Sestrin2 promotes Unc-51-like kinase 1 mediated phosphorylation of p62/sequestosome-1

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Autophagy is a homeostatic process that is important for degrading protein aggregates, nutrient deposits, dysfunctional organelles and several signaling molecules. p62/sequestosome-1 is a protein that binds to several autophagy substrates, such as ubiquitinated proteins, damaged mitochondria and signaling molecules such as an Nrf2 inhibitor Keap1, promoting their autophagic degradation. Sestrin2, a stress-inducible protein, has recently been shown to bind to p62 and promote autophagic degradation of such p62 targets. Because Sestrin2 is a metabolic regulator that suppresses diverse age- and obesity-associated pathologies, the autophagy-controlling function of Sestrin2 may be important for its other physiological functions. However, the molecular mechanism of how Sestrin2 can promote clearance of p62-associated proteins has been unclear. Here we show that Sestrin2 physically associates with Unc-51-like protein kinase 1 (ULK1) and p62 to form a complex in which both Sestrin2 and p62 become phosphorylated by ULK1 at multiple sites. Ser403 of p62, whose phosphorylation is known to promote autophagic degradation of p62 and its targets, is among the sites phosphorylated by ULK1. ULK1-mediated p62 phosphorylation was facilitated by Sestrin2 in cells as well as in *in vitro* kinase assays. Consistent with this finding, oligomycin-induced energy deprivation, which strongly activates ULK1, provoked a robust Ser403 phosphorylation of p62 in wild-type mouse embryonic fibroblasts. However, in ULK1/2- and Sestrin2-deficient mouse embryonic fibroblasts, oligomycin-induced p62 phosphorylation was dramatically attenuated, suggesting that endogenous Sestrin2-ULK1/2 mainly mediates p62 phosphorylation in response to energetic stress. Taken together, this study identifies ULK1 as a new p62 Ser403 kinase and establishes Sestrin2 as a promoter of ULK1-mediated p62 phosphorylation.

Structured digital abstract

- ULK1 physically interacts with Sestrin2 by pull down (View interaction)
- p62 physically interacts with Sestrin2 by anti tag coimmunoprecipitation (1, 2)
- p62 physically interacts with ULK1 by anti tag coimmunoprecipitation (View interaction)
- Sestrin2 physically interacts with p62 and ULK1 by anti bait coip (View interaction)
- Sestrin2 physically interacts with ULK1, FIP200 and Atg13 by anti tag coimmunoprecipitation (View interaction)
- p62 physically interacts with ULK1 and Sestrin2 by anti tag coimmunoprecipitation (View interaction)
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Introduction

Autophagy is a cellular process critical for digesting nutrient deposits, eliminating toxic protein inclusion and dysfunctional organelles and regulating signal transduction machinery by selective degradation of signaling molecules [1]. Autophagy occurs constitutively, but can also be induced in response to cellular stresses in order to maintain metabolic homeostasis [2]. p62/sequestosome-1 protein is an important adaptor protein that binds to diverse autophagy substrates, such as ubiquitinated proteins, damaged mitochondria, microorganism-containing endosomes and an Nrf2 inhibitor Keap1, and promotes their autophagic degradation [3,4]. p62-mediated degradation of Keap1 leads to activation of Nrf2, which is a transcription factor that promotes expression of various antioxidant molecules to defend cells against oxidative damage [5,6].

Sestrin2 is a stress-inducible molecule that is important for maintenance of metabolic homeostasis [7]. Genetic deficiency of Sestrin2 in mice or of its homolog in *Drosophila* results in diverse age- and obesity-associated metabolic pathologies such as accumulation of lipid droplets and protein aggregates, mitochondrial dysfunction, muscle degeneration and insulin resistance [8,9]. Some of these pathologies are found to be caused by defective autophagy, which can happen due to misregulation of AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin complex 1 (mTORC1) signaling. Sestrin2 associates with AMPK and promotes its activation by an upstream kinase LKB1 [10,11]. This activation of AMPK can result in mTORC1 inhibition and subsequent autophagy activation [12]. Consistent with their identified biological functions, Sestrin2-deficient cells are unable to promote autophagy under certain stress conditions [12].

In addition to regulating autophagy through the AMPK–mTORC1 axis, Sestrin2 can have a direct control over autophagy. A recent study showed that Sestrin2 physically associates with p62 and promotes degradation of its substrate Keap1 [13]. During oxidative stress, Sestrin2/p62-mediated Keap1 degradation is important for boosting Nrf2-dependent antioxidant gene transcription to ensure cell survival [13]. However, the molecular mechanism of why p62–Sestrin2 association is important for autophagic degradation of Keap1 and other p62 targets has been obscure. In this study, we demonstrate that Sestrin2 and p62 physically associate with Unc-51-like protein kinase 1 (ULK1), an autophagy-initiating protein kinase. ULK1 phosphorylates p62, and it is Sestrin2′s role to promote the phosphorylation of p62 by ULK1. Because p62 phosphorylation is known to facilitate its own degradation [14–16], our results explain how Sestrin2 can promote autophagic degradation of p62 and its substrates.

Results

Physical association between Sestrin2, ULK1 and p62

To understand how Sestrin2 regulates autophagy, we tested for physical interaction between Sestrin2 and known autophagy-regulating proteins. As a result, we identified ULK1 as a new binding partner for Sestrin2; ULK1 and p62, but not ULK1′s regulatory subunits Atg13 and FIP200, were shown to physically bind to Sestrin2 in co-immunoprecipitation assays (Fig. 1A–C). We found that p62 also interacted with ULK1 in cells (Fig. 1D). The associations between Sestrin2 and ULK1 and between Sestrin2 and p62 were also observed *in vitro*; recombinant Sestrin2 proteins expressed in and purified from *Escherichia coli* were able to bind to ULK1 and p62 in mammalian cell lysates (Fig. 1E,F).

Each of the three Sestrin2 subdomains was sufficient to bind to ULK1 or p62

As previously determined through phylogenic analyses [17], Sestrin proteins contain three subdomains designated as Sesn-A, Sesn-B and Sesn-C (Fig. 2A). Thus, we determined which subdomain of Sestrin2 is required for association with ULK1 and p62. Truncated Sestrin2 mutants lacking Sesn-B and Sesn-C domains (Sesn2$^{\Delta BC}$), Sesn-A domain (Sesn2$^{\Delta A}$) or Sesn-A and Sesn-B domains (Sesn2$^{\Delta AB}$) were used in the protein binding assays. Sesn2$^{\Delta BC}$, Sesn2$^{\Delta A}$ and Sesn2$^{\Delta AB}$, as well as the full-length form of Sestrin2 (Sesn2^{WT}), were able to physically associate with ULK1 (Fig. 2B,C) and with p62 (Fig. 2D), suggesting that each of the subdomains of Sestrin2 can independently interact with
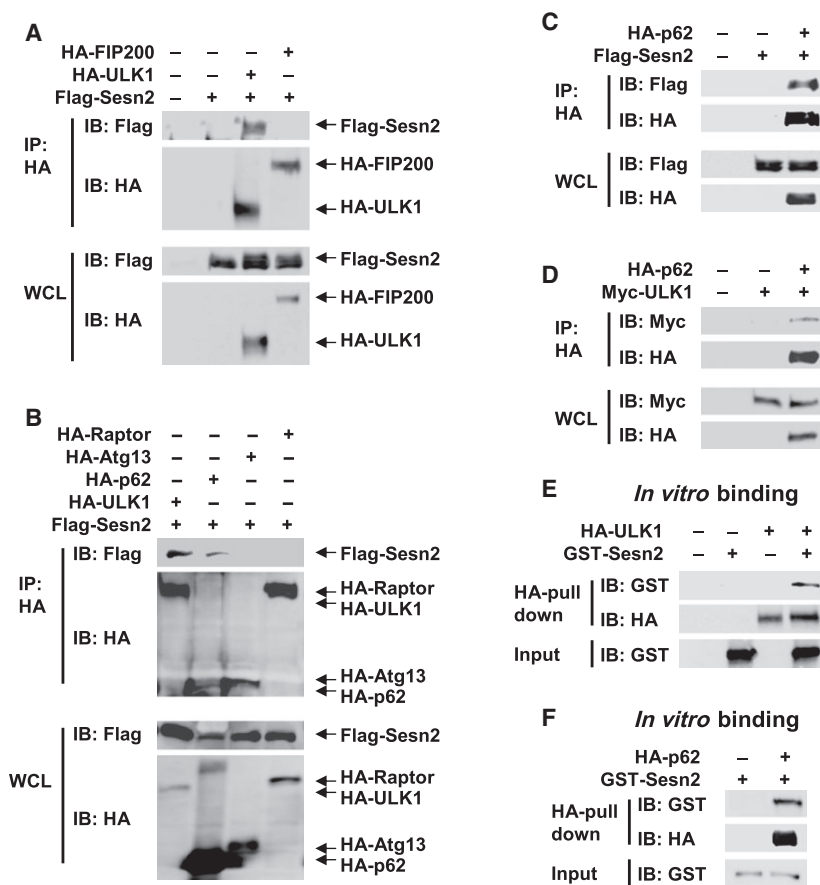
Fig. 1. Sestrin2 physically associates with ULK1 and p62. (A)–(D) ULK1 and p62 are associated with Sestrin2 in cells. HEK293 cells were transfected with plasmids expressing indicated proteins. HA-tagged proteins were immunopurified (IP) from whole cell lysates (WCL) and subjected to immunoblotting (IB). FIP200 and Atg13 are regulatory subunits of ULK1, and Raptor was used as a negative control. (E), (F) ULK1 and p62 bind to Sestrin2 *in vitro*. HEK293 cells were transfected with plasmids expressing HA-tagged ULK1 (E) or p62 (F). Cell lysates were incubated with GST-Sestrin2 recombinant protein expressed and purified from *E. coli* and immunoprecipitated using anti-HA antibody. Pulled-down GST-Sestrin2 proteins were assayed by immunoblotting.

ULK1 and p62. Interestingly, truncated Sestrin2 proteins associated with ULK1 more strongly than the full-length form (Fig. 2B,C), introducing the possibility that each subdomain may have an inhibitory effect on the others when interacting with ULK1.

C-terminal domain of ULK1 is necessary and sufficient to bind to Sestrin2

It was then determined which subdomains of ULK1 and p62 were required for association with Sestrin2. ULK1 consists of three subdomains: N-terminal kinase domain (KD), central serine/threonine-rich domain (STD) and C-terminal domain (CD) [18]. We utilized truncated ULK1 mutants containing each of these domains and a kinase-inactive (KI) ULK1 mutant [18] (Fig. 3A). The catalytic activity of ULK1 was not required for interaction with Sestrin2, as ULK1^{KI} was able to bind to Sestrin2 *in vitro* as efficiently as wild-type (WT) ULK1 (Fig. 3B). Although CD of ULK1 was able to bind to Sestrin2, KD and STD were unable to associate with Sestrin2 (Fig. 3C), suggesting that Sestrin2 specifically binds to CD of ULK1. Because CD interacts with many proteins that regulate

ULK1 and is important for ULK1 function and localization [19], it can be speculated that Sestrin2 may control ULK1 activity through physical association.

Functionality of PB1, LR and UBA domains is dispensable for p62 binding to Sestrin2

p62 contains three notable protein–protein interaction domains: N-terminal Phox/Bemp1 domain (PB1) which associates with diverse signaling molecules, central leucine-rich domain (LR) which associates with an autophagosome marker LC3 and C-terminal ubiquitin-binding-associated domain (UBA) [3] (Fig. 3D). Deletion or mutation of each p62 domain (Fig. 3D) did not abolish its ability to interact with Sestrin2 (Fig. 3E), suggesting that the functionality of these domains is generally dispensable for association with Sestrin2.

Sestrin2 interacts with ULK1–Atg13–FIP200 complex

In mammalian cells, ULK1 is present in an autophagy-initiating protein kinase complex containing Atg13 and FIP200 [20–22]. Although Sestrin2 did not
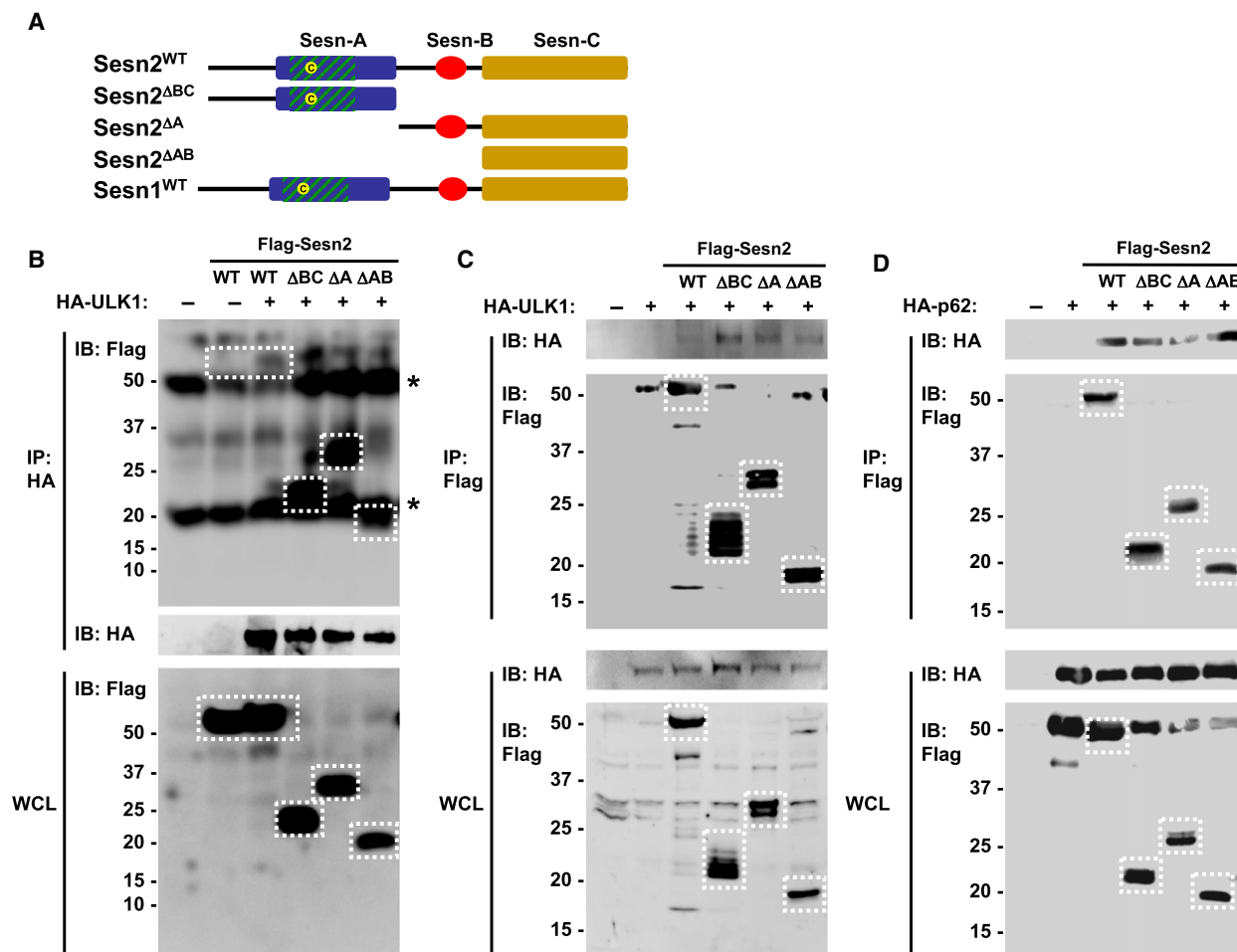
Fig. 2. Mapping the ULK1- and p62-interacting domains of Sestrin2. (A) Diagram depicting the domain structure of WT and truncated mutants of Sestrin2. (B)–(D) HEK293 cells were transfected with indicated proteins and subjected to immunoprecipitation (IP) using indicated antibodies. Whole cell lysates and purified immunocomplexes were analyzed by immunoblotting (IB). Stars denote non-specific bands corresponding to immunoglobulin heavy or light chain in the immunocomplexes.

show direct physical interaction with Atg13 and FIP200 (Fig. 1A,B), we wondered if these subunits could associate with Sestrin2 through ULK1. Indeed, ULK1 co-transfection enabled Atg13 and FIP200 to interact with Sestrin2 (Fig. 4A,B), indicating that Sestrin2 can bind to the ULK1–Atg13–FIP200 complex as a whole.

ULK1 phosphorylates p62 at the Ser403 site

After transfection of Sestrin2, ULK1 and p62, we noticed that co-transfection of ULK1 induced an electromobility retardation (band shift) of p62 and Sestrin2 (Fig. 4B,C), indicating post-translational modification of the proteins. The band shift of p62 became slightly more pronounced when both ULK1 and Sestrin2 were expressed, compared with ULK1

presence alone (Fig. 4C; note that the bottom half of the p62 bands have been shifted up by Sestrin2 co-expression). As ULK1 is a protein kinase, it was investigated further whether the band shift of p62 was caused by ULK1-mediated phosphorylation. Indeed, ULK1^{KI} was unable to produce the band shift of p62 (Fig. 5A), suggesting that it is dependent on the catalytic activity of ULK1. The band shift of p62 was reverted by calf intestinal alkaline phosphatase (CIP) treatment (Fig. 5A), confirming that phosphorylation took place and caused the electromobility retardation.

It has been shown that the Ser403 site on p62 is phosphorylated by casein kinase 2 (CK2) and Tank-binding protein kinase 1 (TBK1) [14,15], while Thr269 and Ser272 sites are phosphorylated by cyclin-dependent kinase 1 (CDK1) [23]. We tested if ULK1 could induce p62 phosphorylation at these known sites.
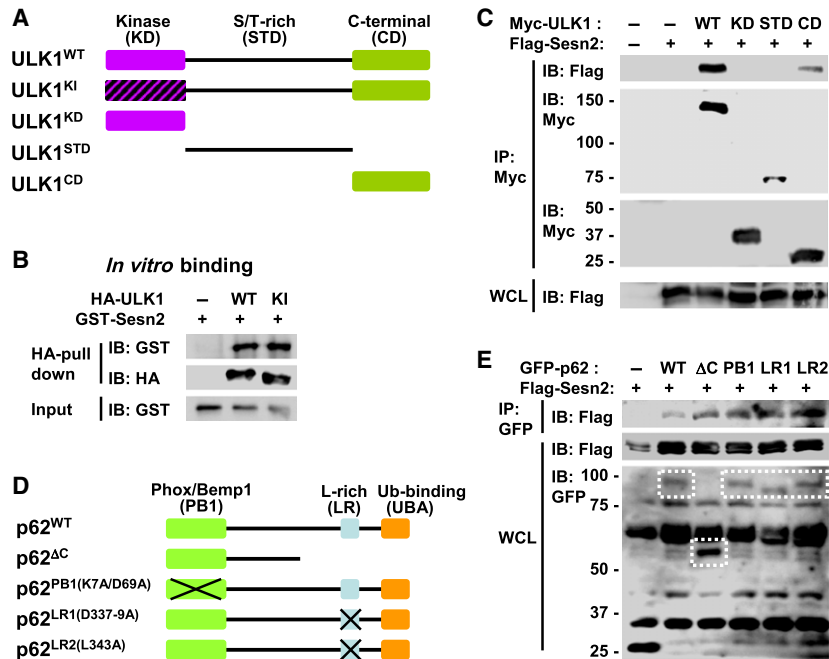
Fig. 3. Mapping the Sestrin2-interacting domains of ULK1 and p62. (A) Diagram depicting the domain structure of ULK1 and its mutant proteins. Kinase-inactive (KI) mutants as well as truncated proteins with kinase domain (KD), Ser/Thr-rich domain (STD) and C-terminal domain (CD) are illustrated. (B) Both ULK1^{WT} and ULK1^{KI} were able to pull down GST-Sestrin2 *in vitro*. HEK293 cells were transfected with plasmids expressing HA-tagged ULK1^{WT} or ULK^{KI}. Cell lysates were incubated with GST-Sestrin2 recombinant protein and immunoprecipitated using anti-HA antibody. Pulled-down GST-Sestrin2 proteins were assayed by immunoblotting (IB). (C) HEK293 cells were transfected with plasmids expressing indicated proteins. Myc-tagged proteins were immunopurified (IP) from whole cell lysates (WCL) and subjected to immunoblotting. (D) Diagram depicting the domain structure of p62 and its mutant proteins. PB1 (Phox/Bemp1), LR (leucine-rich) and UBA (ubiquitin-binding) domains were truncated or mutated. (E) HEK293 cells were transfected with plasmids expressing indicated proteins. GFP-tagged proteins were immunopurified from whole cell lysates and subjected to immunoblotting.
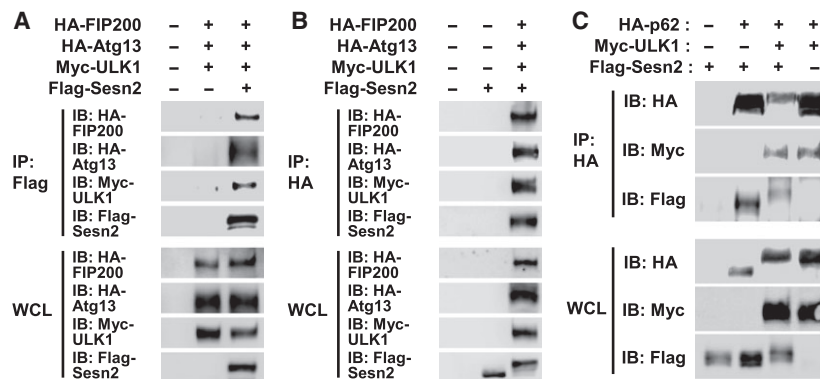

Fig. 4. Association between Sestrin2, ULK1-Atg13-FIP200 and p62. (A)–(C) Sestrin2 associates with Atg13-FIP200 and p62 when ULK1 is co-expressed. HEK293 cells were transfected with plasmids expressing indicated proteins. Immunoprecipitated proteins (IP) and whole cell lysates (WCL) were subjected to immunoblotting (IB) with indicated antibodies. Note that ULK1 co-transfection induces electromobility retardation (band shift) of Sestrin2 (B), (C) and p62 (C).

ULK1 co-transfection with p62 prominently increased phosphorylation of p62 at the Ser403 site, whereas no such effect was observed at the Thr269 and Ser272 sites (Fig. 5B), demonstrating that ULK1 promoted phosphorylation of p62 at the Ser403 site.

We then performed *in vitro* kinase assays to confirm that ULK1-mediated p62 phosphorylation was the result of a direct biochemical reaction. Immunopurified ULK1 was able to induce Ser403 phosphorylation of p62 *in vitro* (Fig. 5C). When ULK1 was co-purified
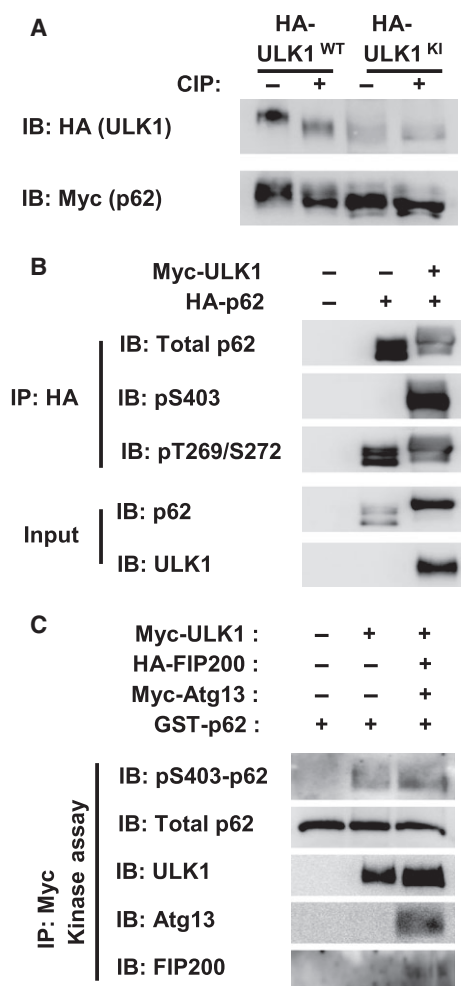
A

B

C

Fig. 5. ULK1 phosphorylates p62 at Ser403. (A) ULK1-induced band shift of p62 is phosphatase-sensitive. HEK293 cells were transfected with HA-tagged ULK1^{WT} or ULK1^{KI}. After treatment with CIP, cell lysates were analyzed by immunoblotting. (B) ULK1 induces Ser403 phosphorylation of p62. HEK293 cells were transfected with plasmids expressing indicated proteins. Anti-HA immunocomplexes (IP) and whole cell lysates (WCL) were analyzed by immunoblotting. (C) HEK293 cells were transfected with Myc-tagged ULK1 and its regulatory subunits FIP200 and Atg13. ULK1 kinase activity of anti-Myc immunocomplexes was assayed using recombinant GST-p62 protein. Amounts of phosphorylated and total proteins were assayed through immunoblotting.

with its regulatory subunits Atg13 and FIP200, the level of phosphorylation was increased (Fig. 5C), reflecting the enhanced catalytic activity of ULK1 by these subunits [20–22].

Sestrin2 promotes ULK1-mediated phosphorylation of p62

Because the ULK1-mediated band shift of p62 was slightly enhanced upon Sestrin2 co-transfection

(Fig. 4C), we tested if Sestrin2 could promote this phosphorylation. Ser403 phosphorylation of p62, which was induced by ULK1^{WT} but not ULK1^{KI}, became much stronger upon co-expression with Sestrin2 (Fig. 6A,B). We also confirmed this finding using immunocytochemistry where total p62 and phospho-Ser403 p62 were stained green and red respectively. Red phospho-p62 signal was hardly detectable in ULK1^{KI}-transfected cells while cells with ULK1^{WT} transfection showed considerable red signals (Fig. 6C,D). Merged images of ULK1^{WT}-transfected cells revealed many yellow spots that represented phosphorylated p62 aggregates in addition to some remaining green spots that implied that not all p62 aggregates were phosphorylated by ULK1^{WT} (Fig. 6C,D). However, when Sestrin2 was co-transfected with ULK1, most p62 aggregates were either yellow or even reddish yellow (Fig. 6C,D), suggesting stronger ULK1-mediated p62 phosphorylation. These data demonstrate that Sestrin2 can promote ULK1-mediated phosphorylation of p62 in mammalian cells.

Because Sestrin2 can activate AMPK and inhibit mTORC1 [10], which can together increase ULK1 activity, it was not clear whether the direct physical association between Sestrin2, ULK1 and p62 was what contributed to the enhanced p62 phosphorylation. To rule out any involvement of indirect signaling pathways, we immunopurified ULK1 from transfected HEK293 cells and performed a kinase reaction with the addition of recombinant Sestrin2 and p62. Through this assay, we tested if Sestrin2 could directly promote ULK1-mediated p62 phosphorylation *in vitro*. ULK1-mediated Ser403 phosphorylation of p62 was strongly enhanced upon addition of recombinant Sestrin2 (Fig. 6E), suggesting that Sestrin2 can directly facilitate p62 phosphorylation by ULK1 without the involvement of indirect signaling pathways such as AMPK and mTORC1. The *in vitro* promotion of ULK1-mediated p62 phosphorylation by Sestrin2 was dose-dependent (Fig. 6F).

Endogenous Sestrin2 and ULK1 control p62 phosphorylation

Through a co-immunoprecipitation assay, we found that endogenous Sestrin2 also associated with endogenous ULK1 and p62 in mammalian cells (Fig. 7A,B). Thus, we asked if endogenous Sestrin2 and ULK1 could regulate p62 phosphorylation as well. Consistent with our findings, Sestrin2-deficient mouse embryonic fibroblasts (MEF) exhibited a substantial decrease in Ser403 phosphorylation of p62 compared
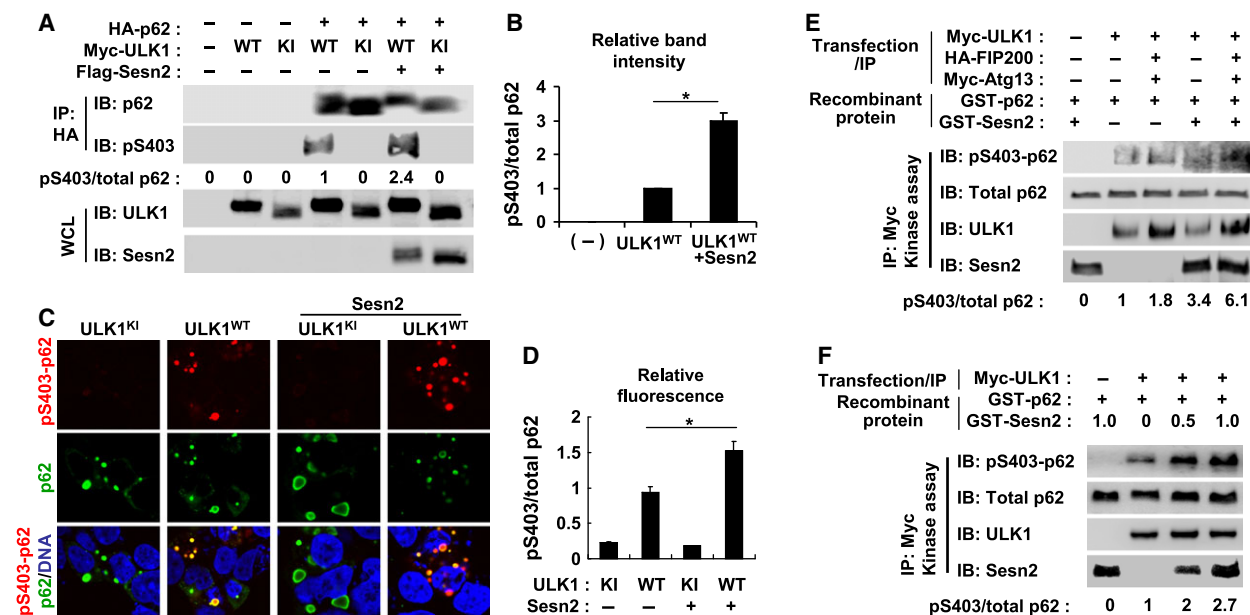
Fig. 6. Sestrin2 promotes ULK1-mediated phosphorylation of p62. (A), (B) HEK293 cells were transfected with plasmids expressing indicated proteins. Anti-HA immunocomplexes (IP) and whole cell lysates (WCL) were subjected to immunoblotting (IB). Relative phosphorylation of p62 at Ser403 (pS403/total p62) was quantified by densitometry and is presented as numbers below each lane (A) and as a graph (B) ($n = 3$). (C), (D) HEK293 cells were transfected with plasmids expressing indicated proteins and immunostained with anti-phospho Ser403 p62 (red) and anti-total p62 (green) antibodies and DAPI (DNA, blue). Relative fluorescence of phosphorylated and total p62 in each aggregate was quantified and is presented as a graph ($n \geq 20$). (E), (F) HEK293 cells were transfected with Myc-tagged ULK1 and its regulatory subunits FIP200 and Atg13. ULK1 kinase activity of anti-Myc immunocomplexes was assayed using recombinant GST-p62 protein. GST-Sesn2 protein (0.5 μg or as indicated) was directly added to the kinase reaction mixture. Amounts of phosphorylated and total proteins were assayed through immunoblotting. Relative phosphorylation of p62 at Ser403 (pS403/total p62) was quantified by densitometry and is presented as numbers below each lane. In graphs, data are shown as means ± standard errors. *$P < 0.05$.

with the control MEF (Fig. 7C). However, very unexpectedly, genetic deletion of FIP200, which completely nullifies activities of both ULK1 and its close homolog ULK2 in regulating autophagy [24], or concomitant deficiency of ULK1 and ULK2 modestly increased Ser403 phosphorylation of p62 compared with the WT control MEF (Fig. 7D,E). Because Ser403 residue of p62 can be phosphorylated by other kinases such as CK2 and TBK1 [14,15], it is possible that absence of ULK1 activity may have induced compensatory upregulation of these kinases. A recent report supported this idea by demonstrating that ULK1 inhibits TBK1 [25] and, consistent with this report, we found that TBK1 is prominently activated in FIP200-deficient MEF (Fig. 7D). Furthermore, treatment of ULK1/2-deficient MEF with a TBK1 inhibitor BX-795 dramatically suppressed the p62 phosphorylation increased by the ULK1/2 loss (Fig. 7E). Therefore, even though ULK1 can directly phosphorylate p62, complete absence of ULK1 provokes slightly more p62 phosphorylation through indirect feedback activation of TBK1.

Sestrin2 and ULK1/2 mediate p62 phosphorylation under energetic stress

Cellular energy deprivation leads to activation of AMPK, which phosphorylates and subsequently activates ULK1 [26]. Consistent with previous reports, oligomycin, which depletes cellular ATP, elevated AMPK-mediated activatory phosphorylation of ULK1 at Ser555 (Fig. 7F). Intriguingly, Ser403 phosphorylation of p62 was very robustly increased by oligomycin treatment in WT MEF (Fig. 7F,G). The oligomycin-induced Ser403 phosphorylation of p62 was dramatically attenuated in ULK1/2-deficient MEF and Sestrin2-deficient MEF (Fig. 7F,G), indicating that endogenous Sestrin2–ULK1/2 mediates p62 phosphorylation during energetic stress.

ULK1 phosphorylates Sestrin2 at multiple sites

Finally, we examined if the ULK1-induced electromobility retardation of Sestrin2 (Fig. 4B,C) was caused by ULK1-mediated phosphorylation as well. The band
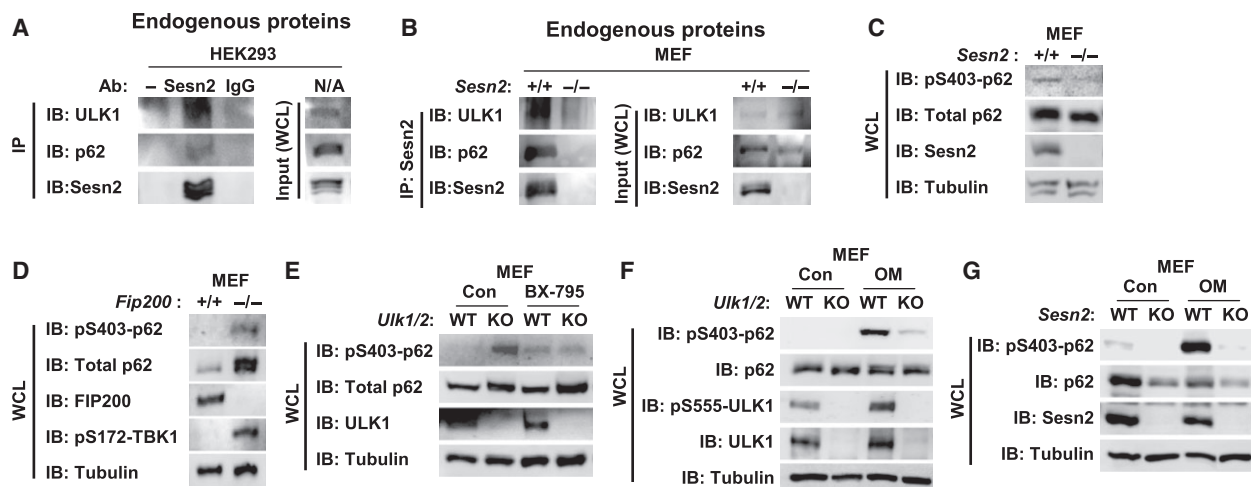
Fig. 7. Effect of Sestrin2 and FIP200 deficiencies on p62 phosphorylation levels. (A), (B) Physical association between endogenous Sestrin2, p62 and ULK1. HEK293 (A) and *Sesn2*^{+/+} and *Sesn2*^{−/−} MEF (B) were treated with 100 μM etoposide for 16 h to maximize endogenous Sestrin2 expression. Sestrin2 was immunopurified using protein G/A-conjugated anti-Sestrin2 antibody. Protein G/A-conjugated pre-immune IgG and empty protein G/A beads (−) were used as negative controls. Both anti-Sestrin2 immunocomplexes (IP) and whole cell lysates (WCL) were assayed through immunoblotting (IB). (C) Reduction of p62 Ser403 phosphorylation in Sestrin2-deficient MEF. Whole cell lysates from WT (*Sesn2*^{+/+}) and *Sesn2*^{−/−} MEF were subjected to immunoblotting with indicated antibodies. (D) Induction of p62 Ser403 phosphorylation in FIP200-deficient MEF. Whole cell lysates from WT (*Fip200*^{+/+}) and *Fip200*^{−/−} MEF were subjected to immunoblotting with indicated antibodies. (E) Induction of basal p62 Ser403 phosphorylation in ULK1/2-deficient MEF was suppressed by BX-795, an inhibitor of TBK1. WT (*Ulk1*^{+/+}/*Ulk2*^{+/+}) and *Ulk1*^{−/−}/*Ulk2*^{−/−} (KO) MEF were treated with NaCl/P$_i$ (Con) or 10 μM BX-795 for 6 h, and subjected to immunoblotting with indicated antibodies. (F), (G) Oligomycin-mediated energy depletion strongly induced Ser403 phosphorylation of p62 in WT MEF, but not as robustly in ULK1/2- or Sestrin2-deficient MEF (KO). WT (*Ulk1*^{+/+}/*Ulk2*^{+/+} and *Sesn2*^{+/+} controls), *Ulk1*^{−/−}/*Ulk2*^{−/−} and *Sesn2*^{−/−} MEF were treated with NaCl/P$_i$ (Con) or 10 μg·mL^{-1} oligomycin for 6 h, and subjected to immunoblotting with indicated antibodies.

shift of Sestrin2 was induced only by ULK1[WT] but not by ULK1[KI] (Figs 6A and 8A,B), showing that the shift was dependent on the catalytic activity of ULK1. CIP treatment reverted the band shift (Fig. 8A), further demonstrating that the shift was caused by ULK1-mediated phosphorylation. We then attempted to identify the ULK1-mediated phosphorylation site(s) on Sestrin2. Because no post-translational modification has been previously reported for Sestrin2, we subjected truncated mutants of Sestin2 (Sesn2[ΔA], Sesn2[ΔAB] and Sesn2[ΔBC]) to band shift assays to identify possible domain(s) of phosphorylation. Sesn2[ΔA] exhibited a band shift upon ULK1[WT] expression while Sesn2[ΔAB] did not (Fig. 8A), suggesting that Sesn-B domain contains ULK1-dependent phosphorylation sites. However, Sesn2[ΔBC] also exhibited slight band shift upon ULK1[WT] expression, which is diminished by either CIP treatment or ULK1[KI] mutation (Fig. 8A), showing that there may be additional phosphorylation sites in the Sesn-A domain. Mutating all Ser/Thr residues in the Sesn-B domain (Sesn2[B2A]) did not abolish the band shift of Sestrin2 (Fig. 8B), confirming that ULK1 phosphorylates Sestrin2 at multiple sites.
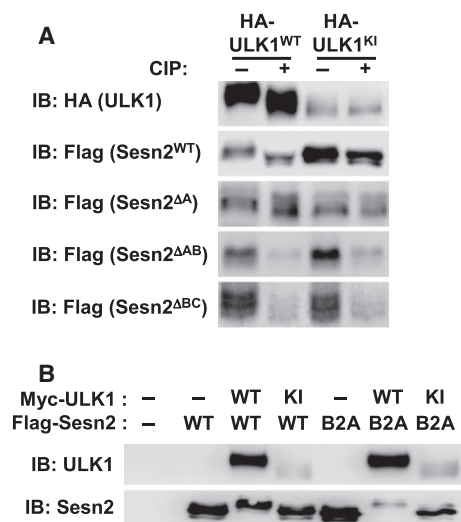

Fig. 8. ULK1 phosphorylates Sestrin2 at multiple sites. (A) HEK293 cells were transfected with HA-tagged ULK1[WT] or ULK[KI] and Flag-tagged WT or truncated Sestrin2 proteins. Cell lysates were treated with CIP and assayed through immunoblotting (IB). (B) HEK293 cells were transfected with plasmids expressing indicated proteins. Cell lysates were assayed through immunoblotting. Sesn2[B2A] is a Sestrin2 mutant protein whose Ser/Thr residues in the Sesn2-B domain were all substituted with Ala.

Discussion

In this study, we revealed a new function of Sestrin2 in promoting ULK1-dependent phosphorylation of p62. In addition to previously known functions of Sestrin2 in regulating AMPK and mTORC1 signaling pathways (Fig. 9A), this new function provides an explanation of how Sestrin2 specifically contributes to selective autophagy of ubiquitinated proteins, dysfunctional mitochondria and a signaling molecule Keap1, which are all mediated by ULK1 and p62 [4–6,27]. Thus we can now explain why Sestrin2-deficient cells and tissues can accumulate a large amount of ubiquitinated protein aggregates and dysfunctional mitochondria [8] and exhibit defective Nrf2 signaling that exacerbates oxidative damage during environmental stresses [13]. By controlling ULK1-mediated Ser403 phosphorylation of p62 that is required for efficient removal of p62 targets [14–16], Sestrin2 can promote selective autophagy (Fig. 9B). The Sestrin2/p62-mediated direct activation of selective autophagy can be further enhanced upon indirect upstream modulation of AMPK and mTORC1 signaling pathways, which is already known to promote both general and selective autophagy processes [18].
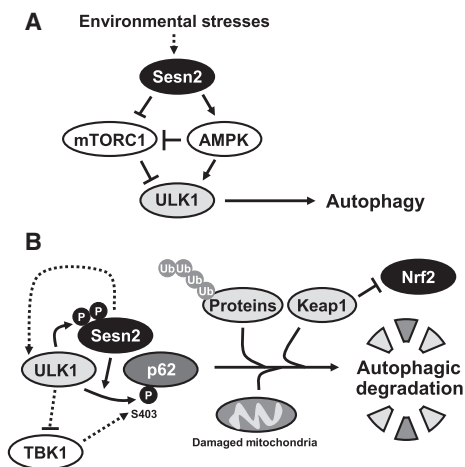

Fig. 9. Proposed roles of Sestrin2 and ULK1 in regulation of p62-mediated selective autophagy. (A) From previous studies, Sestrin2 was shown to be a mediator of stress-induced autophagy. Stress-inducible Sestrin2 can provoke ULK1 activation through activating AMPK and inhibiting mTORC1. (B) In this study, Sestrin2 was shown to associate with ULK1 and p62 and directly promote ULK1-dependent phosphorylation of p62 at Ser403. Because phosphorylation of this site is known to facilitate autophagic degradation of p62 targets, our study provides an explanation of how Sestrin2 is required for proper elimination of ubiquitinated protein aggregates and dysfunctional mitochondria and for appropriate regulation of the Keap1–Nrf2 signaling pathway.

Although ULK1 is one of the first molecules to be identified as an autophagy mediator [27,28], finding its biochemical substrates has been extremely difficult [29]. It has only recently been discovered that ULK1 phosphorylates several autophagy regulators, such as AMBRA1 [30], Beclin-1 [31], ZIPK [32] and Atg9 [33]. In addition to these known substrates, we showed that p62 can be another important substrate of ULK1 mediating its selective autophagy-regulating function. Because ULK1 can associate with p62 even when autophagosome formation is blocked by Vps34 inhibition [34], ULK1-mediated p62 regulation may be one of the early steps of selective autophagy prior to ULK1-mediated Beclin-1/Vps34 [30,31] or Atg9/myosin II [32,33] regulation. Therefore, in addition to contributing to carrying out autophagy through autophagosome membrane formation and growth [30–33], ULK1 can initiate the selective autophagy process by phosphorylating p62 and bringing both p62 and its substrates to the autophagosome formation sites.

p62 is a signaling adaptor protein that has many cellular functions [3]. Recent reports suggest that phosphorylation of p62 can alter its function in regulating autophagy [14–16] and cell cycle [23]. Specifically, phosphorylation of p62 at the Ser403 site is important for degradation of p62 and its target molecules [14,15]. Since ULK1 is a well-known autophagy initiating kinase [27], and Sestrin2 is a recently characterized promoter of autophagy [8,12,13], our study provides a mechanism of how ULK1 and Sestrin2 can come together to facilitate selective autophagy by inducing Ser403 phosphorylation of p62. This mechanism seems to have strong physiological significance in the context of energy deprivation where Ser403 phosphorylaion of p62 is dramatically increased in a Sestrin2- and ULK1/2-dependent manner.

Materials and methods

Plasmids, antibodies and chemicals

Flag-Sesn2^{WT}, Flag-Sesn2^{ΔBC}, Flag-Sesn2^{ΔA} and Flag-Sesn2^{ΔAB} were subcloned into pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). HA-ULK1^{WT}, HA-ULK1^{KI}, Myc-ULK1^{WT}, Myc-ULK1^{KD}, Myc-ULK1^{STD} and Myc-ULK1^{CD} were from K.L. Guan (UC San Diego) and M. Aghajan (Isis Pharm. Inc., Carlsbad, CA, USA) [18]. GFP-p62^{WT}, GFP-p62^{ΔC}, GFP-p62^{PB1(K7A/D69A)}, GFP-p62^{LR1 (D337-9A)} and GFP-p62^{LR2 (L343A)} were from Addgene, Cambridge, MA, USA [34]. Plasmids expressing Myc-ULK1^{WT}, Myc-ULK1^{KI}, Myc-Atg13 and HA-Atg13 were from D.H. Kim (University of Minnesota) [22]. Plasmids expressing HA-FIP200 and HA-p62 were from J.L. Guan (University

of Cincinnati) [24,35]. Antibodies to detect ULK1 (4773S), p62 (5114S), phospho-Thr269/Ser272 p62 (13121S), phospho-Ser555 ULK1 (5869), phospho-Ser172 TBK1 (5483P) and TBK1 (3013S) were from Cell Signaling Technology, Danvers, MA, USA. Anti-Sestrin2 (10795-1-AP) IgG was from Proteintech, Chicago, IL, USA. For immunoprecipitation experiments, we generated additional anti-Sestrin2 antibodies from guinea pigs (PRF&L, Canadensis, PA, USA) using recombinant GST-Sestrin2 proteins purified from *E. coli*. Control IgG was prepared from pre-immune serum of the same animals. Anti-phospho-Ser403 p62 antibody (MABC186) was from Millipore, Billerica, MA, USA. Anti-FIP200 antibody was from J.L.Guan (University of Cincinnati) [36]. Anti-hemagglutinin (HA) antibody (3F10) was from Roche, Penzberg, Upper Bavaria, Germany. Anti-Myc (9E10) and anti-GFP (12A6) antibodies were from Developmental Studies Hybridoma Bank, Iowa City, IA, USA. Anti-GST antibody (554805) was from BD Biosciences, Franklin Lakes, NJ, USA. Anti-Flag (M2) and anti-Tubulin (T5168) antibodies, as well as etoposide and oligomycin, were from Sigma, St Louis, MO, USA. BX-795 was obtained from Selleckchem, Houston, TX, USA.

Cell culture and transfection

HEK293 and MEF cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C in 5% CO_2. $Sesn2^{-/-}$, $Fip200^{-/-}$ and $Ulk1^{-/-}/Ulk2^{-/-}$ MEF cells were previously described [9,24,37]. For transient expression of proteins, HEK293 cells were transfected with purified plasmid constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were harvested 2 days after transfection for immunoblotting, immunoprecipitation, immunocytochemistry or other biochemical assays.

Immunocytochemistry

Two days after transfection, HEK293 cells were seeded onto glass coverslips. On the following day, cells were washed with $NaCl/P_i$ and fixed with 4% paraformaldehyde. After permeabilizing the cells with 0.3% Triton X-100, they were incubated overnight with primary antibodies. The cells were then washed with $NaCl/P_i$ and were incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen) for 30 min and counterstained with DAPI (Invitrogen) [38]. Samples were analyzed under a FluoView 500 laser confocal microscope (Olympus, Tokyo, Japan).

Immunoprecipitation

Cell lysates were prepared in a lysis buffer (20 mM Tris/Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM

NaPPi, 1 mM β-glycerophosphate and 1 mM Na_3VO_4) containing 0.3% CHAPS and protease inhibitor cocktail (Roche), and immunoprecipitated with anti-HA (A2095, Sigma) or anti-Flag (A2220, Sigma) agarose bead or other antibodies conjugated to a protein G/A bead (Calbiochem, Darmstadt, Germany). The immunocomplexes were then washed four times with the lysis buffer and analyzed through immunoblotting [39].

In vitro pull-down assay

Full-length mouse Sestrin2 cDNA was cloned in pGEX-4T-1 (Amersham) and expressed in BL21 (DE3) *E. coli*. The recombinant proteins were purified using a glutathione-Sepharose 4B column (Amersham, Amersham, UK). Cell lysates prepared from HEK293 cells transfected with HA-ULK1 or HA-p62 were incubated with GST-Sestrin2 at 37 °C for 3 h. After the incubation, HA-ULK1 or HA-p62 was immunoprecipitated, and the presence of GST-Sestrin2 in the immunocomplexes was examined through immunoblotting (see below).

In vitro kinase assay

Myc-ULK1[WT] was purified from transfected HEK293 cells through immunoprecipitation using a protein G/A bead-conjugated anti-Myc antibody. The kinase reaction was performed in a solution of 25 mM MOPS, pH 7.5, 1 mM EGTA, 0.1 mM Na_3VO_4, 15 mM $MgCl_2$ and 100 μM ATP. GST-p62 (BML-UW1035; Enzo Life Sciences, Farmingdale, NY, USA) was used as a substrate. GST-Sestrin2 was directly added to the reaction. The reaction mixtures were incubated at 37 °C for 1 h.

Calf intestinal alkaline phosphatase (CIP) assay

Cell lysates were prepared in the lysis buffer described above and incubated with 1 unit·μL^{-1} CIP (M0290S, New England Biolabs, Ipswich, MA, USA) at 30 °C overnight [39].

Immunoblotting

Cell lysates, immunocomplexes and enzymatic reaction mixtures were boiled in SDS sample buffer for 5 min, separated by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes and probed with primary antibodies. After incubation with secondary antibodies conjugated with horseradish peroxidase, chemiluminescence was detected using LAS4000 (GE, Fairfield, CT, USA) systems [9]. All of the contrast adjustments were linear and uniformly applied to the whole image in each panel. Quantification of relative band intensities was done by densitometry. *P* values were calculated using Student's *t* test.

Acknowledgements

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