Autophagy: RNA comes from the vault to regulate p62/SQSTM1 and selective autophagy

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The selective autophagy receptor and signaling adapter p62/Sequestosome-1 (SQSTM1) is regulated directly in its oligomerization by vault RNA (vtRNA). This riboregulation negatively affects the aggregation state and thereby autophagic degradation of p62 and its role as a selective autophagy receptor.

Autophagy involves several pathways culminating in the degradation of cytoplasmic components in the lysosome. This cell autonomous process serves two main purposes; macromolecular and organelle quality control and replenishment of nutrients and energy to maintain cellular homeostasis. Macroautophagy (hereafter referred to as autophagy) is initiated by formation of a crescent shaped double membrane structure, the phagophore, that grows and closes upon itself to form the autophagosome which subsequently fuses with lysosomes generating an autolysosome where the content is degraded and recycled [1, 2]. The discovery of selective autophagy receptors, which on one hand can recognize cargo, and on the other hand bind to the phagophore to include the cargo in the forming autophagosome, has demonstrated that autophagy can be a very selective process [3, 4]. Hence, selective removal of damaged or surplus organelles (i.e. mitochondria, endosomes, lysosomes and peroxisomes), protein aggregates and intracellular pathogens is mediated by both distinct and overlapping sets of autophagy receptors. The "garbage" to be disposed of is often tagged with ubiquitin to mediate recognition by autophagy receptors containing ubiquitin-binding domains [4, 5].

The first selective autophagy receptor discovered, p62/SQSTM1 [6, 7], contains an N-terminal PB1 domain capable of homopolymerization and heterodimerization with a selection of other PB1 domain proteins [8]. Following the PB1 domain is a ZZ type zinc finger domain, the LC3 interacting region (LIR) which binds to ATG8 family proteins, the KEAP1 interacting

region (KIR) and the C-terminal UBA domain binding to mono- or polyubiquitin (Figure 1A) [9]. p62 is itself degraded by autophagy and binds ubiquitinated cargo via its UBA domain, and anchors to LC3B in the membrane of the concave side of the forming autophagosome through its LIR motif. The PB1 domain can polymerize into long helical filaments *in vitro* [10]. In cells, p62 form round bodies or droplets that have liquid-liquid phase transition properties most likely caused by a modulation of filament length by ubiquitin polymers [9]. p62 is member of a group of selective autophagy receptors called sequestosome-1-like receptors (SLRs) that bind ubiquitinated cargo [11]. The ability of p62 to be degraded by autophagy, and bring cargo to the forming autophagosome, depends on PB1 domain polymerization and the binding to ATG8 family proteins via its LIR motif (Figure 1B) [7, 12].

Vault RNAs (vtRNA) are small, 88-100 nt long, non-coding RNAs transcribed by RNA polymerase III and associated with giant cytoplasmic ribonucleoprotein particles (RNPs) called vaults [13]. The four human paralogs are called *vtRNA1-1*, *vtRNA1-2*, *vtRNA1-3* and *vtRNA2-1* while there is only a single murine vtRNA. The 40 x 40 x 70 nm vault particle present in 10^4 to 10^5 copies per cell is made up of the major vault protein (MVP), and two minor components; telomerase associated protein (TEP-1) and vault poly(ADP)ribose polymerase (vPARP) [14]. MVP spontaneously forms vault particles alone. Vaults are evolutionary conserved and found from *Dictyostelium* to mammals, except in plants, yeast, *C. elegans* and *Drosophila*. Although they have been implicated in drug resistance, regulation of apoptosis, and nuclear transport their exact functions remain unknown [14]. Only a fraction of vtRNAs is associated with vault particles. Thus, vtRNAs may clearly act in functions independent of MVP and vaults. The vtRNAs are upregulated by virus infections and are involved in the inactivation of PKR signaling by these viruses to inhibit interferon responses [15]. Other than that, the functions of these abundant ncRNAs are largely unexplored.

Mammalian cells contain more than thousand RNA binding proteins. Perhaps as much as half of these do not possess known RNA-binding domains (RBDs). To identify these proteins and map the RNA-binding regions the group of Mathias W. Hentze at the EMBL in Heidelberg developed RBDmap [16]. The method is based on UV-light induced crosslinking of RNA and protein in cells. The samples are cleaved with proteases LysC and ArgC and mass spectrometry is used to identify native peptides neighboring the crosslinked peptides. The RNA-bound peptide is inferred *in silico* based on the predicted protease cleavage pattern. This way, RBDmap identifies RNA-binding domains of previously unknown RBPs. Employing RBDmap on the human liver cell line (HuH-7) Horos et al. [17] turned up a peptide from p62 suggesting

p62 is an RNA-binding protein. By sequencing RNAs that was UV-crosslinked to and co-immunopurified with p62 they could verify p62 as an RNA-binding protein using two different antibodies. The most prominent RNA species bound by p62 was *vtRNA1-1*, although p62 bound to all four human vtRNA (and the single murine vault RNA *mVR1*). The peptide from p62 that binds is from the ZZ zinc finger domain. p62 binds to loop regions of the vtRNAs in a nucleotide sequence-independent way. Perhaps higher order structure and electrostatic interactions may be important for the binding specificity? Mutation of two basic residues in the zinc finger domain nearly abolished binding to *vtRNA1-1*. Polymers of p62 show very little binding suggesting that monomers, dimers or short oligomers of p62 are the forms that bind *vtRNA1-1* (Figure 1B). Intriguingly a triple mutation in the PB1 domain that renders p62 monomeric also strongly reduce binding.

What is the biological role(s) of vtRNA binding to p62? Horos et al. [17] found that vtRNA is not a substrate for selective autophagic degradation mediated by p62. Neither siRNA-mediated knockdown (KD) or CRISPR/CAS9 knockout (KO) of p62 affected steady state levels of the vault RNAs. However, *vtRNA1-1* KD increased LC3B conjugation (generation of the lipidated form of LC3B required for autophagy) and number of LC3B puncta (read out for autophagosome formation) compared to controls. The fraction of p62 that co-localized with LC3B also increased upon vtRNA1-1 KD. Concomitantly, the levels of p62 also decreased suggesting increased degradation of p62 by autophagy. Interestingly, polymerized p62 that is degraded most efficiently by autophagy, both with and without cargo, does not bind RNA. KO of *vtRNA1-1* increased the interaction between p62 and LC3B in cells as did expression in p62 KO cells of the p62 double mutant in the zinc finger domain that does not bind vtRNAQ1-1. Starvation induces autophagy and the degradation of p62. Using a small molecule binding to the ZZ domain of p62 to induce polymerization and activate p62-dependent autophagy [18], Horos et al demonstrated strongly increased LC3B conjugation in *vtRNA1-1* KO cells. Interestingly, prolonged starvation for 6 hours reduced *vtRNA1-1* levels almost 50%. Together, these data suggest that RNA binding inhibits the function of p62 as an autophagy receptor by inhibiting its PB1 domain-mediated polymerization (Figure 1B). This way *vtRNA1-1* acts as a modulator of p62 function.

p62 is unique among the SLRs by being able to form filamentous polymers. The vtRNA interaction also seems specific to p62 as none of the other SLRs (NBR1, NDP52, TAX1BP1 or OPTN) were detected in the RBDmap screen conducted. To determine the precise interaction mode it will be necessary to solve structures of *vtRNA1-1* binding to p62. Are any of the residues

that were mutated in the PB1 domain to make it monomeric involved in the binding to RNA? Or is it the dimer, trimer or other low complexity oligomer of p62 that binds RNA and not the momomer? Since vtRNA was not the only RNA found crosslinked to p62 one may ask if p62 may have the ability to sense RNA as part of an innate immunity mechanism? p62 is acting both in the autophagic defense against invading bacteria and viruses and in regulation of inflammatory pathways [11]. As mentioned above, vtRNAs are upregulated by virus infections leading to inhibition of interferon responses [15]. Together with the present finding this may mean that p62 is also targeted by viruses indirectly via upregulation of vtRNAs.

The present study implicates the free vtRNA in regulating p62. One may still ask if MVP and vault particle have any role in regulating p62? A previous study found that the intracellular pathogen *Listeria monocytogenes* uses one of its proteins to help decorate its surface with MVP in order to escape recognition by the autophagy machinery and selective autophagy receptors such as NDP52 and p62 [19]. Selective autophagy is also acting as a cell-autonomous innate immunity pathway that protects cytosol against invasive pathogens. Intracellular pathogenic bacteria and viruses have developed various defense strategies against autophagy. Perhaps the vtRNAs and MVP have been hijacked in this evolutionary race between pathogens and the host? The fascinating findings by Horos et al. are likely to spur new interest into the biological roles played by the vtRNAs and perhaps also the enigmatic vault particle itself?

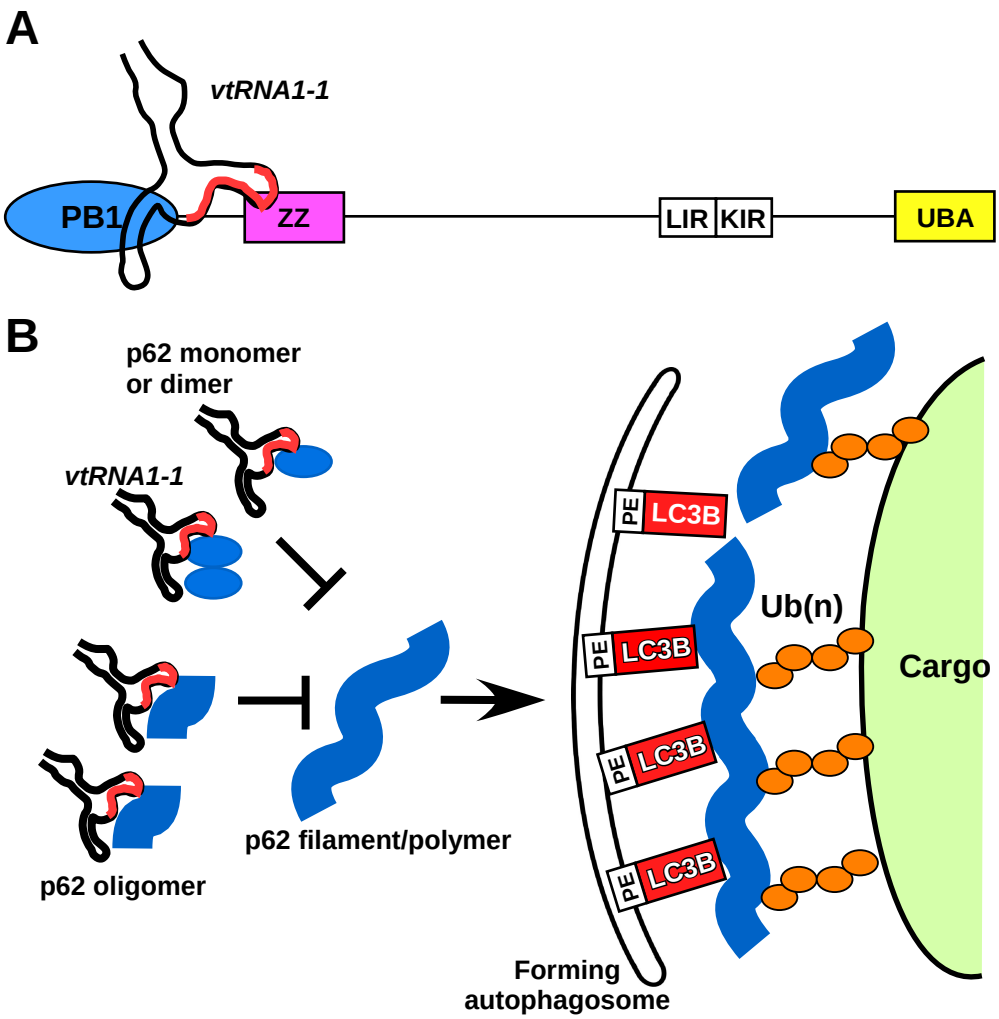
REFERENCES

1. Lamb, C.A., Yoshimori, T., and Tooze, S.A. (2013). The autophagosome: origins unknown, biogenesis complex. Nat Rev Mol Cell Biol *14*, 759-774.
2. Mizushima, N., and Komatsu, M. (2011). Autophagy: renovation of cells and tissues. Cell *147*, 728-741.
3. Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy *7*, 279-296.
4. Rogov, V., Dotsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol Cell *53*, 167-178.
5. Stolz, A., Ernst, A., and Dikic, I. (2014). Cargo recognition and trafficking in selective autophagy. Nat Cell Biol *16*, 495-501.
6. Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol *171*, 603-614.
7. Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

8. Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., Bjørkøy, G., and Johansen, T. (2003). Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. J Biol Chem *278*, 34568-34581.
9. Sanchez-Martin, P., and Komatsu, M. (2018). p62/SQSTM1 - steering the cell through health and disease. J Cell Sci *131*.
10. Ciuffa, R., Lamark, T., Tarafder, A.K., Guesdon, A., Rybina, S., Hagen, W.J., Johansen, T., and Sachse, C. (2015). The Selective Autophagy Receptor p62 Forms a Flexible Filamentous Helical Scaffold. Cell Rep *11*, 748-758.
11. Deretic, V., Saitoh, T., and Akira, S. (2013). Autophagy in infection, inflammation and immunity. Nature reviews. Immunology *13*, 722-737.
12. Itakura, E., and Mizushima, N. (2011). p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. J Cell Biol *192*, 17-27.
13. Kedersha, N.L., and Rome, L.H. (1986). Isolation and characterization of a novel ribonucleoprotein particle: large structures contain a single species of small RNA. J Cell Biol *103*, 699-709.
14. Berger, W., Steiner, E., Grusch, M., Elbling, L., and Micksche, M. (2009). Vaults and the major vault protein: novel roles in signal pathway regulation and immunity. Cell Mol Life Sci *66*, 43-61.
15. Li, F., Chen, Y., Zhang, Z., Ouyang, J., Wang, Y., Yan, R., Huang, S., Gao, G.F., Guo, G., and Chen, J.-L. (2015). Robust expression of vault RNAs induced by influenza A virus plays a critical role in suppression of PKR-mediated innate immunity. Nucleic Acids Res *43*, 10321–10337.
16. Castello, A., Fischer, B., Frese, C.K., Horos, R., Alleaume, A.M., Foehr, S., Curk, T., Krijgsveld, J., and Hentze, M.W. (2016). Comprehensive Identification of RNA-Binding Domains in Human Cells. Mol Cell *63*, 696-710.
17. Horos, R., Buscher, M., Kleinendorst, R., Alleaume, A.M., Tarafder, A.K., Schwarzl, T., Dziuba, D., Tischer, C., Zielonka, E.M., Adak, A., et al. (2019). The Small Non-coding Vault RNA1-1 Acts as a Riboregulator of Autophagy. Cell *176*, 1054-1067 e1012.
18. Cha-Molstad, H., Yu, J.E., Feng, Z., Lee, S.H., Kim, J.G., Yang, P., Han, B., Sung, K.W., Yoo, Y.D., Hwang, J., et al. (2017). p62/SQSTM1/Sequestosome-1 is an N-recognin of the N-end rule pathway which modulates autophagosome biogenesis. Nat Commun *8*, 102.
19. Dortet, L., Mostowy, S., Samba-Louaka, A., Gouin, E., Nahori, M.A., Wiemer, E.A., Dussurget, O., and Cossart, P. (2011). Recruitment of the major vault protein by InlK: a Listeria monocytogenes strategy to avoid autophagy. PLoS Pathog *7*, e1002168.

Figure 1. vtRNA act as a negative riboregulator of the selective autophagy function of p62.

vtRNA1-1 binds to the ZZ zinc finger domain and the PB1 domain of p62 (A) to inhibit polymerization of p62 either from a monomeric state or a low complexity oligomeric state (B). This will inhibit the formation of helical filaments of p62 and thereby efficient cargo binding and recruitment of the membrane of the forming autophagosome via multivalent interactions with LC3B. The region of *vtRNA1-1* likely involved in direct contacts with p62 is indicated in red. PE indicates the lipid phosphatidylethanolamine linking LC3B to the membrane. Ub(n) denotes ubiquitin chains. The domains of p62 are explained in the text.


A
vtRNA1-1
PB1
ZZ
LIR
KIR
UBA
B
p62 monomer
or dimer
vtRNA1-1
p62 oligomer
p62 filament/polymer
PE
LC3B
PE
LC3B
PE
LC3B
PE
LC3B
Ub(n)
Cargo
Forming
autophagosome