrial outer membrane (MOM) is breached, resulting in the release of cytochrome *c* and other intermembrane space proteins into the cytoplasm. The released proteins trigger the activation of multiple pathways that result in the demise of the cell. Thus, a series of protein-protein and protein-membrane interactions control a process that is largely mechanical and that occurs in membranes.

The Bcl-2 family of proteins plays a major role in both sensing different types of cellular stress and regulating MOMP. To accomplish these tasks, different members of the Bcl-2 family are located in multiple parts of the cell and function as both cytoplasmic and membrane proteins by adopting distinct conformations that dictate their function (Figure 1A). Because of the distributed nature of this regulation and the involvement of complex membrane interactions, this process deviates from the classical "lock and key" enzyme-substrate interactions that are fundamental to biochemistry and pharmacology. Moreover, even though apoptosis involves dramatic cell morphology changes, the techniques of observational molecular biology are not easily applicable to the analysis of apoptosis because it comprises a series of hierarchical stochastic events governed by complex reversible equilibria. Yet together, both approaches have led to tremendous insights (and blind alleys) in our quest to understand and therapeutically exploit apoptosis.

As Bcl-2 family proteins target to both the endoplasmic reticulum (ER) and the mitochondria, the cell fate decision is regulated by both complex binding equilibria between the proteins and the local concentration of active binding partners, which is very different at the two organelles (Figure 1A). Moreover, active Bax, a proapoptotic Bcl-2 family member, has also been found sequestered at the Golgi in embryonic stem cells, providing a large pool of death effectors primed to cause MOMP upon the first indication of DNA damage. Therefore, Golgi are another membrane system at which the Bcl-2 family proteins can interact, contributing further to the complexity of regulating apoptosis (Dumitru et al., 2012). Thus, for the Bcl-2 family, the crucial role played by membranes makes the traditional approach of examining the underpinnings of particular cellular phenotypes with "grind and find" biochemistry problematic. To bridge this gap, several in vitro model systems of varying complexity have been exploited, and in this Review, we will highlight some of the insights gained by this approach, with a focus on studies in which fluorescence techniques are being used to integrate observations from purified proteins with those from live cells and even animals. The ongoing theme is that molecular characterization of phenotypes observed at a cellular, tissue, or organism level requires rigorous analysis with increasingly sophisticated model systems.

In Vitro Models Elucidate the Core Mechanisms

The oncogene *bcl2* was originally discovered more than 20 years ago as a chromosomal translocation partner in human follicular B cell lymphoma that resulted in the protein being overexpressed. Experiments using transgenic mice and overexpressing cell lines demonstrated that its function was to inhibit the then novel process of apoptosis. By using Bcl-2 as a binding target in immunoprecipitation studies and analyzing other cellular models of apoptosis, many other proteins related to Bcl-2 have been found, including those with the opposite function of promoting apoptosis. The Bcl-2 family was subsequently divided into three groups based on function and the presence of conserved Bcl-2 homology (BH) regions. The proapoptotic proteins comprise two groups: those with multiple BH regions, such as Bax and Bak, and those that contain only the BH3 region. Bax and Bak undergo complex conformational changes that result in their oligomerization in the MOM and, in turn, cause MOMP, thereby releasing apoptogenic factors such as cytochrome *c* and SMAC. These conformational changes are initiated by binding BH3-only

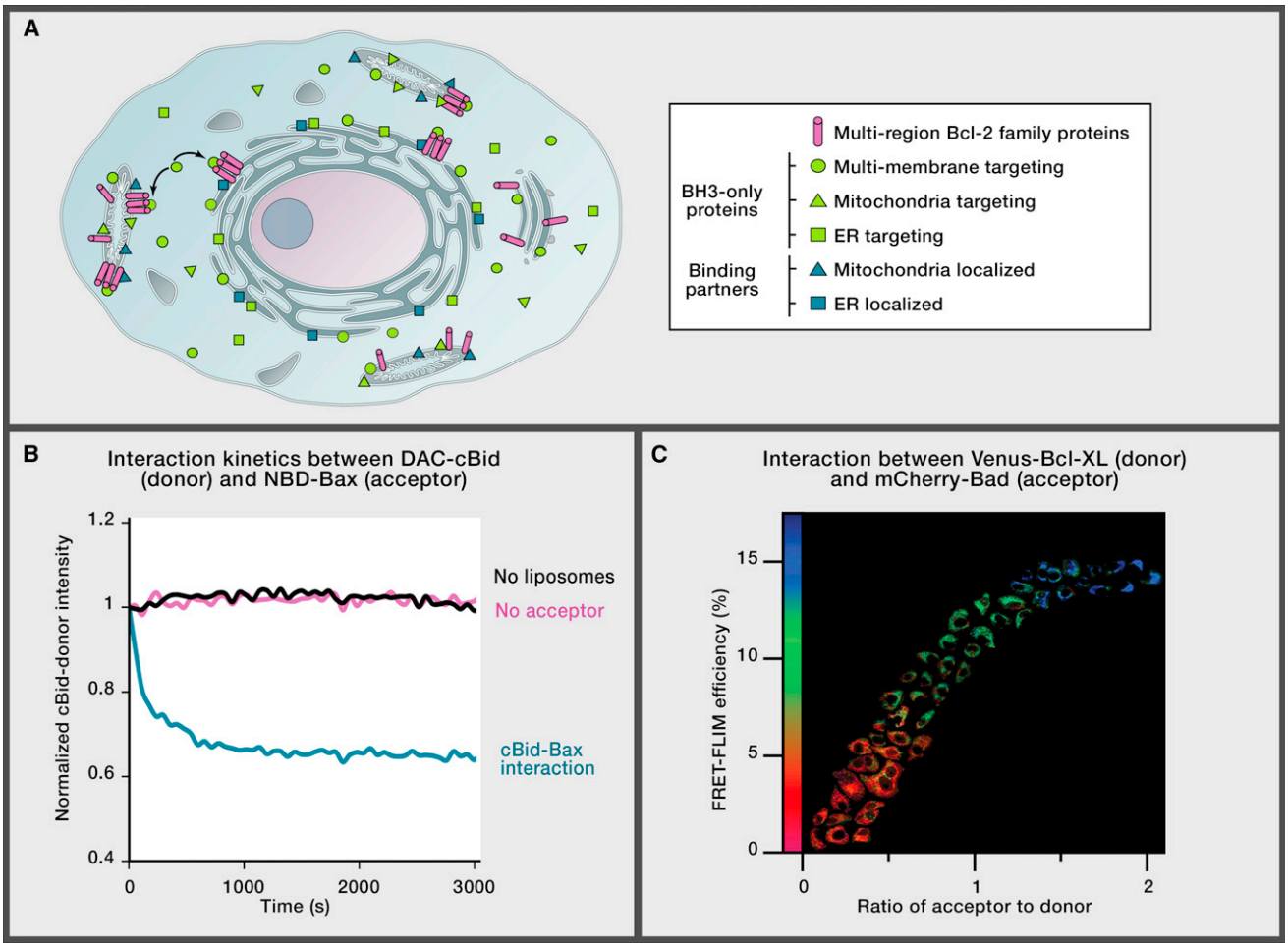

Figure 1. Interactions of Bcl-2 Family Proteins Quantified Using Fluorescence

The Bcl-2 family of proteins modulates apoptosis through a series of intricate protein-protein and protein-membrane interactions that exist in equilibrium. Fluorescence-based assays uniquely permit quantitative analyses in real time of interaction mechanisms that govern the biology of complex systems at membranes.

(A) Bcl-2 family proteins are located at multiple membranes within the cell and undergo a series of complex interactions that regulate apoptosis. Multiregion Bcl-2 family proteins (red cylinders) are found at ER and mitochondrial membranes, with a small subset also at Golgi. BH3-only proteins (yellow) are found in many areas of the cell and are recruited to single (squares, ER; triangles, mitochondria) or multiple subcellular membranes (circles). The specific sets of binding partners are different at each subcellular membrane (blue squares, ER; blue triangles, mitochondria). The balance of interactions between all of these proteins governs the fate of the cell.

(B) Attaching fluorescent dyes to cBid (DAC, donor) and Bax (NBD, acceptor) and measuring FRET via a decrease in donor fluorescence in real time reveals that a cBid-Bax interaction requires the presence of a membrane. Such measurements allow both affinities and the kinetics of the interaction to be quantified.

(C) Fluorescence techniques can be used to extend in vitro results to live cells by expressing Bcl-2 family proteins fused with fluorescent proteins and measuring protein-protein interactions and binding dynamics by FRET. The observed FRET FLIM efficiency increases (according to the color scale) as the acceptor:donor ratio increases until the donors are saturated by bound acceptors generating a binding curve, permitting the determination of binding constants.

proteins such as Bid, Bim, and Puma, which are termed activators. The third group includes the antiapoptotic proteins such as Bcl-2, Bcl-XL, and Mcl-1 that contain three or four BH regions and prevent MOMP by binding either activator BH3-only proteins or activated Bax and Bak. Bad and Noxa are examples of sensitizer BH3-only proteins that promote apoptosis indirectly by competing for binding to antiapoptotic proteins, consequently displacing activated Bax/Bak or activator BH3-only proteins to allow Bax/Bak activation. Thus, the core mechanism of apoptosis includes the conformational changes in Bax or Bak that permit MOMP and interactions with other Bcl-2 family members that either enhance or inhibit this process.

Though studies using immunoprecipitation of whole-cell lysates identified some of the potential interactions noted in the scheme above, this method has limitations, as nonionic detergents such as Triton cause heterodimerization between Bax and Bcl-2 that does not occur constitutively in situ; conversely, CHAPS dramatically decreases Bax binding to Bid. To circumvent these problems, one can study interactions between purified proteins. However, many of these proteins

have hydrophobic C-terminal tails, which considerably complicates purification of full-length and functional proteins, and in the case of some proteins that are constitutively localized to intracellular membranes (e.g., Bcl-2 and Bak) or are difficult to purify for various reasons (e.g., Mcl-1), this has not yet been accomplished. An alternative approach was to use peptides and/or truncated versions of Bcl-2 family proteins lacking the C-terminal tail for biophysical and structural studies, including nuclear magnetic resonance (NMR) and crystallography. These approaches generated data indicating that Bcl-2 family proteins function as ligand-receptor pairs relying on the BH3 region of one protein binding to a hydrophobic groove, termed the BH3 pocket, on the other member. Beyond this consensus, the limitations of the approaches resulted in both controversy and confusion in the field.

Many of these problems have been bypassed in an elegant series of studies (Kim et al., 2009) using a detergent-free system with full-length proteins synthesized by in vitro translation, which confirmed the hierarchical nature and presumed order of interactions as indicated above. These experiments also confirmed Bid, Bim, and PUMA (the latter previously contentious) as activators of Bax or Bak and demonstrated that antiapoptotic proteins bound to all three activators but differentially to sensitizer-BH3-only proteins. For example, the sensitizer Bad displaced activator BH3-only proteins from Bcl-2 and Bcl-XL, but not Mcl-1, whereas the sensitizer Noxa was effective against Mcl-1, but not Bcl-2/Bcl-XL.

However, it is not possible to study the kinetics of or to measure the affinity of binding with such an approach. Therefore, we developed an in vitro system using liposomes of defined composition and included fluorescently labeled versions of the relevant recombinant full-length proteins. This system possesses several unique advantages. First, by using Förster resonance energy transfer (FRET), the binding of the proteins to each other and to membranes could be *measured quantitatively* at physiologic concentrations in an environment with liposomes that mimic the composition of the mitochondrial membrane (Figure 1B). Furthermore, by using combinations of fluorophores whose excitation/emission spectra allowed simultaneous analyses, the interactions could be ordered in real time. Finally, using fluorescent dyes for which the emission is sensitive to the presence of water allowed the insertion of these proteins into the hydrophobic environment of the membrane to be monitored (Lovell et al., 2008). The data indicated that the permeabilization of MOM by tBid-activated Bax proceeds in a discrete series of steps: caspase-mediated cleavage of the BH3-only protein Bid resulted in the cleaved protein cBid, containing a p7 and p15 fragment, binding rapidly to membranes. This binding causes the amino-terminal p7 fragment to dissociate from the membrane-bound form of the p15 fragment, tBid. Membrane-bound tBid, which contains the BH3 region, then binds to Bax, causing it to insert into the membrane. Studies of the binding between mutant proteins suggest that the basis of binding on the membrane likely involves structural changes in tBid and the N-terminal helix of Bax, which stabilizes the C-terminal transmembrane helix in the BH3 pocket by masking this binding surface on Bax in solution (Kim et al., 2009). After binding by tBid, the interaction with Bax is diminished, allowing the transmembrane helix to disengage from the pocket and target Bax to membranes. Bax then oligomerizes in the MOM, permeabilizing it and releasing apoptogenic factors (e.g., cytochrome *c*, SMAC) from the intermembrane space.

Using fluorescence to measure the rates of these individual interactions revealed that insertion of Bax into the membrane was the rate-limiting step for MOMP (Lovell et al., 2008). The antiapoptotic proteins interfere with MOMP at several steps. Similar to Bax, Bcl-XL is recruited to the membrane by tBid, and by binding to it at this location, Bcl-XL prevents tBid from interacting with Bax (Billen et al., 2008). However, Bad binding to Bcl-XL frees tBid to activate Bax (Lovell et al., 2008). This exchange of binding partners is governed by the affinities and therefore the relative abundance of the membrane-bound proteins, both factors that are hard to measure in cells. That the various interacting partners are exchangeable and in equilibrium is the mechanistic basis for Bad functioning as a sensitizer.

Bcl-XL interacts with Bax in multiple ways. It binds directly to membrane-inserted, activated Bax, thereby preventing propagation of the oligomers required for MOMP. Perhaps more importantly, Bcl-XL also causes the retrotranslocation of Bax from the MOM to the cytoplasm before Bax attains the membrane-inserted conformation competent for oligomerization (Edlich et al., 2011). In this way, Bcl-XL intercedes very early in the multistep Bax activation sequence to shuttle peripherally bound Bax from the MOM to the cytoplasm, preventing incidental Bax activation in healthy cells due to Bax proximity to the membrane.

The above model of how the Bcl-2 family regulates MOMP is based on observations from a variety of cell-free systems made by many laboratories. Collectively, the results emphasize the importance of protein-protein and protein-membrane interactions. Binding to membranes shifts the equilibria between the conformations of Bcl-2 proteins. Therefore, the function of the proteins is markedly different in the cytoplasm and within the membrane. To reflect these observations, the model was given the name ''embedded together'' (Leber et al., 2010).

The Next Step: Regulation of the Core Mechanism in Cells

Because of the paramount importance of dysregulated apoptosis in many disease processes (too little in cancer, too much in neurodegenerative diseases and ischemia), understanding the core mechanism is critical for drug development. The Bcl-2 family is considered to be an excellent target for eliciting or enhancing an anticancer response because of a large body of cell-based evidence indicating that cancer cells are ''addicted'' to the presence of antiapoptotic proteins (i.e., they are required for ongoing cellular survival; Ni Chonghaile and Letai, 2008). Based on structural studies, initial small-molecule screens focused on disrupting the interaction between peptides corresponding to BH3 regions and truncated versions of Bcl-2 family proteins. Because such assays only partially mimic the in vivo environment of these proteins, it is not surprising that few of the first generation of compounds actually hit their target in cells. In an elegant series of experiments testing these compounds using cell lines with both Bax and Bak knocked

out as controls for cell death via nonapoptotic mechanisms, only ABT-737 (a peptidomimetic based on the Bad BH3 region) killed cells primarily by inducing apoptosis (Vogler et al., 2009). An orally available form of this drug (ABT-263, Navitoclax; Tse et al., 2008) has rapidly moved into clinical trials for cancer, and consistent with the differential binding properties of Bad to antiapoptotic proteins observed in vitro, the presence of Mcl-1 in the tumor mediates resistance to the drug.

Recent sophisticated murine models of lymphoma addicted to specific antiapoptotic Bcl-2 family members have suggested an additional Achilles' heel: ABT-737 was not able to kill tumors dependent on Bcl-XL inhibiting the BH3-only activator Bim (Mérino et al., 2012). Current animal studies shed no light on molecular mechanisms, and this feature of ABT-737 could not have been predicted from previous structural studies with peptides and protein fragments. However, the finding was independently predicted in a cellular model system using fluorescence lifetime imaging microscopy (FLIM) with fluorescent proteins functioning as FRET pairs fused to different Bcl-2 family members (Aranovich et al., 2012,). By measuring the lifetime decrease of FRET donor Venus in cells stably expressing Venus-Bcl-XL and expressing different (but measurable) amounts of mCherry acceptor (mCherry-Bad, -tBid, or -Bim), it was possible to generate a binding curve and thereby link biochemical observations with molecular mechanisms for proteins in live cells (Figure 1C). Relative Kd values can then be calculated in the presence or absence of a drug to test the effects of exogenous agents on the interaction. Using this approach, it was shown that ABT-737 did not displace Bim from Bcl-XL but was effective at disrupting interactions with tBid and Bad. As an explanation for this difference, it was noted that mutations in the BH3 regions that disrupted the binding of tBid or Bad to Bcl-XL (and to Bcl-2) did not affect Bim binding to Bcl-XL. These results strongly suggest that, in its physiologic membrane environment, Bim binds to Bcl-XL by a novel mechanism using other regions besides the traditional BH3 region, which confers its resistance to ABT-737 and ABT-263 (Aranovich et al., 2012; Liu et al., 2012). Moreover, this approach provides a means for identifying the residues required for protein-protein interactions in live cells, indicating that FRET-based FLIM can effectively link studies using purified proteins and analyses in live cells to reveal novel insights in biochemistry and pharmacology.

Small molecules and fluorescence have been used to link cell-free observations with the activity of proteins in cells. One example comes from studies of the novel binding site for a stapled Bim BH3 α-helical peptide on purified Bax. The binding site identified, located opposite of the canonical BH3 pocket, is referred to as the rear pocket (Gavathiotis et al., 2008). Using a competitive fluorescence polarization assay and a library of small molecules predicted to dock to the rear pocket of Bax, a Bax-specific activator was identified that does not show appreciable affinity for the antiapoptotic proteins or Bak. This activator can only cause apoptosis in Bax-containing cells, illustrating how in vitro studies can be used to identify a specific protein mechanism(s) and can thereby lead to the development of small molecules that can manipulate the core mechanism in live cells (Gavathiotis et al., 2012).

Cell Death and Survival beyond the Core Mechanism of MOMP

Beyond the canonical pathways of BH3-only proteins sensing different types of cell stress and then activating Bax/Bak at the MOM, other cellular processes regulated by the Bcl-2 family also prime the cell for death. Much attention has been paid to the ER as a source of these pathways (reviewed in Heath-Engel et al., 2008).

The ER is the platform on which Bcl-2 regulates autophagy by interacting with Beclin 1, an autophagy promoter (Pattingre et al., 2005). The development of fluorescent protein fusions for the autophagy regulatory protein LC3 greatly facilitated the examination of autophagy at the molecular level in live cells, suggesting that this process is amenable to the precise ordering of regulatory events at membranes by using fluorescence techniques similar to the studies investigating MOMP described above. An obvious goal of these studies would be to reveal the molecular mechanisms that switch autophagy from a cell survival to a cell death process.

Calcium is one of the important mediators of intracellular signals, emanating from the ER that is modulated by the Bcl-2 family (He et al., 1997). Sudden changes in calcium concentrations within the cell can trigger apoptosis, but it is unknown whether cell death is triggered via a sudden decrease or increase calcium levels within the ER or mitochondria, respectively. Additionally, the calcium uniporter on the MOM has a low affinity for calcium, and it was unclear how it could respond to calcium leakage from the ER during signaling or stress, as the measured global increase in cytoplasmic calcium was not enough to allow calcium import into the mitochondria. Recently, an important facet of this intraorganellar communication has been elucidated in live cells via fluorescence imaging measurements. Using a complex system with multiple fluorophores that allowed simultaneous measurements of the size of the ER-mitochondrial contacts and the local concentration of calcium at these points, experiments were conducted in cells that unequivocally demonstrated the existence of microdomains between these organelles (Csordás et al., 2010). Furthermore the high local concentration of calcium caused by stimulation of IP3 receptors, which can be regulated by Bcl-2 at the ER, was enough to allow import of calcium into the mitochondrion. The stage is now set to precisely measure these changes, determine the magnitude of the response required to elicit mitochondrial dysfunction and cell death, and resolve how the Bcl-2 family can regulate this process.

Aside from interactions at different organelles, other mechanisms that modulate the core protein-protein interactions governing MOMP include posttranslational modifications of the component proteins (reviewed in Kutuk and Letai, 2008). Under normal cellular conditions, Bim is located on microtubules. However, upon induction of apoptosis, Bim is phosphorylated by JNK1 releasing it from microtubules, whereupon it targets to the MOM and activates Bax and Bak, leading to MOMP and apoptosis. Interestingly, phosphorylation of other Bcl-2 family members results in inhibition of apoptosis. Phosphorylation of Bad by AKT promotes Bad interaction with 14-3-3 proteins, preventing the induction of apoptosis. AKT can also phosphorylate Bax, resulting in its inhibition via an unknown

mechanism. Although functionally important posttranslational modifications are generally identified using cells or tissues, determining how they affect the core molecular mechanisms of protein function requires the use of sophisticated cell-free systems. Deciphering the molecular mechanism is particularly important for proteins like Bax that are potential therapeutic targets.

Future Challenges: Regulation of Apoptosis in Animals

With the emergence of new therapeutic agents that inhibit Bcl-2 protein interactions with the aim of regulating apoptosis in tumor development and resistance to chemotherapy, whole-animal models would be an invaluable aid in determining the most rational way to combine these drugs with conventional cancer treatments. Because of the historic role of human B cell lymphoma in the discovery and identification of Bcl-2 as the first mammalian apoptotic regulator, it is perhaps fitting that the Bad BH3-mimetic drug Navitoclax may find its first use in this type of cancer. The murine lymphoma model alluded to previously (Mérino et al., 2012) suggests its promise in this context. In the clinic, these cancers are traditionally treated with a complex schedule of multiple chemotherapy agents. Surprisingly, recent analysis of the mechanism of action of two important drugs in the regime (Ehrhardt et al., 2011) indicated that the simultaneous administration schedule used for the last 30 years actually leads to mutual antagonism of the Bcl-2-dependent cytotoxic effect! Using luminescence or fluorescence imaging of whole tumors in mice allows potential readouts of "final" effects of antitumor treatment combinations. Although still not generally used, recent developments indicate that we may be able to be more precise and analytical with this approach. FRET probes have been developed with a caspase-3 cleavage site to monitor the activity of the final protease effector of apoptosis. This probe permits real-time monitoring of apoptosis in animals following antitumor therapy (Zhou et al., 2010). These studies report distinct time courses of apoptosis after different single-agent anticancer treatments, underlining the importance of investigating these effects in the most natural context available. Therefore, adding an inhibitor of Bcl-2 at an arbitrary time to current treatment schedules may not be the most rational way to modulate apoptosis. Monitoring apoptosis in animals using FRET is not without its limitations and is currently restricted to studying cancer cell lines and tumors at subcutaneous sites amenable to fluorescence measurements. Regardless, one can imagine ways of extending this technique to look at protein-protein interactions within live animals in multiple tissues with and without chemotherapy treatment. Using FRET may not only permit observation of binding between proteins, but may also provide binding dynamics as well. With the appropriate B cell lymphoma model, such an approach would identify the best time to switch off Bcl-2 to maximize the proapoptotic effects of the other chemotherapy drugs.

Thus, at all relevant levels of analysis in apoptosis research, from protein-protein interactions using increasingly sophisticated in vitro systems to investigation of whole-animal models, the application of novel fluorescence techniques suggests that the future is bright!

ACKNOWLEDGMENTS

The work in the authors' laboratory is supported by grant FRN12517 from the Canadian Institute of Health Research (CIHR) to D.W.A. and B.L. and by a Tier I Canada Research Chair in Membrane Biogenesis to D.W.A. J.K. and Q.L. are recipients of a doctoral and a postdoctoral fellowship, respectively, from the Canadian Breast Cancer Foundation, Ontario Division.

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