Cell Research - Research Highlight

Love laughs at Locksmiths: UBE2D2/UBE2D3-mediated ubiquitylation of the ubiquitin-binding domain of p62/sequestosome-1 unlocks its autophagy receptor potential

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Word count: 1267

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The multimodular adapter p62/sequestosome-1 plays prominent roles in physiology and disease by mediating cell signaling and cargo degradation. The work by Peng *et al.* in this issue of *Cell Research* provides mechanistic insights into activation of its autophagy receptor function critical for maintaining cell homeostasis during various forms of stress.

Main text

The discovery made a decade ago that the signaling adapter p62/sequestosome-1 (p62) plays a role in selective degradation of ubiquitylated targets paved the way for today's understanding of how the autophagy pathway handles bulky cargos, such as protein aggregates, damaged mitochondria, and intracellular bacteria[1]. As the prototypic receptor for selective autophagy, p62 (**Figure 1**, inset) contains a C-terminal ubiquitin (Ub)-binding domain (UBA) and an intermediate LC3-interacting region (LIR) responsible for binding Ub-like proteins (UBLs) of the LC3/GABARAP family, which are covalently conjugated to the membrane of autophagic vesicles (autophagosomes). By tethering the ubiquitylated cargo to the nascent autophagosome (phagophore), p62 stabilizes the lipid membrane and promotes its local expansion on the surface of the cargo[2]. The scaffolding and tethering roles of p62 are reinforced by its oligomeric nature: its N-terminal Phox and Bem1 (PB1) domain interacts with itself in a front-to-back fashion driving formation of helical filaments that can extend for 20-60 nm *in vitro*[3]. One important implication of p62 oligomerization is however the fact that its UBA domain can become locked in an inactive confirmation, which results from a partial occlusion of the Ub-binding surface through the UBA dimerization interface[4,5]. The work by Peng *et al.* may have identified the 'key' for the UBA dimer 'lock' (reference to Peng et al).

The authors started by asking how cells regulate p62 levels during conditions that cause rapid Ub accumulation (Ub overload, or Ub^+, stress). Overexpression of Ub, heat shock, or prolonged treatment with the proteasomal inhibitor bortezomib resulted in increased levels of the p62 protein, which is a consequence of a boost in stress-mediated gene transcription. At the same time, upregulation of substrate ubiquitylation and an increased autophagic flux were observed. By using p62-null mouse embryonic fibroblasts (MEFs), Peng *et al.* confirmed the leading role of p62 in the clearance of ubiquitylated proteins following the induction of Ub stress, which they operationally define as an increase in the Ub concentration above 500 picomoles of free Ub per milligram of total protein in the cell. An important implication of this finding is that overexpression of Ub will induce selective autophagy, which should be taken into consideration when experimenting with cellular levels of Ub.

During the analysis of stressed cells, the authors noticed that p62 was itself extensively ubiquitylated. Using a lysine-less Ub mutant, they showed that monoubiquitylation at multiple sites is likely to be the predominant post-translational modification of p62 in response to Ub stress and that it did not depend on an intact UBA domain. The authors then asked which proteins involved in the ubiquitylation cascade mediated these multiple monoUb modifications. In a yeast-two-hybrid screen using p62 as a bait, two highly conserved E2 enzymes, UBE2D2 and UBE2D3, were identified as novel p62 interactors. The specificity of this interaction was consequently confirmed using a range of assays, such as GST pull down, co-immunoprecipitation, and co-localization, and a E2-interacting region (EIR) was mapped to residues 294-320 of the human p62, which precedes and is adjacent to the LIR. In subsequent *in vitro* and *in vivo* experiments, Peng *et al.* confirmed the specificity of the E2 enzymes to the p62 substrate and the dependence of p62 ubiquitylation on its EIR. They also explored the functional significance of UBE2D2/UBE2D3's binding to p62 for selective autophagy by reconstituting p62-null MEFs with a p62 mutant lacking EIR. Importantly, similar to the UBA domain that is essential for binding ubiquitylated cargos, the EIR was required for p62's ability to induce protein degradation in response to the Ub stress. As the ultimate proof for the role of the E2 enzymes in the p62-mediated autophagy, the authors knocked out the *UBE2D2/UBE2D3* in HEK293T cells and saw that p62 failed to undergo ubiquitylation and exert its autophagy role in response to Ub overexpression, heat shock, or proteasome inhibition.

So, what does monoubiquitylation do to p62? To answer this question, Peng *et al.* first defined lysine residues within p62 that are the most likely substrates of Ub conjugation. Mass spectrometry of ubiquitylated p62 revealed four potential sites *in vivo* and pointed to the lysine-420 (K420) as the most interesting one. The K420, in conjunction with the E409 from the opposite UBA moiety, is one of the amino acid residues implicated in stabilization and auto-inhibition of the UBA dimers[4,5]. Indeed, in a pulldown experiment, the K420R/E409E mutant, unlike the wild-type p62, could precipitate polyUb chains. Inspired by this finding, the authors now prepared ubiquitylated p62, using their established *in vitro* ubiquitylation protocol, and employed it in a pulldown assay with polyUb. Satisfyingly, greatly enhanced interaction between ubiquitylated p62 and polyUb chains, as compared to unmodified p62, could be achieved *in vitro*. That unlocking UBA is critical for binding between p62 and Ub was also demonstrated by Peng *et al.* in experiments involving dynamic light scattering and electron microscopy, in which increased complex formation between the purified ubiquitylated p62 and polyUb chains could be visualized.

The model that the authors put forward (**Figure 1A**) suggests that different forms of Ub stress induce high levels of p62, Ub, and the E2s. Monoubiquitylation of, amongst other lysine residues, the K420 within the UBA domain reduces the inhibitory UBA dimer formation and promotes p62-polyUb interactions, thereby positively regulating autophagic degradation of ubiquitylated targets. This simple view will surely need thorough testing *in vivo* where many more factors are at play. While UBE2D2 and UBE2D3 seem to be essential for p62 ubiquitylation in response to the Ub stress, multiple E3 enzymes are known to directly interact with p62, with TRAF6 and KEAP1 being among the most prominent ones[6]. It is therefore likely that different Ub chains of different complexity and topology will affect p62's conformation, oligomerization, and interaction landscape. For example, in a recent study, the E3 ligase TRIM21 was found to polyubiquitylate the K7 within the PB1 domain of p62, leading to disabled p62 oligomerization and p62-mediated protein sequestration[7]. Similarly, other functions of p62 may be influenced by ubiquitylation at different lysine residues. It is for instance possible that modification of the K295 and K313 adjacent to the LIR might impact the ability of p62 to bind LC3/GABARAP with far-reaching consequences for its role as a selective autophagy receptor. Yet another mind-boggling prospect is that p62 ubiquitylation creates high-complexity scaffolds that await their characterization. Thus, another Ub-binding adapter, Optineurin, when ubiquitylated, recruits p62 as previously shown by the same group of authors[8]. A very tight interplay between Ub-binding receptors, i.e. p62, NBR1, Optineurin, NDP52, TAX1BP1, and Tollip/Cue5 can now be expected (**Figure 1B**).

In addition to ubiquitylation, other post-translational modifications may modify p62 functions. Phosphorylation of S403 was previously described to affect the UBA affinity for Ub[9,10]. While Peng *et al.* did not see any effect of S403 mutation on Ub binding in their study, combination of different modifications will likely have profound impact on p62 functions.

The role of p62 in human physiology and disease is exceedingly significant, with its prominent oncogenic role in epithelial tumor cells and a tumor-suppressor function in stromal cells[6]. Therefore, studies of the involvement of p62 and its modifications in cell signaling and autophagy will be extremely important for therapeutic exploitation of this versatile adaptor protein. The study of Peng et al. is yet another bright example of how a seemingly small discovery unlocks the door to a broad avenue of biomedical research.

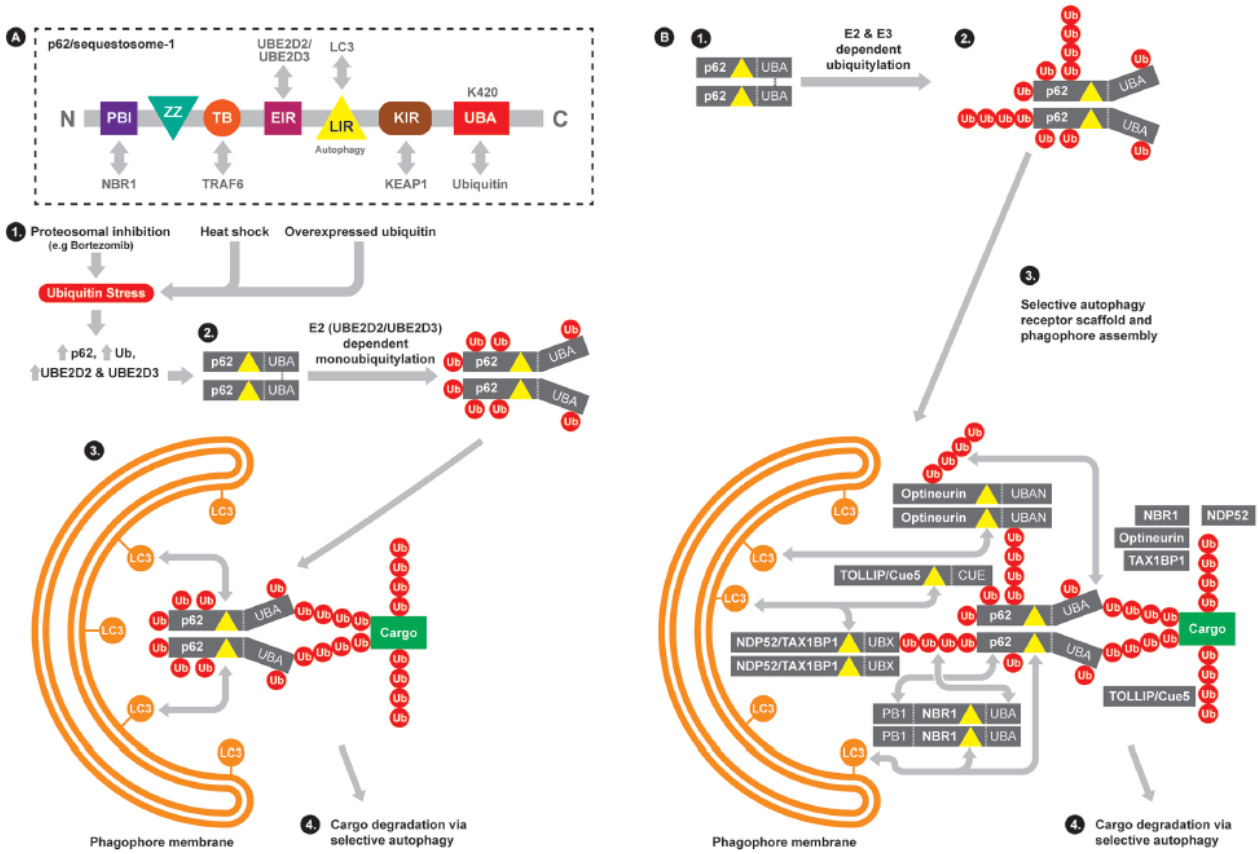
Figure 1. Implications of p62 ubiquitylation for its role as a selective autophagy receptor.

Figure legend

Implications of p62 ubiquitylation for its role as a selective autophagy receptor. (Inset) A schematic of p62 domain organization. (A) Peng et al. model for UBA domain activation via UBE2D2/UBE2D3-mediated monoubiquitylation of K420. [1] Ub stress induces levels of Ub, p62 and UBE2D2/UE2D3; [2] p62 becomes monoubiquitylated at multiple sites and UBA dimer becomes unlocked by ubiquitylation at K420; [3] Activated p62 tethers ubiquitylated cargo to LC3/GABARAP (shown as LC3 only) conjugated to the phagophore; [4] Autophagic

degradation of the engulfed complexes takes place. (B) The model for the Ub-driven formation of molecular scaffolds for selective autophagy. p62 oligomer (shown as a dimer) [1] can be mono- and polyubiquitylated by the E2 and E3 enzymes (KEAP1 or TRAF6, not shown) [2]. As a result, other LIR-containing selective autophagy receptors, such as NBR1, Optineurin, NDP52, TAX1BP1, and TOLLIP/Cue5 may bind ubiquitylated p62 (in addition to the ubiquitylated cargo itself) via their Ub-binding domains to build a scaffold for LC3-mediated autophagosome formation around the ubiquitylated cargo [3]. Multimodular scaffold mediates autophagic degradation of the ubiquitylated cargo [4]. CUE, coupling of ubiquitin to ER degradation; EIR, E2-Interacting Region; KIR, KEAP1 Interacting Region; LIR, LC3-Interacting Region; NLS, nuclear localization sequence; PB1, Phox and Bem1 domain; TB, TRAF6-binding domain; UBA, Ubiquitin-Associated Domain; UBAN, Ubiquitin binding in ABIN and NEMO domain; UBX, ubiquitin-like domain; ZZ, ZZ-type zinc finger domain.

Figure 1. **Implications of p62 ubiquitylation for its role as a selective autophagy receptor.** (A) A schematic of p62 domain organization (inset). Model by Peng et al. for UBA domain activation via UBE2D2/UBE2D3-mediated monoubiquitylation of K420. ❶ Ub stress induces increased levels of Ub, p62 and UBE2D2/UE2D3; ❷ p62 becomes monoubiquitylated at multiple sites and UBA dimer becomes unlocked by ubiquitylation at K420; ❸ Activated p62 tethers ubiquitylated cargo to LC3/GABARAP (shown as LC3 only) conjugated to the phagophore; ❹ Autophagic degradation of the engulfed complexes occurs. (B) Model for the Ub-driven formation of molecular scaffolds for selective autophagy. p62 oligomer (shown as a dimer) ❶ can be mono- and polyubiquitylated by the E2 and E3 enzymes (KEAP1 or TRAF6, not shown) ❷. As a result, other LIR-containing selective autophagy receptors, such as NBR1, Optineurin, NDP52, TAX1BP1, and TOLLIP/Cue5 may bind ubiquitylated p62 (in addition to the ubiquitylated cargo itself) via their Ubbinding domains to build a scaffold for LC3-mediated autophagosome formation around the ubiquitylated cargo ❸. Multimodular scaffold mediates autophagic degradation of the ubiquitylated cargo ❹. CUE, coupling of ubiquitin to ER degradation; EIR, E2-Interacting Region; KIR, KEAP1-Interacting Region; LIR, LC3-Interacting Region; PB1, Phox and Bem1 domain; TB, TRAF6-binding domain; UBA, Ubiquitin-Associated Domain; UBAN, Ubiquitin binding in ABIN and NEMO domain; UBX, ubiquitin-like domain; ZZ, ZZ-type zinc finger domain.



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