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SPOTLIGHT

# NCOA4: More than a receptor for ferritinophagy

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**Liquid–liquid phase separation (LLPS) triages protein cargoes for autophagic degradation. In this issue, Ohshima et al. (2022. *J. Cell Biol.* https://doi.org/10.1083/jcb.202203102) demonstrate that the autophagy receptor NCOA4 interacts with ferritin particles to form liquid-like condensates via LLPS. The NCOA4-ferritin condensates are delivered to lysosomes for degradation via either canonical macroautophagy or endosomal microautophagy to maintain intracellular iron homeostasis.**

Iron is critical for numerous biological processes, such as ATP production, DNA synthesis and repair, enzymic catalysis, and erythropoiesis (1). Excess free iron causes cellular toxicity by producing reactive oxygen species and triggering ferroptosis (1). Cellular iron levels are tightly controlled via a network of iron-sensing factors, among which ferritin stands center stage (Fig. 1). Typical ferritins in higher eukaryotes are composed of 24 subunits (a mixture of ferritin heavy chain [FTH1] and light chain [FTL]) that assemble into a spherical shell to store, oxidize, and mineralize up to 4,500 iron ions (1). During iron deficiency, ferritin is delivered to lysosomes via autophagy pathways to release stored iron (2–6). Autophagy includes autophagosome-mediated macroautophagy, endosome-mediated microautophagy, and chaperone-mediated autophagy (7, 8). Macroautophagy involves the initiation of an isolation membrane that further expands and closes to form a double-membraned autophagosome, which then fuses with late endosomes/lysosomes for degradation of sequestrated cargos. Microautophagy refers to direct encapsulation of cargoes by the endosomal membrane to form intraluminal vesicles (ILVs) for subsequent delivery to lysosomes (7–9). Ferritin particles can be degraded by macroautophagy, which requires autophagy proteins such as the LC3-lipidation machinery (2–4), and also via LC3-lipidation-independent but ESCRT-dependent endosomal microautophagy (6). Both degradation pathways require the receptor NCOA4 that interacts with ferritin via FTH1, and the adaptor TAX1BP1 that binds to NCOA4 (3–6). It is not completely understood how NCOA4 and TAX1BP1 act in these two morphologically and mechanistically distinct degradation pathways. In this issue, Ohshima et al. (10) show that during iron deficiency, NCOA4 drives ferritin particles to undergo liquid–liquid phase separation (LLPS) to form liquid-like condensates, and TAX1BP1 mediates the recognition of condensates for subsequent degradation (10).

Protein LLPS refers to the concentration of uniformly dissolved proteins into liquid-like condensates, which can further transition into less dynamic material states such as gels or solid fibers (11). LLPS can triage protein cargoes for macroautophagic degradation (12). To investigate whether ferritin particles undergo LLPS, Ohshima et al. (10) observed mGFP-FTH1 and mGFP-FTL, whose expression levels are comparable to those of endogenous proteins, in WT cells and cells with knockout (KO) of *FIP200*, an essential macroautophagy gene. mGFP-FTH1 and mGFP-FTL form punctate structures in WT cells and larger ones in *FIP200* KO cells under normal and iron-overloaded conditions. mGFP-FTH1 puncta are spherical and are not enclosed by membranes in *FIP200* KO cells (revealed by transmission electron microscopy), show mobile interior molecules (examined by fluorescence recovery after photobleaching assays [FRAP]), and also undergo fusion upon encounter in WT cells (10). These characteristic liquid-like properties indicate that large puncta containing ferritin in living cells are assembled via LLPS.

Next, the authors examined the role of NCOA4 in ferritin LLPS. NCOA4 colocalizes with ferritin puncta. Formation of mGFP-FTH1 puncta in WT and *FIP200* KO cells is abolished by NCOA4 depletion. FTL knockdown (KD) does not affect mGFP-NCOA4 punctum formation (10). To overcome the complexity caused by the diminished NCOA4 protein level in *FTH1* KD cells, the authors exogenously expressed NCOA4 and FTH1 in yeast cells, in which ferritin and NCOA4 homologs are absent. Expression of FTH1 and NCOA4 together, but not individually, resulted in formation of large punctate structures (10). Therefore, NCOA4 and FTH1, but not FTL, are required for formation of phase-separated ferritin condensates.

Multivalent interactions between biomacromolecules drive LLPS (11). Ohshima et al. (10) next mapped the inter- and intramolecular interactions required for assembly of NCOA4-ferritin condensates. By analyzing the ability of a series of NCOA4 truncations to restore mGFP-FTH1 puncta in *NCOA4* KO cells, the authors found that the N-terminal self-oligomerization coiled-coil domain and

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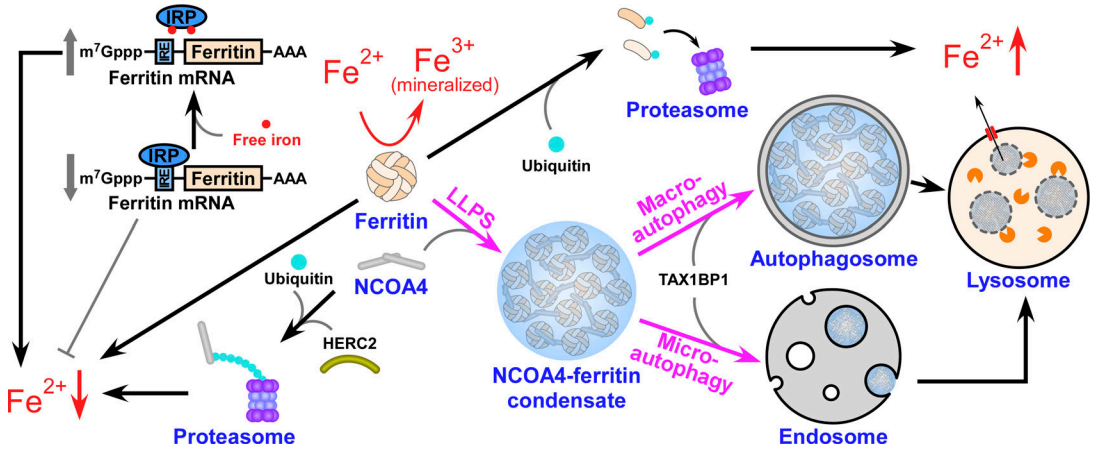


Figure 1. **Model showing how ferritin turnover maintains intracellular iron homeostasis.** Expression of ferritin is subjected to translational regulation. Iron Responsive Proteins (IRP1 and IRP2) bind to the Iron Regulatory Element (IRE) located in the 5′UTR of *ferritin* mRNAs to repress their translation. This repression is relieved by iron binding to IRPs (1). The ferritin subunits assemble into particles that mineralize $Fe^{2+}$ to $Fe^{3+}$ for storage. Ferritin can undergo ubiquitin-mediated proteasomal degradation, macroautophagy, or ESCRT-mediated endosomal degradation known as microautophagy. Lysosomal degradation requires NCOA4-mediated LLPS of ferritin to form liquid-like condensates, and TAX1BP1 for selective cargo engulfment. NCOA4 itself is subject to ubiquitin-proteasome degradation and autophagic degradation to modulate the level of ferritin turnover (3–5). HERC2 is an ubiquitin ligase that mediates NCOA4 turnover via the proteasome system (5).

the FTH1-interacting intrinsically disordered region (IDR2) are required (10). Point mutations in the N-terminal domain (I56E or L63R) that disrupt self-oligomerization of NCOA4 also abolish its ability to drive formation of GFP-FTH1 puncta (10). Thus, multivalent interactions involving NCOA4 self-oligomerization and NCOA4-FTH1 interaction mediate formation of NCOA4-ferritin condensates.

Ohshima et al. (10) further examined autophagic degradation of ferritin condensates. Using immunofluorescent imaging and three-dimensional correlative light and electron microscopy (3D-CLEM), they found that a portion of the large ferritin condensates is sequestrated by cup-shaped autophagosomal membranes in a piecemeal manner with the remaining portion retaining a spherical shape (10). This may be caused by a wetting effect resulting from contact between liquid condensates and membranes (13). The authors observed that ferritin condensates are also included in ILVs inside endosomes (10), consistent with degradation of ferritin condensates by endosomal microautophagy. *NCOA4* depletion abolished the incorporation of ferritin into endosomal ILVs, which is rescued by expression of WT NCOA4 but not LLPS-deficient truncations and mutations of NCOA4. This indicates that formation of ferritin condensates is required for microautophagic degradation.

Selective incorporation of protein condensates into autophagosomes or ILVs involves cargo recognition. TAX1BP1 binds directly to NCOA4 and is required for lysosomal turnover of ferritin under basal and iron-depleted conditions (6). mGFP-TAX1BP1 co-localizes with mRuby3-FTH1 puncta in WT and *FIP200* KO cells, implying that TAX1BP1 is a component of NCOA4-ferritin condensates. Ohshima et al. (10) found that *TAX1BP1* KO does not affect the formation of ferritin puncta but impairs their incorporation into autophagosomes and endosomal ILVs (10). Thus, TAX1BP1 is an adaptor for specific recognition of NCOA4-ferritin condensates by autophagosomal and endosomal membranes.

This work adds another example of the role of LLPS in sorting cargos for macroautophagic degradation and also demonstrates, for the first time, LLPS of protein condensates in microautophagy. The interaction between TAX1BP1 and LC3 or FIP200 mediates selective incorporation of NCOA4-ferritin condensates into autophagosomes. However, it has yet to be elucidated how TAX1BP1 links the condensates to endosomal membranes. It is also unknown whether the formation of ILVs is driven by a wetting effect. Under iron deficiency and iron overloaded conditions, NCOA4-ferritin condensates are preferentially degraded by macroautophagy and microautophagy, respectively (2). Is the degradation route specified by the size and/or material states of NCOA4-ferritin condensates, which could be modulated by the stoichiometry of NCOA4 and FTH1 subunits or free iron level? The requirement for receptor and adaptor proteins appears to be a general mechanism for autophagic degradation of phase-separated protein condensates. For example, during autophagic degradation of PGL granules in *C. elegans*, the receptor SEPA-1 promotes LLPS of PGL-1/-3 cargo proteins, while the adaptor EPG-2 decorates the surface and elicits gelation of SEPA-1/PGL condensates, which still exhibit a certain degree of liquidity shown by FRAP (14). This study establishes a model for investigating phase separation and transition in mediating degradation of protein condensates by macroautophagy or microautophagy.

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