Fine-tuning of ULK1 mRNA and protein levels is required for autophagy oscillation

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Autophagy is an intracellular degradation pathway whose levels are tightly controlled to secure cell homeostasis. Unc-51–like kinase 1 (ULK1) is a conserved serine–threonine kinase that plays a central role in the initiation of autophagy. Here, we report that upon autophagy progression, ULK1 protein levels are specifically down-regulated by the E3 ligase NEDD4L, which ubiquitylates ULK1 for degradation by the proteasome. However, whereas ULK1 protein is degraded, ULK1 mRNA is actively transcribed. Upon reactivation of mTOR-dependent protein synthesis, basal levels of ULK1 are promptly restored, but the activity of newly synthesized ULK1 is inhibited by mTOR. This prepares the cell for a new possible round of autophagy stimulation. Our results thus place NEDD4L and ULK1 in a key position to control oscillatory activation of autophagy during prolonged stress to keep the levels of this process under a safe and physiological threshold.

Introduction

Autophagy is a catabolic process occurring in all eukaryotic cells so as to maintain cellular viability and homeostasis during basal conditions by controlling long-lived proteins and damaged organelles. Autophagy can also be stimulated to maintain cell survival in response to sublethal stresses, such as nutrient or growth factor deprivation, hypoxia, reactive oxygen species, or viral and pathogen invasion (Choi et al., 2013). This process requires both the ULK1 serine–threonine kinase and the BECLIN 1–VPS34 core complex for its upstream signaling to generate double-membrane vesicles, the autophagosomes, which transfer portions of cytosolic content to lysosomes (Wirth et al., 2013).

ULK1 is one of the most upstream autophagy-related factors; in fact, it forms a stable complex with ATG13, FIP200, and ATG101, playing a crucial role in the initiation steps of autophagy (Noda and Fujioka, 2015). Furthermore, ULK1 regulates its substrates and is itself regulated by phosphorylation events. mTOR and 5′ AMP-activated protein kinase are among its well-known upstream regulators (Kim et al., 2011). Other posttranslational modifications, including ubiquitylation and acetylation, have been reported to modulate the pace of ULK1 turnover and kinase activity in different cellular contexts (Lin et al., 2012; Kuang et al., 2013; Jiao et al., 2015). Indeed, Hsp90 and Cdc37 are chaperones that regulate ULK1 stability and activity by forming a complex with ULK1, which subsequently influences ATG13-mediated mitophagy (Joo et al., 2011). Further, soon after autophagy induction, the stability of ULK1 is regulated by K63-linked ubiquitin chains, which are mediated by the AMBRA1 complex with the E3 ligase TRAF6 (Nazio et al., 2013). Of note, it has been found that AMBRA1 protein levels are also significantly modulated by E3 ligases during the autophagy response (Antonioli et al., 2014; Xia et al., 2014). Several additional E3 ligases have been identified as ULK1 regulators (Nazio et al., 2013; Li et al., 2015; Liu et al., 2016), and it is now apparent that activation of protein kinases can initiate irreversible down-regulation by ubiquitin proteasome system
Here, we report that ULK1 levels are finely regulated during an autophagy response at the transcriptional, translational, and degradation levels. We show that ULK1 protein is down-regulated during the first few hours of starvation through the activity of the E3 ligase NEDD4L (neural precursor cell-expressed developmentally down-regulated 4–like) and restored to basal levels during prolonged starvation. This ULK1 rescue requires a constant transcription of $\text{ULK1}$ mRNA and mTOR-dependent de novo protein synthesis reactivation.

mTOR may then inhibit ULK1 activity and block autophagy progression in the absence of new stimuli. Autophagy, if re-induced, can proceed, through this novel pathway of regulation, by tightly controlled pulses, by which a cell can avoid excessive self-digestion culminating in cell death.

**Results**

**ULK1 mRNA and protein levels are regulated in a controlled time course during autophagy.**

Because the protein levels of AMBRA1, a key regulator of ULK1 protein stability, are regulated by UPS during autophagy (Nazio et al., 2013; Antonioli et al., 2014), we first analyzed ULK1 protein levels in HeLa cells under starvation conditions (lacking both amino acids and serum). We found that ULK1 protein levels are reduced during the first 4 h of starvation, being later restored to almost basal levels after starvation for 6 h (Fig. 1 A). Autophagy flux is analyzed in the presence or absence of the lysosomal inhibitor chloroquine (Clq) by LC3 lipidation. We also analyzed the levels of some ULK1-interacting proteins, such as FIP200 and ATG13. Protein levels of ULK1 and TUBULIN were detected by WB. In A and B, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. In C, data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison post test. ***, $P < 0.001$.
ULK1 interacts with the E3 ligase NEDD4L

Prompted by this finding, we then moved to the identification of the E3 ubiquitin ligase responsible for the first steps of ULK1 proteasome-dependent degradation. Given the role of TRAF6 on ULK1 positive regulation and our preliminary data on ULK1 interactions based on mass spectrometry (MS) analysis, and because NEDD4L acts as an E3 ligase in the opposite way of TRAF6 in regulating the nerve growth factor receptor TrkA, another kinase and ULK1 interactor (Geetha et al., 2005; Zhou et al., 2007; Georgieva et al., 2011), we decided to investigate the existence of a putative NEDD4L–ULK1 interaction by a biochemical approach. To verify this, coimmunoprecipitation (co-IP) analyses were performed in HeLa cells using both NEDD4L and ULK1 antibodies. These analyses reveal the interaction between endogenous NEDD4L and ULK1 (Fig. 2 A). Moreover, by analyzing the interaction between ULK1 and NEDD4L after autophagy induction by starvation, we found an increase in their binding both in endogenous or overexpression conditions (Fig. 2, B and C). Next, because NEDD4L substrates interact specifically with the WW domains of Homologous to E6AP C terminus (HECT) E3 ligase to be ubiquitylated (Harvey et al., 1999), we tested which NEDD4L WW domains mediate the interaction with ULK1. As shown in Fig. 2 D, the WW1 domain of NEDD4L is capable of binding ULK1, with WW2 showing only a very modest interaction. Unfortunately, no canonical WW-binding PPXY motifs are present in ULK1, suggesting that critical phosphorylated residues could mediate this binding or some adaptor proteins bearing PPXY-like sequences could be involved (Lu et al., 1999; Shearwin-Whyatt et al., 2006; Léon and Haguenauer-Tsapis, 2009).

NEDD4L ubiquitylates ULK1 and induces its degradation via the proteasome

As NEDD4L is a HECT domain–containing E3 ubiquitin ligase, we then tested whether NEDD4L could regulate the protein levels of ULK1. Indeed, wild-type NEDD4L (NEDD4LWT) efficiently promotes ULK1 protein decrease, but at variance with its C821A mutant lacking ubiquitin ligase activity (NEDD4LCA; Bruce et al., 2008; Yang and Kumar, 2010; Fig. 3, A and B). This effect is specific because overexpression of NEDD4L does not show any effects on other known ULK1-binding proteins, such as ATG13, AMBRA1, and BECLIN 1 (Fig. S2 A). The decrease in ATG13 levels that we find during starvation is therefore most likely regulated by another degradation mechanism.

Because of the high degree of homology between NEDD4 and NEDD4L, and the fact that NEDD4 is involved in the degradation of another key proautophagic protein BECLIN 1 (Platta et al., 2012), we tested whether NEDD4 could also regulate ULK1 protein degradation. However, at variance with NEDD4L, NEDD4 overexpression is not able to induce ULK1 protein decrease (Fig. S2 B).
Next, we found that down-regulation of the ULK1 protein by NEDD4L is blocked by proteasome inhibitors (MG132 and lactacystin), but not by lysosomal inhibitors (leupeptin and Clq; Figs. 3 C and S2 C), indicating that NEDD4L triggers ULK1 degradation exclusively through the proteasome pathway. Prompted by this finding, we investigated whether NEDD4L could induce ULK1 ubiquitylation. We found that NEDD4L WT, but not NEDD4L CA mutant, efficiently promotes ULK1 ubiquitylation in vivo (Fig. 3 D). To exclude the possibility that NEDD4L might act indirectly on ULK1, we investigated whether NEDD4L could directly ubiquitylate ULK1 in vitro. Indeed, the ubiquitylation assay in vitro reveals a direct ubiquitylation of ULK1 by NEDD4L (Fig. 3 E).

K48-linked ubiquitylation is mainly believed to target substrates for proteasome degradation, whereas K63-linked ubiquitylation is involved in regulating protein activity. It has been reported that NEDD4L is able to promote K11-, K48-, K63-, K29-, K27-, and K6-linked ubiquitylation to mediate the degradation of its substrates (Fotia et al., 2006; Ding et al., 2013). This raised the intriguing question as to what type of degradative ubiquitylation NEDD4L induces on ULK1. To address this, we used a series of HIS-tagged ubiquitin constructs possessing a
ULK1S1047A (Dorsey et al., 2009) mutated form in its autophos-
tylation (Alers et al., 2012), also undergoes a similar
nutrient starvation. Interestingly, we found that ULK2, another
down-regulation induces an increase in ULK1 protein levels
NEDD4L. As shown in Fig. 4 (A–C) and Fig. S3 A, NEDD4L
and that ULK1 is degraded by UPS during starvation, we de-
given that NEDD4L induces ULK1 proteasomal degradation
the wild-type (ULK1 WT), the kinase-dead (ULK1 K46I), and the
activation (Nazio et al., 2013).
Ten sites (S342 and S448) flanking the NEDD4L WW domains can be phosphory-
lated by several AGC kinase family members, including SGK1,
Akt, and PKA (Lee et al., 2007; Gao et al., 2009). These phos-
phorylation events inhibit NEDD4L function as a regulator of
ENACs and TGF-β signaling. For this reason, we analyzed the
phosphorylation status of NEDD4L during autophagy induc-
tion by starvation at different time points. As shown in Fig. 4 F,
NEDD4L phosphorylation is reduced during the first 4 h of star-
vation. Interestingly this modification pattern parallels that of
ULK1 expression levels (Fig. 1 A). Moreover, using qPCR, we
found a significant increase in NEDD4L mRNA during starva-
tion (Fig. 4 G), supporting that NEDD4L is positively regulated
during autophagy progression.
Collectively, both of these findings support the hypothesis
that NEDD4L is more active during autophagy than in standard
conditions, and this facilitates ULK1 proteasomal degradation.

NEDD4L is a negative regulator
of autophagy
Based on the evidence that NEDD4L degrades ULK1 during
autophagy, we decided to explore whether NEDD4L inactiva-
tion, and the resulting stabilization of ULK1 in prolonged
starvation, can alter the duration of the autophagy response. To
this aim, we down-regulated NEDD4L by RNAi and induced auto-
phagy for different time periods; we then analyzed autophagy
using three different methods: LC3 lipidation and p62 protein
levels by Western blotting (WB; Fig. 5, A and B) and both LC3
and ATG16L puncta detection by immunofluorescence (Fig. 5,
C and D). As shown, in control cells, autophagy rapidly in-
creases at 1–2 h and then declines at 4–6 h of starvation. In
NEDD4L-silenced cells, an increased and persistent autophagy
flux is observed, suggesting a correlation between ULK1 pro-
tein degradation and the on-rate of autophagy.

NEDD4L ubiquitylates ULK1 at lysine 925
and lysine 933
To finely dissect ULK1 regulation by NEDD4L at a structural
level, we next determined the NEDD4L-dependent ubiquityla-
tion sites on ULK1. By MS analysis, we identified two lysine
residues on the same ULK1 peptide (K925 and K933) that
proved to be ubiquitylated (Table S1). By mutating the lysine
residues to arginines, we generated three mutant constructs
(ULK1K925R, ULK1K933R, and ULK1K925R+K933R). First, we ana-
yzed the half-life of the single-mutant constructs in the presence
of cycloheximide (CHX), a protein translation inhibitor,
or CHX+MG132, and found that the ULK1K925R and ULK1K933R
constructs are as active and functional as ULK1 WT but more sta-
ble than ULK1 WT (Fig. 6 A and Fig. S4, A and B).
Then, we evaluated the capability of all the mutant con-
structs to be degraded by NEDD4L in standard conditions
(Fig. 6, B and C) or during autophagy (Fig. 6, D and E), in the
presence or not of MG132. We thus found that both mutations
affect NEDD4L-dependent degradation of ULK1 during auto-
phagy. Finally, we analyzed the capability of NEDD4L to ubiqui-
ty late ULK1K925R, ULK1K933R, and ULK1K925R+K933R (Fig. 6, F
and G) and found a significant decrease in the ubiquitylation
status of all the mutant constructs analyzed when compared
with the wild type. However, a residual ubiquitylation is found
in this context, arguing for the presence for other unknown
ubiquitylation site(s).
Because the NEDD4L-dependent ubiquitylation sites are
located in the region of ULK1 that is known to mediate the inter-
action with ATG13 (Jung et al., 2009), we analyzed whether the
ULK1-ATG13 binding is affected during autophagy induction

NEDD4L degrades ULK1 protein during
prolonged starvation
Given that NEDD4L induces ULK1 proteasomal degradation
and that ULK1 is degraded by UPS during starvation, we de-
cided to analyze ULK1 protein levels in cells knocked down for
NEDD4L. As shown in Fig. 4 (A–C) and Fig. S3 A, NEDD4L
down-regulation induces an increase in ULK1 protein levels
in fed conditions and prevents its degradation after prolonged
nutrient starvation. Interestingly, we found that ULK2, another
Atg1 orthologue in mammals often shown to compensate for
ULK1 functions (Alers et al., 2012), also undergoes a similar
regulation (Fig. S3 B).
Because ULK1 kinase is active during autophagy induc-
tion, we set out to find whether NEDD4L is able to distinguish
the active form of ULK1 from the inactive one for the purpose
of triggering its proteasomal degradation. We thus cotransfected
the cells with three different Myc-tagged ULK1 constructs,
the wild-type (ULK1 WT), the kinase-dead (ULK1 K46I), and the
ULK1K1047A (Dorsey et al., 2009) mutated form in its autophos-
tylation (Kim and Huijbregts, 2009; Maspero et al., 2013), we
also checked the capability of NEDD4L to promote this kind of
modification on ULK1. Indeed, by overexpressing NEDD4L, an
increase of K63 ubiquitylation is detectable on ULK1 (Fig. S2
E), implying a putative additional role for ubiquitin K63 chains
in regulating ULK1 activity in this context of regulation. Of note,
we previously showed that the alternative E3 ligase TRAF6 could
favor a similar modification on ULK1 early during autophagy ac-
tivation (Nazio et al., 2013).

Moreover, we analyzed the capability of NEDD4L to
bind the kinase-dead form of ULK1, and we found that both the
wild-type and mutant form of ULK1 are able to interact with
NEDD4L (Fig. S3 E), suggesting that ULK1 needs activation
to undergo conformational changes functional to its NEDD4L-
degradation site(s), but not to interact with NEDD4L.
Last, because NEDD4L is regulated by self-ubiquitylation
(Bruce et al., 2008) and phosphorylation (An et al., 2014),
we decided to investigate whether these modifications are
regulated during autophagy. First, we analyzed NEDD4L
self-ubiquitylation in basal conditions and after autophagy
induction by starvation. As shown in Fig. 4 E, NEDD4L displays
higher self-ubiquitylation levels after autophagy induction than
in control conditions. Next, we noticed that two sites (S342
and S448) flanking the NEDD4L WW domains can be phosphory-
lated by several AGC kinase family members, including SGK1,
or after NEDD4L overexpression. As shown in Fig. S4 (C and D), no impairment is found, suggesting that ATG13–ULK1 binding is not necessary for ULK1 degradation.

Finally, we decided to analyze autophagy in the presence of ULK1 mutant constructs. Thus, we expressed, in independent experiments, ULK1<sup>WT</sup> and ULK1<sup>KK46I</sup> or ULK1<sup>K925R+K933R</sup>mCherry-tagged plasmids in ULK1 knockdown cells, and we analyzed ATG16L puncta formation after autophagy induction by starvation at two different time points. As shown in Figs. 6 H and S4 E, the ULK1<sup>WT</sup> mutant construct increases autophagy with respect to the ULK1<sup>WT</sup>, as we found after NEDD4L down-regulation. A similar autophagy alteration is also found by only increasing ULK1 levels by expression of ULK1<sup>WT</sup> or ULK1<sup>K925R+K933R</sup> in control cells (Fig. S4 F). Moreover, also using the single-mutant
constructs (ULK1^K923R and ULK1^K933R), LC3 lipidation is increased and stable during the starvation period (Fig. S4 G). Further, this effect is not additive to NEDD4L down-regulation (Fig. S4 H). All these experiments support the idea that there is a correlation between ULK1 protein degradation and termination of the autophagy response.

Figure 5. **NEDD4L down-regulation increases autophagy.** (A) NEDD4L expression was down-regulated in HeLa by RNAi. Cells were nutrient-starved for the indicated time periods in the presence or not of Cq. Protein extracts were analyzed by WB for the expression of LC3, ACTIN, and NEDD4L. Densitometric analysis of LC3II+Clq-LC3II control over ACTIN band is shown. Data are expressed as the mean ± SEM of three independent experiments (n = 3). (B) HeLa cells were treated as in A. Protein extracts were analyzed by WB for the expression of p62 and ACTIN. (C and D) NEDD4L expression was down-regulated by RNAi as in A; cells were nutrient-starved for the indicated time periods and fixed and labeled with anti-ATG16L or anti-LC3 antibodies (red puncta) and visualized by confocal microscopy. Analysis of the number of both ATG16L and LC3 puncta occurrence per cell is shown in the graph. Data are expressed as the mean ± SEM (n = 3); representative images are shown. Bar, 20 µm (>50 cells analyzed per sample). In A, data were analyzed by one-way ANOVA followed by Tukey post hoc test. * P < 0.05. In C and D, data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison post test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

ULK1 restoration requires de novo mTOR-dependent protein synthesis. Last, we investigated the mechanism underlying the restoration of ULK1 during prolonged starvation. Because it is known that mTOR is reactivated during prolonged starvation (Yu et al., 2010; Sahani et al., 2014) and its activity also reported as
Figure 6. NEDD4L ubiquitylates ULK1 at lysine 925 and lysine 933. (A) HeLa cells were transfected with ULK1 WT, ULK1K925R, and ULK1K933R HA-tagged proteins and treated with CHX (50 µM) for different time periods. Levels of ULK1 and TUBULIN were detected by WB. Densitometric analysis of ULK1 over TUBULIN is also shown (right graph). Data are expressed as the mean ± SEM (n = 3). (B and C) HeLa cells were transfected ULK1 WT, ULK1K925R, ULK1K933R, and ULK1K925R+K9333R HA-tagged proteins together with NEDD4L-HA in the presence or not of MG132 (4 h) and all were treated with CHX for 3 h. Levels of ULK1, NEDD4L, and TUBULIN were detected by WB. (B) Densitometric analysis of ULK1+MG132/ULK1 control over TUBULIN bands is also shown. Data are expressed as the mean ± SEM (n = 3). (D and E) HeLa cells were transfected with ULK1 WT, ULK1K925R, ULK1K933R, and ULK1K925R+K933R HA-tagged proteins in the presence or not of MG132 and autophagy was induced with EBSS for 4 h. The levels of ULK1 and TUBULIN were detected by WB.
oscillatory in lymphocytic subpopulation in response to proliferative stimuli (Procaccini et al., 2010), we first analyzed the involvement of mTOR kinase.

As shown in Fig. 7 A and Fig. S5 A, mTOR is active after 6 h with both amino acid and serum starvation or with amino acid alone. We analyzed both the autophosphorylation of mTOR and the mTOR-mediated phosphorylation of ULK1 and found that the newly synthesized pool of ULK1 is directly inhibited by mTOR phosphorylation, contributing to maintain autophagy inhibition after prolonged starvation. Next, when cells were treated with two different mTOR inhibitors, rapamycin and Torin1 at 4 h of starvation, we found that mTOR inhibition effectively suppressed both ULK1 protein level restoration and ULK1 neophosphorylation by mTOR (Fig. 7, B and C), suggesting an involvement of mTOR reactivation in ULK1 restoration. Then, to evaluate whether the restoration of ULK1 required de novo protein synthesis, we used CHX after 4 h of starvation and found that this treatment also abolished ULK1 restoration, even though CHX is known to secondarily activate mTOR (Fig. 7 C).

Because we found an increase in ULK1 mRNA level transcription during autophagy (Fig. 1 B), we analyzed whether transcriptional up-regulation was required for ULK1 protein restoration. As shown in Fig. 7 D, Act D is able to inhibit ULK1 restoration after 6 h of starvation, supporting the role of ULK1 mRNA transcription as propaedeutical for de novo synthesis of ULK1 protein.

To determine whether increased translation of ULK1 mRNA accounted for the increased protein levels at 6 h of starvation, we performed sucrose gradients to profile ULK1 mRNA distribution in translationally active polysomes or in translationally inactive RNPs (Bianchini et al., 2008). Autophagy was induced by starvation for 2 and 6 h, and polysome–mRNA complexes were fractionated by sucrose gradient centrifugation. Figs. 7 E and S5 B show a decrease in polysome-associated ULK1 mRNA in cells grown under starvation conditions for 2 h, indicating translational repression of the mRNA. However, a strong recruitment of ULK1 mRNA was observed after 6 h of starvation, strongly suggesting that increased translation of ULK1 mRNA at 6 h of starvation (after mTOR reactivation) contributes to de novo synthesis of ULK1 protein.

Because prolonged autophagy is known to influence cell death (Füllgrabe et al., 2013; Antonioli et al., 2014), we analyzed cell viability in both control conditions and after NEDD4L down-regulation during prolonged starvation and in the presence or not of a low dose of bafilomycin A1 (Baf A1). As shown in Figs. 7 F and S5 C, when NEDD4L is down-regulated and autophagy remains high, we can detect an increase in starvation-induced cell death, which is blocked by Baf A1, suggesting that persistent autophagy may trigger cell demise.

**Autophagy restimulation requires mRNA preaccumulation and is amplified by NEDD4L down-regulation**

To gain insight into the physiological relevance of ULK1 protein and mRNA modulation during prolonged starvation, we first decided to analyze ULK1 protein levels after reinduction of autophagy in the system by nutrient replenishment. As shown in Fig. 8 A, ULK1 protein levels can be down-regulated again as soon as autophagy is reinduced, indicating an oscillating regulation for this autophagy key factor. Then, to evaluate the importance of mRNA transcription in general (and of ULK1 mRNA in particular; Fig. 1 D) in this oscillation, we used Act D for the first 4 h of starvation and analyzed both ULK1 protein levels and autophagy occurrence. As shown in Fig. 8 B, the temporary inhibition of mRNA transcription is sufficient to block autophagy reinduction by starvation after nutrient replenishment, as analyzed by counting ATG16L1 puncta. On the contrary, when NEDD4L is down-regulated and ULK1 protein levels cannot be decreased, we observed a stronger reinduction of autophagy (Fig. 8 C) when compared with the control. These results, collectively, confirm the importance of ULK1 fine-tuning in autophagy restimulation, which confers an oscillatory behavior to autophagy progression.

**Discussion**

Autophagy is the last resource to help cells survive during starvation, but self-degradation has a limit, beyond which the cell will die. For instance, after birth, in neonatal tissues, a rapid reprogramming of cell metabolism is necessary, and autophagy is activated soon after the transplacental nutrient supply is suddenly interrupted. However, autophagosome formation after birth is completely prevented, suggesting that neonates use the amino acids produced by autophagy for energy homeostasis (Schiaffino et al., 2008).

Displaying a negative control mechanism will thus help cells avoid overactivation of autophagy, enabling them to calibrate autophagy to an optimal level and ensuring that they can survive prolonged starvation. In fact, this study supports a mechanism of cross-regulation of ULK1 mRNA transcription, protein translation, and degradation in response to the oscillatory activation of mTOR that regulates autophagy induction (see our model in Fig. 9). It is well known that the mTOR pathway activity is also regulated by oscillation in other contexts, such as in regulatory T cell proliferation, where another proautphagic

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[D] Densitometric analysis of ULK1+MG132/ULK1 control over TUBULIN bands is also shown. Data are expressed as the mean ± SEM (n = 3). [F] HeLa cells were transfected with a vector encoding a 6xHis-tag ubiquitin together with ULK1WT, ULK1K925R and ULK1K933R HA-tagged proteins in the presence of NEDD4L. Protein extracts were prepared in a denaturing urea buffer and subjected to Ni-NTA purification. The amount of ubiquitylated ULK1 copurified with 6xHis-ubiquitin was evaluated by WB. The intensity of ULK1 bands is shown. Data are the mean ± SEM (n = 3). [G] HeLa cells were transfected with a vector encoding a 6xHis-tag ubiquitin together with ULK1WT and ULK1K925R–K933R mCherry-tagged proteins in the presence of NEDD4L. Protein extracts were prepared in a denaturing urea buffer and subjected to Ni-NTA purification. The amount of ubiquitylated ULK1 copurified with 6xHis-ubiquitin was evaluated by WB. [H] Endogenous ULK1 was silenced by RNAi; cells were then transfected with ULK1WT or ULK1K925R–K933R mCherry-tagged plasmids. Cells were nutrition-starved for the indicated time periods, fixed and labeled with anti-ATG16L1 antibody (green puncta), and visualized by confocal microscopy. Analysis of the number of ATG16L1 occurrence per cell is shown in the graph. Data are expressed as the mean values ± SEM (n = 3). Representative images are shown. Bars, 20 µm (>50 cells analyzed per sample). In A, B, D, and F, data were analyzed by one-way ANOVA followed by Tukey post hoc test. * P < 0.05; ***, P < 0.0001. In H, data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison post test. ***, P < 0.01; ****, P < 0.0001. For all ULK1 mutant constructs the same amount of DNA is used for the transfection; the differences are caused by the increased stability of these mutants.

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Figure 7.ULK1 restoration requires de novo mTOR-dependent protein synthesis. (A) HeLa cells were treated with EBSS for the indicated time periods. The levels of p-mTOR, mTOR, ULK1, p-ULK1 S757, and ACTIN were detected by WB. Densitometric analysis of p-mTOR over ACTIN is also shown (right graph). Data are expressed as the mean ± SEM (n = 4). (B) HeLa cells were treated with EBSS for the indicated time periods; at 4 h of starvation, rapamycin (100 nM) was added. Levels of p-mTOR, mTOR, ULK1, p-ULK1 S757, and ACTIN were detected by WB. Densitometric analysis of ULK1 over ACTIN is also shown (right graph). Data are expressed as the mean ± SD (n = 3), and statistical analysis was performed using unpaired Student’s t test. *, P < 0.05. (C) HeLa cells were treated with EBSS for the indicated time periods and at 4 h of starvation, 250 nM Torin1 or 50 µM CHX was added. Levels of p-mTOR, mTOR, p-p70S6K, ULK1, and ACTIN were detected by WB. (D) HeLa cells were treated with EBSS for 6 h in the presence or not of Act D. Levels of ULK1 and ACTIN were detected by WB. Densitometric analysis of ULK1 over ACTIN is also shown (right graph). Data are expressed as the mean ± SD (n = 3) and statistical analysis was performed using unpaired Student’s t test. *, P < 0.05. (E) qPCR analysis of polysomal recruitment of ULK1 and ACTIN mRNAs in control (CTRL) and EBSS-starved (2 h EBSS and 6 h EBSS) HeLa cells. Densitometric analysis of the signal in each fraction was performed, and the results were represented as the percentage of total signal in all fractions. Data represent mean ± SEM (n = 3). (F) NEDD4L expression was down-regulated by RNAi. Cells were nutrient-starved for 12 h in the presence or not of Baf A1 and analyzed by MTS assay. NEDD4L down-regulation was detected by WB. In A and C, data were analyzed by one-way ANOVA followed by Tukey post hoc test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. In E and F, data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison post test. ***, P < 0.001; ****, P < 0.0001.
stimulus, the mTOR inhibitor rapamycin, might differentially affect regulatory T cells reactivity depending on their metabolic state (Procaccini et al., 2010). Moreover, the ULK1 gene has been found to be a target of C/EBPβ protein, a transcription factor that links autophagy to the circadian pacemaker and maintains nutrient homeostasis throughout light/dark cycles; this is consistent with the fact that autophagy is highly sensitive to nutritional status (Ma et al., 2011). In sum, ULK1 complex regulation may represent a hinge point in a cell response to stress, and its oscillatory nature, intended as the key to the remitting and relapsing nature of autophagy, can be considered as an essential element of the cell’s struggle between death and survival.

NEDD4L is an ubiquitin ligase implicated in several cellular or physiological processes (Goel et al., 2015). It is known that two members of the NEDD4 family, NEDD4 and Smurf1 (SMAD-specific E3 ubiquitin protein ligase 1), are involved in autophagy. NEDD4 has been found to promote Becn1 degradation by the proteasome (Platta et al., 2012). Further, RNAi analyses revealed that NEDD4 down-regulation leads to an autophagy increase (Behrends et al., 2010). SMURF1
plays multiple physiological functions through targeting a variety of substrates acting in both viral autophagy and mitophagy (Orvedahl et al., 2011).

We previously described ubiquitylation of ULK1 by a K63-linked chain mediated by the TRAF6–AMBA1 complex during short-term starvation and at early time points (30 min), an event stabilizing the active ULK1 complex (Nazio et al., 2013). In a recent paper, we found that the other autophagy activator and ULK1 regulator AMBA1 is degraded by the Cullin4–DDB1 complex during prolonged starvation and that AMBA1–TRAF6 binding to ULK1 decreases during prolonged autophagy (Antonioli et al., 2014). An obvious hypothesis is that when the AMBA1–TRAF6 complex dissociates from ULK1, the ULK1 carboxy-terminal domain becomes unmasked and available for NEDD4L-dependent ubiquitylation.

Moreover, the NEDD4L-mediated ULK1 ubiquitylation is reminiscent of another target of TRAF6 and NEDD4L ligases, TrkA; TRAF6-dependent ubiquitylation is supposed to function nonproteolytically, whereas NEDD4L-mediated ubiquitylation results in the degradation of this plasma membrane receptor (Geetha et al., 2005; Georgieva et al., 2011). We can thus speculate that, similarly to TrkA, ULK1 can be alternatively regulated by these two ligases with two opposite effects, implicating the existence of an ULK1-targeting E3 ligase network regulating cell fate. Last, the recent finding that Cullin3–KHLH20 can also degrade ULK1, Beclin 1, and Vps34 during prolonged autophagy may reflect the need for the cell to possess alternative backup systems for autophagy termination, highlighting the absolute need for a tight control of this event in the general cellular homeostasis (Liu et al., 2016).

As a matter of fact, E3 ligases can promote the formation of polyubiquitin chains on substrates through any of the seven lysines present on ubiquitin molecules. Interestingly, the NEDD4L-mediated ubiquitylation of ULK1 is K27- and K29-linked, but not typical K48-linked, ubiquitylation. Our finding adds to the recent and relatively small amount of existing data on this type of posttranslational modification.

A final issue of interest is the fact that NEDD4L is able to regulate autophagy also in basal conditions. Although basal autophagy has been shown to be independent of ULK1 in a few instances (Petherick et al., 2015; Joo et al., 2016), this does not seem to be the case in cancer cell lines that are characterized by a strong basal autophagy, including HeLa cells (Mizushima, 2010; Wong et al., 2015). Indeed, we found that NEDD4L interacts with ULK1 also in basal conditions and also when the ULK1 kinase domain is disrupted. However, NEDD4L is able to degrade only activated ULK1. Several general principles apply to the degradation of activated protein kinases or their regulators for ubiquitylation and degradation (Lu and Hunter, 2009). Autophosphorylation of ULK1 could change its conformation for NEDD4L-mediated ubiquitylation or, alternatively, the NEDD4L pool binding ULK1 in the absence of autophagy induction is in an inactive form. The trans-phosphorylation of a protein kinase could, indeed, create a phosphodegron that is necessary for ubiquitylation by a phospho-dependent ligase, such as NEDD4L. In addition, because phosphorylation on S342 of NEDD4L is necessary for its subsequent ubiquitylation activity (Lee et al., 2007; Gao et al., 2009), ULK1 could also be involved in regulating other kinases (such as SGK1, Akt, and PKA) known to phosphorylate the NEDD4L residue S342.
A comprehensive analysis of the autophagy-related posttranslational modifications is therefore of the highest importance in biomedicine and may represent the forefront of autophagy research.

Materials and methods

Cell culture and autophagy assays

HeLa and HEK293 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 2 mM L-glutamine, and 1% penicillin/streptomycin solution at 37°C under 5% CO₂.

The induction of autophagy by nutrients starvation was obtained washing the cells with PBS and incubating them with Earle’s balanced salt solution (EBSS; Sigma-Aldrich). Proteasome activity was inhibited for indicated time points with 5 µM MG132 (Sigma-Aldrich) or 10 nM Baf A1, and autophagy was induced with 100 nM rapamycin (Sigma-Aldrich) or 250 nM Torin1 leupeptin (Sigma-Aldrich), or 10 nM Baf A1, and autophagy was induced with 100 nM rapamycin (Sigma-Aldrich) or 250 nM Torin1 leupeptin (Sigma-Aldrich), or 10 nM Baf A1, and autophagy was induced with 100 nM rapamycin (Sigma-Aldrich) or 250 nM Torin1 leupeptin (Sigma-Aldrich). Transcription was inhibited with 0.5 µM Act D (Sigma-Aldrich). HeLa cells were transiently transfected with Lipo-fectamine 2000 (Thermo Fisher Scientific) as provided by the supplier.

Ni-NTA assay

For detection of whole-cell ubiquitylation, HeLa cells were transfected with a 6xHIS-ubiquitin expression plasmid along with equal amounts of the indicated plasmids. Thereafter, cells were collected in PBS, resuspended in 1 ml lysis buffer (8 M urea, 0.1 M NaH₂PO₄, 0.05 M Tris, pH 8.0, 40 mM imidazole, 0.5% CHAPS, and 50 mM 2-mercaptoethanol), sonicated, and centrifuged. To lysates containing equal amounts of whole-cell protein, 30 µl nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) were added, and the mixture was incubated at room temperature for 4 h with rotation. Subsequently, the beads were washed for 5 min at room temperature with 800 µl lysis buffer containing 40 mM imidazole. Ubiquitylated proteins were eluted by incubating the beads in 30 µl buffer containing 400 mM imidazole and 2x Laemmli buffer and resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies.

In vitro ubiquitylation assays

HeLa cells were independently transfected with plasmids encoding NEDD4L-HA and Myc-ULK1. 24 h later, cells were lysed in buffer (20 mM Hapes, pH 7.4, 2 mM EDTA, 0.5% CHAPS, and 50 mM 2-mercaptoethanol), sonicated, and centrifuged. To lysates containing equal amounts of whole-cell protein, 30 µl nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) were added, and the mixture was incubated at room temperature for 4 h with rotation. Subsequently, the beads were washed for 5 min at room temperature with 800 µl lysis buffer containing 40 mM imidazole. Ubiquitylated proteins were eluted by incubating the beads in 30 µl buffer containing 400 mM imidazole and 2x Laemmli buffer and resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies.

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Antibodies

The primary antibodies used in this study were: mouse and rabbit anti-HA tag antibody (Sigma-Aldrich), mouse anti-Myc (Santa Cruz Biotechnology, Inc.), rabbit anti-BECLIN 1 (Santa Cruz Biotechnology, Inc.), rabbit anti-LC3 (Cell Signaling Technology), rabbit anti–human AMBRA1 (Novus Biologicals), mouse anti-multiprotein ubiquitin (MLB), rabbit anti-ULK1 (Santa Cruz Biotechnology, Inc.), goat anti-ULK2 (Santa Cruz Biotechnology, Inc.), rabbit anti-pS757 ULK1 (Cell Signaling Technology), mouse anti-ULK2 (Abcam), rabbit anti-NEDD4L (Cell Signaling Technology), mouse anti-NEDD4L (Santa Cruz Biotechnology, Inc.), rabbit anti-pS342 NEDD4L (Cell Signaling Technology), rabbit anti-mTOR (Cell Signaling Technology), rabbit p-S2448 mTOR (Cell Signaling Technology), rabbit anti-p62 (Santa Cruz Biotechnology, Inc.), rabbit anti-ATG13, rabbit p-S318 ATG13 (Rockland), rabbit anti-Actin (Sigma-Aldrich), mouse anti-Tubulin (Sigma-Aldrich), rabbit cleaved-PARP (Cell Signaling Technology), rabbit anti-Atg16L (Cell Signaling Technology), and rabbit p-p70S6K and p70S6K (Cell Signaling).

Immunoprecipitation and WB

HeLa cells were rinsed with ice PBS and lysed in CHAPS lysis buffer (20 mM Hapes, pH 7.4, 2 mM EDTA, 0.3% CHAPS, and protease and phosphatase inhibitor cocktail; Sigma-Aldrich). 0.5 mg lysates for co-IP in overexpression and 2 mg lysates for co-IP in endogenous conditions were then incubated with 2 µg primary antibody for ULK1 and NEDD4L or 1 µg primary antibody for MYC at 4°C with rotation overnight before addition of 30 µl protein A agarose beads (Roche) and then incubated at 4°C for 1 h.

For the co-IP of HA-tagged proteins, lysates were incubated with 20 µl agarose-coupled antibodies against HA at 4°C for 2 h.

To detect ubiquitin, after lysis and before immunoprecipitation, 1% SDS was added, and the lysates were incubated for 5 min at 90° to dissociate protein–protein interactions. The samples were diluted 10-fold with the same buffer, and the immunoprecipitation assay was prepared as described above in this paragraph.

Immunoprecipitates were then washed 5 x 5 min with lysis buffer (20 mM Hapes, pH 7.4, 2 mM EDTA, 0.3% CHAPS, and NaCl 150 mM) and then denatured by adding 1 vol of 4x Laemmli SDS sample buffer with β-mercaptoethanol and incubated at 95° for 10 min.

Proteins were separated on acrylamide gels (Bio-Rad Laboratories) and electrophoresed onto nitrocellulose (Protran; Schleicher & Schuell) or polyvinylidene difluoride (EMD Millipore) membranes. Blots were incubated with primary antibodies in 5% nonfat dry milk or 5% BSA in TBS plus 0.1% Tween-20 overnight at 4°C. Detection was achieved using horseradish peroxidase–conjugated secondary antibody (Bio-Rad Laboratories) and visualized with ECL (EMD Millipore).

Of note, in some cases, Western blots were captured using a digital camera (Fluor Chem SP; Alpha Innotech; see Fig. 1, A and E, bottom panels; Fig. 2 C; Fig. 3 E, HA, bottom panel; Fig. 3 F, bottom panels; Fig. 4, B and D; Fig. 5 A, bottom panels; Fig. 6 G; Fig. 7, B–E; and Fig. 8, A–C), whereas in others, they were captured on film (Aurogene). Backgrounds in Western blot images from different
experimental approaches were then equalized by changing their expression postacquisition.

RNAi
RNAi was performed using the following RNA oligonucleotide (oligo) duplex from Thermo Fisher Scientific: NEDD4L#1 HSI118597, 5'-CCCAAGACUGCAGAACCCGCUAU-3' (10620318); 5'-AAUAGCUUGUGUUCUCAGUCUGGG-3' (10620319); NEDD4L#2 ID: 22855 (3' UTR), 5'-GGCAGAUUAUUACAU-3'; 5'-AUUGAUGUCAUUAGCCGC-3'; ULKI HSI140824, 5'-GAGAACUGCUCCAAAGUGCAAGCU-3' (127303D05); 5'-ACAGCUUGCUACUUGUGUACCUUC-3' (127303D06); ULKI (3' UTR) ID: 118261, 5'-CCCAAGACCUUUUAUCA-3'; 5'-UAUGCAUAAGGCUUUGGG-3'.

qPCR
Total RNA was isolated by using the RNeasy mini kit (QIAGEN). 2 µg RNA was retrotranscribed using M-MLV enzyme and oligodT (Promega). qPCR was then performed using SYBR Green Mix (Roche) on 1 µl of cDNA and 0.5 µM of each primer. Melting curve analysis was used to confirm primer specificity. Background absorbance was calculated on set of control wells (without cells) containing the same volumes of medium and Baf A1.

MTS assay
For the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay, cells were incubated for 30 min at 56°C with 10 mM DTT for cysteine reduction and 20 min at room temperature with 55 mM iodoacetamide for cysteine alkylation.

Immunocytochemistry
Cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 15 min. After permeabilization with 0.4% Triton X-100 in PBS for 5 min, cells were blocked in 3% horse serum in PBS and incubated overnight at 4°C with primary antibodies. Cells were then washed and incubated for 1 h with labeled anti–mouse (Alexa Fluor 488; Molecular Probes) or anti–rabbit (FITC; Jackson ImmunoResearch Laboratories, Inc.; Alexa Fluor 647 donkey anti–rabbit) secondary antibodies. Images were examined under a confocal laser scanning microscope (FV1000; Olympus) interfaced with 1 x 81 inverted microscope equipped with a 60x (NA 1.35) oil-immersion objective. A 405-nm diode laser was used for the blue channel, a 488-nm argon laser was used for the green channel, and a 543-nm HeNe laser was used for the red channel.

Polysome fractionation
Polysome separation was performed as previously described (Paronetto et al., 2006). In brief, HeLa cells were grown overnight to 60–70% confluence and incubated for the indicated time period (2 and 6 h) in EBSS or control medium. After extensive washing in cold PBS, cells were scraped in lysis buffer (100 mM NaCl, 10 mM MgCl2, 30 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM DTT, 0.5 mM Na2VO3, and 25 U/ml RNasin [Promega]) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and lysed by 10-min incubation in ice. Cell lysates were centrifuged for 10 min at 12,000 g at 4°C, and the supernatants were collected (cell extracts). 1 mg cell extracts was loaded on a 15–50% (wt/vol) sucrose gradients (10 ml) and sedimented by ultracentrifugation for 2 h at 37,000 rpm in a rotor (SW41; Beckman Coulter). UV-absorption (A260) profiles of polysome gradients were measure by UV detector (UVis-920; GE Healthcare), and each gradient was collected in 10 fractions of 1 ml each. For RT-PCR analysis, 500 µl of fractions 1–5, 6 and 7, and 8–10 were pooled to generate polysomal, 80S, and RNP samples, respectively, and RNA was isolated with RNeasy Mini kit (QIAGEN). Proteins were isolated from the remaining 500 µl of each gradient fraction by adding 50 µl of 72% trichloroacetic acid. Suspensions were mixed, incubated for 15 min in ice, and centrifuged at 12,000 g for 10 min at 4°C. Protein pellets were washed with −20°C chilled acetone and resuspended in Laemmli buffer for Western blot analysis.

Statistical analysis
For all experiments shown, n is indicated in the figure legends.

Densitometric analysis was performed using ImageJ software; the mean of the values from different experiments (as indicated) related to the control ratio was arbitrarily defined as 1.00. Each point value represents the mean ± SD or mean ± SEM (as indicated in the figure legends) from three independent experiments unless specified otherwise. Comparison between control and sample in the WB intensity measurement was made from the same Western blot.


References


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