ULK1 cycling: The ups and downs of the autophagy response

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The Ser/Thr kinase ULK1/Atg1 controls autophagy initiation under nutrient starvation conditions. In this issue, Nazio et al. (2016. *J. Cell Biol.* https://doi.org/10.1083/jcb.201605089) demonstrate that oscillatory modulation of NEDD4L-mediated proteasomal degradation and mTOR-dependent de novo protein synthesis of ULK1 ensures the proper amplitude and duration of the autophagy response during prolonged starvation, thus maintaining cellular homeostasis.

Autophagy involves the engulfment of a portion of cytosol in the double-membrane autophagosome and its subsequent delivery to lysosomes for degradation (Nakatogawa et al., 2009). Autophagy is essential for cell survival in response to starvation or energy deprivation. However, excessive self-digestion by autophagy can lead to cell death (Levine and Kroemer, 2008). The amplitude and duration of autophagy are thus tightly controlled to maintain cellular homeostasis.

The serine/threonine kinase ULK1/Atg1, which forms a complex with FIP200/Atg17 and ATG13, acts at multiple steps of autophagy and executes its function in part by phosphorylating ATG proteins, including ATG13, Beclin 1, and ATG9 (Papinski and Kraft, 2016). The kinase activity of ULK1/Atg1 is regulated by phosphorylation mediated by mTOR and AMP kinase (AMPK), which sense nutrients and energy availability. mTOR-mediated phosphorylation inhibits, whereas AMPK-mediated phosphorylation activates, ULK1 kinase activity (Kim et al., 2011). The AMBRA1–TRAF6 (E3 ligase) complex mediates K63-linked polyubiquitination of ULK1 during early stages of autophagy to maintain its stability, self-association, and kinase activity (Nazio et al., 2013). Conversely, Cullin/KLHL20 catalyzes K48-linked polyubiquitination of ULK1 for proteasome degradation during prolonged nutrient starvation, thus providing negative feedback control of the autophagy response (Liu et al., 2016). In this issue, Nazio et al. provide evidence that levels and activity of ULK1 are temporally controlled by NEDD4L-mediated degradation and mTOR-dependent de novo protein synthesis to modulate the duration of the autophagy response during prolonged starvation.

Nazio et al. (2016) analyzed the kinetics of ULK1 protein levels and activity in HeLa cells under starvation conditions. ULK1 protein levels are reduced during the first 4 h of starvation and restored to basal levels by 6 h. Conversely, *ULK1* mRNA is gradually increased during starvation, reaching 15-fold at 6 h, whereas ULK1-dependent phosphorylation of ATG13 at serine 318, an indicator of ULK1 kinase activity, increases during the first hour and then gradually decreases after starvation for 4–6 h. The decrease in ULK1 protein levels is inhibited by treatment with the proteasome inhibitor MG132, indicating that turnover of ULK1 is mediated by the ubiquitin–proteasome pathway. Consistent with this, ULK1 is ubiquitinated and this modification exhibits a rapid increase 1–2 h after starvation.

Nazio et al. (2016) further identified the E3 ubiquitin ligase responsible for ubiquitination and degradation of ULK1 during prolonged starvation. The HECT domain–containing E3 ubiquitin ligase NEDD4L interacts with ULK1 and the interaction is enhanced after autophagy induction. Overexpression of wild-type NEDD4L, but not the C821A mutant lacking ubiquitin ligase activity, NEDD4L(C821A), efficiently promotes ULK1 ubiquitination both in vivo and in vitro and also promotes ULK1 protein degradation, whereas NEDD4L down-regulation results in an increase in ULK1 protein levels and prevents its degradation after prolonged starvation.

Conjugation of distinct ubiquitin chain configurations has different effects on substrates. For example, K48-linked polyubiquitination targets substrates for proteasome degradation, whereas K63-linked ubiquitination regulates protein activity (Kuang et al., 2013). In addition, K27- and K29-linked ubiquitination has been associated with lysosomal degradation (Kuang et al., 2013). Although NEDD4L triggers ULK1 degradation by the proteasome pathway, it also promotes K27- and K29-linked polyubiquitination of ULK1. Overexpression of NEDD4L also leads to increased K63 ubiquitination of ULK1, but the physiological role of this NEDD4L-mediated modification remains unknown.

Upon autophagy induction, ULK1 kinase activity is activated and ULK1 undergoes autophosphorylation at Serine 1047 (mouse ULK1). Both wild-type ULK1 and kinase-inactive ULK1(K46I) interact with NEDD4L; however, NEDD4L induces ubiquitination and subsequent degradation of only the active form of ULK1. The ULK1(S1047A) mutant, which cannot be autophosphorylated, is also refractory to NEDD4L-mediated ubiquitination and degradation. The selectivity could be conferred by a change in ULK1 conformation induced by autophosphorylation or posttranslational modification elicited by autophagy stimuli such as AMPK-mediated phosphorylation.

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The Rockefeller University Press $30.00
J. Cell Biol. Vol. 215 No. 6 757–759
https://doi.org/10.1083/jcb.201611177

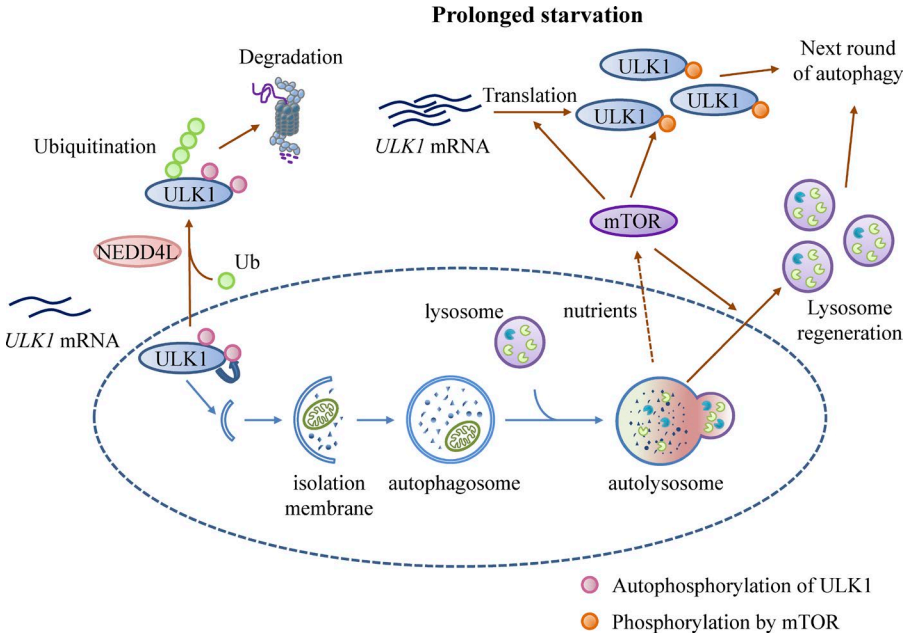

Figure 1. **Model for the cycling of ULK1 protein levels, which modulates the oscillatory mode of the autophagy response during prolonged starvation.** Upon autophagy induction, ULK1 is activated and undergoes autophosphorylation, which triggers autophagosome formation. The activated ULK1 is then ubiquitinated by NEDD4L and targeted to the proteasome for degradation. *ULK1* mRNA is gradually increased during starvation. Release of nutrients from autolysosomes after the degradation of sequestrated materials activates mTOR, which promotes de novo ULK1 protein synthesis. The newly synthesized ULK1 is inhibited by mTOR phosphorylation. Reactivation of mTOR also promotes lysosome regeneration from autolysosomes to maintain the lysosome pool for the next round of autophagy. Steps in the autophagy pathway are enclosed within the dashed oval.

NEDD4L is regulated by self-ubiquitination and phosphorylation. Compared with control conditions, self-ubiquitination levels of NEDD4L after autophagy induction are higher. Phosphorylation on S342 of NEDD4L inhibits its binding to substrates (Lee et al., 2007; Gao et al., 2009). Here, Nazio et al. (2016) find that NEDD4L phosphorylation is reduced during the first 4 h of starvation, a pattern that parallels ULK1 levels, whereas *NEDD4L* mRNA is significantly increased. It is therefore possible that ULK1 regulates the kinases that phosphorylate S342 of NEDD4L, such as SGK1, Akt, and PKA. Thus, NEDD4L is positively regulated upon autophagy induction to facilitate ULK1 proteasome degradation.

The authors further explored whether NEDD4L inactivation, which results in stabilization of ULK1 in prolonged starvation, alters the amplitude and duration of the autophagy response. The formation of ATG16 puncta, which label early autophagic structures known as isolation membranes, and LC3 puncta, which label all stages of autophagic structures, were analyzed to measure the rate of autophagosome formation at different time points. In controls cells, autophagy activity is transiently activated; ATG16L and LC3 puncta increase at 1–2 h, then decline at 4–6 h of starvation, and return to basal levels after 6–8 h. The decline of ATG16 puncta and LC3 puncta at late time points of the autophagy response is significantly reduced in *NEDD4L* KD cells, suggesting persistent autophagy flux.

By mass spectrometry analysis, Nazio et al. (2016) identified that lysine 925 and lysine 933 in ULK1 are ubiquitinated by NEDD4L. Mutant ULK1(K925R) and ULK1(K933R) proteins exhibit a significant decrease in ubiquitination and are more stable than wild-type ULK1. Both mutations affect NEDD4L-dependent degradation of ULK1 during autophagy. Compared with wild-type ULK1, expression of the ubiquitination-defective mutant ULK1 increases autophagy activity and this effect is not further enhanced by *NEDD4L* down-regulation. These results reveal a correlation between ULK1 protein degradation and termination of the autophagy response.

Nazio et al. (2016) further investigated the mechanism underlying the restoration of ULK1 during prolonged starvation. mTOR activation phosphorylates translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 to promote protein synthesis (Laplante and Sabatini, 2012). mTOR is reactivated during prolonged starvation (Yu et al., 2010). Treatment with the mTOR inhibitors Rapamycin and Torin 1 at 4 h of starvation effectively suppressed both restoration of ULK1 protein levels and mTOR-mediated ULK1 phosphorylation. This indicates an involvement of mTOR reactivation in restoration of ULK1 protein levels. Cycloheximide treatment, which inhibits translation, or actinomycin D treatment, which inhibits mRNA transcription, inhibits ULK1 restoration after 6 h of starvation. The newly synthesized ULK1 is inhibited by mTOR phosphorylation, contributing to autophagy inhibition after prolonged starvation (Fig. 1). Consistent with the involvement of de novo protein synthesis for ULK1 restoration, analysis of the distribution profile of *ULK1* mRNA in translationally active polysomes or in inactive ribonucleoproteins reveals a decrease in polysome-associated *ULK1* mRNA in cells 2 h after starvation, but a dramatic increase in cells by 6 h.

Finally, Nazio et al. (2016) determined ULK1 protein levels after reinduction of autophagy during prolonged starvation. ULK1 protein levels are down-regulated when autophagy is reinduced by starvation after nutrient replenishment. The inhibition of mRNA transcription by actinomycin D blocks autophagy reinduction. In *NEDD4L* KD cells, ULK1 protein levels fail to reduce, resulting in a stronger reinduction of autophagy than in control cells. Persistently high autophagy resulting from impairment of autophagy termination potentiates prolonged starvation-induced cell death that can be blocked by the autophagy inhibitor bafilomycin A1.

The ULK1 complex acts as a node for integrating nutrient status and also represents a hinge point in determining the oscillatory nature of autophagy activity. Levels and activity of ULK1 are explicitly controlled during autophagic flux via multiple mechanisms. Yeast Atg1 is transported to and degraded in the vacuole via its direct interaction with autophagosome-associated Atg8 (Kraft et al., 2012), whereas mammalian ULK1 is mainly degraded by the proteasome (Alemu et al., 2012). K63-linked polyubiquitination of ULK1 mediated by the AMBRA1–TRAF6

complex in the early stage of autophagic flux facilitates ULK1 stabilization and function (Nazio et al., 2013). During prolonged autophagy, ULK1 undergoes K48-linked polyubiquitination mediated by Cullin3/KLHL20 for degradation (Liu et al., 2016). The interaction of KLHL20 with ULK1 is greatly enhanced by ULK1 autophosphorylation induced by autophagy (Liu et al., 2016). Thus, KLHL20 and NEDD4L mediate degradation of autophagy-activated ULK1. This negative feedback regulation mechanism restrains the amplitude and duration of autophagy. Moreover, selective degradation of autophagy-activated ULK1 does not impair other ULK1-mediated cellular functions. Further investigations are needed to determine how the NEDD4L- and KLHL20-mediated modifications of ULK1 interact. They may act in different physiological settings or act coordinately to regulate autophagy oscillation. Different E3 ligases mediate ubiquitination and degradation of ULK1 under different conditions. The mitochondrial outer-membrane E3 ligase MUL1 mediates the K48-linked polyubiquitination of ULK1 for degradation in selenite-induced mitophagy (Li et al., 2015). During prolonged nutrient deprivation, reactivation of mTOR induced by degradation of sequestrated materials in autolysosomes triggers lysosome regeneration to maintain the lysosome pool for the next round of autophagy (Yu et al., 2010). The study of Nazio et al. (2016) shows that reactivation of mTOR allows resynthesis of ULK1 to establish a new pool of ULK1. Thus, mTOR ensures the rapid and transient induction of autophagy, preventing excessive cellular degradation (Fig. 1). It will be interesting to determine how oscillation of ATG proteins and regeneration of lysosomes is coordinately regulated to maintain the proper autophagy level in response to prolonged nutrient starvation.

Acknowledgments

Work in the authors' laboratory is supported by the National Natural Science Foundation of China (31630048, 31421002, 31561143001, and 31225018 to H. Zhang and 31401184 and 31671430 to Y.G. Zhao) and a grant from the National Basic Research Program of China (2013CB910100) to H. Zhang. The research of H. Zhang is supported in part by an International Early Career Scientist grant from the Howard Hughes Medical Institute.

The authors declare no competing financial interests.

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