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PROBLEMS & PARADIGMS

Prospects & Overviews

Cell–cell fusion: To lose one life and begin another

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Abstract

As life extended into eukaryota, a great host of strategies emerged in the pursuit of cellular life. Some cells have been successful in solitude, some moved into cooperatives (i.e., multicellular organisms), but one additional strategy emerged. Throughout eukaryotes, many of the diverse multicellular cooperatives took life in partnership one step further. These cells came together and lost their singularity in the expanse of syncytial life. Recently in our search for this elusive "how", we discovered the intriguing peculiarity of a nuclear, RNA-binding protein living a second life as a fusion manager at the surface of developing osteoclasts, ushering them into syncytia 1. It is from here that we will develop several thoughts about the advantages of multinucleated cells and discuss how these fusing cells pass through several hallmarks of cell death. We will propose that cell fusion shares much with cell death because cell fusion is a death of sorts for the cells that undergo it – a death of the life that was and the beginning of new life in a community without borders.

KEYWORDS
apoptosis, cell-cell fusion, membrane fusion, myoblast, osteoclast, syncytia

INTRODUCTION

Cell fusion is a key stage in the formation of a fertilized egg, multinucleated skeletal myofibers, osteoclasts, and placental syncytiotrophoblast (reviewed in Ref.[1]). The sizes of fusion-generated multinucleated cells in human bodies vary widely from ~5–10 nuclei/osteoclast,[2] to hundreds of nuclei/skeletal muscle fiber,[3] to the truly unimaginable tens of billions of nuclei/syncytiotrophoblast. The physiological importance of the cell fusion processes is emphasized by the numerous human diseases associated with alterations in cell fusion. Impaired trophoblast fusion has been linked to placental-derived pregnancy diseases, such as preeclampsia and intrauterine growth restriction.[4,5] Defective myoblast fusion is associated with muscular dystrophies and myopathies.[6,7] In the case of osteoclasts, the number of fusion events that generated each cell and, thus, the size of the multinucleated osteoclast, directly correlates with the cell's ability to resorb bone, and elevated bone loss activity in osteolytic diseases correlate with increased fusion potential and sizes of osteoclasts.[8] Excessive osteoclast fusion also accompanies SNX10-linked autosomal recessive osteopetrosis.[9] In addition to the important cell fusion processes in normal physiology, cell fusion has been also suggested to play a key role in viral infections and during the development and progression of cancer.[1,10]

Although all cell fusions share the key stage of fusion of two plasma membranes (PMs) into one, the fusion event is preceded by complex and multistep processes that prepare the cells for fusion and is followed by complex multistep processes that expand the nascent membrane connection between the aqueous volumes of the cells and complete the formation of a new multinucleated cell from the mononucleated precursors. We will begin our discussion with a very brief summary of fusion pathways and the proteins involved in initiating and driving fusion. We refer the readers to more comprehensive reviews

of these exciting topics.[1,11,12] We will limit our discussion of multinucleated cells to those formed by fusion between cells and not consider multinucleated cells formed by endomitosis with incomplete cytokinesis; or by endocycles without entry into mitosis producing mononucleated polyploid cells; or joining cytoplasm of many cells by utilizing gap junction proteins without membrane breaches.[13] Our focus will be on the general biological significance and consequences of the cell fusion process and on the intriguing link between cell fusion and cell death pathways. Programmed cell death (PCD) is ubiquitous in multicellular organisms, and each of the death pathways, including apoptosis, necroptosis, autophagy, and pyroptosis, is characterized by distinct PCD-triggering events, signaling cascades, biochemical mechanisms, and morphological characteristics. The shared and specific features of these self-destruction pathways are discussed in depth in many excellent reviews.[14,15] Importantly, early stages of PCD are reversible and cells can be rescued if PCD stimulus is removed (reviewed in Refs.[14, 16]).

CONSERVED PATHWAY OF MEMBRANE FUSION AND DIVERSE PROTEIN MACHINES

Membranes that separate our cells from their environment and divide the intracellular volumes into distinct compartments (membrane organelles) consist of a continuous lipid bilayer. Diverse biological membrane fusion processes apparently converge at a conserved fusion-through hemifusion pathway of lipid arrangements. Both establishing immediate pre-fusion contact between the bilayers and their remodeling in fusion intermediates require energy input. Fusion pore opening and, especially, expansion are the most energy-demanding steps in the pathway. Cells can drive fusion by coupling fusion events with energy-releasing conformational changes of the proteins that initiate and drive membrane rearrangements, by locally bending the membranes, and/or by generating and concentrating fusion-supporting lipids.

Protein machines identified as necessary and sufficient for inducing a local merger of two membranes that normally do not fuse are strikingly diverse in numbers of involved proteins, structures of these proteins, and regulatory mechanisms (reviewed in Refs.[1, 11, 12, 17]). In an important conserved motif, opening of nanometer-sized fusion pores that connect the volumes of fusing cells does not necessarily lead to full unification of the cells. Nascent fusion pores can close or extend giving rise to long-living and many microns-long tunneling membrane nanotubes connecting cells. In contrast to nanotubes, the formation of multinucleated cells requires an expansion of the fusion pores. Importantly, in many cell fusion processes the transition from early hemifusion intermediates to opening of an expanding fusion pore involves many proteins neither required nor sufficient for hemifusion. In myoblast fusion, hemifusion, and opening of a fusion pore depend on the function of two essential muscle-specific proteins: Myomaker and Myomerger (also referred to as Myomixer and Minion), respectively.[12]

In myoblasts, in osteoclasts, and in cell fusion initiated by viral fusogens, the final expansion of fusion pores and formation of multinucleated cells depend on dynamin 2 and phosphatidylinositol 4,5-bisphosphate and does not proceed in ATP-depleted cells.[18–20]

TO FUSE OR NOT TO FUSE?

In many cases, the large sizes that syncytial formation brings facilitate specialized cellular functions. Larger osteoclasts more efficiently form an isolated acidic sealing zone of resorption between osteoclast and its underlying bone matrix. The seamless single-celled placental syncytiotrophoblast controls maternal/fetal nutrient and gas exchange and grows during pregnancy by fusion of additional cells reaching ~10 square meters at term. A potential advantage of fusion-formed multinucleated cells includes decreasing the distance that nuclear products must travel to the cytoplasmic domain considering that, at least for myofibers, muscle gene mRNA transcripts and proteins products remain in the vicinity of their originating myonuclei (reviewed in Ref.[3]). Syncytial cells also permit the specialization of nuclei and their transcriptional tuning, for example, permitting the disfavoring of nuclei with DNA damage. Having multiple genome copies within a syncytium allows diversification of the genome without suffering damaging consequences of loss of function mutations.[21] Thanks to their high plasticity, bone marrow-derived multinucleated cells have been proposed to play an important role in tissue regeneration.[22] Furthermore, acquisition of new myonuclei by an additional fusion event can be a more cost-effective way of reacting to a localized injury than global upregulation of transcription in all nuclei of the myofiber and protein expression throughout the cytoplasm.[3] On the other hand, cells that have joined into a multinucleated cell can share the fate of the whole in cases of cell injury. Indeed, the appearance of just one apoptotic nucleus in a multinucleated giant cell in foreign-body granulomas can commit this cell to apoptosis.[23] However, the placental syncytial layer challenged with ischemic injury has been suggested to rescue itself by deporting damaged syncytial nuclei.[24] Furthermore, osteoclasts, myofibers, and syncytiotrophoblast are terminally differentiated cells, and while some polyploid cells can undergo error-prone division,[25] normally, fusion-generated multinucleated cells do not undergo mitosis. However, the joining of multiple cells into a single one is not always an irreversible union. Syncytia can undergo fission releasing smaller cells. Osteoclasts can divide into daughter cells called osteomorphs.[26,27] Senescent multinucleated giant cells divide in various cancers, becoming the source of highly aggressive mononucleated stem-like cells.[28] Finally, even skeletal muscle fibers can divide into mononucleated cells in urodele amphibians.[29] While skeletal muscle fiber fission and dedifferentiation is not an attribute observed in mammals, amphibian muscle extracts have been demonstrated to confer this ability to murine skeletal muscle fibers.[30] So, while we do believe the transition from mononucleated to multinucleated life is a momentous transition in the life of a eukaryotic cell, it does seem that life always finds a way back when motivated.

We recently had a serendipitous introduction to the resourcefulness of eukaryotic cells in our discovery of osteoclasts' repurposing of a ubiquitous nuclear RNA chaperone as a fusion regulator.[31] This finding and recent studies on the role of PM asymmetry loss in cell fusion in muscle and bone development and regeneration[31–34] have motivated us to see the development of the fusion competence in cells as a kind of "railroad switch" that drastically changes their biochemical identity. The "decision" to fuse and unite is almost as existential for a cell as the "decision" to die in PCD, both dependent on a radical trajectory shift from one set of pathways to another set of pathways. In the diverse cell fusions we have studied over the years, we now see a common theme. Precursors undergo a complex and demanding transition from one to another type of cells (i.e., differentiation) that brings them to the edge of death in order to fulfil a physiological requirement their resident tissue faces. It is at this edge where cellular life leaves them with but two choices: (1) stay under these extreme stressors and die or (2) come together as a community and transform. To illustrate this idea, we will use a new friend – Lupus La protein (La).

LA PROTEIN AS A GUIDE INTO THE LIFE OF FUSING OSTEOCLASTS

La plays a well characterized, essential role in RNA metabolism required for all eukaryotic life. In its conventional, RNA metabolism role, La binds to the 3′ UUU-OH termini of newly synthesized RNA polymerase III (RNA Pol III) transcripts (e.g., tRNA, small nuclear RNA, and small nucleolar RNA), protects them from premature degradation, and retains nascent transcripts within the nucleus, facilitating their assembly into mature, functional RNA-protein complexes. La does so through two highly conserved N-terminal RNA binding domains – the La motif and RNA recognition motif 1 (RRM1). In this context, La has been well characterized as an abundant (estimates of $\sim 2 \times 10^7$ copies per human cell) phosphoprotein primarily retained in the nucleus of all eukaryotic cells (typically >95% of the total protein is nuclear). La's nuclear import in eukaryotes is facilitated by a C-terminal nuclear localization sequence (NLS), and once imported, its retention within the nucleus is further facilitated by a nuclear retention element. While La is known to play other RNA-binding roles in more specific contexts (e.g., the maturation of a special class of mRNA through a mechanism independent of UUU-OH binding), La has been widely accepted as a nuclear chaperone for small RNA biogenesis for decades (reviewed in Refs.[35, 36]).

In Ref.,[31] we reported that in differentiating osteoclasts La shifts from its well-characterized role of RNA chaperone and lives a second life at the exofacial surface of osteoclasts – where it promotes the membrane fusion events that set the size and resorptive function of multinucleated osteoclasts. Strikingly, we found osteoclast La to behave very differently from La that was studied in the context of RNA metabolism.[31,34] First, while its RNA chaperone function is carried out by a reduced, phosphorylated (particularly at Ser366), full-length species of La, the fusion-regulating function is performed by an oxidized, dephosphorylated, cleaved species of La.[31,34] Second, La's role in osteoclast fusion is independent of its N-terminal RNA binding motifs. While both the La motif and RRM1 are required for transcript recognition and RNA binding in La's conventional role in the maturation of RNA Pol III transcripts, we found that both the La motif and RRM1 were not required for La's role in promoting membrane fusion in osteoclasts. Instead, we demonstrated that the C-terminal half of the La protein between RRM1 and the C-terminal NLS are sufficient to promote osteoclast multinucleation and an accompanying increase in resorptive function. Third, and possibly most importantly, La's role in osteoclast fusion lies at the exofacial surface of the PM, where membrane contact and fusion occur, not in the nucleus or cytoplasm.[31,34] In synchronized membrane fusion experiments,[31] we found that anti-La antibodies and recombinant La applied outside the cell within an hour inhibit and promote osteoclast fusion, respectively. In contrast, cytoplasmic overexpression of an "uncleavable" La that retains its NLS had no effect on osteoclast fusion or resorption, while a truncated "cleaved" La construct promoted both (Figure 1).

Although repurposing an important house-keeping protein to carry out a novel, seemingly unrelated function can be just one more example of many moonlighting proteins,[37] we propose an alternative hypothesis that the general importance of La as an RNA chaperone is significant for its selection as a cell fusion regulator. In a thought-provoking twist, the switch to La's fusion-regulating function is accompanied by the downregulation of the phosphorylated, full-length form of La responsible for the RNA-chaperoning function of the protein. A dramatic loss of La protein may indicate that this differentiation process requires the concerted downregulation of a specific La-regulated pool of mRNAs. Indeed, interference of the La gene in cancer cells results in major changes in their properties, including a significant decrease in the proliferation and migration of cancer cells, an increase in apoptosis, and changes in the expression of many regulatory proteins.[38] Moreover, La is not the only nucleic acid-binding protein implicated in cell fusion. Acheron, a member of La protein family,[39] HuR, a member of ELAV-family of RNA-binding proteins,[40] and essential nuclear DNA-binding high mobility group box 1 protein, known to also exist in a secreted form[41] are all involved in the formation of multinucleated myotubes. Future work will show whether the ability of nucleic acid-binding proteins to control multiple distinct aspects of cell physiology is important for their role as regulators of cell fusion.

In continuing our work on the role of La in osteoclast formation and function, we have come to realize that La is not an oddity, but rather a guide in our efforts to better appreciate how eukaryotic cells have repurposed seemingly disparate biological players and systems to manage cell fusion processes. We – the authors – have spent many years imagining a world where cell fusion is governed by protein machines and signaling processes that were developed specifically to control cell fusion. Perhaps we came to expect dedicated fusion machines because this is what we observe in better-characterized membrane fusion systems like the entry of enveloped viruses into host cells and in intracellular fusion events. In these systems, one or a few proteins are masters of membranes and bend them into fusion pores in response to direct stimuli and biological triggers. While the discovery of Myomaker and Myomerger as the two-part "fusogen machine"

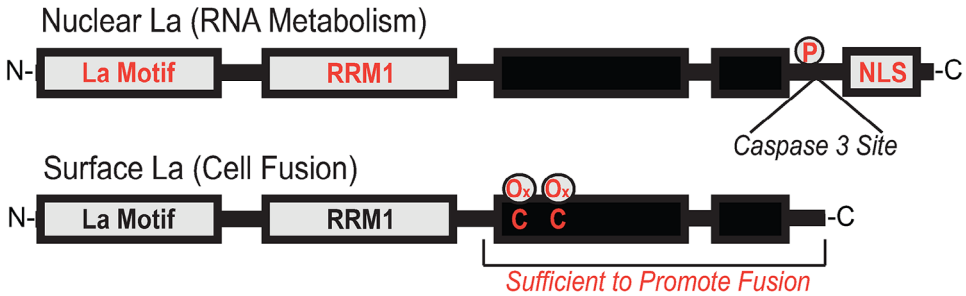

FIGURE 1 Osteoclast fusion depends on the unconventional species of La. (Top) Phosphorylated, full-length, reduced La is highly abundant in the nuclei of all eukaryotic cells where it plays a key role in RNA Pol III transcript maturation. (Bottom) Dephosphorylated, cleaved, oxidized La decorates the surface of fusing osteoclast precursors. Brackets denote a region we found sufficient to promote osteoclast fusion. NLS, nuclear localization sequence; P, phosphorylated-Serine366; RRM1, RNA recognition motif 1.

that drives skeletal muscle multinucleation[12] is seemingly consistent with this hope, we now see diverse cell fusion systems as not fully controlled by protein machines specific for cell fusion. Instead, in many cases the pathways and proteins that render one syncytial cell from many precursors appear to be a concert of repurposed tools eukaryotic evolution found a second role for. Perplexingly, one biological pathway fusion seems to share many commonalities with appears to be cell death.

LIFE AT THE EDGE: LINKS BETWEEN CELL FUSION AND APOPTOSIS

The work of many laboratories on diverse cell fusion processes, including our "La La Land" journey in osteoclasts, suggests an intriguing and thought-provoking link between cell fusion and PCD. To start with, both fusion and apoptosis depend on intracellular calcium signaling,[42–44] oxidizing stresses,[34,45–47] and actin cytoskeleton remodeling.[48–51] Syncytial life in eukaryotes also shares with this queerest of bedfellows – death, several more specific dependences, including the involvement of caspases, the alterations in the trafficking and molecular species of La, and the importance of phosphatidylserine (PS) signaling.

Caspases and La: Much of the conversation around the cysteine proteases of family Caspase revolves around their role as enzymatic harbingers of the end – death; however, syncytial life has its own dance in mind. Caspases are endoproteases that are produced as inactive zymogens and freed toward their snippy proclivities via oligomerization and/or cleavage. Classical understanding, from the bench to the classroom, sees caspases organized into Initiator (8, 10, 9, and 2) and Executioner (3, 6, and 7) classes in PCD (apoptosis). While much nuance exists in this world of death making, Initiator caspases meet through an "induced proximity model" where a variety of input signals can promote the dimerization, autocleavage, and stabilization of an apoptosis-initiating complex. These initiating complexes can then conscript through cleavage Executioner caspase dimers that proceed through the cell, dismantle its various systems, and prepare the carcass for pickup. In addition to their essential roles in apoptosis,[52] caspases play non-conventional roles when activated at sub-apoptotic thresholds in cell fusion, another addition to the growing list of non-apoptotic roles for these enzymes.[53,54]

Sub-apoptotic caspase signaling triggers a "trial by fire" in the preparation of fusogenic precursors for their rebirth into syncytial life. In exploring the formation of syncytial muscle fibers, placental trophoblasts, osteoclasts, and, likely, other fusogenic eukaryotic cell types, sub-apoptotic caspase activity appears to play roles in both the differentiation programs required for the pre-fusion preparations and the membrane fusion stage of cell–cell fusion.

Although the role of sub-apoptotic caspases can be seen in many processes of mammalian syncytial cell formation (myoblast fusion,[47] trophoblast fusion[55]) perhaps this relationship in the formation of multinucleated cells can be most clearly appreciated in the preparation of osteoclast precursor macrophages and their subsequent fusion into multinucleated osteoclasts.[31] The role of the sub-apoptotic caspase 3 activation in the RANKL-mediated transition of macrophage precursors to fusogenic osteoclasts has been noted for decades.[56] Most recently, our work demonstrates that this caspase 3 plays a vital role in proteolytic processing that redirects La protein from its conventional nuclear localization to the surface of committed osteoclasts, where La promotes fusion and formation of multinucleated, resorption competent osteoclasts.[31] La has a caspase 3 cleavage site just before its NLS and the cleavage frees the low molecular weight protein lacking NLS to traffic to the PM. Mutation of this La cleavage site or inhibition of caspase 3 blocked La's ability to traffic and promote osteoclast membrane fusion. Apoptosis also stimulates the rapid dephosphorylation, caspase-mediated cleavage of La (Figure 2A[57 58]), and its relocation to the surface of the cells.[59] In contrast, elevated levels of full-length La maintain the survival of cancer cells by protecting them from apoptosis.[60] These findings emphasize the striking similarities between cells committed to either death or fusion as it relates to their cleavage and trafficking of La protein (Figure 2[31,57,58]). Note that for fusing osteoclasts (Figure 2B[31]), in contrast to apoptotic cells (Figure 2A[57]), the cleaved form of La is observed only for a limited time and at day 5 of osteoclastogenic differentiation is replaced by a full-length La, when the cells approach a mature size and fusion slows down.

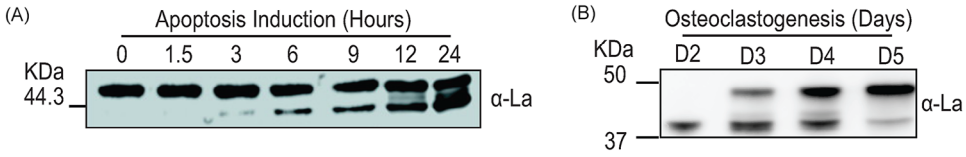

FIGURE 2 La cleavage in apoptosis and osteoclast multinucleation. (A) Western blot from Ayukawa et al., 2000.[57] HL-60 cells were incubated with camptothecin for the indicated times to induce apoptosis. (B) Western blot from Whitlock et al., 2023.[31] Macrophage precursors derived from primary human monocytes were incubated with recombinant MCSF + RANKL for the indicated times to induce osteoclastogenesis.

Phosphatidylserine: "I'm dying, I'm fusing". Eukaryotic cells pay a massive energy cost to asymmetrically organize the lipids that make up their PMs. This cellular hallmark is facilitated primarily through collections of ATPases and ABC transporters that "flip" and "flop" various species of membrane lipids across the hydrophobic core of the membrane via ATP hydrolysis. The result is an exofacial leaflet enriched in uncharged lipid species (e.g., phosphatidylcholine and sphingolipids) with saturated fatty acid tails opposed to a cytofacial leaflet enriched in anionic lipids and, especially, PS and phosphoinositols with unsaturated tails. PM asymmetry plays vital roles in the biophysical characteristics of the PM, regulates membrane trafficking, facilitates the ordering of lipid microdomains, and supports the activities of countless proteins and biological pathways. Nevertheless, eukaryotic cells possess the ability to open proteinaceous gates (lipid scramblases) that allow PM lipids to locally and transiently run down their concentration gradients. Why would a cell throw all that organization away? Well, it appears they do so to trigger radical intercellular collaborations, including, photoreceptor maintenance, synaptic pruning, thrombus formation, and the clearance of cellular carcasses – to name a few.

In the early 90s, Fadok et al. made the foundational observation that apoptotic cells lose their PM lipid asymmetry, exposing PS across the exofacial leaflet of cell carcasses.[61] Vermes et al. extended these observations, demonstrating that necrotic cells also lost asymmetry, exposing PS, and that a relatively simple assay employing a fluorescently labeled PS-binding protein – annexin V – could quickly mark dying and dead cells and facilitate their rapid identification.[62] Even though PS exposure was identified a decade earlier as a nucleating event in hemostasis, the broad recognition of PS exposure as a feature of apoptotic cells vital for their recognition, engulfment, and removal via macrophage led to the universal adoption of PS exposure as an "eat me" signal in apoptosis.

Although it is commonly referred to as an "eat me" apoptotic signature, the exposure of PS at the surface of fusogenic precursors plays a major role in cell-cell fusion events in the formation of virtually all syncytial cell types in eukaryotes (as reviewed in Ref.[17]). Moreover, PS at the surface of fusing cells is often decorated by the endogenous PS-binding protein annexin V, which also plays a fairly universal role in promoting cell–cell fusion in eukaryotes. While the mechanism by which cell surface PS promotes or triggers cell fusion processes in the formation of diverse multinucleated cell types in eukaryotes remains to be characterized, the analogy to apoptotic pathways that already at their early stages deliver PS to the surface of the cells is clear. Of note, PS exposure in apoptotic cells and in the cells transiently expressing PS can be mediated by different scramblases (XKR proteins activated by caspase cleavage in apoptosis[63] versus TMEM16 proteins activated by a rise in intracellular Ca^{2+} in non-apoptotic, fusing cells ([33,64,65] but see Ref.[66]).

Returning to our recent work in osteoclast fusion,[31,64] we found that the role of PS exposure in facilitating membrane fusion may be at least partially reliant on La protein. Our discovery that La promotes the fusion of committed osteoclasts and facilitates its cell–cell fusion function at their exofacial surface was initially puzzling. Setting aside the mystery of how this nuclear resident would even get to the PM, how could La sit at the surface of these cells? It is a globular protein with no transmembrane domain nor previously noted membrane binding ability. We also saw that La alone showed little to no membrane affinity when introduced to liposomes even though we could introduce the same recombinant protein to fusing osteoclasts and see it decorate their PMs. Strikingly, we found that La's PM association in osteoclasts is at least partially reliant on its relationship with annexin V. We found that endogenous La and annexin V were in a supermolecular complex in fusing osteoclasts, that recombinant La and annexin V directly bind each other, and that annexin V could facilitate La-liposome association. Consistent with previous findings, annexin V and La's association with the surface of these fusion osteoclasts was reliant on the non-apoptotic exposure of PS.

Utilization of the pathways and players involved in apoptosis in drastic changes of living cell physiology is not limited to cell fusion processes, as suggested by contributions of caspases and PS externalization in some non-apoptotic developmental processes.[54,67] Why would eukaryotic cells pair syncytium formation and cell death by utilizing closely related biochemical strategies and regulators (Figure 3)? Perhaps we should dispense with seeing death and syncytial life as fully separate but recognize their kinship in this – a radical and fateful cellular "choice" in service of their tissue, a choice they likely will not return from. In the end, syncytial life is a sort of death – a death to what came before in mononucleated existence and a rebirth into an interconnected whole. Perhaps certain ancient physiological systems lost to time (e.g., skeletal muscle beyond nematodes) were routinely placed under such rigorous demands that these cells regularly died and required replacement like our own epidermis. This constant energy-demanding replacement of specialized cells would consume an exceptional amount of energy. Under these conditions, some organism "discovered" tissue-specific advantages in the union of the dying cells. An ancestor with the ability to unite these cells – with the tools of a captured retrovirus and/or with specialized protein

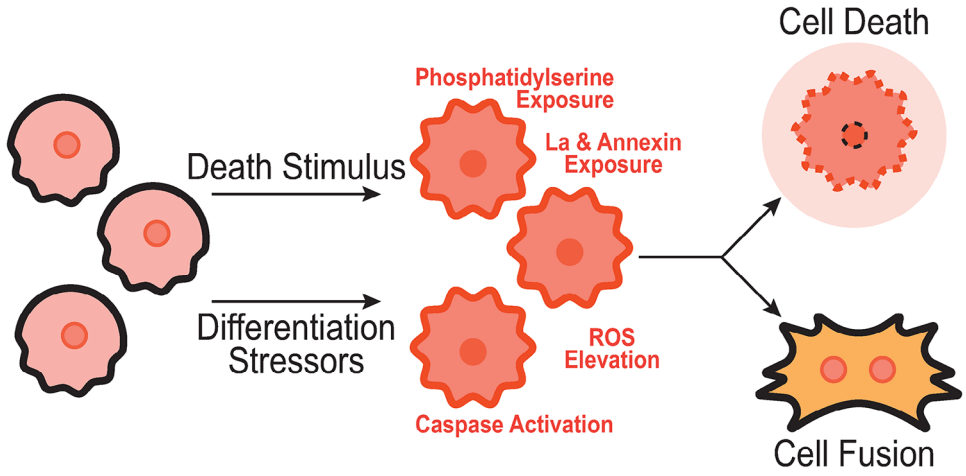

FIGURE 3 Some of the shared hallmarks of cell death and cell fusion pathways.

fusogens – produced larger multinucleated cells better suited to certain functions than mononucleated cells. A drastic change in a multitude of signaling pathways and physiological processes characteristic for the programmed death commitment not only promotes their fusion but also changes the properties of emerging syncytial cells to better fulfil their function. In the pressures of a challenging environment, these cells thrive on what might kill their neighbor. It is here – surrounded by death – that we imagine syncytial cells were born.

AUTHOR CONTRIBUTIONS

Jarred M. Whitlock and Leonid V. Chernomordik conceived, drafted and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicting of interest.

DATA AVAILABILITY STATEMENT

While we are happy to make data from our published work available upon request, no novel data are reported in this manuscript. Should someone be interested in the cartoons, graphics files are available upon request.

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