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To cite this article: Do Hoon Kwon, Leehyeon Kim & Hyun Kyu Song (2018): pH-dependent regulation of SQSTM1/p62 during autophagy, *Autophagy*, DOI: [10.1080/15548627.2018.1532264](https://doi.org/10.1080/15548627.2018.1532264)

To link to this article: <https://doi.org/10.1080/15548627.2018.1532264>



Accepted author version posted online: 05 Oct 2018.



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**Publisher:** Taylor & Francis & Taylor and Francis Group, LLC

**Journal:** *Autophagy*

**DOI:** 10.1080/15548627.2018.1532264

**pH-dependent regulation of SQSTM1/p62 during autophagy**

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## ABSTRACT

During macroautophagy/autophagy, SQSTM1/p62 plays dual roles as a key mediator of cargo selection and as an autophagic substrate. SQSTM1 links N-degrons and/or ubiquitinated cargoes to the autophagosome by forming homo- or hetero-oligomers, although its N-degron recognition and oligomerization mechanisms are not well characterized. We recently found that SQSTM1 is a novel type of N-recognin whose ZZ domain provides a negatively-charged binding pocket for Arg-charged N-degron (Nt-Arg), a prototype type-1 substrate. Although differences in binding affinity exist for each N-degron, SQSTM1 also interacts with type-2 N-degrons, such as Nt-Tyr and Nt-Trp. Intriguingly, interactions between SQSTM1's ZZ domain and various N-degrons are greatly influenced by pH-dependent SQSTM1 oligomerization *via* its PB1 domain. Because cellular pH conditions vary from neutral to acidic depending on the stage of autophagy, the pH-dependent regulation of SQSTM1's oligomerization must be tightly coupled with the autophagic process.

**Keywords:** aggrephagy, autophagy adaptor, HSPA5/BiP/GRP78, N-end rule, pH-dependent oligomerization, SQSTM1/p62, UBR box, ZZ domain

The autophagy-lysosome system (ALS) is a well-conserved catabolic pathway in eukaryotic cells that maintains cellular homeostasis together with the ubiquitin-proteasome system (UPS). Although a relatively costly process compared with UPS, autophagy generally removes bulky cellular cargo that the UPS cannot accommodate, such as damaged organelles, intracellular

pathogens, and protein aggregates. Recent studies showed that SQSTM1, a key autophagic receptor, not only mediates protein aggregate removal but also self-aggregates and is degraded by autophagy. The central ZZ domain of SQSTM1 recognizes the ER chaperone HSPA5/BiP/GRP78, when it is converted to N-terminal Arg-charged HSPA5 (R\*-HSPA5) by ATE1/arginyl-tRNA protein transferase 1 by stress signals. Post-translationally modified R\*-HSPA5 chaperone molecules complexed with protein aggregates are recognized by SQSTM1 and ultimately removed by ALS. In addition, SQSTM1 forms homo- and hetero-oligomers *via* its PB1 (Phox and Bem1) domain, which is important for the autophagic process. However, the mechanisms of R\*-HSPA5 recognition by the ZZ domain and SQSTM1's self-oligomerization *via* its PB1 domain remain unclear.

To investigate these mechanisms, we obtained high-resolution structures of the ZZ domain complexed with 8 different N-degrons and determined the pH-dependent behavior of SQSTM1 [1]. The ZZ domain can bind both type-1 N-degrons (positively-charged amino acids: Arg, Lys, and His) and type-2 N-degrons (bulky hydrophobic amino acids: Tyr, Trp, Phe, Leu, and Ile). Four key residues (Asp127, Asn132, Asp147, and Asp149) form a negatively-charged pocket that receives the primary residue of the N-degron. The bipolar side chain of Asn132 participates in the specific recognition of positively-charged type-1 N-degrons, N-terminal Tyr, and N-terminal Trp *via* polar atoms in their side chains. Furthermore, ionic interaction between Arg139 and the negatively-charged side chain of N-degron secondary residues, such as Glu, strengthens the ZZ domain's affinity for N-degrons. The adjacent PB1 domain also plays an important role in regulating SQSTM1's interaction with N-degrons. Its apparent binding affinity is markedly enhanced by the avidity effect due to oligomerized SQSTM1's ability to provide multiple N-degron binding sites. The affinity of a recombinant PB1-ZZ domain protein is

approximately 10- to 1000-fold higher (depending on different pH conditions and binding constant measurement method) than that of the ZZ domain only. Moreover, oligomerization-defective mutants (K7A and D69A) have similar affinity with the ZZ domain only and, like structure-based mutants defective in recognizing N-degrons (D127N, N132L, R139D, D147R, and D149R), exhibit dysfunctional SQSTM1-dependent autophagy in HeLa cells. Our results showed that autophagic degradation of the N-end substrate is affected by both the ZZ and PB1 domains of SQSTM1.

Generally, when autophagy is induced, the cellular level of SQSTM1 increases and large SQSTM1 aggregates appear as indicated by the appearance of experimental GFP-SQSTM1 puncta. However, no clear data showing how SQSTM1 aggregates during autophagy have been reported. We unexpectedly determined that SQSTM1 oligomerization is regulated by experimental pH conditions. The oligomeric state of SQSTM1 dramatically changes in specific pH ranges (**Figure 1**). For example, at physiological pH (pH 7.4), SQSTM1 forms small, donut-shaped oligomers, but as the pH decreases in the autophagosome and early endosome (pH 5.5–6.5), SQSTM1 forms large, highly flexible helical filaments. Finally, at lysosomal pH (pH < 5.0), the helical structure disappears, and the filament dissociates into smaller molecules distinctly different from the high-pH oligomer. The smaller SQSTM1 oligomers maintain their protein folding even at acidic pH, whereas R\*-HSPA5 molecules are easily denatured. Indeed, SQSTM1 protects the unfolding of R\*-HSPA5 at mildly acidic pH.

Due to technical limitations, accurately measuring the pH of a specific cellular compartment, especially temporary organelles, such as endosomes and autophagosomes, is challenging. However, based on confocal pH imaging, the pHs of autophagic intermediates are as follows: autophagosome (pH 5.8–6.2), early autolysosome (pH 5.4–5.8), matured

autolysosome (pH 5.0–5.4), and lysosome (pH < 5.0). Because the pH changes at each stage of autophagy, we can attempt to explain the role of pH-dependent SQSTM1 oligomers in the autophagic process. Under physiological conditions, SQSTM1 forms small oligomers that can maintain proper avidity. When SQSTM1 encounters protein aggregates or selective autophagic cargo, it binds them in various ways *via* the ZZ and UBA domains. These cargo clusters, including SQSTM1, are targeted for transport to the phagophore assembly site. As the phagophore membrane elongates, the local pH may gradually decrease due to negatively-charged molecules in the membrane's vicinity. In parallel, SQSTM1 interacts with membrane-anchored LC3 (LC3–PE) *via* the LIR motif. We speculate that filamentous SQSTM1 may be important for strong binding to multiple LC3 molecules, thus enabling the cargo molecules to attach to the inner phagophore membrane. After fusion with the lysosome, filamentous SQSTM1 converts into a trimer or tetramer at a low pH and subsequently releases the cargo rapidly due to low avidity. These small molecules must be the appropriate substrates for various lysosomal proteases. Although the pH-dependent behavior of SQSTM1 under *in vitro* conditions needs to be verified *in vivo*, our findings provide a framework for further experiments to understand the molecular mechanism of the aggregation and regulation of the important autophagy adaptor SQSTM1.

#### **Disclosure of potential conflicts of interest**

Authors declare no potential conflict of interest.

## **Funding**

This work was supported by National Research Foundation grants from the Korean government (NRF-2016R1E1A1A01942623 and BRL: No. 2015041919). L.K. is a recipient of a POSCO Science Fellowship of POSCO TJ Park Foundation.

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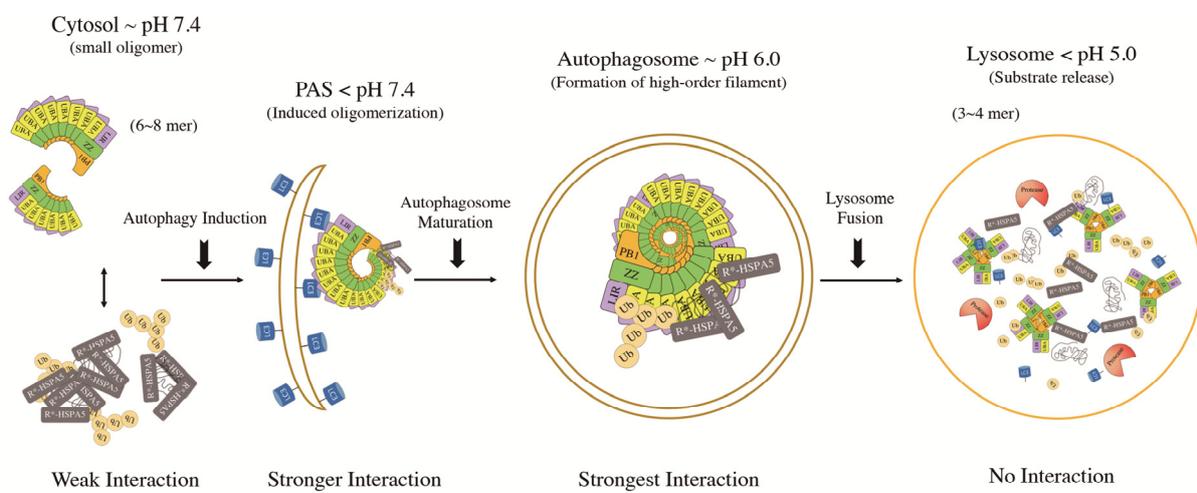
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## Figure legend

**Figure 1.** pH-dependent regulation of SQSTM1 during autophagy. The ZZ domain of SQSTM1 recognizes cargo-bound R\*-HSPA5 or unknown N-degron (type-1 or type-2) proteins. The PB1, ZZ, LIR, and UBA domains in SQSTM1 are shown as orange, green, purple, and yellow, respectively. The R\*-HSPA5 chaperone (gray rectangle) binds to the ubiquitinated aggregate (Ub, beige circle) under certain conditions, such as ER stress. At physiological pH, SQSTM1 forms small oligomers composed of 6 to 8 monomers bearing a relatively weak affinity to the cargo. Phagophore assembly site (PAS) formation, followed by autophagy induction, results in a decreased pH, which promotes further oligomerization of SQSTM1 and facilitates a stronger interaction with the cargo. Autophagosomal pH is lowered even further and generates a higher-order and more flexible filament, which may represent the form of SQSTM1 with the strongest cargo interaction *via* enhanced avidity. Finally, following fusion of the autophagosome with the lysosome, lysosomal pH facilitates separation of the large, strongly bound complexes (aggregates and SQSTM1) into smaller molecules easily degraded by lysosomal proteases (red crescent). LIR, LC3-interacting region.



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