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Normal and disordered reticulocyte maturation

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Abstract

Purpose of review—Reticulocyte remodeling has emerged as an important model for the understanding of vesicular trafficking and selective autophagy in mammalian cells. This review covers recent advances in our understanding of these processes in reticulocytes and the implications for erythroid development.

Recent findings—Enucleation is a key event, caused by chromatin condensation, vesicular trafficking, and microfilaments. Mitochondrial elimination is achieved through selective autophagy, in which mitochondria are targeted to autophagosomes, and undergo subsequent degradation and exocytosis. The mechanism involves an integral mitochondrial outer membrane protein, and general autophagy pathways. Plasma membrane remodeling, and the elimination of certain intracellular organelles, is achieved through the exosomal pathway.

Summary—Vesicular trafficking and selective autophagy have emerged as central processes in cellular remodeling. In reticulocytes, this includes enucleation and the elimination of all membrane-bound organelles and ribosomes. In contrast to yeast where certain ubiquitin-like conjugation pathways are required for all types of autophagy, in reticulocytes inactivation of these pathways has only a partial effect. Thus, in higher eukaryotes, there appears to be redundancy between these pathways and other processes. Future studies will address the relationship between autophagy and vesicular trafficking, and the significance of both for cellular remodeling.

Keywords

Exosomes; autophagy; mitochondria; enucleation; vesicles

Introduction

Erythrocytes are highly specialized cells, which are dedicated to hemoglobin transport and gas exchange in the lungs and the peripheral tissues. Erythrocytes develop from reticulocytes, which are defined (in mammals) as erythroid cells that have lost their nucleus but still retain residual RNA. Reticulocytes in turn are generated in the bone marrow from mature erythroblasts by enucleation. After a brief period of time in the bone marrow, reticulocytes are released into circulation, where they eliminate their residual RNA, and mature into erythrocytes. Under homeostatic conditions, maturation of rodent reticulocytes takes 2–3 days, equally divided between the bone marrow compartment and circulation [1–4]. Compared to the average life-span of an erythrocyte (120 days in humans), the reticulocyte stage of erythroid development is brief; however, it is a period of rapid change. Reticulocytes undergo extensive membrane remodeling, volume changes, and eliminate all internal membrane-bound organelles and ribosomes. These transformative processes ensure that critical cellular functions, such as hemoglobin production, oxygen transport, and

deformability, are optimized in mature erythrocytes. This review will cover recent advances in our understanding of these processes, and some of the biological implications.

Enucleation: birth of the reticulocyte

Enucleation has several beneficial effects. Among these, it definitively prevents dedifferentiation and transformation of mature erythroid cells. It also has a potential impact on organismal fitness. The nucleus comprises two-fifths of erythroid cell weight; accordingly, enucleation reduces the circulatory work of the heart by two-fifths [5]. Apart from these theoretical benefits, enucleation plays a direct role in reticulocyte maturation. Enucleation of a late stage erythroblast generates a nascent reticulocyte with abundant cytoplasm and hemoglobin, and a membrane-bound nucleus with a thin rim of cytoplasm, designated the pyrenocyte [6]. Enucleation is accompanied by a sorting process, which directs membrane proteins to either the reticulocyte or the pyrenocyte. Cytoskeletal proteins important for erythrocyte function, such as spectrin, ankyrin, and protein 4.1, are directed to the nascent reticulocyte [7;8]. Integral membrane proteins that are associated with the cytoskeleton, such as glycophorin A [9], are also directed to the reticulocyte. By contrast, proteins that mediate intercellular attachments, such as erythroblast macrophage protein and $\beta 1$ integrin are directed to the pyrenocyte. The mechanism is not known, but is thought to involve connectivity to the underlying membrane cytoskeleton. Consistent with this interpretation, mutations of cytoskeletal proteins, such as those seen in hereditary spherocytosis and elliptocytosis, are associated with misorting and deficiencies of nonmutant proteins [10].

Nuclear condensation, which occurs during terminal erythroid differentiation, is a prerequisite for efficient enucleation. In mammalian erythroid cells, nuclear condensation is associated with histone H3K9 dimethylation and H4K12 deacetylation [11]; however, in contrast to avian species, it is not associated with changes in nucleosome structure or the accumulation of special structural proteins. Histone deacetylases (HDAC) 2 and 5 are upregulated during erythroid differentiation, and a loss-of-function analysis implicates HDAC2 in nuclear condensation [11;12]. Further, miR-191 regulates enucleation through its effect on *Riok3*, *Mxi1*, and the histone acetyltransferase *Gcn5* [13]. These studies support the notion that histone deacetylation plays an important role in nuclear condensation and elimination. However, it does not appear to be sufficient. For example, Retinoblastoma 1 (Rb1) regulates cell cycle and differentiation. Rb1-deficient erythroblasts exhibit defective nuclear condensation and enucleation [14], which may reflect a primary role of Rb in chromatin condensation [15]. Downregulation of vimentin intermediate filaments releases the nucleus from its moorings, setting the stage for enucleation [16]. Regarding enucleation, several cell autonomous mechanisms have been proposed. Caspases are involved in enucleation in keratinocytes and lens epithelial cells; however, there is no evidence for broad caspase activation in terminally differentiated erythroblasts [17]. Microtubules and microfilaments have also been implicated [7;18], and during enucleation a constrictive actin ring forms between the nucleus and the nascent reticulocyte. Formation of this ring, and enucleation, requires Rac GTPases and the downstream effector mDia2 [19]. Also, Sox6 appears to have a role in this process [20]. Recently, Keerthivasan *et al.* provided evidence that enucleation is driven by the clathrin-dependent generation and coalescence of vacuoles at the nuclear-cytoplasmic junction [21]. This study suggests that vesicular trafficking has an under appreciated role in enucleation. Vesicle recruitment by the multisubunit tethering exocyst complex is also required for asymmetrical abscission at the midbody ring in cytokinesis [22]. Given that enucleation may represent an asymmetrical form of cytokinesis, perhaps the exocyst complex is also involved in erythroid cell enucleation.

Membrane remodeling during reticulocyte maturation: role of vesicular trafficking

Nascent reticulocytes are motile, multilobulated cells [23]. Initially, the membranes of these cells are rigid and unstable. As they mature, reticulocytes lose volume and surface area, assume the shape of a biconcave disk, and acquire stability and deformability [24;25]. To a large extent, these functional and morphological changes can be attributed to maturational changes in the membrane cytoskeleton, as discussed elsewhere in this issue. However, there are also changes in the expression of membrane proteins during reticulocyte maturation; prominent among these is downregulation of the transferrin receptor. The transferrin receptor-1 (TfR1) participates in a cycle that transports extracellular iron into erythroid cells (reviewed by Hentze et al. [26]). In this cycle, iron-loaded transferrin binds to its receptor, and is imported via clathrin-mediated endocytosis into the early endosomal compartment. Internalization is followed by endosomal acidification, release of iron from transferrin, iron reduction, and iron transport into the cytoplasm. Finally, TfR1 returns to the plasma membrane and releases apo-transferrin, completing the cycle. Some TfR1 is recycled rapidly to the membrane, whereas the rest is slowly recycled after passing first through the endosomal recycling compartment. Notably, a naturally-occurring mutation of *Sec151l*, a homolog of a yeast exocyst component, is associated with decreased rapid recycling of the TfR, an iron utilization defect, and microcytic anemia [27–30].

Once hemoglobin synthesis is complete, reticulocyte TfR1 is rapidly downregulated to prevent continued iron importation, and iron-mediated toxicity. This is accomplished by a switch in TfR1 trafficking from the recycling pathways to the multivesicular endosomal (MVE) pathway [31]. MVE are formed by membrane invagination in late endosomes, which is mechanistically related to the process of viral budding [32]. Budding in MVE is initiated by ESCRT complexes (reviewed by Saxena et al. [33]), although ESCRT-independent mechanisms have been described that are mediated by lipid rafts or lectin-induced aggregation [34;35]. ESCRT complexes bind ubiquitylated proteins, providing one mechanism for protein sorting into MVE. Regarding TfR1, an ESCRT-associated protein, Alix, may play a role in its sorting into exosomes [36]. MVE undergo fusion with lysosomes, followed by degradation, or fusion with the plasma membrane, which leads to the release of intraluminal vesicles into the extracellular space. The release of such vesicles, known as exosomes, was initially described in the context of TfR1 downregulation [37;38], but was subsequently shown to be involved in other biological processes, such as antigen presentation and the transmission of infectious agents [39–41]. Exosome secretion provides a means of rapid TfR1 downregulation. Besides this, it is involved in plasma membrane elimination from reticulocytes [42;43], and volume regulation through downregulation of Na/K-ATPase and aquaporin-1 [38;44]. Secretion of aquaporin-1 is suppressed by a proteasome inhibitor, suggesting that its sorting in MVE may be regulated by ubiquitylation. Other membrane proteins, such as glucose, nucleoside, and amino acid transporters are also downregulated through this pathway [38;44;45].

Mitochondrial elimination

Nascent reticulocytes consume oxygen, and have a functioning tricarboxylic acid cycle, which is downregulated during maturation [46;47]. Since one of the main functions of mature erythrocytes is oxygen transport, and mitochondria consume oxygen, it is not surprising that mitochondria are targeted for inactivation during reticulocyte maturation. Indeed, mitochondria are eliminated from reticulocytes as they develop, and are absent in mature erythrocytes. Early ultrastructural studies implicated two mechanisms of elimination, structural deterioration by a degradative process [48], and exocytosis [49–51]. Ultrastructurally, mitochondrial clearance is initiated at the same time as enucleation and

continues as the reticulocyte matures[50]. Mitochondria undergoing clearance are surrounded by double-membraned structures, similar in appearance to autophagosomes, and subsequently undergo degradation and elimination by exocytosis. Mitochondria free in the cytoplasm also show signs of deterioration. In this regard, the enzyme 15-lipoxygenase inserts into mitochondrial membranes in reticulocytes and allows access of lytic enzymes to mitochondria[52;53]. However, as will be discussed, studies from our laboratory suggest that mitochondrial degradation and clearance is primarily mediated through vacuolar structures.

NIX is an atypical member of the BH3-only subgroup of BCL2-related proteins (reviewed by Zhang and Ney [54]). NIX is upregulated during terminal erythroid differentiation [55]. To determine the role of NIX in erythroid development several groups generated mice with targeted disruption of *Nix*. Consistent with its documented proapoptotic role, Diwan et al. found that NIX-deficient erythroblasts are resistant to cytokine deprivation-induced cell death[56]. Two other groups found that NIX-deficient reticulocytes exhibit a striking defect in mitochondrial clearance [57;58]. Mitochondria in NIX-deficient reticulocytes are not eliminated, but remain in the cytoplasm in association with isolation membranes and the cytoplasmic face of autophagosomes. Ultrastructurally, autophagy membranes are not properly targeted to mitochondria in the absence of NIX; consequently, mitochondria are not incorporated into autophagosomes, and they are not eliminated. NIX-deficient mice are anemic, which is likely related to an increase in caspase activity and the shortened life-span of NIX-deficient erythrocytes. Another likely consequence of the mitochondrial clearance defect, which may be of functional relevance, is that blood from NIX-deficient mice undergoes accelerated deoxygenation *ex vivo* (Ney PA, unpublished data).

There are several models of the mechanism NIX-mediated mitochondrial clearance. Recent studies implicate defective mitochondrial clearance in the pathogenesis of Parkinson's disease. The genes *Parkin* and *Pink1* are frequently mutated in autosomal recessive Parkinson's disease[59;60]. PINK1 is a mitochondrial integral membrane protein that is stabilized by mitochondrial depolarization. PINK1 recruits Parkin, an E3 ligase [61;62]. Ubiquitylation of mitochondrial proteins by Parkin further leads to the recruitment of adaptors and the autophagy machinery[63;64]. Thus, one proposal is NIX acting in its capacity as a proapoptotic protein causes mitochondrial depolarization and consequent elimination by autophagy [58]. Against this possibility, mitochondrial clearance in reticulocytes proceeds in the absence of the proapoptotic proteins BAX and BAK, and independent of the mitochondrial permeability transition pore [57]. Furthermore, the rate of mitochondrial depolarization is greatly diminished when autophagosome formation is impaired, indicating that mitochondrial depolarization in reticulocytes is not the event that initiates mitochondrial clearance[65]. A second model is that NIX directly recruits autophagy proteins to mitochondria. Consistent with this model, NIX has a microtubule-associated protein-1, light chain-3 (LC3)-interaction region near its amino terminus, and a point mutation of this motif diminishes, but does not eliminate NIX-dependent mitochondrial clearance in reticulocytes [66;67]. A third possibility is that NIX promotes autophagy by competing with Beclin-1 for binding to other BCL2-related proteins [68]; however, at present there is no evidence to suggest that this is the mechanism of mitochondrial clearance in reticulocytes. At present, none of these models can fully account for the activity of NIX in mitochondrial clearance.

Role of autophagy in reticulocyte maturation

Autophagy, or "self-digestion", is a process for recycling cellular components. In contrast to the other major proteolytic pathway, proteasome-dependent degradation, autophagy can recycle complete subcellular structures, such as cytoplasm, protein aggregates, and

organelles. This is accomplished by packaging these structures in double-membranes for delivery to lysosomes. Autophagy was originally described as an ultrastructural phenomenon, in mammalian cells [69;70]. However, in the past decade there has been considerable progress defining the pathways that regulate autophagy in yeast (reviewed by Xie and Klionsky [71]). Starvation induces nonselective autophagy (macroautophagy, or simply autophagy); however, metabolic changes can also cause selective degradation of organelles, such as peroxisomes and mitochondria. In this regard, Atg32, an integral mitochondrial outer membrane protein (like NIX), mediates mitochondrial autophagy in yeast[72;73].

Most autophagy proteins identified in yeast have one or more mammalian homologs. Atg1 is a serine-threonine kinase, itself a target of Tor kinase, and a critical regulator of autophagy. The mammalian homologs of Atg1 are ULK1 and ULK2; however only ULK1 is expressed at high levels in erythroid cells. ULK1-deficient reticulocytes exhibit a moderate defect in mitochondrial clearance, less severe than that caused by deficiency of NIX, and a mild defect in ribosomal clearance[74]; otherwise, reticulocyte maturation is not affected. Atg7 is an E1-like ubiquitin ligase, and key autophagy enzyme in two parallel ubiquitin-like conjugation pathways. Atg7-deficient reticulocytes exhibit a mild-moderate defect in mitochondrial clearance[65;75], less severe than that caused by deficiency of ULK1, and have no other apparent defect in maturation. Together, these studies demonstrate that autophagy has a role in reticulocyte maturation; however, the absence of a more severe defect suggests that functionally redundant pathways exist.

Clearance of other organelles

To create an optimal cell for hemoglobin transportation, reticulocytes jettison all their unnecessary components. Besides the nucleus and mitochondria, this includes the smooth and rough ER, golgi apparatus remnants, ferritin particles, and ribosomes. Compared with the nucleus and mitochondria, less is known about the elimination of other structures and organelles, but similar mechanisms appear to be involved. Lamp-2, a marker of the endolysosomal compartment, is present in secreted exosomes[35], suggesting that this structure is eliminated in part through MVE. Ferritin is observed in cytoplasmic aggregates, in vacuoles, and in MVE-like structures [76;77], although its mechanism of elimination is not yet known. Regarding ribosomes, their degradation by selective autophagy is regulated by a deubiquitinating enzyme in yeast [78]. The mechanism in higher eukaryotes remains to be elucidated. In reticulocytes, ribosomal RNA is degraded by ribonucleases into nucleotides, which in turn are broken down into nucleosides by erythroid pyrimidine 5'-nucleotidase [79]. Deficiency of this enzyme is associated with basophilic stippling, and inherited hemolytic anemia.

Conclusions

The creation of an erythrocyte, a highly specialized cell, requires the upregulation of dedicated proteins, like hemoglobin, and the elimination of all detrimental or simply unnecessary proteins and structures (Figure 1). There is a balance, but both types of processes are required. Because the changes are dramatic and easily studied, the reticulocyte provides an excellent model for the study of cellular remodeling. As discussed here, cellular remodeling in the reticulocyte itself appears to occur through two central processes: vesicular trafficking and autophagy. Although they are considered distinct processes, they may overlap at the cellular and molecular levels. At the cellular level, they can both terminate with exocytosis, suggesting a common mechanism. Further, autophagosomes can directly fuse with MVE, leading to hybrid organelles [80]. At the molecular level, there is recent evidence of shared components between the vesicular trafficking and autophagy

pathways [81;82]. In this regard, in higher eukaryotes, inactivation of the ubiquitin-like conjugation pathways leads to only a partial loss of autophagy activity [65;81]. Whether a component of the vesicular trafficking machinery supplies this redundant activity remains to be determined.

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References

1. Gronowicz G, Swift H, Steck TL. Maturation of the reticulocyte in vitro. *J Cell Sci.* 1984; 71:177–197. [PubMed: 6097593]
2. Noble NA, Xu QP, Ward JH. Reticulocytes. I. Isolation and in vitro maturation of synchronized populations. *Blood.* 1989; 74:475–481. [PubMed: 2752124]
3. Koury MJ, Koury ST, Kopsombut P, Bondurant MC. In vitro maturation of nascent reticulocytes to erythrocytes. *Blood.* 2005; 105:2168–2174. [PubMed: 15528310]
4. Wiczling P, Krzyzanski W. Flow cytometric assessment of homeostatic aging of reticulocytes in rats. *Exp Hematol.* 2008; 36:119–127. [PubMed: 18206724]
5. Jandl, JH. *Textbook of Hematology.* 2. New York: Little, Brown and Company; 1996.
6. McGrath KE, Kingsley PD, Koniski AD, Porter RL, Bushnell TP, Palis J. Enucleation of primitive erythroid cells generates a transient population of “pyrenocytes” in the mammalian fetus. *Blood.* 2008; 111:2409–2417. [PubMed: 18032705]
7. Koury ST, Koury MJ, Bondurant MC. Cytoskeletal distribution and function during the maturation and enucleation of mammalian erythroblasts. *J Cell Biol.* 1989; 109:3005–3013. [PubMed: 2574178]
8. Wickrema A, Koury ST, Dai CH, Krantz SB. Changes in cytoskeletal proteins and their mRNAs during maturation of human erythroid progenitor cells. *J Cell Physiol.* 1994; 160:417–426. [PubMed: 8077279]
9. Lee JC, Gimm JA, Lo AJ, Koury MJ, Krauss SW, Mohandas N, Chasis JA. Mechanism of protein sorting during erythroblast enucleation: role of cytoskeletal connectivity. *Blood.* 2004; 103:1912–1919. [PubMed: 14563645]
- *10. Salomao M, Chen K, Villalobos J, Mohandas N, An X, Chasis JA. Hereditary spherocytosis and hereditary elliptocytosis: aberrant protein sorting during erythroblast enucleation. *Blood.* 2010; 116:267–269. This study provides evidence that sorting of reticulocyte membrane proteins during enucleation is clinically significant. [PubMed: 20339087]
- *11. Popova EY, Krauss SW, Short SA, Lee G, Villalobos J, Etzell J, Koury MJ, Ney PA, Chasis JA, Grigoryev SA. Chromatin condensation in terminally differentiating mouse erythroblasts does not involve special architectural proteins but depends on histone deacetylation. *Chromosome Res.* 2009; 17:47–64. This study provides evidence that histone deacetylation is required for chromatin condensation, during terminal erythroid differentiation. [PubMed: 19172406]
- *12. Ji P, Yeh V, Ramirez T, Murata-Hori M, Lodish HF. Histone deacetylase 2 is required for chromatin condensation and subsequent enucleation of cultured mouse fetal erythroblasts. *Haematologica.* 2010; 95:2013–2021. This study shows through loss-of-function studies that HDAC2 is required for chromatin condensation. [PubMed: 20823130]
- *13. Zhang L, Flygare J, Wong P, Lim B, Lodish HF. miR-191 regulates mouse erythroblast enucleation by down-regulating Riok3 and Mxi1. *Genes Dev.* 2010 This study shows that histone deacetylation, chromatin condensation, and enucleation are regulated by a micro-RNA during erythroid development.

14. Spike BT, Dirlam A, Dibling BC, Marvin J, Williams BO, Jacks T, Macleod KF. The Rb tumor suppressor is required for stress erythropoiesis. *EMBO J.* 2004; 23:4319–4329. [PubMed: 15457215]
15. Coschi CH, Martens AL, Ritchie K, Francis SM, Chakrabarti S, Berube NG, Dick FA. Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. *Genes Dev.* 2010; 24:1351–1363. [PubMed: 20551166]
16. Ngai J, Capetanaki YG, Lazarides E. Differentiation of murine erythroleukemia cells results in the rapid repression of vimentin gene expression. *J Cell Biol.* 1984; 99:306–314. [PubMed: 6376521]
17. Krauss SW, Lo AJ, Short SA, Koury MJ, Mohandas N, Chasis JA. Nuclear substructure reorganization during late-stage erythropoiesis is selective and does not involve caspase cleavage of major nuclear substructural proteins. *Blood.* 2005; 106:2200–2205. [PubMed: 15933051]
18. Chasis JA, Prenant M, Leung A, Mohandas N. Membrane assembly and remodeling during reticulocyte maturation. *Blood.* 1989; 74:1112–1120. [PubMed: 2752157]
19. Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol.* 2008; 10:314–321. [PubMed: 18264091]
20. Dumitriu B, Patrick MR, Petschek JP, Cherukuri S, Klingmuller U, Fox PL, Lefebvre V. Sox6 cell-autonomously stimulates erythroid cell survival, proliferation, and terminal maturation and is thereby an important enhancer of definitive erythropoiesis during mouse development. *Blood.* 2006; 108:1198–1207. [PubMed: 16627753]
- *21. Keerthivasan G, Small S, Liu H, Wickrema A, Crispino JD. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood.* 2010; 116:3331–3340. This study provides evidence that vesicular trafficking plays a central role in enucleation. [PubMed: 20644112]
22. Gromley A, Yeaman C, Rosa J, Redick S, Chen CT, Mirabelle S, Guha M, Sillibourne J, Doxsey SJ. Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. *Cell.* 2005; 123:75–87. [PubMed: 16213214]
23. Mel HC, Prenant M, Mohandas N. Reticulocyte motility and form: studies on maturation and classification. *Blood.* 1977; 49:1001–1009. [PubMed: 324534]
24. Waugh RE, McKenney JB, Bauserman RG, Brooks DM, Valeri CR, Snyder LM. Surface area and volume changes during maturation of reticulocytes in the circulation of the baboon. *J Lab Clin Med.* 1997; 129:527–535. [PubMed: 9142049]
25. Waugh RE, Mantalaris A, Bauserman RG, Hwang WC, Wu JH. Membrane instability in late-stage erythropoiesis. *Blood.* 2001; 97:1869–1875. [PubMed: 11238131]
26. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010; 142:24–38. [PubMed: 20603012]
27. Lim JE, Jin O, Bennett C, Morgan K, Wang F, Trenor CC III, Fleming MD, Andrews NC. A mutation in Sec1511 causes anemia in hemoglobin deficit (hbd) mice. *Nat Genet.* 2005; 37:1270–1273. [PubMed: 16227995]
28. White RA, Boydston LA, Brookshier TR, McNulty SG, Nsumu NN, Brewer BP, Blackmore K. Iron metabolism mutant hbd mice have a deletion in Sec1511, which has homology to a yeast gene for vesicle docking. *Genomics.* 2005; 86:668–673. [PubMed: 16289749]
29. Zhang AS, Sheftel AD, Ponka P. The anemia of “haemoglobin-deficit” (hbd/hbd) mice is caused by a defect in transferrin cycling. *Exp Hematol.* 2006; 34:593–598. [PubMed: 16647565]
30. Garrick MD, Garrick LM. Loss of rapid transferrin receptor recycling due to a mutation in Sec1511 in hbd mice. *Biochim Biophys Acta.* 2007; 1773:105–108. [PubMed: 17087999]
31. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol.* 1983; 97:329–339. [PubMed: 6309857]
32. Martin-Serrano J, Zang T, Bieniasz PD. Role of ESCRT-I in retroviral budding. *J Virol.* 2003; 77:4794–4804. [PubMed: 12663786]
33. Saksena S, Sun J, Chu T, Emr SD. ESCRTing proteins in the endocytic pathway. *Trends Biochem Sci.* 2007; 32:561–573. [PubMed: 17988873]
34. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwillle P, Brugger B, Simons M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science.* 2008; 319:1244–1247. [PubMed: 18309083]

- *35. Barres C, Blanc L, Bette-Bobillo P, Andre S, Mamoun R, Gabius HJ, Vidal M. Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. *Blood*. 2010; 115:696–705. This study provides evidence for Lamp-2 clearance by galectin-5-induced MVE formation through the exosomal pathway, suggesting that this is a mechanism of endolysosomal clearance. [PubMed: 19903899]
36. Geminard C, De GA, Blanc L, Vidal M. Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. *Traffic*. 2004; 5:181–193. [PubMed: 15086793]
37. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983; 33:967–978. [PubMed: 6307529]
38. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem*. 1987; 262:9412–9420. [PubMed: 3597417]
39. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996; 183:1161–1172. [PubMed: 8642258]
40. Nguyen DG, Booth A, Gould SJ, Hildreth JE. Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. *J Biol Chem*. 2003; 278:52347–52354. [PubMed: 14561735]
41. Fevrier B, Vilette D, Archer F, Loew D, Faigle W, Vidal M, Laude H, Raposo G. Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A*. 2004; 101:9683–9688. [PubMed: 15210972]
42. Johnstone RM, Bianchini A, Teng K. Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood*. 1989; 74:1844–1851. [PubMed: 2790208]
43. Vidal M, Sainte-Marie J, Philippot JR, Bienvenue A. Asymmetric distribution of phospholipids in the membrane of vesicles released during in vitro maturation of guinea pig reticulocytes: evidence precluding a role for “aminophospholipid translocase”. *J Cell Physiol*. 1989; 140:455–462. [PubMed: 2777884]
- *44. Blanc L, Liu J, Vidal M, Chasis JA, An X, Mohandas N. The water channel aquaporin-1 partitions into exosomes during reticulocyte maturation: implication for the regulation of cell volume. *Blood*. 2009; 114:3928–3934. This study demonstrates the presence of aquaporin-1 in exosomes, and shows its regulation by ubiquitylation and osmotic conditions. [PubMed: 19724054]
45. Blanc L, Vidal M. Reticulocyte membrane remodeling: contribution of the exosome pathway. *Curr Opin Hematol*. 2010; 17:177–183. [PubMed: 20173636]
46. Gasko O, Danon D. The metabolism of maturing reticulocytes. I. The existence of a functional tricarboxylic acid cycle. *Br J Haematol*. 1972; 23:525–533. [PubMed: 4636185]
47. Gasko O, Kanon D. The metabolism of maturing reticulocytes. II. Decline in activity of the tricarboxylic acid cycle associated with reticulocyte maturation. *Br J Haematol*. 1972; 23:535–539. [PubMed: 4636186]
48. Gasko O, Danon D. Deterioration and disappearance of mitochondria during reticulocyte maturation. *Exp Cell Res*. 1972; 75:159–169. [PubMed: 4635876]
49. Simpson CF, Kling JM. The mechanism of mitochondrial extrusion from phenylhydrazine-induced reticulocytes in the circulating blood. *J Cell Biol*. 1968; 36:103–109.
50. Heynen MJ, Tricot G, Verwilghen RL. Autophagy of mitochondria in rat bone marrow erythroid cells. Relation to nuclear extrusion. *Cell Tissue Res*. 1985; 239:235–239. [PubMed: 3967280]
51. Gronowicz G, Swift H, Steck TL. Maturation of the reticulocyte in vitro. *J Cell Sci*. 1984; 71:177–197. [PubMed: 6097593]
52. van LK, Duvoisin RM, Engelhardt H, Wiedmann M. A function for lipoxygenase in programmed organelle degradation. *Nature*. 1998; 395:392–395. [PubMed: 9759730]
53. Grullich C, Duvoisin RM, Wiedmann M, van LK. Inhibition of 15-lipoxygenase leads to delayed organelle degradation in the reticulocyte. *FEBS Lett*. 2001; 489:51–54. [PubMed: 11231012]
54. Zhang J, Ney PA. Mechanisms and biology of BNIP3 and NIX. *Antioxid Redox Signal*. 2010

55. Aerbajinai W, Giattina M, Lee YT, Raffeld M, Miller JL. The proapoptotic factor Nix is coexpressed with Bcl-xL during terminal erythroid differentiation. *Blood*. 2003; 102:712–717. [PubMed: 12663450]
56. Diwan A, Koesters AG, Odley AM, Pushkaran S, Baines CP, Spike BT, Daria D, Jegga AG, Geiger H, Aronow BJ, Molkentin JD, Macleod KF, Kalfa TA, Dorn GW. Unrestrained erythroblast development in Nix^{-/-} mice reveals a mechanism for apoptotic modulation of erythropoiesis. *Proc Natl Acad Sci U S A*. 2007; 104:6794–6799. [PubMed: 17420462]
57. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, Kundu M, Opferman JT, Cleveland JL, Miller JL, Ney PA. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci U S A*. 2007; 104:19500–19505. [PubMed: 18048346]
58. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, Wang J. Essential role for Nix in autophagic maturation of erythroid cells. *Nature*. 2008
59. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 1998; 392:605–608. [PubMed: 9560156]
60. Deas E, Plun-Favreau H, Wood NW. PINK1 function in health and disease. *EMBO Mol Med*. 2009; 1:152–165. [PubMed: 20049715]
61. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. 2008; 183:795–803. [PubMed: 19029340]
- *62. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol*. 2010; 8:e1000298. This study establishes the mechanism of Parkin recruitment to depolarized mitochondria. [PubMed: 20126261]
- *63. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W. PINK1/ Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol*. 2010; 12:119–131. This study and Ding et al. (*J Biol Chem* 2010; 285:27879–27890) provide evidence that ubiquitylation of mitochondrial proteins by Parkin leads to recruitment of the adaptor p62 and the autophagy machinery. [PubMed: 20098416]
64. Ding WX, Ni HM, Li M, Liao Y, Chen X, Stolz DB, Dorn GW, Yin XM. Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J Biol Chem*. 2010; 285:27879–27890. [PubMed: 20573959]
- *65. Zhang J, Randall MS, Loyd MR, Dorsey FC, Kundu M, Cleveland JL, Ney PA. Mitochondrial clearance is regulated by Atg7-dependent and -independent mechanisms during reticulocyte maturation. *Blood*. 2009; 114:157–164. This study from our laboratory and that of Mortensen et al. (*Proc Nat Acad Sci* 2010; 107:832–837) show that Atg7 activity, which is essential for dual ubiquitin-like autophagy pathways, is not essential for mitochondrial clearance in reticulocytes. [PubMed: 19417210]
- *66. Schwarten M, Mohrluder J, Ma P, Stoldt M, Thielmann Y, Stangler T, Hersch N, Hoffmann B, Merkel R, Willbold D. Nix directly binds to GABARAP: a possible crosstalk between apoptosis and autophagy. *Autophagy*. 2009; 5:690–698. This study and Novak et al. (*EMBO Rep* 2010; 11:45–51) shows that NIX recruits Atg8 homologs through its amino terminus. [PubMed: 19363302]
- *67. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, Rogov V, Lohr F, Popovic D, Occhipinti A, Reichert AS, Terzic J, Dotsch V, Ney PA, Dikic I. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep*. 2010; 11:45–51. This study correlates loss of Atg8 recruitment to NIX with diminished mitochondrial clearance in reticulocytes. [PubMed: 20010802]
68. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol*. 2009; 29:2570–2581. [PubMed: 19273585]
69. CLARK SL Jr. Cellular differentiation in the kidneys of newborn mice studies with the electron microscope. *J Biophys Biochem Cytol*. 1957; 3:349–362. [PubMed: 13438920]
70. Ashford TP, PORTER KR. Cytoplasmic components in hepatic cell lysosomes. *J Cell Biol*. 1962; 12:198–202. [PubMed: 13862833]

71. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol.* 2007; 9:1102–1109. [PubMed: 17909521]
- **72. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell.* 2009; 17:98–109. This study and Okamoto et al. (*Dev Cell* 2009; 17:87–97) demonstrates the role of Atg32 in selective mitochondrial autophagy in yeast. [PubMed: 19619495]
73. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev Cell.* 2009; 17:87–97. [PubMed: 19619494]
74. Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, Zhang J, Selak MA, Ney PA, Thompson CB. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood.* 2008; 112:1493–1502. [PubMed: 18539900]
75. Mortensen M, Ferguson DJ, Edelmann M, Kessler B, Morten KJ, Komatsu M, Simon AK. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. *Proc Natl Acad Sci U S A.* 2010; 107:832–837. [PubMed: 20080761]
76. Heynen MJ, Verwilghen RL. A quantitative ultrastructural study of normal rat erythroblasts and reticulocytes. *Cell Tissue Res.* 1982; 224:397–408. [PubMed: 7105141]
77. Tanaka Y. Bi-directional transport of ferritin in guinea pig erythroblasts in vitro. *Blood.* 1970; 35:793–803. [PubMed: 5427249]
78. Kraft C, Deplazes A, Sohrmann M, Peter M. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat Cell Biol.* 2008; 10:602–610. [PubMed: 18391941]
79. Valentine WN, Fink K, Paglia DE, Harris SR, Adams WS. Hereditary hemolytic anemia with human erythrocyte pyrimidine 5'-nucleotidase deficiency. *J Clin Invest.* 1974; 54:866–879. [PubMed: 4372252]
80. Fader CM, Sanchez D, Furlan M, Colombo MI. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic.* 2008; 9:230–250. [PubMed: 17999726]
- **81. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature.* 2009; 461:654–658. This study establishes the existence Atg5- and Atg7-independent autophagy in mammalian cells, and confirms the finding of Zhang et al. (*Blood* 2009; 114:157–164) that the ubiquitin-like conjugation pathways are not essential for mitochondrial clearance in reticulocytes. The authors provide evidence that an alternate autophagy pathway exists downstream of Beclin-1 and ULK1, which involves vesicular trafficking pathways. [PubMed: 19794493]
- **82. Bodemann BO, Orvedahl A, Cheng T, Ram RR, Ou YH, Formstecher E, Maiti M, Hazelett CC, Wauson EM, Balakireva M, Camonis JH, Yeaman C, Levine B, White MA. RalB and the Exocyst Mediate the Cellular Starvation Response by Direct Activation of Autophagosome Assembly. *Cell.* 2011; 144:253–267. This study shows that RalB and the exocyst complex, involved in vesicular trafficking, are required for starvation-induced autophagy. This study provides direct evidence of cross-talk between autophagy and vesicular trafficking. [PubMed: 21241894]

Key points

- Vesicular trafficking plays a central role in reticulocyte remodeling and remains poorly understood.
- Mitochondrial elimination from reticulocytes depends on an integral membrane protein, NIX, and to a lesser extent on core autophagy pathways.
- In higher eukaryotes, there is redundancy between autophagy pathways and other pathways that remain to be defined.
- Cross-talk exists between autophagy and vesicular trafficking pathways.

Figure 1.

Normal reticulocyte maturation. Four steps in reticulocyte maturation are shown, in roughly sequential order. 1) Iron import-transferrin receptor recycling. Iron-loaded transferrin bound to the transferrin receptor is internalized via clathrin-mediated endocytosis into the early endosomal compartment. After acidification of the endosome and release of iron from transferrin, transferrin-transferrin receptor complex recycles to the cell surface. 2) Enucleation. Vesicles coalesce at the nuclear cytoplasmic junction, creating a new limiting membrane. The sides are pinched inwards by the combined action of vesicle trafficking and microfilaments. 3) Mitochondrial clearance. Vesicles are recruited and surround mitochondria in a process that requires NIX, and is partially dependent on autophagy. Mitochondria are subsequently degraded and eliminated by exocytosis. 4) Secretion of exosomes. Membrane is lost and transferrin receptor and other membrane proteins are downregulated through the MVE-exosomal pathway. MVE are generated by membrane invagination in late endosomes. Intraluminal vesicles are released into the extracellular space by exocytosis.