RESEARCH HIGHLIGHT

A new route for EV biogenesis

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Exosomes are known to be generated in multivesicular bodies, a subset of endosomes. A recent study by Gao and colleagues uncovers a new type of small extracellular vesicle, which is generated by a different route involving non-canonical autophagy and a previously unknown intermediate organelle named by the authors as RAB22A-mediated non-canonical autophagosome fused with early endosome (Rafeesome).

For cell biologists, the rule of thumb is that the neatly drawn models in the literature seldomly reflect the reality of the bewildering complexity inside the cell. For example, in most models, small extracellular vesicles (EVs) are generated by specialized late endosomes named multivesicular bodies (MVBs). In these models, exosomes are generated by invagination of the MVB membrane, which gives rise to the intraluminal vesicles of the MVB. When the MVB fuses with the plasma membrane, the intraluminal vesicles are released and become exosomes.[1] Unfortunately, this picture does not fit well with the extreme heterogeneity of small EVs,[2] which argues for alternative mechanisms of small EV generation. Autophagy is another good example: historically, autophagy was proposed as a lysosome-dependent degradation process, which is driven by a well-defined machinery.[3] However, there is increasing evidence that autophagy has many roles other than lysosome-based degradation, and the list of non-canonical autophagy pathways gets longer and longer.[4] In a recent paper published in *Cell Research*, Gao et al.[5] report a new type of non-canonical autophagy by which autophagosome cargo is delivered to a new intermediate organelle, ultimately giving rise to a new type of EV. Even in the world of cell biology where people are used to seeing new structures and new mechanisms, these data are striking.

STING/MITA is an endoplasmic reticulum (ER) transmembrane protein which plays essential roles in innate immunity.[6,7] STING has been shown to be degraded by autophagy.[8] The story started with a surprising finding that treatment of various cancer cells with a STING agonist can make these cells release small EVs containing active STING. Moreover, when incubated with recipient cells, these activated STING-containing EVs can induce cytokine production in the recipient cells, promoting antitumor immunity. These data prompted the authors to study the underlying mechanism. Using a panel of 62 constitutively active Rab proteins, they found that the active STING proteins are included in the intraluminal vesicles of MVB-like structures which are positive for Rab22A. These structures can be induced by overexpression of wild-type or constitutively active Rab22A. Importantly, these Rab22A-positive, STING-containing MVB-like structures can be observed in cells treated with STING agonists, indicating that they are not artifacts of Rab22 overexpression. Formation of activated STING-containing EVs is reduced in Rab22-knockout cells, but is not affected by the canonical MVB biogenesis pathway. These observations suggest that the STING-containing vesicles may be a new type of MVB-like structure, which the authors named RAB22A-mediated non-canonical autophagosome fused with early endosome (Rafeesome).

Activated STING is known to induce autophagy, which prompted the authors to ask whether autophagy in general, and STING-activated autophagy in particular, play a role in the formation of these Rab22-positive, STING-containing MVB-like structures. After a long list of experiments, the answer is yes and no. The formation of Rafeesomes does depend on a subset of core autophagy genes; however, it does not depend on core components of the activated STING-driven autophagy degradation pathway. Moreover, "Rafeesome autophagy" is dependent on Rab22, which is clearly required for the formation of Rafeesomes, and is likely required for the formation of autophagosomes destined to become Rafeesomes. More interestingly, "Rafeesome autophagy" involves PI4P, which appears to be required for recruitment of core autophagy machinery to the ER, thus initiating formation of autophagosomes destined to become Rafeesomes. Once formed, rather than fusing with lysosomes for degradation, these autophagosomes fuse with Rab22A-positive early endosomes to generate Rafeesomes. This is likely because the presence of Rab22A on autophagosomes prevents them from fusing with lysosomes by inactivating Rab7, which is known for its essential roles in autophagosome/lysosome fusion. Once the double-membraned autophagosome fuses with Rab22A-positive early endosomes, the outer membrane of the autophagosome becomes part of the Rafeesome membrane, while the inner membrane and contents of the autophagosome become an intraluminal vesicle of Rafeesome, which can be released from the cell as an RAB22A-induced extracellular vesicle (R-EV) when Rafeesome fuses with the plasma membrane.

The heterogeneity of small EVs is being increasingly recognized; for example, the same group has reported an alternative exosome pathway, which depends on Rab31, but not ESCRT.[9] What is unusual about this new study is that it reveals a completely different route for biogenesis of small EVs, which starts from non-canonical autophagy, and goes through a previously unknown intermediate organelle, where the intraluminal vesicles are formed by a mechanism different from the one for MVB formation. These facts raise many interesting possibilities. For example, ER proteins other than activated STING may also be selectively delivered into R-EVs. Moreover, one can speculate that other types of autophagosomes may also be able to fuse with

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Rab22A-positive early endosomes, giving rise to different subtypes of R-EVs. Furthermore, the invagination-based mechanism may generate other types of intraluminal vesicles in Rafeesomes, thus contributing to the diversity of the R-EV family. In summary, the Rafeesome-R-EV route can be viewed as a parallel pathway to the MVB-exosome route, which has the potential to generate a family of small EVs.

Of course, for the authors and for researchers interested in R-EVs, a long list of exciting questions is waiting to be answered. The generality of this new route needs to be evaluated, and markers for R-EVs need to be identified so that R-EVs can be monitored in vivo. Identifying *RAB22A* and *PI4K2A* as R-EV essential genes offers a good starting point for understanding the biogenesis of R-EVs; more work clearly needs to be done on this front. Last but not least, R-EV-deficient animal models need to be established so that the physiopathological relevance of this route can be investigated. It is my personal view that all these time-consuming, difficult future efforts will prove worthwhile, as such an elaborate route is unlikely to have evolved without possessing important functions.

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ADDITIONAL INFORMATION

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