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1 **SUMMARY**

2 Vault RNAs (vtRNA) are small non-coding RNAs transcribed by RNA polymerase III found in
3 many eukaryotes. Although they have been linked to drug resistance, apoptosis and viral
4 replication, their molecular functions remain unclear. Here we show that vault RNAs directly
5 bind the autophagy receptor sequestosome-1/p62 in human and murine cells. Overexpression of
6 human *vtRNA1-1* inhibits, while its antisense LNA-mediated knock down enhances p62-
7 dependent autophagy. Starvation of cells reduces the steady state and p62-bound levels of vault
8 RNA1-1, and induces autophagy. Mechanistically, p62 mutants that fail to bind vtRNAs display
9 increased p62 homo-oligomerization and augmented interaction with autophagic effectors. Thus,
10 *vtRNA1-1* directly regulates selective autophagy by binding p62 and interference with
11 oligomerization, a critical step of p62 function. Our data uncover a striking example of the
12 potential of RNA to control protein functions directly, as previously recognized for protein-
13 protein interactions and post-translational modifications.

1 **INTRODUCTION**

2 Vault RNAs (vtRNA) have been described as small non-coding RNA components of
3 giant ribonucleoprotein particles (RNPs), termed vaults (Kedersha and Rome, 1986). Humans
4 express four vtRNA paralogs (*vtRNA1-1*, *vtRNA1-2*, *vtRNA1-3*, *vtRNA2-1*), which are 88-100nt
5 long and transcribed by RNA polymerase III. Vaults are found in a broad spectrum of eukaryotes
6 ranging from protists to mammals (Stadler et al., 2009). Although vaults can occur at 10,000 to
7 100,000 particles per cell and have been linked to cellular processes like drug resistance,
8 apoptosis and nuclear transport (Berger et al., 2009), their function remains unclear.
9 Sedimentation experiments showed that only a fraction of vtRNAs is incorporated into vaults
10 (Kickhoefer et al., 1998), suggesting that vtRNAs may have roles outside of vault RNPs.
11 Overexpression of *vtRNA1-1* was shown to be protective against apoptosis in a cellular model of
12 Epstein Barr Virus infection (Amort et al., 2015), and to favor influenza virus replication via
13 PKR deactivation (Li et al., 2015). Yet, the molecular functions of vault RNAs remain
14 undefined.

15 Macroautophagy (referred to further as autophagy) is an essential cellular process
16 responsible for the recognition, removal and degradation of intracellular components, organelles
17 and pathogens within membrane vesicles termed autophagosomes (Klionsky et al., 2016). The
18 molecular and functional details of the multiprotein complexes that regulate the formation and
19 growth of autophagosomal double-membranes have been intensively studied (for review see
20 (Yin et al., 2016)). After encompassing the cargos, autophagosomes close and fuse with
21 lysosomes, and degrade their contents to supply amino acids, lipids and nucleotides for the
22 anabolic needs of cells.

1 Autophagy was considered to be a non-selective mechanism until the discovery of
2 autophagic receptors with the ability to bind specific autophagic substrates, and bring them to the
3 forming autophagosomal membranes via interaction with Atg8-like proteins, including LC3B
4 and GABARAP (Galluzzi et al., 2017). The protein p62 (also known as sequestosome-1,
5 SQSTM1) is such an autophagic receptor with a C-terminal ubiquitin binding domain (UBA) and
6 a LC3-interaction motif (LIR) (Pankiv et al., 2007). p62 co-localizes with LC3-positive
7 autophagosomes and is itself degraded in autophagolysosomes (Pankiv et al., 2007, Sahani et al.,
8 2014, Bjorkoy et al., 2005). Thus, determination of p62 protein levels can serve as a proxy for
9 autophagic flux (Klionsky et al., 2016). p62 mostly serves in selective autophagy for the removal
10 of intracellular pathogens (Zheng et al., 2009), and the degradation of intracellular aggregates
11 marked by ubiquitin (Ub) (Pankiv et al., 2007). Amongst the autophagy receptors, p62 has the
12 distinct property to oligomerize via its N-terminal PB1 (Phox and Bem1p) domain (Ciuffa et al.,
13 2015). Oligomerization is functionally important, as it increases p62 affinity for LC3-positive
14 membranes (Wurzer et al., 2015), and is thought to help align p62 to forming autophagosomal
15 structures (Ciuffa et al., 2015). Importantly, oligomerization-deficient p62 is dysfunctional in
16 autophagy (Itakura and Mizushima, 2011). Yet, it is not well understood how the oligomerization
17 of p62 is controlled.

18 Here we uncover that the autophagy receptor p62 is an RNA-binding protein. We show
19 that p62 binds the short, non-coding RNA Pol III transcript vault RNA1-1 *in vivo* and *in vitro*.
20 We demonstrate that vault RNA1-1 inhibits p62-dependent autophagy and Ub aggregate
21 clearance, and we show that vault RNA1-1 expression is diminished when autophagy is activated
22 during starvation. Mechanistically, *vtRNA1-1* appears to inhibit p62 oligomerization,

- 1 consequently impairing the binding of p62 to the Atg8-like autophagic effectors LC3B and
- 2 GABARAP. Thus, vault RNA1-1 emerges as a riboregulator of targeted autophagy.

1 RESULTS

2 **The autophagy receptor p62/SQSTM1 is an RNA-binding protein**

3 We recently developed a method for the proteome-wide identification of RNA-interacting
4 peptides in RNA-binding proteins (RBPs), termed RBDmap (Castello et al., 2016). We
5 performed RBDmap on human hepatic HuH-7 cells and identified peptides from both known and
6 previously unknown RBPs (<http://www.hentze.embl.de/public/RBDmapHuh7/vignettes/result/>,
7 **Table S1**). A peptide mapping to the autophagy receptor p62/sequestosome-1 suggested that p62
8 interacts with RNA. While lysosome-mediated RNA degradation was described long ago
9 (reviewed in (Frankel et al., 2016)), and autophagy has been implicated in the degradation of
10 ribosomal (Kraft et al., 2008) and retrotransposon RNA (Guo et al., 2014), none of the known
11 mammalian autophagy receptors have been shown to bind RNA directly, which we therefore
12 explored further.

13 We first validated the p62-RNA interaction. HuH-7 cells were exposed to UV-C light,
14 and covalently bound RNA-binding proteins were recovered from lysates using oligo-(dT)
15 coupled beads (Castello et al., 2013). Western blotting confirmed specific p62 binding to
16 polyadenylated RNA (**Figure 1A**). In an alternative approach, we UV-C treated cells followed
17 by lysis and RNase treatment. We then immunoprecipitated (IP) p62 and used the IP for
18 radioactive labeling of RNA 5' ends with T4 polynucleotide kinase (PNK) (Baltz et al., 2012).
19 We observed a typical, smeared signal corresponding to RNA-containing complexes, which was
20 reduced to a sharper band depending on the RNase treatment, confirming the p62-crosslinked
21 entity as RNA (**Figure 1B**). Thus, p62 is a bona fide RNA-binding protein.

22 To determine the RNAs bound by p62, we performed iCLIP (Huppertz et al., 2014). We
23 sequenced RNAs that UV-crosslinked to and co-immunopurified with p62 using two

1 independent antibodies (and the respective controls, **Figure S1A and B**), identifying 165 high
2 confidence RNA hits. Amongst these are numerous polyadenylated transcripts, explaining the
3 initial identification of p62 as an RBP by RBDmap. However, RNA polymerase III transcripts
4 including tRNAs and vault RNAs emerged as the top categories specifically enriched in the IPs
5 (**Figure 1C, Table S2**). Differential crosslink site occurrence of individual RNAs isolated from
6 p62 or control IPs, respectively, placed all four human vtRNAs prominently on the p62 target list
7 (**Figure 1D, Table S3**). More detailed analysis of the iCLIP data revealed that p62 preferentially
8 interacts with looped regions of the central domains of the vtRNAs (**Figure 1E and Figure S1C**)
9 without an apparent binding motif.

10 Thus, p62 predominantly interacts with a subset of Pol III transcripts, and shows
11 preferential binding to the central domains of vault RNAs.

12

13 **Vault RNA 1-1 is the prime p62-interacting RNA**

14 To complement the results of iCLIP and to quantify RNA binding to p62 under steady-state
15 conditions, we performed p62 RIP-qPCR (RNP immunoprecipitation followed by cDNA
16 synthesis and quantitative real-time PCR) from HuH-7 cells, using a p62 antibody or control
17 IgG, respectively. We observed prominent and specific enrichment of *vtRNA1-1* relative to the
18 other vault and control RNAs (**Figure 2A, Figure S2A**). Thus, vault RNA1-1 appears to be a
19 prime interacting RNA of p62.

20 Next, we tested a series of cell lines for the interaction between p62 and *vtRNA1-1* (for
21 human HeLa cells) or the single murine vault RNA *mVR1* in hepatic Hepa1-6 and Hep-56.1D
22 cells, NIH3T3 fibroblasts and monocytic RAW264.7 cells by RIP-qPCR. We confirmed p62
23 binding to *vtRNA1-1* in HeLa cells (**Figure 2B**), and to *mVR1* in the different mouse cell lines

1 (Figure 2C). Thus, the binding of p62 to vault RNAs is conserved between human and mouse
2 cells.

3 We next investigated the interaction of p62 and *vtRNAI-1* *in vitro* using an
4 electrophoretic mobility shift assay (EMSA) with purified recombinant protein and radiolabeled
5 *in vitro*-transcribed RNA. Since native p62 tends to oligomerize spontaneously *in vitro* (Ciuffa et
6 al., 2015), we tagged the N-terminus with MBP (maltose binding protein; 40 kDa) to prevent
7 spontaneous oligomerization and to facilitate native gel electrophoresis. We observed that MBP-
8 p62 forms complexes with *vtRNAI-1* (Figure S2B). To test whether the interaction between
9 MBP-p62 and *vtRNAI-1* is specific, we used unlabeled *vtRNAI-1* as specific, and the iron-
10 responsive element (IRE) from human *FTH1* mRNA or a mixture of bacterial tRNAs,
11 respectively, as non-specific competitors. Unlabeled *vtRNAI-1* effectively competes with the
12 labeled *vtRNAI-1* from p62 ($K_i=200.7$ nM) as compared to IRE ($K_i=856.6$ nM) or bacterial
13 tRNAs ($K_i=1531$ nM) (Figure 2D). We next determined the apparent K_d of p62-*vtRNAI-1*
14 complex formation in the presence of non-specific competitor and determined it to be 546.3
15 ± 106.6 nM, while the MBP tag alone did not yield any shifted complexes (Figure 2E and
16 Figure S2C). These findings confirm that p62 specifically binds *vtRNAI-1* *in vitro*,
17 complementing our *in cellulo* data (Figure 1 and Figure 2A-C).

18

19 ***vtRNAI-1* inhibits p62-mediated autophagy and Ub aggregate clearance**

20 To uncover the functional significance of the *vtRNAI-1*/p62 interaction, we first tested whether
21 p62 might mediate the lysosomal degradation of vault RNAs. However, no difference in the
22 steady-state levels of the vtRNAs could be seen in cells depleted of p62 by siRNA treatment

1 (Figure 2F and G) or in p62 KO cells (Figure S2D), respectively. Thus, neither acute nor
2 sustained p62 deficiency affects the steady state levels of the vault RNAs.

3 Since p62 binding does not overtly affect the expression of its major class of RNA
4 binders, we next explored whether *vtRNAI-1* conversely might affect p62 function in autophagy.
5 We knocked-down *vtRNAI-1* with antisense LNAs, and monitored autophagic flux by assessing
6 two parameters, LC3B conjugation from LC3B-I to LC3B-II during autophagosome assembly,
7 and p62 levels reflecting its autolysosomal degradation. Interestingly, *vtRNAI-1* knockdown
8 (KD) (Figure S3A and B) stimulated LC3B conjugation (compare the ratios of LC3BII over
9 LC3BI, Figure 3A and B), suggesting increased autophagic flux. In keeping with this notion, we
10 observed a dose-dependent decrease in p62 levels (Figure 3A). To examine whether this effect is
11 dependent on p62, we concurrently removed p62 and *vtRNAI-1*. We observed that removal of
12 p62 partially restores the LC3B conjugation ratio after *vtRNAI-1* depletion compared to controls
13 (Figure 3C, compare lanes 3 and 4 with 1 and 2). Treatment with bafilomycin A₁ (BafA), an
14 inhibitor of autophagosome-lysosome fusion and lysosomal degradation that leads to the
15 accumulation of autophagosomes (Klionsky et al., 2016), restored the LC3B conjugation ratio
16 and led to the expected accumulation of total LC3B in *vtRNAI-1* KD cells (Figure 3C, lanes 5
17 and 6). In addition, immunofluorescence microscopy of cells depleted for *vtRNAI-1* revealed an
18 increased number of LC3B punctae compared to controls (Figure 3D and E), and the fraction of
19 p62 co-localizing with LC3B increased upon *vtRNAI-1* KD (Figure 3D and F), providing
20 independent evidence for the stimulation of autophagic flux upon *vtRNAI-1* KD. We also
21 corroborated these results by assessing the expression levels of another Atg8-like protein,
22 GABARAP (Figure S3C).

1 The phosphorylation of the mTORC1 targets ULK1 or 4E-BP1 was not noticeably
2 affected by *vtRNA1-1* KD, suggesting that the effect on autophagy is not the result of decreased
3 mTORC1 activity (**Figure S3C**). Thus, multiple lines of evidence show that sequestration of
4 *vtRNA1-1* induces autophagic flux in a p62-dependent way.

5 We also tested whether the increased expression of *vtRNA1-1* by transfection affects
6 autophagy. Elevation of *vtRNA1-1* levels suppresses LC3B conjugation and yields a concomitant
7 accumulation of p62 in a dose-dependent manner (**Figure 3G and S3D**), both suggesting
8 decreased autophagic flux. Overexpression of other vault RNAs did not consistently affect the
9 LC3B conjugation ratio (**Figure 3H and S3D**). BafA treatment restored the LC3B conjugation
10 ratio in cells overexpressing *vtRNA1-1* (**Figure 3H**), suggesting that *vtRNA1-1* overexpression
11 does not disturb LC3 conjugation per se, but rather restricts autophagic flux.

12 Lastly, we investigated whether p62-dependent Ub aggregate clearance is affected by
13 *vtRNA1-1*. We treated HuH-7 wt and *vtRNA1-1*KO cells (**Figure S6B-D**), respectively, with the
14 proteasome inhibitor MG132 and assessed Ub-positive aggregate levels by Western blotting. In
15 cells lacking *vtRNA1-1*, aggregate accumulation is significantly reduced when p62 wt protein is
16 present (**Figure 3I**). However, the p62 S407A variant that does not undergo ULK1-dependent
17 phosphorylation and activation of the UBA domain (Lim et al., 2015) fails to mediate better
18 aggregate clearance in *vtRNA1-1*KO cells (**Figure 3I**), showing that the observed difference
19 between wt and *vtRNA1-1*KO cells in aggregate clearance is mediated by p62.

20 Taken together, experimental up- or down-modulation of *vtRNA1-1* levels inversely
21 affect p62-dependent autophagy and p62-dependent aggregate clearance.

22
23 ***vtRNA1-1* levels and p62 RNA binding are dynamically regulated by starvation**

1 Next, we explored whether the regulatory potential of the p62 and *vtRNAI-1* interaction is used
2 physiologically. Amino acid and serum starvation induce autophagy, where p62 supports the
3 increased autophagic flux (Bjorkoy et al., 2005) and undergoes degradation itself (Sahani et al.,
4 2014). HuH-7 cells cultivated in minimal media lacking amino acid and serum respond with a
5 major drop in *vtRNAI-1* levels after 6 hours of starvation (**Figure 4A and S4A**). This decrease in
6 *vtRNAI-1* levels is not a result of autophagic co-degradation with p62, because neither the KD of
7 p62 nor BafA treatment significantly restored *vtRNAI-1* levels (**Figure 4A**). Interestingly, the
8 specific, starvation-induced decrease in *vtRNAI-1* levels is also observed when the RNA is
9 expressed from a heterologous (H1) promoter (**Figure S4B**), suggesting that the regulatory
10 information is contained within the gene body of *vtRNAI-1*. Whether regulation is exerted
11 transcriptionally via the internal Pol III promoter sequences and/or post-transcriptionally at the
12 RNA level remains to be determined. We also assessed a starvation time course of the levels of
13 several tRNAs, which further attests to the specificity of the response of *vtRNAI-1* (**Figure**
14 **S4C**).

15 RNA binding of p62 follows the diminished *vtRNAI-1* expression in the course of
16 starvation (**Figure 4B and C**). Cells starved in the presence of BafA, which prevents p62
17 degradation, show that bulk autophagy decreases the fraction of RNA-bound p62 relative to total
18 p62. BafA treatment per se in complete medium does not change the RNA binding properties of
19 p62 (**Figure S4D**), suggesting that the effect on RNA binding is not driven by p62 protein levels.
20 Therefore, p62 active in autophagy and destined for lysosomal degradation apparently does not
21 bind RNA, in keeping with the notion that RNA binding inhibits p62 function in autophagy.

22 Of note, the starvation-induced decrease in *vtRNAI-1* levels correlates with a decrease in
23 the fraction of RNA-bound p62 (**Figure S4E**). Importantly, starvation reduces the interaction of

1 p62 and *vtRNA1-1* (**Figure 4D**), and removal of *vtRNA1-1* by LNA KD prior to starvation further
2 potentiates interaction of p62 with LC3B (**Figure 4E and F**). It thus appears that at least part of
3 the starvation-mediated activation of autophagy includes the removal of inhibitory *vtRNA1-1*
4 from p62.

5 Collectively, these data show that *vtRNA1-1* levels and the interaction of *vtRNA1-1* with
6 p62 are physiologically regulated by amino acid and serum starvation.

7

8 **p62 binds *vtRNA1-1* primarily via its zinc finger domain**

9 To study the mechanism of how *vtRNA1-1* inhibits p62 function, we wanted to generate an RNA
10 binding-deficient mutant of p62. p62 possesses several domains with assigned functions and
11 interaction partners, but no classical RNA-binding motif (**Figure 5A**). To identify the RNA-
12 binding region of p62, we utilized our RBDmap data and inspected the neighboring region of the
13 RBDpep (Castello et al., 2017) that we had identified within the ZZ domain (**Figure 5A and B**).
14 We found that substitution of the conserved K141 within the ZZ domain of p62 by alanine
15 appeared to decrease RNA binding (**Figure S5A**), but complex formation between the
16 endogenous wild type p62 with the stably expressed p62 K141A variant interfered with the
17 analysis. We therefore depleted endogenous p62 from HuH-7 cells by RNAi, and transfected
18 tagged p62 variants, resistant to the siRNAs by synonymous mutations. As expected, siRNA KD
19 of p62 effectively removed its radiolabeling signal (**Figure 5C**, lanes 1, 2). The p62 K141A
20 variant displays strongly reduced RNA binding compared to the wild type, which is further
21 diminished in the R139/K141/AA variant (referred to as p62 RK/A, **Figure 5C**, lanes 3-5). The
22 shift of tagged p62 by 2.5 kDa, both on the PNK assay and the Western blot, also provides
23 evidence that RNA binding of p62 is direct.

1 Next, we generated HuH-7 p62 KO clones using CRISPR/Cas9 gene inactivation (**Figure**
2 **S5B and S5C**), and confirmed the decreased RNA binding of p62 RK/A in a p62 null
3 background (**Figure S5D**). To investigate whether oligomerization of p62 may affect RNA
4 binding, we expressed an oligomerization-deficient variant of p62 (Lamark et al., 2003), a triple
5 mutation R21A, D69A, D73A, referred to as p62 PB1m. p62 PB1m also showed diminished
6 RNA binding (**Figure 5D**). Even if RNA binding of p62 PB1m is less compromised than that of
7 the p62 RK/A variant (**Figure S5D**), its diminished RNA interaction compared to the wild type
8 protein suggests that the PB1 domain contributes to RNA binding.

9 To quantify the RNA binding of the p62 variants to vault RNA, we expressed HA-tagged
10 p62 variants in HuH-7 p62 KO cells (**Figure S5E**), and IP-ed these followed by qPCR. As is the
11 case for endogenous p62 protein, tagged wt p62 strongly interacts with *vtRNA1-1*, while the p62
12 RK/A mutant and the PB1m variant display strongly reduced *vtRNA1-1* binding (**Figure 5E**),
13 suggesting that both domains contribute to full vault RNA1-1 binding.

14

15 ***vtRNA1-1* regulates the interaction of p62 with Atg8-like proteins**

16 With the RK/A mutant available, we could address the mechanism of *vtRNA1-1* function. We
17 first tested whether RNA binding affects interactions of p62 with the Atg8-like proteins LC3B
18 and GABARAP by co-immunoprecipitation. Both proteins co-purify with p62 and display the
19 expected increase in interaction when autophagy is induced by starvation (**Figure S6A**, compare
20 the lanes 2 and 4). Following KD of *vtRNA1-1* p62 displays increased interaction with LC3B
21 (**Figure 6A**), corroborating data obtained by IF microscopy. We also prepared HuH-7 *vtRNA1-1*
22 KO cell lines by CRISPR/Cas9 gene deletion. We deleted the *vtRNA1-1* transcription unit
23 (**Figure S6B**) and verified the specificity of the deletion by genomic PCR (**Figure S6C**). We

1 confirmed the deletion of *vtRNA1-1* using qRT-PCR on total RNA prepared from the *vtRNA1-*
2 1KO clones (**Figure S6D**). We also observed increased interaction between p62 and LC3B in
3 cells deleted for the *vtRNA1-1* locus (**Figure 6B and S6E**). These data suggest that *vtRNA1-1*
4 regulates the interaction of p62 with LC3B.

5 To test this further, we conducted co-IPs in HuH-7 p62 KO cells reconstituted with the
6 p62 wt, RK/A or PB1m variants, respectively. Although the interaction with LC3B is not
7 significantly changed, the p62 RK/A variant shows increased complex formation with the Atg8-
8 like protein GABARAB compared to wt p62 (**Figure 6C and D**). As a control, the interaction
9 with the PB1 domain-binding protein NBR1 is unchanged between the p62 wt and RK/A variant
10 (**Figure 6C and D**). As expected, the p62 PB1m variant displays decreased binding to LC3,
11 GABARAP and NBR1 (**Figure 6C and D**), reflecting the requirement for p62 oligomerization
12 for efficient interaction with the autophagic machinery (Itakura and Mizushima, 2011).

13 Since removal of vault RNA1-1 or rendering p62 RNA binding-deficient both increase
14 p62's engagement with LC3B and GABARAP, the data suggest that vault RNA1-1 binding
15 affects the interactions of p62 with Atg8-like proteins.

16

17 **p62 RNA binding inversely correlates with its oligomerization state *in cellulo***

18 We then wanted to determine whether the above regulation was a direct or an indirect effect of
19 *vtRNA* binding to p62. Further analysis of the data shown in **Figure 5D** revealed that UV-C
20 exposure of cells to crosslink RNAs to p62 also induces a laddering of the protein on a Western
21 blot that persists through denaturing SDS-PAGE (lower panel). While p62 oligomerization has
22 been studied extensively *in vitro* (Wurzer et al., 2015, Ciuffa et al., 2015, Zaffagnini et al.,
23 2018), little is known about the precise oligomeric state in the cellular context (Carroll et al.,

1 2018). Since the oligomerization-deficient PB1m variant completely lacks this laddering (**Figure**
2 **5D**), serendipitously, we appear to have found direct biochemical evidence for p62
3 oligomerization in cells and a simple assay for this process. Although UV-treatment typically
4 does not induce protein-protein crosslinks (Pashev et al., 1991, Suchanek et al., 2005), the local
5 topology of p62 oligomers may favor reactive oxygen species-induced covalent bond formation
6 between the p62 subunits (Donohue et al., 2014).

7 To evaluate this laddering assay in a physiological context, HuH-7 cells were starved for
8 2 hours before UV crosslinking, and cell lysates were assessed by the laddering assay and for
9 RNA binding by PNK assay. As expected, starvation reduces p62 RNA binding (**Figure 7A**,
10 upper panel) and, importantly, increases the laddering of p62 visualized by Western blotting
11 (**Figure 7A**, lower panel, compare lane 5 with lane 8). The RK/A variant strikingly displays this
12 enhanced laddering even under basal culture conditions (lane 6), which is not increased further
13 by starvation (lane 9). Thus, the RK/A mutant oligomerizes under basal conditions like wt p62
14 after starvation, strongly suggesting that *vtRNA1-1* interferes with p62 oligomerization. We also
15 note that the p62 oligomers display profoundly reduced RNA binding compared to monomeric
16 p62 (**Figure 7A and Figure 5D**, compare the upper and the lower panels), which indicates that
17 p62 monomers or low complexity oligomers represent the RNA-binding forms of p62.

18 Finally, we used XIE62-1004-A (XIE, in short) (**Figure 7B**), a small molecule that binds
19 to the ZZ-domain of p62, inducing p62 oligomerization and activating p62-dependent autophagy
20 (Cha-Molstad et al., 2017). XIE thus affords an opportunity to directly test p62-dependent
21 autophagy in addition to starvation-induced autophagy, which is more pleiotropic. HuH-7 wt or
22 *vtRNA1-1*KO cells were treated with XIE, or solvent control, and autophagy was assessed by
23 LC3B conjugation. As expected, the *vtRNA1-1*KO cells display increased LC3B conjugation

1 compared to their wt counterparts already under control conditions (**Figure 7C**). In keeping with
2 its original description (Cha-Molstad et al., 2017), XIE treatment induces LC3B lipidation in
3 control cells, and its effect is significantly enhanced in the *vtRNA1-1*KO cells (**Figure 7D**).
4 Thus, the cellular response to this p62-specific activator of autophagy is strongly affected by
5 *vtRNA1-1* (**Figure 7C, D**), meeting a prediction for a riboregulator of p62 function.

6 7 **DISCUSSION**

8 Our experiments show that the small non-coding RNA *vtRNA1-1* contributes to the regulation of
9 autophagic flux by direct interaction with the autophagy receptor protein p62/sequestosome-1.
10 Thus, we can assign a mechanism-based function to the first member of the family of vault
11 RNAs, which was described more than 30 years ago (Kedersha and Rome, 1986). Our data also
12 show that a small non-coding RNA can regulate a biological process by directly affecting the
13 function of a protein, which is typically expected of protein-protein interactions.

14 15 **Vault RNAs, p62 and the regulation of autophagy**

16 Autophagy is a highly regulated process, especially at the level of initiation (Sica et al., 2015).
17 We show that starvation triggers a reduction of the steady state levels of *vtRNA1-1* (**Figure 4A**
18 **and S4A-C**) and diminishes *vtRNA1-1* binding to p62 (**Figure 4B, C and D**). Gain- and loss-of-
19 function experiments demonstrate that *vtRNA1-1* negatively controls p62 in autophagy (**Figure**
20 **3**). This conclusion is corroborated by analyses of the RNA binding-deficient RK/A mutant of
21 p62, which shows characteristics of activated p62 even under non-starvation conditions (**Figure**
22 **6D, E and Figure 7A**). The starvation-induced drop in *vtRNA1-1* levels thus contributes to the
23 physiological, p62-mediated increase in autophagic flux.

1 Future work will address how starvation signals control *vtRNA1-1* expression.
2 Conceivably, both transcriptional and/or post-transcriptional mechanisms could contribute to this
3 control. Pol III transcription is controlled by the repressor protein MAF1, which in turn is
4 inhibited by phosphorylation via mTORC1 (Orioli et al., 2016). Whether MAF1-inhibition may
5 be selective for *vtRNA1-1* transcription compared to the other vault RNA genes remains to be
6 investigated. Alternatively, cytosine-5 methylation at position C69 of *vtRNA1-1* was shown to
7 promote processing of *vtRNA1-1* into small svRNAs (Hussain et al., 2013), effectively reducing
8 the pool of *vtRNA1-1*. It will be interesting to investigate *vtRNA1-1* processing in the context of
9 starvation. In any case, since *vtRNA1-1* expressed from a heterologous promoter is specifically
10 and strongly regulated by starvation (**Figure S4B**), the cis-regulatory information is embedded
11 within the DNA and/or RNA sequence of *vtRNA1-1* itself.

12 While our experiments explore starvation, proteasome inhibition and pharmacological
13 induction by XIE62-1004-A as triggers of p62-dependent autophagy, we have not tested other
14 conditions that affect autophagy so far. It will be informative to investigate whether e.g. p62-
15 dependent xenophagy (Galluzzi et al., 2017) is affected by *vtRNA1-1*. It will also be relevant to
16 investigate possible functions of the other RNAs that bind p62, including the three additional
17 human vault RNAs. Upregulation of a *vtRNA2-1* fragment (svtRNA2-1a) was found to mark
18 early stages of Parkinson disease (PD) and to induce neuronal dysfunction (Minones-Moyano et
19 al., 2013). Intracellular protein inclusions that are typical of PD commonly contain p62
20 (Zatloukal et al., 2002), and it is possible that RNA binding by p62 could play a role in PD.

21 Vault RNAs and vault particles are highly expressed in cells and processes that are
22 strongly linked to autophagy. These include *Dictyostelium discoideum* (Vasu et al., 1993) and
23 macrophages (Izquierdo et al., 1996), highly autophagic/phagocytic cells, and several multi-drug

1 resistant cancer cells (Berger et al., 2009). Epstein-Barr virus (EBV) potently induces *vtRNAI-1*
2 expression, which potentiates viral replication and protects infected cells from apoptosis (Amort
3 et al., 2015, Nandy et al., 2009). Induced expression of *vtRNAI-1* also increases viral titers of
4 influenza A in cell culture models and *in vivo* (Li et al., 2015). Interestingly, both EBV and
5 influenza A infections can block autophagosome maturation and turnover, respectively (Jackson,
6 2015). Whether p62 is involved in these responses remains to be investigated. Since p62 is
7 involved in other cellular processes including oxidative stress response (Komatsu et al., 2010),
8 mTORC1 regulation (Duran et al., 2011), or NF- κ B signaling (Duran et al., 2004), it will also be
9 meaningful to explore the role of RNA binding in these settings.

10

11 **A mechanistic model for p62 regulation by *vtRNAI-1***

12 Mutagenesis experiments based on RBDmap data identified a critical role of the ZZ
13 domain of p62 in RNA binding (**Figure 5**). Interestingly, a PB1 domain mutant of p62 bearing
14 an intact ZZ domain also displays compromised RNA-binding activity (**Figure 5D, E**),
15 suggesting that the PB1 domain may also interact with RNA, or that oligomerization is required
16 for RNA binding. In favor of the former explanation, PNK assays show strong RNA binding for
17 monomeric and low complexity oligomeric p62, but hardly any for higher complexity multimers
18 (**Figure 5D and Figure 7**). The results of EMSA assays support this interpretation (**Figure S2B**
19 **and C**). We suggest that *vtRNAI-1* binds to monomeric, dimeric or low complexity oligomeric
20 p62 and interferes with subsequent p62 multimerization. This interference is evident from the
21 increased oligomerization of p62 when *vtRNAI-1* expression is diminished during starvation
22 (**Figure 7A**), when *vtRNAI-1* binding is impaired due to the RK/A mutation (**Figure 7A**), and
23 when the oligomerization-inducing ligand XIE is used (**Figure 7C, D**). Therefore, we propose

1 that *vtRNA1-1* interferes with p62 multimerization, which is in turn required for the localization
2 of p62 to autophagosomes and its interaction with the Atg8-like proteins LC3B and GABARAP
3 (Itakura and Mizushima, 2011).

4 We have no evidence to believe that *vtRNA1-1* acts as a dominant “on-off switch” of p62
5 activity. We rather envisage *vtRNA1-1* to function as a “p62 activity dial” that is itself regulated
6 by biological cues such as starvation, and that modulates the activity of p62 as an autophagy
7 receptor by controlling its multimerization. While our data implicate p62 as the direct and
8 primary target of *vtRNA1-1* in autophagy, we cannot exclude additional regulatory roles of
9 *vtRNA1-1* in this process. In fact, knock-down of p62 by RNAi did not fully restore the LC3B
10 lipidation ratio following *vtRNA1-1* knock-down (**Figure 3C**), reflecting the incomplete removal
11 of p62 by RNAi, and/or implicating additional autophagic targets of *vtRNA1-1*.

12 We currently do not know the precise features of *vtRNA1-1* that determine its specific
13 binding to p62. Our iCLIP data indicate that p62 primarily binds to the central regions of the
14 different human vault RNAs (**Figure 1E and S1C**). We also found that the single murine *mVRI*
15 *RNA* binds p62 in several cell lines that we tested (**Figure 2C**). We have not yet recognized a
16 shared feature of *vtRNA1-1* and *mRV1* RNAs that distinguishes these from the other three human
17 vault RNAs. While iCLIP showed that p62 interacts with all four human vault RNA paralogs
18 (**Figure 1D and E**), the RIP-qPCR data show that *vtRNA1-1* is the predominant p62 target
19 (**Figure 2A and 5E**). We hypothesize that a cellular factor may contribute to the selectivity of
20 p62 for *vtRNA1-1* under steady state conditions. Mutagenesis experiments and high resolution
21 structural analyses are expected to shed light on the exact mode of interaction between p62 and
22 *vtRNA1-1*, with implications for both the question of determinants of binding specificity and the
23 mechanistic model discussed above.

1 It will also be important to determine whether *vtRNA1-1* binds to and inhibits p62 as a
2 ‘free’ RNA or as part of the vault particle. A large fraction of vault RNAs is not associated with
3 the vault particle in different cell types (Kickhoefer et al., 1998), and both possibilities therefore
4 need to be considered. At least in vitro, non-particle associated *vtRNA1-1* can bind to p62.
5 Moreover, p62 co-immunoprecipitation experiments that yield *vtRNA1-1* and the Atg8-like
6 proteins (**Figure 6**), have failed to identify the major vault protein (data not shown) as an
7 interactor. While this indirect evidence points to the free, non-particle associated RNA as the
8 regulator of p62, the question remains to be answered formally.

9

10 **Beyond p62 and vault RNA**

11 Recent work from several laboratories identified hundreds of proteins that had previously
12 escaped detection as RNA-binding proteins (reviewed in (Hentze et al., 2018)). Many of these
13 RBPs lack classical RNA-binding domains and assigned functions in RNA biology; we
14 collectively refer to these as enigmRBPs (Beckmann et al., 2015). The intensively studied
15 p62/sequestosome-1 is an excellent example of such an enigmRBP, whose RNA-binding
16 properties was previously undiscovered.

17 The regulation of protein function by an RNA, as seen for p62/*vtRNA1-1*, could represent
18 a general principle of biological control, complementing well-recognized forms of regulation
19 such as by protein-protein interactions and post-translational modifications. In bacteria, 6S
20 RNAs are 180-200 nucleotide-long RNAs that bind and regulate RNA polymerase activity
21 during the stationary growth phase (Wassarman and Storz, 2000). In mammalian cells, innate
22 immune effector proteins such as Toll-like receptors or the protein kinase PKR sense the
23 presence of viral RNAs, leading to their activation (Kato et al., 2011, Meurs et al., 1990).

1 *vtRNAI-1* acts as a physiological “riboregulator” that controls a cellular process, such as
2 autophagy, by direct binding to the autophagy receptor p62. We predict that the regulatory
3 principle employed by p62/*vtRNAI-1* will be found to be more widespread in biology, especially
4 amongst enigmRBPs.

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13
14 **AUTHORS CONTRIBUTIONS**

15 R.H. and M.W.H. designed the project; R.H., M.B., R.K. and A-M.A. performed most of the
16 experiments; A.K.T. performed protein in vitro purifications; T.S. analyzed the sequencing data;
17 D.D. synthesized XIE62-1004-A; C.T. analyzed the imaging data; E.Z. set up
18 immunofluorescence microscopy; A.A. performed interactome capture; A.C. performed
19 RBDmap; R.H., M.B., W.H., C.S. and M.W.H. performed the data analysis; R.H. and M.W.H.
20 wrote the manuscript with input from all authors.

21
22 **DECLARATION OF INTEREST**

23 The authors declare no competing financial interests. Aspects of this work have been included in
24 patent application #EP3263704A1.

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4
5

1 **MAIN FIGURE LEGENDS**

2 **Figure 1. The autophagy receptor p62 is an RNA-binding protein.**

3 (A) Western blot analysis of input and eluate samples from interactome capture experiment.
4 TDP43 serves as a positive control for RNA binding, whereas actin serves as negative control.
5 (B) Lysates from UV-treated or control cells were treated with dilutions of RNaseA and used for
6 immunoprecipitation followed by radioactive labeling (upper panel) and Western blotting (lower
7 panel). (C) Log₂ odds ratios of the enrichment of different RNA classes in p62 IPs over the
8 control IPs (Fisher exact test, Benjamini-Hochberg (BH) adjusted $P < 0.05$). (D) Volcano plot of
9 differential crosslink site (CS) occurrences; each dot corresponds to a genomic region (exons,
10 introns), black coloring indicates significant enrichment in p62 IPs (BH adjusted $P < 0.05$). The
11 data were normalized for background and CS enrichment in p62 IPs over controls was tested
12 with DESeq2. Open circles indicate vault RNAs. (E) Predicted RNA secondary structures of
13 vtRNAs. Mean CS count values in p62 IPs are shown by the indicated color code. See also
14 Figure S1.

15 **Figure 2. p62 binds vault RNA1-1.**

16 (A) Differential enrichment of RNAs in native p62 IPs from HuH-7 cells as measured by qRT-
17 PCR. RNA enrichments were normalized by *5S rRNA* and compared between p62 and IgG (=1)
18 IPs. Significant differences are indicated by p values. (B) RNA enrichment in p62 IPs compared
19 to the respective input levels. (C) A representative native p62 IP followed by cDNA synthesis
20 and qRT-PCR measurement. RNA enrichment in p62 IPs compared to the respective input
21 levels. (D) Competition electromobility shift assay (EMSA) using 10nM of radiolabeled *vtRNA1-*
22 *1*, 1500 nM of MBP-p62 protein and unlabeled competitor RNAs as indicated on the plot; n=3,
23 (E) Determination of RNP complex K_d using EMSA assay with radiolabeled *vtRNA1-1* and

1 MBP-p62 or MBP tag only. n=3 (F) Cells were transfected with indicated siRNAs and lysed
2 after 48 hours. Total protein lysates were analyzed by Western blotting; a representative image is
3 shown. (G) Total RNA extracted from cells as in (F) was analyzed by Northern blotting. Probe
4 signals recognizing respective vtRNAs were quantified and normalized by 5S rRNA. Data shown
5 are RNA levels in p62 siRNA-treated cells relative to respective siRNA control. See also Figure
6 S2.

7 **Figure 3. *vtRNA1-1* regulates p62-dependent autophagy and aggregates clearance.**

8 (A) HuH-7 cells were transfected with a control LNA oligo, or with increasing amounts of an
9 LNA oligo targeting *vtRNA1-1*, and lysed after 48 hours. Lysates were analyzed by Western
10 blotting with the indicated antibodies. (B) Quantification of LC3 conjugation from independent
11 experiments using 50 nM LNA transfections. (C) Cells were transfected with the indicated LNAs
12 and control or p62 siRNA, and incubated for 48 hours. Where indicated, cells were treated with
13 BafA. Lysates were analyzed by Western blotting with the indicated antibodies. (D)
14 Representative IF images of cells treated as in (B). LC3B cyan; p62 magenta; DNA blue,
15 p62/LC3B co-localization white. Scale bar represents 20 μ M. (E,F) Quantitative image analysis
16 of 2 independent experiments as in (D), n indicates the number of cells analyzed. (G) HuH-7
17 cells were transfected with empty vector (ctrl) or plasmid encoding *vtRNA1-1*, and lysed after 24
18 hours. Lysates were analyzed by Western blot with the indicated antibodies. (H) Cells were
19 transfected with indicated vtRNA-encoding plasmids, vehicle treated or treated with BafA at 100
20 nM for 5 hours, and then lysed. Lysates were analyzed by Western blotting and images of LC3B
21 staining were quantified. (I) HuH-7 wt or *vtRNA1-1*KO cells were treated with siRNA against
22 p62, and p62 wt or p62 S407A, respectively, was expressed by transfection of siRNA-resistant
23 cDNA expression vectors. Cells were then vehicle treated or treated with 0.25 μ M of MG132

1 overnight, lysed and analyzed by Western blotting. Quantification of Western blots for anti-
2 ubiquitin normalized by GAPDH expression is plotted as fold changes compared to HuH-7 wt
3 vehicle control sample. n=3. See also Figure S3.

4 **Figure 4. Starvation reduces p62 RNA binding and *vtRNAI-1* expression.**

5 (A) HuH-7 cells were treated with a control siRNA or p62 siRNA for 48 hours, followed by
6 starvation for 6 hours. Total RNA was isolated and analyzed by Northern blotting;
7 phosphorimages were quantified and data were plotted as relative to non-starved samples.
8 Significant differences to respective non-starved samples are indicated by *p*-values. n=6 (B)
9 Cells were starved in medium containing solvent control or BafA at 100 nM for the indicated
10 time, 254nm UV-C light exposed and lysed. Lysates were used for p62 IP and RNA
11 radiolabeling assay. After SDS-PAGE and transfer, the membrane was exposed overnight on
12 film and used subsequently for Western blotting. (C) Phosphorimages and Western blots of 3
13 independent replicates as described in (B) were used for quantification. Significant differences to
14 the 0 hour sample are indicated by *p*-values. (D) Enrichment of *vtRNAI-1* on native p62 IPs from
15 cells cultured in complete media or starvation media for 2 hours. (E) Cells were transfected with
16 the indicated LNAs, incubated for 48 hours, and then starved for 2 hours. Representative IF
17 images of cells are shown; LC3B cyan; p62 magenta; DNA blue; p62/LC3B co-localization
18 white. Scale bar represents 20 μ M. (F) Image analysis and quantification of cells as in (E) using
19 2 independent experiments; n indicates the number of cells analyzed. See also Figure S4.

20 **Figure 5. p62 binds *vtRNAI-1* primarily via its zinc finger domain**

21 (A) RBDmap-enriched peptide (red) and a peptide not enriched in the RNA-bound fraction
22 (blue) positioned on the p62 protein. The X-axis is scaled to protein length. A scheme of the p62
23 domain architecture is drawn below. NLS, nuclear localization signal; NES, nuclear export

1 signal, LIR, LC3 interaction region; KIR, Keap1 interaction region; UBA, ubiquitin associated
2 domain. (B) Human p62 protein region between AA 101-163. Orthologous proteins are aligned
3 below; the dotted region represents the insertion of a longer peptide. The RBDmap-enriched
4 peptide (FDR 1%) is shaded in grey. *Hs*, *Homo sapiens*, *Mm*, *Mus musculus*, *Xl*, *Xenopus laevis*,
5 *Dr*, *Dario rerio*. (C) HuH-7 cells treated with the indicated siRNA were transfected with empty
6 vector (ev) or p62 wt and variants (K141A, RK/A refers to the R139/K141-AA) cDNAs resistant
7 to RNAi. Cells were exposed to 254nm UV-C light, lysed and used for IP followed by the
8 radioactive labeling of RNAs and Western blotting. (D) HuH-7 p62KO cells were transfected
9 with empty vector (ev) or p62 wt and variants (RK/A; PB1m refers to triple mutation in PB1
10 domain). Cells were exposed to 254nm UV-C light, lysed and used for IP followed by the
11 radioactive labeling of RNAs and Western blotting. (E) Differential enrichment of indicated
12 RNAs on HA IPs from p62 KO cells expressing transfected p62 variants. RNA enrichment levels
13 measured by qRT-PCR normalized to 5S rRNA were compared to ev enrichment (=1).
14 Significant differences between the p62 variants are indicated by *p* values. See also Figure S5.

15

16 **Figure 6. *vtRNAI-1* affects p62 interactions with Atg8-like proteins**

17 (A) HuH-7 cells were transfected with the ctrl LNA or LNA targeting *vtRNAI-1*, cultured for 48
18 hours, and then treated with 100nM BafA for 5 hours. Cells were subjected to DSP protein-
19 protein crosslinking, lysed, and used for p62 IPs. Eluates from IPs were analyzed by Western
20 blotting and quantified. (B) p62 co-IP analysis of controls and *vtRNAI-1*KO cells, performed as
21 in (A). (C) HuH-7 p62KO cells were transfected with the indicated p62 variants, cultured for 24
22 hours, and then treated with 100nM BafA for 5 hours. Cells were subjected to DSP protein-
23 protein crosslinking, lysed and used for IPs. Eluates from IP were analyzed by Western blotting.

1 * indicates a non-specific band. **(D)** Quantification of pull down efficiency of replicate
2 experiments as shown in (C). See also Figure S6.

3

4 **Figure 7. Mechanism of *vtRNA1-1* function as a riboregulator of autophagy**

5 **(A)** HuH-7 p62KO cells were transfected with the indicated p62 variants, cultured for 24 hours,
6 and then cultured in starvation media for 2 hours or treated with 100nM BafA in complete media
7 for 5 hours. Cells were exposed to 254nm UV-C light, lysed and used for IP followed by the
8 radioactive labeling of RNAs and Western blotting. **(B)** Chemical structure of XIE62-1004-A
9 (XIE). **(C)** HuH-7 wt or *vtRNA1-1*KO cells were treated with 20 μ M XIE, or solvent, for the
10 indicated times. Cells were then lysed and analyzed by Western blotting. **(D)** HuH-7 control cells
11 (wt and CRISPR ctrl clones) or *vtRNA1-1*KO cells, respectively, were treated with 10 μ M XIE
12 or solvent for 1h, lysed and analyzed by Western blotting. LC3B conjugation ratios from 3
13 independent experiments are plotted. Significant differences are indicated by *p*-values. The effect
14 of XIE was analyzed by the robust F test. **(E)** Working model for the function of *vtRNA1-1* as a
15 riboregulator of p62 protein multimerization and activity.

16

1 **SUPPLEMENTARY FIGURE LEGENDS**

2

3 **Figure S1. iCLIP analysis of p62** Related to Figure 1

4 (A) Lysates from 254nm UV-C light exposed HuH-7 cells were treated with a low (20U/ml
5 RNase I, L) or high concentration of RNase I (200 U/ml, H), and used for IPs with the indicated
6 antibodies and controls. p62-RNA complexes were separated by SDS-PAGE, blotted and excised
7 as indicated by the red dotted rectangles. The underlined area of the blot was used for a
8 subsequent Western blotting shown in panel (B). (C) p62 crosslink site (CS) analysis on
9 vtRNAs. Significant (FDR 5%) CS read counts of p62 IPs displayed on the vtRNAs transcript
10 sequence.

11 **Figure S2. In cellulo and in vitro p62-vtRNAI-1 analysis** Related to Figure 2

12 (A) Raw RT-qPCR Ct values related to Figure 2A. (B) EMSA using MBP-p62 and radiolabeled
13 vtRNAI-1. (C) Representative EMSA using MBP-p62 and radiolabeled vtRNAI-1, with
14 unlabeled non-specific competitor RNA included. (D) qRT-PCR analysis of total RNA from wt
15 or p62 KO HuH-7 cells; n=3.

16 **Figure S3. vtRNAI-1 phenotypic analysis** Related to Figure 3

17 (A,B) Cells were transfected with indicated amounts of LNA and lysed after 48 hours. Total
18 RNA was isolated and analyzed by Northern blotting (A), representative image, and by qRT-
19 PCR (B); n=3. (C) Cells were transfected with control LNA or LNA targeting vtRNAI-1 and
20 incubated for 48 hours. Cells were then subjected to following treatments: control, 100 nM BafA
21 for 5h, or media lacking amino acids (-AA) for 4h. Lysates were analyzed by Western blotting. *
22 indicates a non-specific band. (D) Cells were transfected with the indicated expression vectors,
23 total RNA was extracted after 24h and analyzed by Northern blotting.

1 **Figure S4. Starvation-mediated regulation of *vtRNAI-1* levels** Related to Figure 4

2 (A) Cells were treated as indicated and used for total RNA isolation and analysis by qRT-PCR.
3 n=9 (B) Cells were transfected with the indicated expression vectors ('*vtRNA* o.e.'), and 24
4 hours later were either starved for 6h or control treated. Total RNA was extracted and analyzed
5 by Northern blotting. Probe signals were quantified and plotted as data relative to non-starved
6 control. n=3 (C) Cells were starved for the indicated times, total RNA was extracted, and
7 analyzed by Northern blotting with indicated probes. (D) Cells were starved in minimal medium
8 containing solvent control or BafA at 100 nM for the indicated time, 254nm UV-C light exposed
9 and lysed. Lysates were used for p62 IPs and RNA radiolabeling assay. After SDS-PAGE and
10 transfer, the membrane was exposed overnight on film and used subsequently for Western
11 blotting. (E) Data of quantified RNA-bound p62/total p62 from Figure 4C is plotted together
12 with the total *vtRNAI-1* levels acquired from the HuH-7 cells starved in minimal media for
13 indicated time and measured by Northern blotting; n=3

14 **Figure S5. p62 PNK analysis and HuH-7 p62KO cell generation** Related to Figure 5

15 (A) HuH-7 wt cells stably expressing tagged p62 wt or K141A variant subjected to UV-C
16 254nm, lysed and used for HA IP followed by RNA radiolabeling assay. Arrows indicate the
17 endogenous (lower band) and exogenous (upper band) p62, respectively. (B) Western blotting
18 analysis of stable HuH-7 p62 KO CRISPR clones analyzed for the expression of p62. * indicates
19 a non-specific band. (C) Sanger sequencing analysis of the p62 genomic locus around ATG.
20 Reference genomic sequence ("ref") is displayed on the top. Two lines for each clone represent
21 alleles identified in the TA cloning for the amplified PCR fragment. (D) Quantification of
22 radiolabeling experiments related to Figure 5D. The colors indicate paired replicates. (E)
23 Representative Western blot analysis of HA IP eluates related to Figure 5E.

1 **Figure S6. p62 co-IP analysis and HuH-7 vtRNA1-1KO cells generation** Related to Figure 6
2 (A) HuH-7 cells were treated as indicated (starv.+BafA indicates 5h in starvation media in the
3 presence of 100 nM BafA). Cells were then crosslinked using DSP, lysed and used for p62 IPs.
4 Eluates were analyzed by SDS-PAGE and visualized by Western blotting. * indicates band from
5 previous LC3B antibody stain (B) Schematic representation of the genomic locus of *vtRNA1-1*.
6 Localization of guide RNAs and primers for PCR analysis are indicated. The combinations of
7 guide RNAs for CRIPSR are shown. (C) PCR analysis of genomic loci of the respective vault
8 RNAs for wt, control and CRISPR KO clones. * indicates a non-specific amplicon. (D) qRT-
9 PCR analysis of vault RNAs from total RNA of selected CRISPR clones. “0” indicates no value
10 detected. (E) Western blotting analysis of p62 co-IP eluates. IP from lysates of indicated cell
11 clones cultured in the presence of BafA for 5h at 100 nM and treated with DSP.

12

1 **STAR METHODS**

2

3 **CONTACT FOR REAGENT AND RESOURCE SHARING**

4 Further information and requests for resources and reagents should be directed to and will be
5 fulfilled by the Lead Contact, Matthias W. Hentze (hentze@embl.de).

6

7 **EXPERIMENTAL MODELS AND SUBJECT DETAILS**

8 HuH-7 cells (human male origin) were cultured in low glucose (5mM) DMEM supplemented
9 with 10% heat inactivated FCS (PAA), 2mM L-glutamine (25030081, Thermo Fisher) and 100
10 U/ml PenStrep (15140122, Thermo Fisher). We derived a HuH-7 Flp-In T-REx cell line using
11 published protocols (Flp-In T-Rex, Thermo Fisher), and prepared stably expressing doxycycline-
12 inducible cell lines following manufacturer's instructions. Stable cell lines were grown in
13 medium containing blasticidine (5µg/ml) and zeocin (100 µg/ml) or hygromycin (200 µg/ml).
14 Induction was performed with doxycycline at 100 ng/ml overnight. HeLa (human female origin),
15 Hepa1-6 (mouse origin), Hep-56.1D (mouse female origin), RAW264.7 (mouse male origin) and
16 NIH3T3 cells (mouse origin) were grown in high glucose DMEM supplement as above. Cell
17 lines were not authenticated. *E.coli* BL21(DE3) CodonPlus-RIL cells were pre-cultured
18 overnight at 37°C at 220 rpm in LB broth containing 34µg/ml kanamycin and 34µg/ml
19 chloramphenicol. 25 ml of the saturated overnight culture was added to the ZY autoinduction
20 media (LB broth without NaCl) containing 34µg/ml kanamycin and 34µg/ml chloramphenicol
21 and incubated at 37°C for 6 hours with agitation at 200 rpm.

22

23 **METHOD DETAILS**

1 **Transfections and treatments**

2 Transfections were done using Lipofectamine 3000 (L3000008, Thermo Fisher) for the plasmid
3 DNA, or Lipofectamine RNAiMax (13778075, Thermo Fisher) for the siRNA and LNAs.
4 Bafilomycin A₁ (tlrl-baf1, InvivoGen) was diluted in DMSO to 100 μM and used at 50-100 nM
5 for 4-6 hours. 4-thiouridine (T2933, Biomol) was used at 100 μM for 16 hours. MG132 (tlrl-
6 mg132) was used at 0.25μM concentration overnight. For starvation, cells were washed twice
7 with PBS and starved in low glucose DMEM lacking amino acids (D9800-13, USBiological) and
8 serum.

9

10 **RNA interactome capture**

11 RNA interactome capture was performed with minor modifications in the cell lysis procedure as
12 previously described (Castello et al., 2013). Shortly, cells were washed twice with PBS on ice
13 before UV crosslinking at 150 mJ/cm². Cells were lysed directly with lysis buffer (20 mM pH7.5
14 Tris HCl, 500 mM LiCl, 0.5% LiDS, 1 mM EDTA, 5 mM DTT) on the cell culture plates,
15 scraped and lysates were sheared through a 27G needle before incubation with oligo d(T) beads
16 (volume ratio lysate to beads 15:1) for 1 hour at 4°C. Beads were then washed twice with lysis
17 buffer, twice with wash buffer 1 (20 mM pH7.5 Tris HCl, 500 mM LiCl, 0.1% LiDS, 1 mM
18 EDTA, 5 mM DTT), twice with wash buffer 2 (20 mM pH7.5 Tris HCl, 500 mM LiCl, 1 mM
19 EDTA, 5 mM DTT, 0.01% NP40) and twice with wash buffer 3 (20 mM pH7.5 Tris HCl, 200
20 mM LiCl, 1 mM EDTA, 5 mM DTT). RNAs were eluted using elution buffer (20 mM pH7.5
21 Tris HCl, 1 mM EDTA) and pooled eluates from three rounds of purification were used for
22 RNase treatment with 10 U of RNase A and 10 U of RNase T1 for 30 min at 37°C. Samples were
23 then concentrated using Amicon 3K columns (UFC500396, Merck Millipore) and mixed with 4x

1 sample buffer (4xSB) (200mM Tris-HCl pH6.8; 8% SDS; 40% Glycerol, 0.04% bromophenol
2 blue, 400mM DTT; 10% beta mercaptoethanol) before loading on SDS-PAGE.

3

4 **Polynucleotide kinase (PNK) assay**

5 The cells were washed twice with PBS on ice before UV crosslinking at 150 mJ/cm², lysed in
6 lysis buffer (100mM NaCl; 50mM Tris-HCl pH7.5; 0.1% SDS; 1 mM MgCl₂; 0.1 mM CaCl₂;
7 1% NP40; 0.5% sodium deoxycholate; protease inhibitors (11873580001, Roche)) and
8 homogenized by ultrasound (level 4, 3x 10sec, 50% amplitude) on ice. After homogenization the
9 lysates were treated with 10 ng/μl of RNase A (R5503, Sigma-Aldrich) and 2U/ml Turbo DNase
10 (AM2238, Thermo Fisher) for 15 min at 37°C, cooled on ice and used for IPs. For the Figure 1B,
11 a series of RNaseA dilution was used. After the IP and 3 washes with lysis buffer, beads were
12 washed additionally twice with PNK buffer (50mM NaCl; 50mM Tris-HCl pH7.5; 10mM
13 MgCl₂; 0.5% NP-40; protease inhibitors (11873580001, Roche)), then resuspended in PNK
14 buffer containing 0.1 μCi/μl [γ -³²P] rATP (Hartmann), 1 U/μl T4 PNK (NEB), 1mM DTT and
15 labeled for 15 min at 37°C. After 4 washes with PNK (without DTT) buffer, proteins were eluted
16 at low pH (0.1M glycine pH2.0), neutralized with 0.2M Tris-HCl pH8.5, and mixed with 4xSB
17 buffer. Samples were resolved by SDS-PAGE and blotted on nitrocellulose membrane. The
18 membrane was exposed overnight to phosphorimager screen or to the imaging film (Z350397-
19 50EA, Sigma), followed by Western blotting.

20

21 **p62 iCLIP**

22 iCLIP was performed as published (Huppertz et al., 2014) using following IP procedure. 0.75-1.5
23 μg of p62 antibody or appropriate control IgG was coupled for 1 hour at RT to 12.5 μl of Protein

1 G coupled magnetic beads (10004D, Thermo Fisher). Cells were washed twice with cold PBS,
2 lysed in lysis buffer (100mM NaCl; 50mM Tris-HCl pH7.5; 0.1% SDS; 1 mM MgCl₂; 0.1 mM
3 CaCl₂; 1% NP40; 0.5% sodium deoxycholate; protease inhibitors (11873580001, Roche)) and
4 homogenized by ultrasound (level 4, 3x 10sec, 50% amplitude) on ice. Treatment of the lysates
5 with RNaseI (AM2295, Thermo Fisher) was used at 20 U/ml. Lysates containing 2 mg of total
6 protein were used for IP for 1 hour at 4°C, washed three times with high salt buffer (500mM
7 NaCl; 20mM HEPES pH7.3; 1% NP-40; 0.1% SDS; 1 mM EDTA; 0.5% sodium deoxycholate;
8 protease inhibitors (11873580001, Roche)) and three times with the lysis buffer. Next, beads
9 were incubated for 16 h (1 100 rpm, 16°C) in 20 µL ligation mix (50 mM Tris-HCl pH 7.5, 10
10 mM MgCl₂, 10 mM DTT, 500 U/mL T4 RNA ligase 1, 500 U/mL RNasin, 1.5 µM
11 preadenylated linker L3 [5'-rApp-AGATCGGAAGAGCGGTTCAG-ddC-3'], 20% PEG-400
12 [Sigma]) and washed three times with lysis buffer. Complexes were eluted at low pH (0.1M
13 glycine pH2.0), neutralized with 1M Tris-HCl pH8.5, digested with proteinase K and used for all
14 subsequent steps as described previously (Huppertz et al., 2014). cDNA libraries obtained after
15 PCR amplification with universal Solexa primers (25 cycles) were multiplexed and sequenced
16 using an Illumina HiSeq2000 platform.

17

18 **Native p62 IP and protein-protein co-IP**

19 Cells were washed twice with PBS on ice. For co-IP variant, cells were treated with 0.5mM DSP
20 (Thermo Fisher #22585) for 1h and quenched with TRIS-HCl as recommended by vendor. Cells
21 were lysed directly on the plates with lysis buffer (20mM Tris HCl pH7.4, 100mM NaCl; 1mM
22 EDTA; 1mM EGTA; 1% triton X-100; protease inhibitors (11873580001, Roche); 5 µg/ml
23 RNasin (Promega)) by swelling 5min on ice, followed by scraping. Lysates were homogenized

1 by pipetting against the plate and 5sec vortex. Lysates were centrifuged for 10 min at 11,800xg
2 at 4°C and supernatant was used for IP. IP was performed either using p62 antibodies (as above)
3 or 25 µl HA-beads slurry (Pierce). Lysis buffer washes were applied 6x and tubes were renewed
4 after every second wash. Proteins were eluted as described above and analyzed with Western
5 blotting. Alternatively, eluates were directly resuspended in TRI reagent (T9424, Sigma-Aldrich)
6 and RNA was purified according to manufacturer's protocol. 5µg of linear acrylamide (Thermo,
7 AM9520) was used as carrier and RNA was resuspended in 10 µl water.

8

9 **qRT-PCR**

10 0.5 µg of total RNA or 7 µl of IP RNA was used for cDNA synthesis using Maxima RT kit
11 (Thermo Fisher, K1671). Typically, 5ng of cDNA was used for qPCR. Primers are listed in
12 Supplementary Table S4.

13

14 **RNA isolation and Northern blotting**

15 RNA was isolated using TRI reagent (T9424, Sigma-Aldrich) as recommended by the
16 manufacturer. RNA was dissolved in nuclease-free water and stored at -80°C. Typically, 10 or
17 15 µg of total RNA was mixed with 2x loading dye (95% formamide; 0.025% xylene cyanol and
18 bromophenol blue; 18mM EDTA; 0.025% SDS), denatured for 5 min at 95°C, cooled on ice and
19 loaded on 8% acrylamide (19:1), 7M urea polyacrylamide gels. A semi-dry blotting apparatus
20 was used for blotting on Hybond N⁺ membranes (RPN1520B, GE) which were UV auto-
21 crosslinked, pre-hybridized for 1 hour at 50°C and used for hybridizations with ³²P labelled DNA
22 antisense oligonucleotide probes (Supplementary Table S4) overnight at 50°C. The membranes
23 were then washed three times with high stringency buffer (5X SSC; 5% SDS), three times with

1 low stringency buffer (1X SSC; 1% SDS) and exposed to phosphorimaging screens for 4 hours
2 or overnight. Screens were scanned at Typhoon FLA-7000 (GE) and TIFF images were
3 quantified by ImageJ.

4

5 **RBDmap**

6 Shortly, HuH-7 lysates and oligo-d(T) captures were done as for the RNA interactome capture
7 method. After washes and elution (eluate 1), 3 µg of LysC was added to the eluate 1 and
8 incubated at 37°C for 8h. Afterwards, aliquots for protein and RNA quality controls were
9 harvested. Next, second oligo-d(T) capture was applied on the LysC-treated eluate 1, while the
10 flow-through after oligo-d(T) incubation was saved. Washes were applied and elution (eluate 2)
11 was applied. Next, inputs, eluates and flow-through were used for RNase treatment with 10 U of
12 RNase A and 10 U of RNase T1 for 30 min at 37°C. Proteomics analysis was performed and
13 analyzed as described (Castello et al., 2016). Shortly, cysteines were reduced (5 mM DTT, 56°C,
14 30 min) and alkylated (10 mM Iodoacetamide, 30 min in the dark). Samples were buffer-
15 exchanged into 50 mM triethylammoniumbicarbonate, pH 8.5, using 3 kDa centrifugal filters
16 (Millipore, UFC500396) and digested with sequencing grade trypsin (Promega, V5280; enzyme-
17 protein ratio 1:50) at 37°C for 18 h. Resulting peptides were desalted and labelled using stable
18 isotope reductive methylation (Boersema et al., 2009) on StageTips (Rappsilber et al., 2007).
19 Labels were swapped between replicates. Labeled samples were combined and fractionated into
20 12 fractions on an 3100 OFFGEL Fractionator (Agilent) using Immobiline DryStrips (pH 3–10
21 NL, 13 cm; GE Healthcare) according to the manufacturer's protocol. Isoelectric focusing was
22 carried out at a constant current of 50 mA allowing a maximum voltage of 8000 V. When 20
23 kVh were reached the fractionation was stopped, fractions were collected and desalted using

1 StageTips. Samples were dried in a vacuum concentrator and reconstituted in MS loading buffer
2 (5% DMSO 1% formic acid). Samples were analyzed on a LTQ-Orbitrap Velos Pro mass
3 spectrometer (Thermo Scientific) coupled to a nanoAcquity UPLC system (Waters). Peptides
4 were loaded onto a trapping column (nanoAcquity Symmetry C18, 5 μm , 180 μm \times 20 mm) at a
5 flow rate of 15 $\mu\text{l}/\text{min}$ with solvent A (0.1% formic acid). Peptides were separated over an
6 analytical column (nanoAcquity BEH C18, 1.7 μm , 75 μm \times 200 mm) using a 110 min linear
7 gradient from 7-40% solvent B (acetonitrile, 0.1% formic acid) at a constant flow rate of 0.3
8 $\mu\text{l}/\text{min}$. Peptides were introduced into the mass spectrometer using a Pico-Tip Emitter (360 μm
9 outer diameter \times 20 μm inner diameter, 10 μm tip, New Objective). MS survey scans were
10 acquired from 300–1700 m/z at a nominal resolution of 30000. The 15 most abundant peptides
11 were isolated within a 2 Da window and subjected to MS/MS sequencing using collision-induced
12 dissociation in the ion trap (activation time 10 msec, normalized collision energy 40%). Only
13 2+/3+ charged ions were included for analysis. Precursors were dynamically excluded for 30 sec
14 (exclusion list size was set to 500). The RBDmap dataset and analysis can be accessed at
15 <http://www.hentze.embl.de/public/RBDmapHuH7>.

16

17 **Protein extracts, SDS-PAGE and Western blotting**

18 For Western blotting, cells were washed twice with ice cold PBS on ice and lysed on plate using
19 RIPA lysis buffer (89900, Thermo Fisher) supplemented with protease inhibitor (11873580001,
20 Roche). Lysates were treated with benzonase (100U/ml, 71206, Merck Millipore) for 15 min on
21 ice and protein concentrations were measured. Lysates were mixed with 4xSB, boiled for 5 min
22 and typically 15 μg of lysate was used for SDS-PAGE. Proteins were transferred to
23 nitrocellulose or PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) and

1 blocked for 1 hour at room temperature with 5% milk in PBS; 0,05% Tween (PBS-T). Primary
2 antibodies were incubated in 5% milk PBS-T either overnight at 4°C or 1 hour at RT, followed
3 by 3x PBS-T washes, secondary antibody incubation in 5% milk in PBS-T for 1 hour at RT, 3x
4 PBS-T washes and developed using ECL (WBKLS0500, Millipore). Primary and secondary
5 antibodies used for Western blotting are listed in the Key Resources Table.

6

7 **siRNA, LNAs**

8 An siRNA pool targeting p62 (L-010230-00-0020, GE) was used at 30 nM concentration for 48
9 hours. As control siRNA an equimolar mix of Scramble (5' UUCUCCGAACGUGUCACGUtt
10 3'; s229174, Thermo Fisher), sLuciferase (5' CGGAUUACCAGGGGAUUUCAtt 3'; Thermo
11 Fisher) and SWNeg9 (5' UACGACCGGUCUAUCGUAGtt 3'; s444246, Thermo Fisher) was
12 used. LNAs (Exiqon) targeting *vtRNA1-1* (#1: 5' ttaaagaactgtcgaa 3'; #3: 5'ttaaagaactgtcga 3')
13 and control negA (5' aacacgtctataccg 3') were used at 25 or 50 nM for 48 hours.

14

15 **Cloning**

16 Full length human p62 wild type cDNA was cloned into pcDNA5_FRT/TO vector with N-
17 terminal FLAG/HA tag (MDYKDDDDKSAGGYDPDYAKL...) using HindIII and XhoI
18 sites. Single and double amino acid mutations were done using PCR-mediated mutagenesis.
19 Recognition sites of p62 siRNA were mutated in synonymous fashion (5'
20 GGATCGAGGTAGACATAGA 3'; 5' GAGCAAATGGAATCCGACA 3'; 5'
21 GGACGCACCTCTCATCTAA 3'; 5' CGACTGGCCTCAAAGAGGC 3'), cDNA was
22 synthesized in pUC57 (GenScript) and swapped into p62 cDNA using BamHI and XhoI sites.
23 Vault RNA with T7 or H1(2xTO) promoters were synthesized (GenScript) in pUC57 backbone.

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CRISPR/Cas9 gene deletion of *vtRNA1-1*

Single guide RNAs targeting the *vtRNA1-1* locus (Supplementary Table S4) were predicted using the CRISPR online tool (<http://crispor.tefor.net>; Version May 2017) (Ran et al., 2013), ordered from Sigma-Aldrich, annealed and cloned into pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid #48138 kindly provided by Fang Zhang), pSpCas9(BB)-2A-RFP or pSpCas9(BB)-2A-Cer3 (both kindly provided by Kyung-Min Noh, EMBL) using the BbsI restriction sites. Different combinations of the generated plasmids (see Figure S6B) were nucleofected into HuH-7 cells using the Nucleofector 4D system according to manufacturer's guidelines (Lonza, Cell Line Nucleofector Kit SF, program FF137, 1 million cells and 1µg of DNA per nucleofection). As negative control a mixture of all parental plasmids (without sgRNA) was used. Single cell sort of double/triple positive cells was performed 48h after nucleofection. Upon clone expansion the genomic DNA was isolated (Zymo, Quick-DNA Miniprep Kit) and a PCR was performed to screen for homozygous deletions of the vault RNA1-1 (see Supplementary Table S4 for primers, Figure S6C). Resulting vaultRNA1-1 KO clones were further validated by qPCR (Figure S6D). In order to detect possible off-target effects all vault RNA paralogues were included into the screen.

18

CRISPR/Cas9 mediated p62 gene inactivation

20 Alt-R kit (IDT) was used for p62 gene inactivation. In short, HuH-7 cells were transfected with
21 3nM RNP complexes using 2 different crRNAs (Supplementary Table S4) and cell sorted 48
22 hours later using tracrNA-Atto 550nm. Single cell derived clones were grown out and analyzed
23 by Western blotting. Positive clones were cultured in the presence of 100nM BafA for 5 hours

1 and re-analyzed by Western blotting using 2 different p62 antibodies (Figure S5B). Genomic
2 locus spanning p62 ATG was amplified for selected clones (primers sequences in Supplementary
3 Table S4), cloned into TA plasmid (Thermo Fisher, K204001) and sequenced. For all
4 experiments presented in the main Figures p62KO clone I-E7 was used.

6 **In vitro transcription and EMSA**

7 pUC57 plasmid with T7_vault RNA1-1 was used for in vitro transcription reaction using
8 MEGAscript kit (AM1354, Thermo Fisher) with ³²P- α UTP (SRP-210, Hartmann) according
9 to the manufacturer's protocol. RNA was gel purified, phenol-chloroform extracted, dissolved in
10 water and measured for the specific activity with scintillation counter and concentration with
11 QuBit (Thermo). Before the reaction, RNA was denaturated 10 min at 65°C and cooled down to
12 room temperature. Afterwards MgCl₂ was added to 2.5mM. IRE RNA element production was
13 described (Pantopoulos and Hentze, 1995). EMSA reaction contained typically 0.025-6 μ M of
14 proteins, 15 nM of RNA (150 fmol, 3-5kCPM), 1 mg/ μ l of BSA, 10 mg/ μ l RNAsin, 5mM DTT,
15 0.5mM PMSF, 2.5mM MgCl₂, 100mM KCl; 20mM HEPES pH7.9; 0.2 mM EDTA and
16 20%glycerol. Reactions were incubated 20 min at room temperature. After the reaction samples
17 were loaded on 20cm long 5% acrylamide native gel and ran overnight at 70V. Gel was dried for
18 1 hour at 80°C and exposed 4h or overnight to phosphorimager screen.

20 **p62 protein expression and purification**

21 MBP-p62-his₆ was expressed in *E.coli* BL21(DE3) CodonPlus-RIL cells by autoinduction in ZY
22 media. After pre-culture at 37°C for 6 hours, cell cultures were cooled down to room temperature
23 and incubated for 16 hrs at 20°C. Cells were lysed by resuspension in lysis buffer (50mM

1 HEPES pH 8.0, 1M NaCl, 0.5 mM TCEP, 1x protease inhibitor) followed by four passes through
2 a microfluidizer. Lysate was clarified by centrifugation at 48 000xg and incubated with Ni-NTA
3 beads for 1 hr. Beads were washed extensively in buffer 1 (50mM HEPES pH 8.0, 1M NaCl, 0.5
4 mM TCEP, 50mM Imidazole) and protein eluted with buffer 2 (50mM HEPES pH 8.0, 1M
5 NaCl, 0.5 mM TCEP, 250mM Imidazole).

6

7 **Immunofluorescence microscopy and image analysis**

8 For immunostaining, cells were cultured on ibidi slides (80426; ibidi), fixed for 10 min with
9 methanol, washed with PBS, permeabilized and blocked for 1 hour in 0.1% Triton-X 100 in 1%
10 BSA solution. Cells were then incubated with primary antibodies for 1 hour at room temperature,
11 washed in PBS and incubated with the secondary antibody, DAPI and Cell Mask for 1 h at room
12 temperature in the dark. Slides were washed 3 times in PBS and stored at 4°C in PBS until
13 imaging. Reagents used were anti-p62 (1:1000, #PM045, MBL), anti-LC3B (1:200; #CTB-LC3-
14 2-IC, Cosmo Bio), anti-mouse IgG Alexa Fluor 488 (1:1000; #4408, Cell Signaling), anti-Rabbit
15 IgG Alexa Fluor 555 (1:1000; #4413, Cell Signaling), DAPI (#10236276001, Roche); HSC
16 CellMask Deep Red stain (1:40 000; #H32721, Thermo Fisher). Images of the fluorescent
17 staining were acquired on a Leica SP5 confocal microscope using a 63x/NA1.2 PlanApo water
18 immersion lens. Excitation and emission settings were as follows: Dapi (Ex 405nm / Em 410 -
19 484 nm), Alexa Fluor 488 (Ex 488nm / Em 500 – 551 nm) and Alexa Fluor 555 (Ex 561 nm /
20 Em 564 – 628 nm), and Cell Mask Deep Red (Ex 633 nm / Em 661 – 714 nm). One image data
21 set comprised one z-stack with 24 slices acquired at 335 nm spacing, each slice consisting of
22 1024 x 1024 pixels with a pixel size of 120 nm x 120 nm. Typically, 10 such image data sets
23 were acquired per condition and replicate. For visual inspection and quantitative single cell

1 analysis in CellProfiler 2.2.0 (Carpenter et al., 2006) z-maximum projections were computed
2 using ImageJ (Schneider et al., 2012). Using CellProfiler, nuclei were segmented in the DAPI
3 channel by means of an intensity threshold. Cells were segmented by an image-based watershed
4 algorithm, computed on the Cell Mask Deep Red staining, using segmented nuclei as seeds. LC3
5 and p62 objects were segmented in the respective antibody staining images by applying a
6 morphological tophat filter for enhancement of locally bright structures followed by an intensity
7 threshold. LC3 and p62 objects were counted as co-localizing if the pixel based overlap was
8 greater than or equal to three pixels. To ensure an unbiased analysis all settings of the analysis
9 pipeline such as all intensity thresholds and filter settings were kept unchanged across all
10 conditions and replicates.

11

12 **Chemical synthesis of XIE62-1004-A**

13 XIE62-1004-A (2-((3,4-bis(benzyloxy)benzyl)amino)ethan-1-ol) was chemically synthesized
14 starting from 3,4-dihydroxybenzaldehyde according to the literature procedure described by
15 (Cha-Molstad et al., 2017) with the following modifications. The crude 2-((3,4-
16 bis(benzyloxy)benzyl)amino)ethan-1-ol was purified by silica gel column chromatography using
17 a NH₃(aq.)/MeOH/CH₂Cl₂ mixture for elution (1/1/98 → 1/10/89). The product obtained as
18 colorless oil was re-dissolved in HPLC grade CHCl₃, aliquoted into eppendorf tubes and then
19 the solvent was evaporated in a Speedvac overnight. XIE62-1004-A was used as a 500 mM
20 DMSO stock.

21

22 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1 Data are displayed as single points, mean \pm SEM; Student's *t*-test was used, *n* values are
2 indicated in the respective Figure legends. *p*-values are indicated in the Figures, *p*<0.05 was
3 considered statistically significant. GraphPad Prism v4.0 was used to create plots. Images were
4 quantified with ImageJ.

5

6 **DATA AND SOFTWARE AVAILABILITY**

7 The sequencing data was deposited to Array Express with the accession number E-MTAB-4894:
8 <https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-4894/?page=1&pagesize=500>

9 The analysis of the p62 iCLIP datasets is described at [http://www.hentze.embl.de/public/p62-](http://www.hentze.embl.de/public/p62-iCLIP)
10 [iCLIP](http://www.hentze.embl.de/public/p62-iCLIP). RNA secondary structures were predicted using the ViennaRNA package.

1 **SUPPLEMENTARY TABLES – EXCEL TABLES**

2 **Table S1. List of proteins found in HuH-7 cells RBDmap** Related to Figure 1

3 **Table S2. iCLIP crosslink site data per RNA class** Related to Figure 1

4 **Table S3. iCLIP analysis per single RNA** Related to Figure 1

5 **Table S4. List of oligonucleotides used in the study.** Related to Figures 2, 4, S5 and S6

6

7

Key Resources Table

The small non-coding vault RNA1-1 acts as a riboregulator of autophagy

Horos et al.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-p62	MBL	Cat#:PM045
Mouse monoclonal anti-p62	NOVUS	Cat#:H00008878-M01 RRID:AB_437085
Rabbit polyclonal anti-TDP43	ProteinTech	Cat#:10782-2-AP RRID:AB_615042
Mouse monoclonal anti-B-actin	Sigma-Aldrich	Cat#:A5441 RRID:AB_476744
Rabbit polyclonal anti-LC3B	MBL	Cat#:PM036 RRID:AB_2274121
Rabbit monoclonal anti-GABARAP	CST	Cat#:13733
Rabbit polyclonal anti-NBR1	PTG	Cat#:16004-1-AP RRID:AB_2251178
Rabbit monoclonal anti-phospho-Ser757 ULK1	CST	Cat#:14202 RRID:AB_2665508
Rabbit monoclonal anti-ULK1	CST	Cat#:8054 RRID:AB_11178668
Rabbit polyclonal anti-phosphoThr389 S6K1	CST	Cat#:9205 RRID:AB_330944
Rabbit monoclonal anti-S6K1	CST	Cat#:2708 RRID:AB_390722
Rabbit polyclonal anti-phospho-Ser4E-BP1	CST	Cat#:9451 RRID:AB_330947
Rabbit monoclonal anti-4E-BP1	CST	Cat#:9644 RRID:AB_2097841
Mouse monoclonal anti-Ub (FK2)	Tebu-Bio	Cat#:AB120 RRID:AB_10541840
Rabbit polyclonal anti-GAPDH	Sigma-Aldrich	Cat#:G9545 RRID:AB_796208
Goat anti-mouse IgG-HRP	Santa Cruz	Cat#:Sc-2005 RRID:AB_631736
Goat anti-rabbit IgG-HRP	Santa Cruz	Cat#:Sc-2004 RRID:AB_631746
Mouse anti-LC3B	Cosmo Bio	Cat#:CTB-LC3-2-IC
Anti-mouse IgG Alexa Fluor 488	CST	Cat#:4408 AB_10694704
Anti-rabbit IgG Alexa Fluor 555	CST	Cat#:4413 AB_10694110
Bacterial and Virus Strains		
<i>E. coli</i> BL21(DE3) CodonPlus-RIL	Agilent	Cat#:230240
Chemicals, Peptides, and Recombinant Proteins		
Bafilomycine A1	InVivoGen	Cat#:tlrl-baf1
MG132	InVivoGen	Cat#:tlrl-mg132
4-thiouridine	Biomol	Cat#:T2933
DAPI	Roche	Cat#:10236276001
HSC CellMask Deep Red	Thermo Fisher	Cat#:H32721
Critical Commercial Assays		

TRI-reagent	Sigma-Aldrich	Cat#:T9424
Alt-R	IDT	N/A
Quick DNA Miniprep Kit	Zymogen	Cat#:D4074
Nucleofector 4D	Lonza	Cat#:SF
Lipofectamine 3000	Thermo Fisher	Cat#:L3000008
Lipofectamine RNAi max	Thermo Fisher	Cat#:13778075
MEGAscript	Thermo Fisher	Cat#:AM1354
Anti-HA magnetic beads	Thermo Fisher	Cat#:88836
Maxima RT kit	Thermo Fisher	Cat#:K1671
Deposited Data		
p62 iCLIP data	this paper	Array Express E-MTAB-4894
Experimental Models: Cell Lines		
HuH-7	Beckmann et al., 2015	N/A
HuH-7 Flp-In T-Rex	Beckmann et al., 2015	N/A
HeLa	Castello et al., 2013	N/A
NIH3T3	C. Merten laboratory	N/A
RAW264.7	M.Muckenthaler laboratory	N/A
Hep-56.1D	M.Muckenthaler laboratory	N/A
Hepa1-6	M.Muckenthaler laboratory	N/A
Oligonucleotides		
sgRNAs, crRNAs, PCR primers and Northern blotting probes - please refer to the Supplementary Table S4	this paper	N/A
Recombinant DNA		
pcDNA5-FRT-TO-FLAG/HA-p62 wt and variants	this paper	N/A
pETM43-MBP-3C-p62-His6 wt and variants	this paper	Ciuffa et al., 2015
pUC57-T7-vtRNAs	this paper	N/A
pUC57-H1-vtRNAs	this paper	N/A
pSpCas9(BB)-2A-GFP	Ran et al., 2013	Addgene plasmid#: 48138
pSpCas9(BB)-2A-RFP/Cer3	kind gift from Noh lab, EMBL	N/A
Software and Algorithms		
Vienna fold	N/A	http://rna.tbi.univie.ac.at/
iCOUNT	N/A	https://icount.readthedocs.io/en/latest/
Prism GraphPad	GraphPad Software	Version 4
ImageJ	N/A	https://imagej.net/Welcome
CellProfiler	N/A	http://cellprofiler.org/
HuH-7 RBDmap data analysis	this paper	http://www.hentze.embl.de/public/RBDmapHuh7/vignettes/result/
p62 iCLIP data analysis	this paper	http://www.hentze.embl.de/public/p62-iCLIP

Figure 1

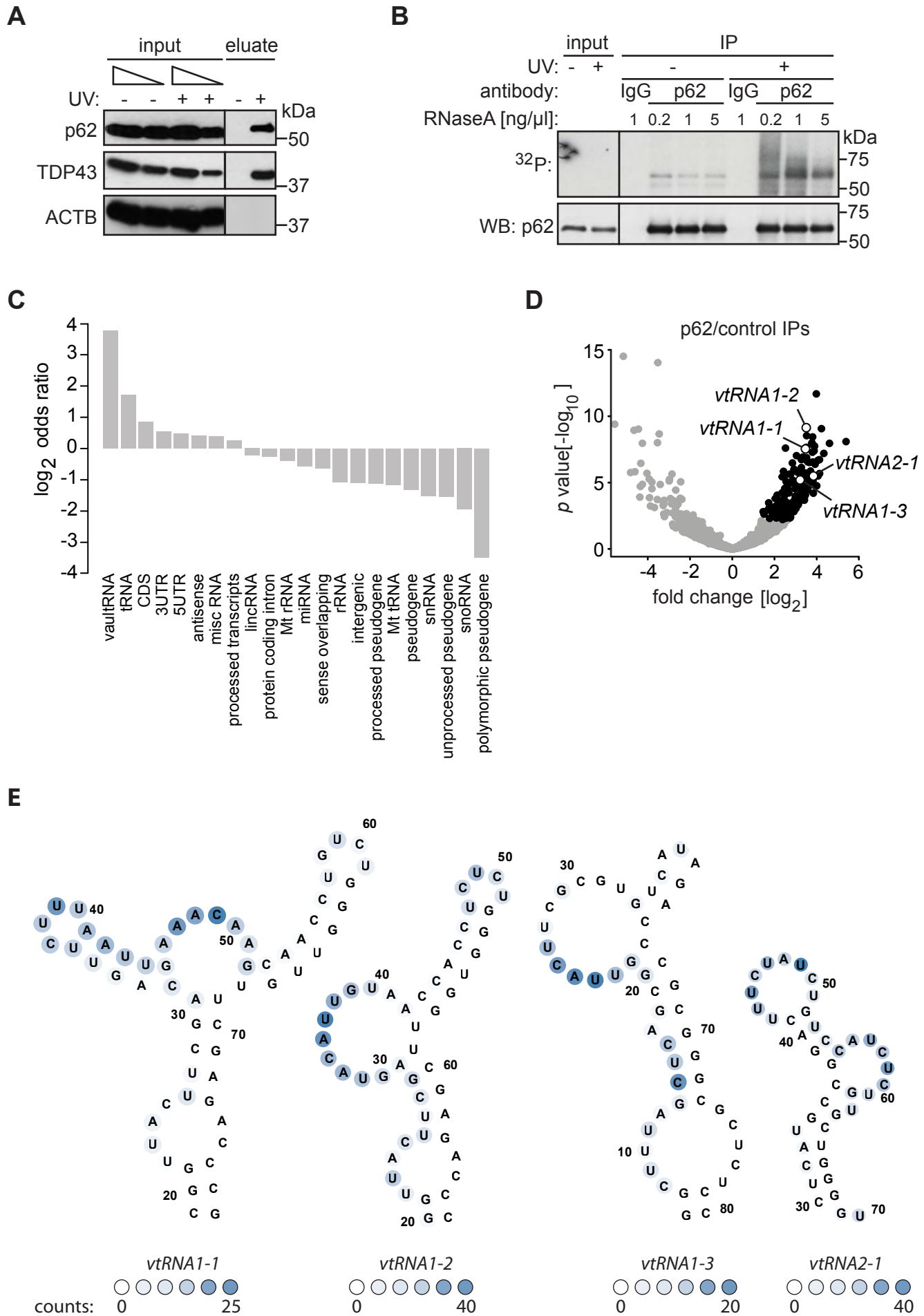


Figure 2

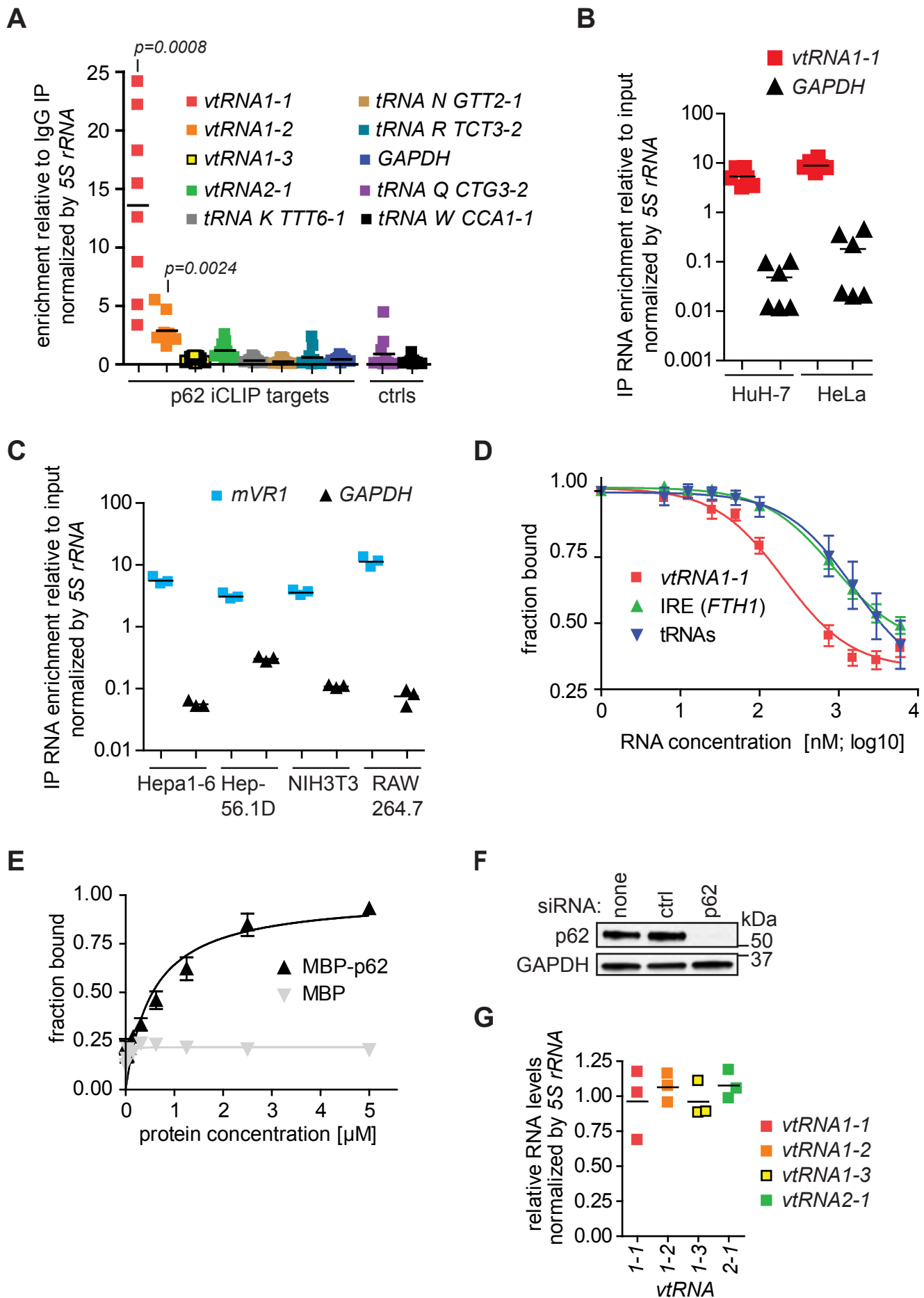


Figure 4

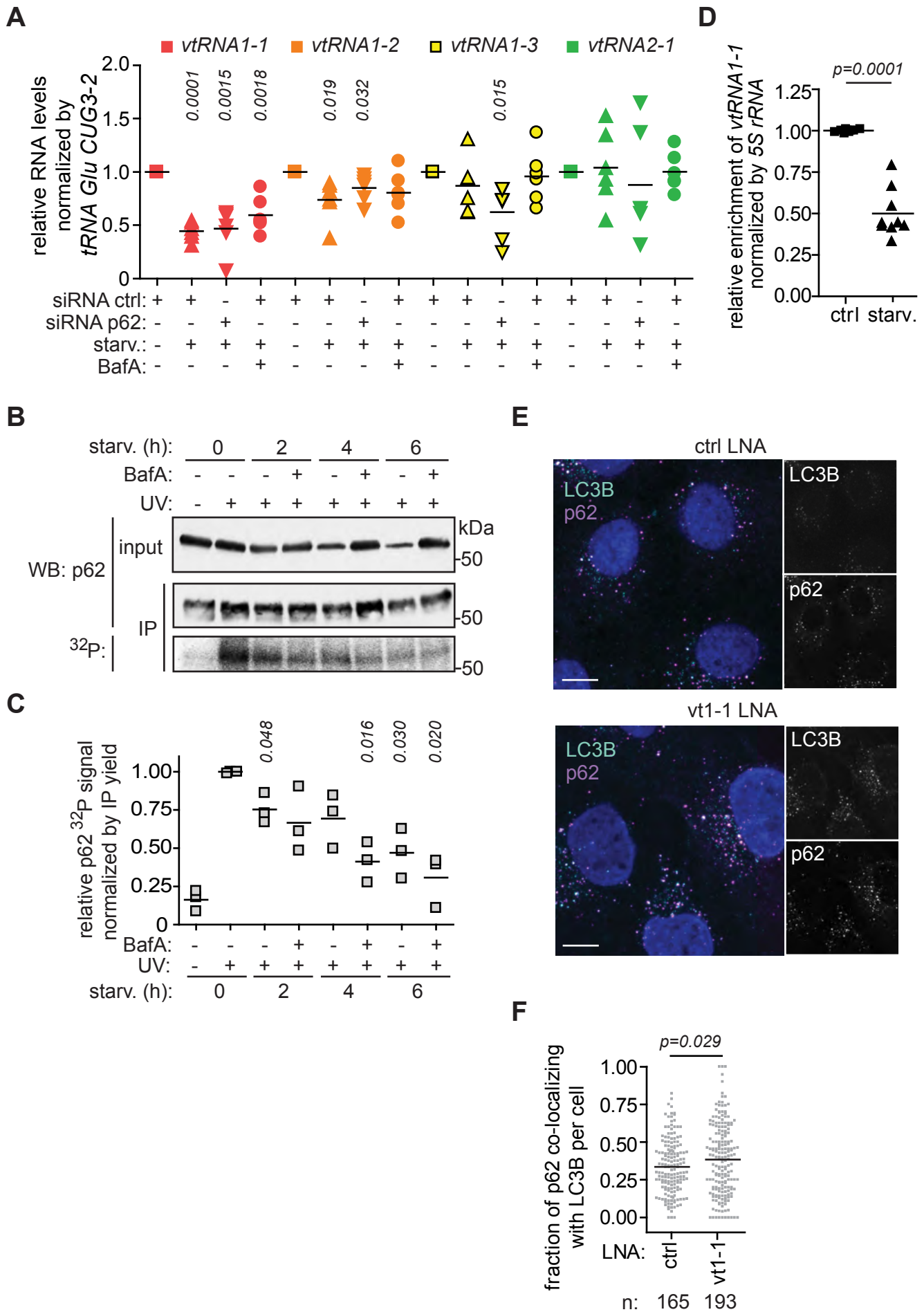


Figure 3

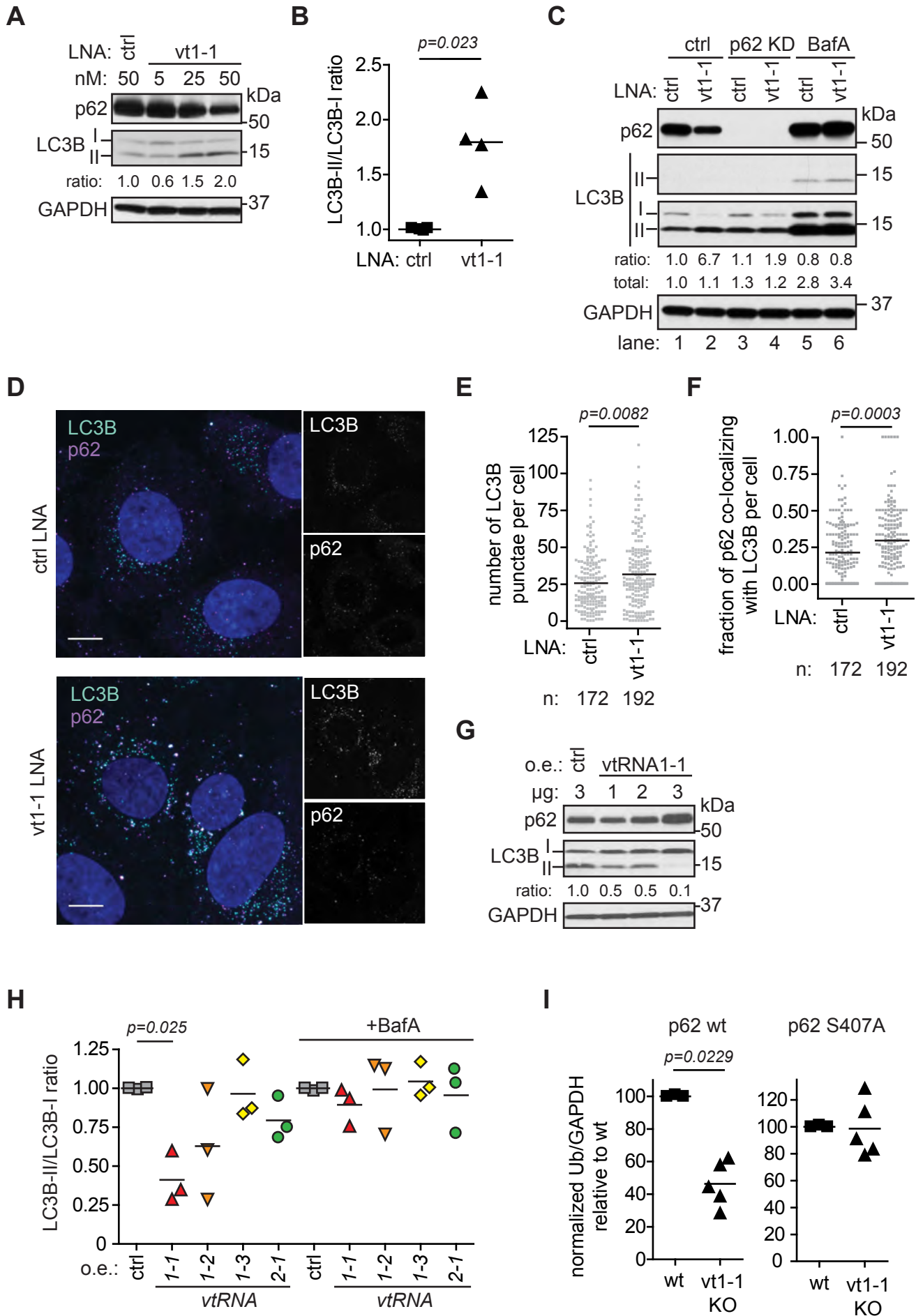


Figure 5

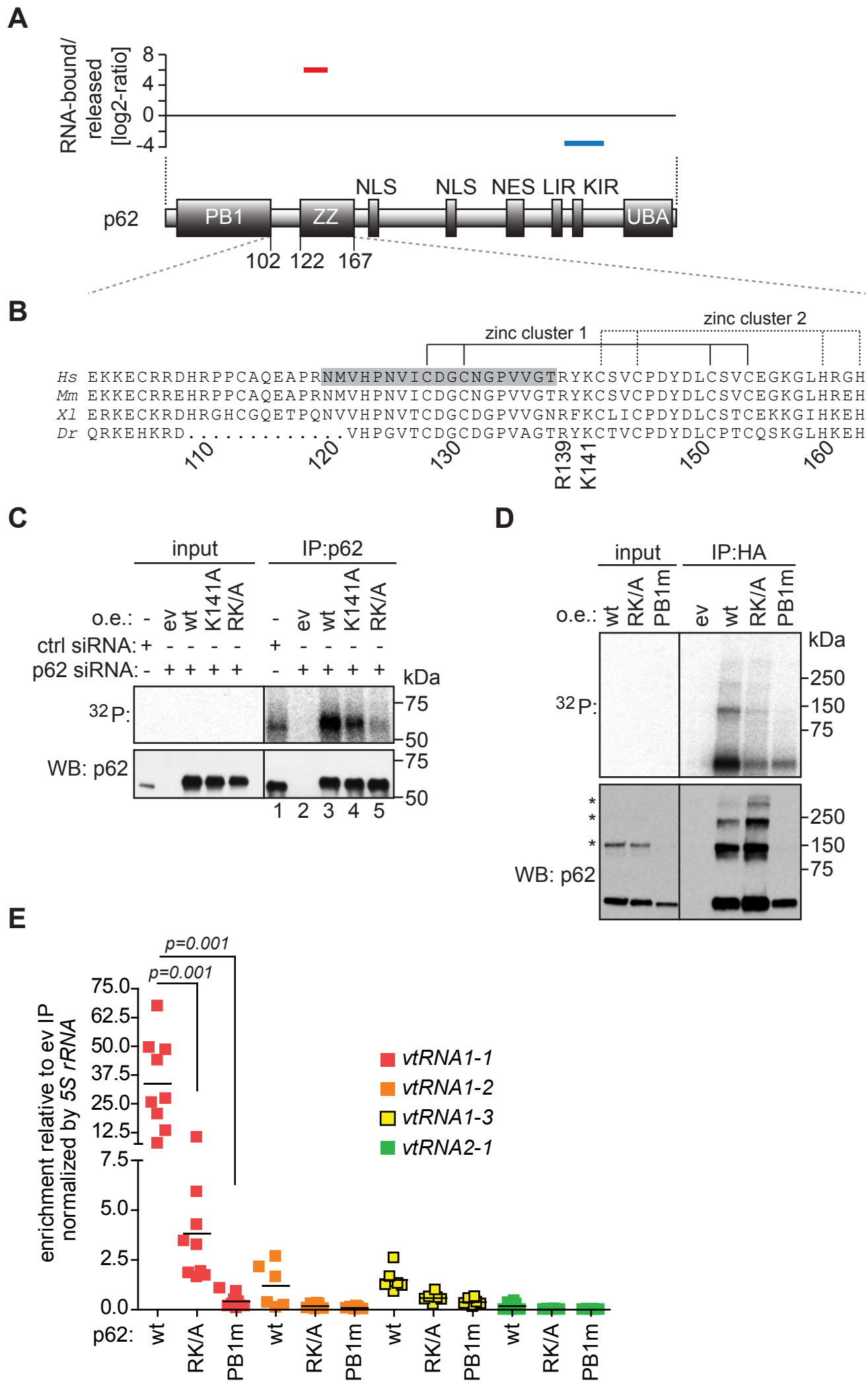


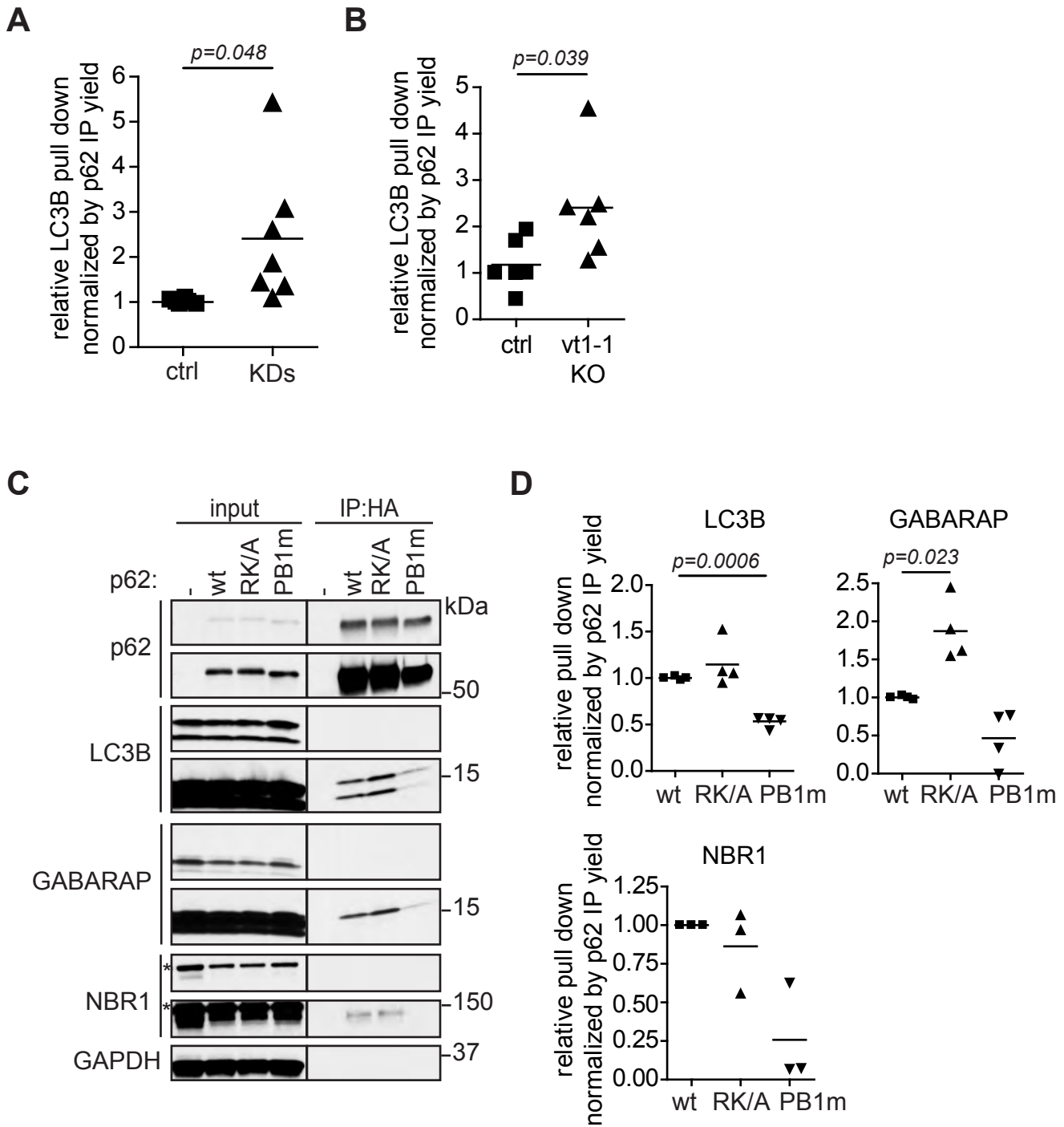
Figure 6

Figure 7

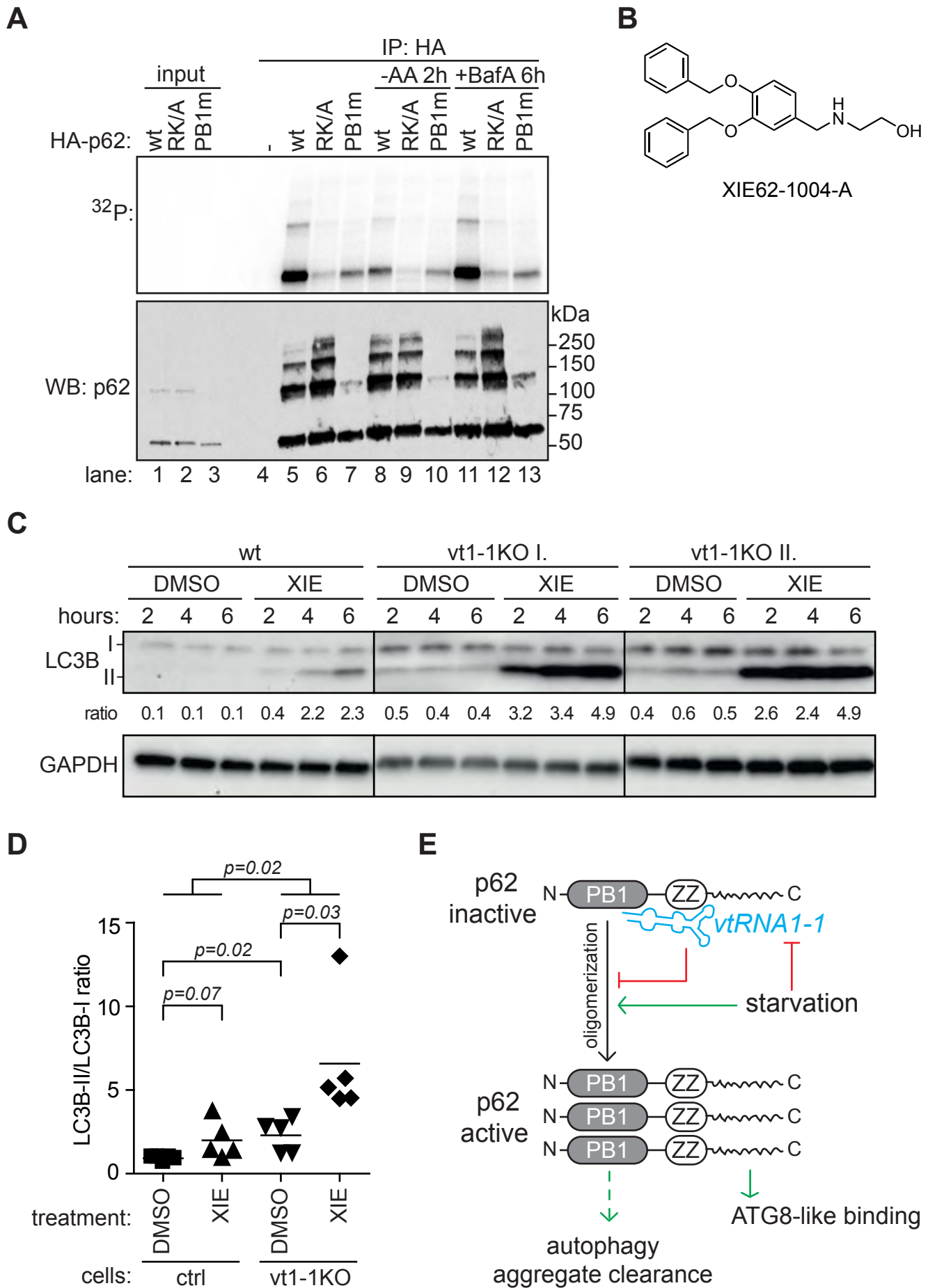


Figure S1

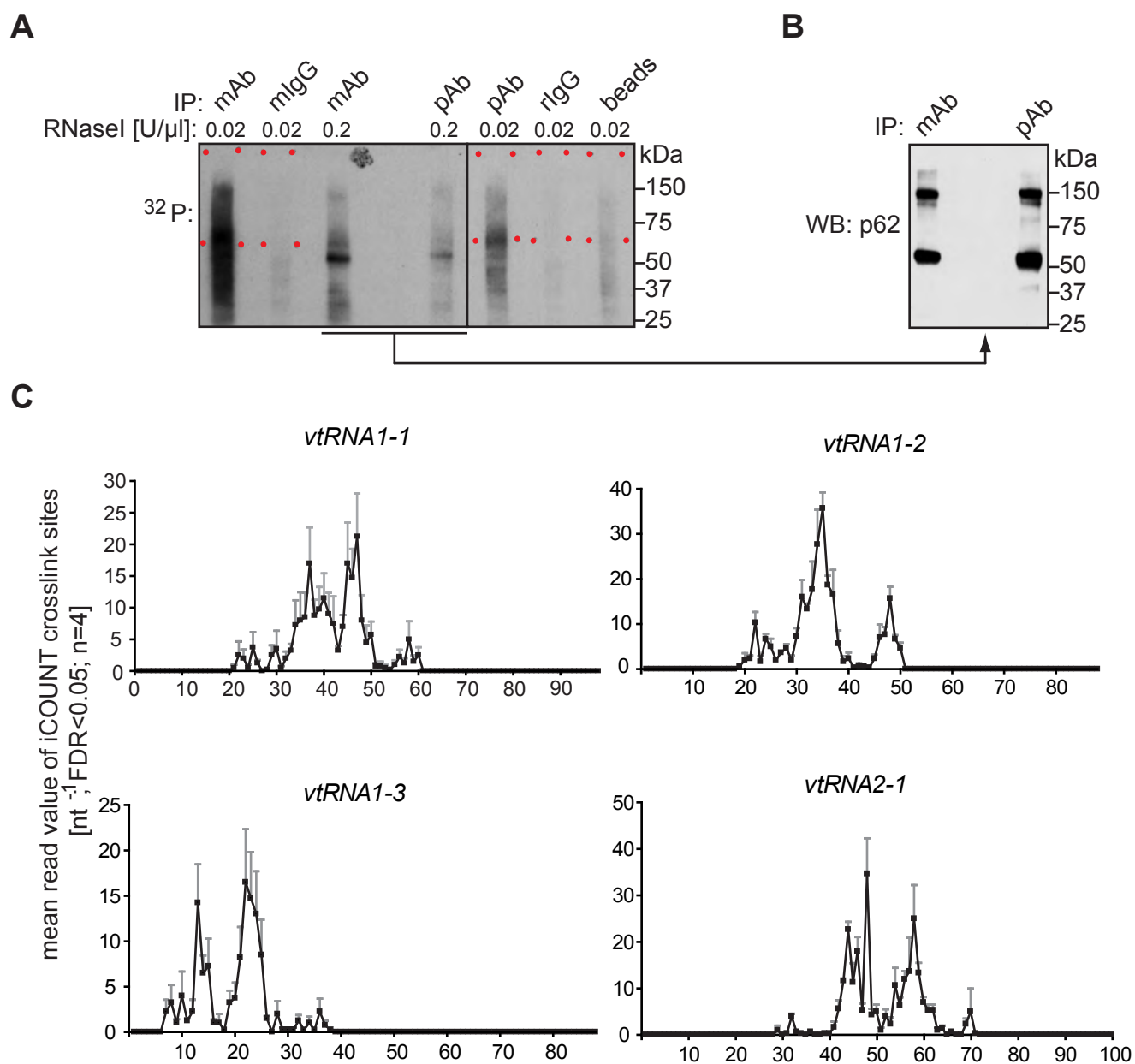


Figure S2

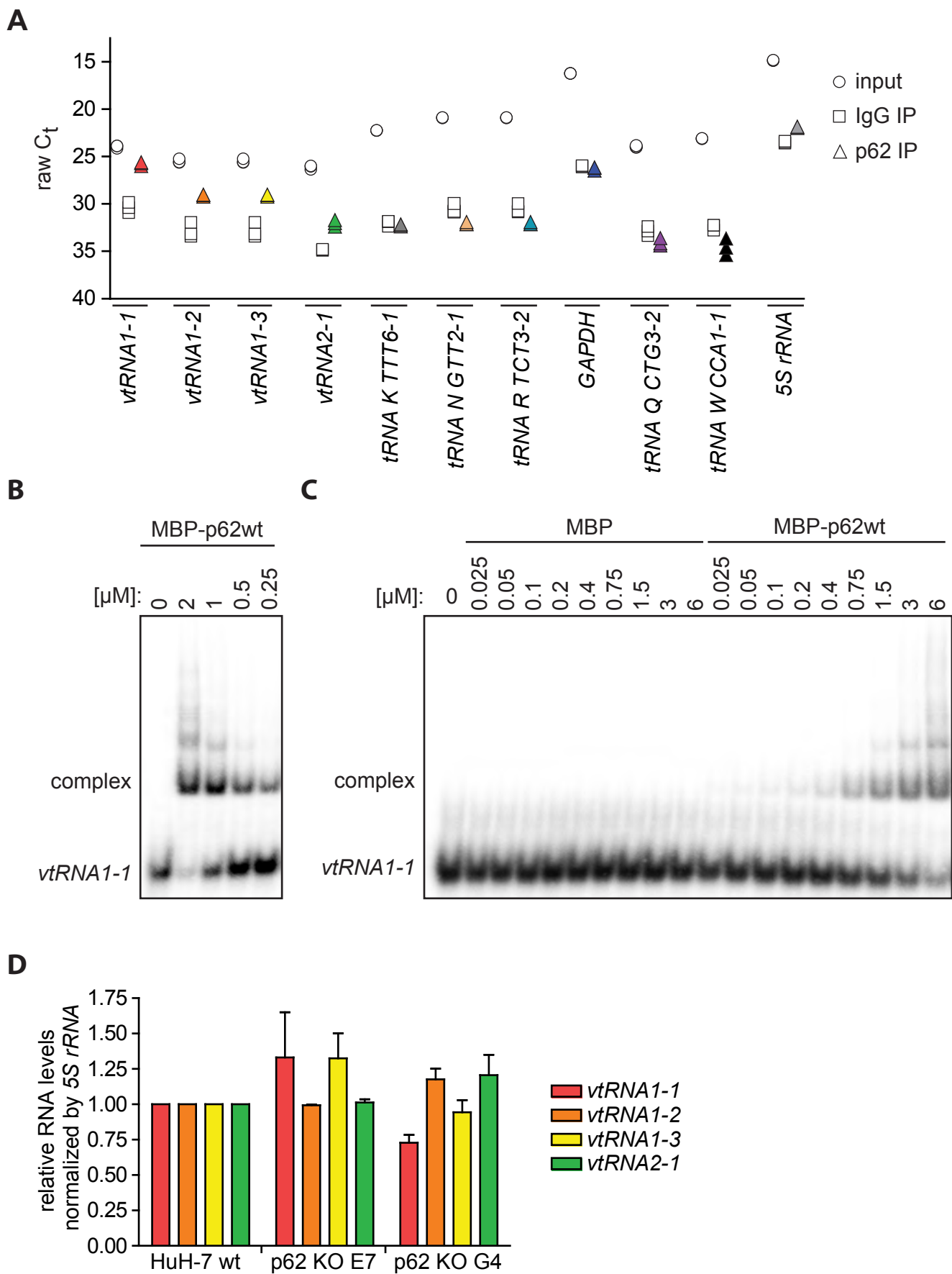


Figure S3

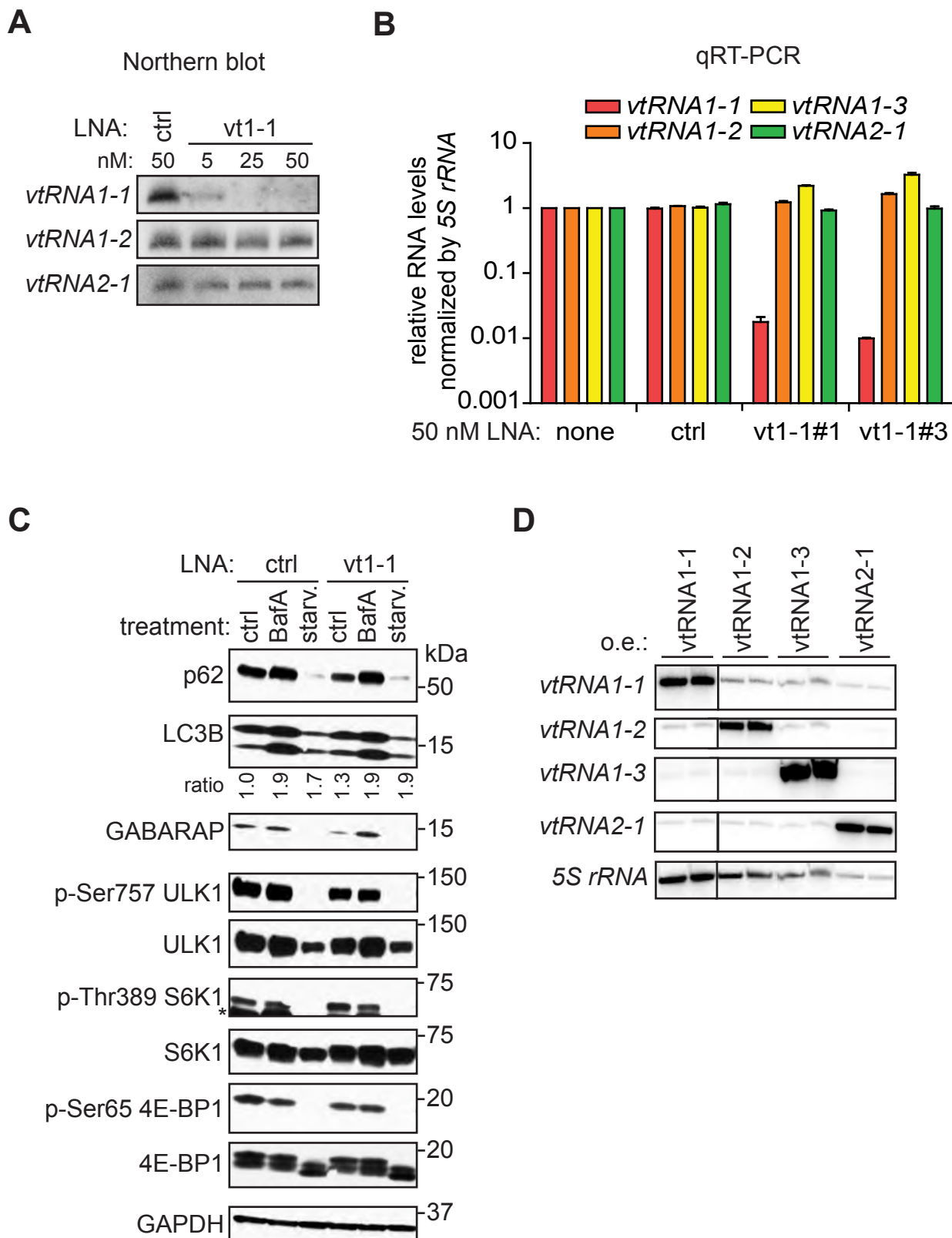


Figure S4

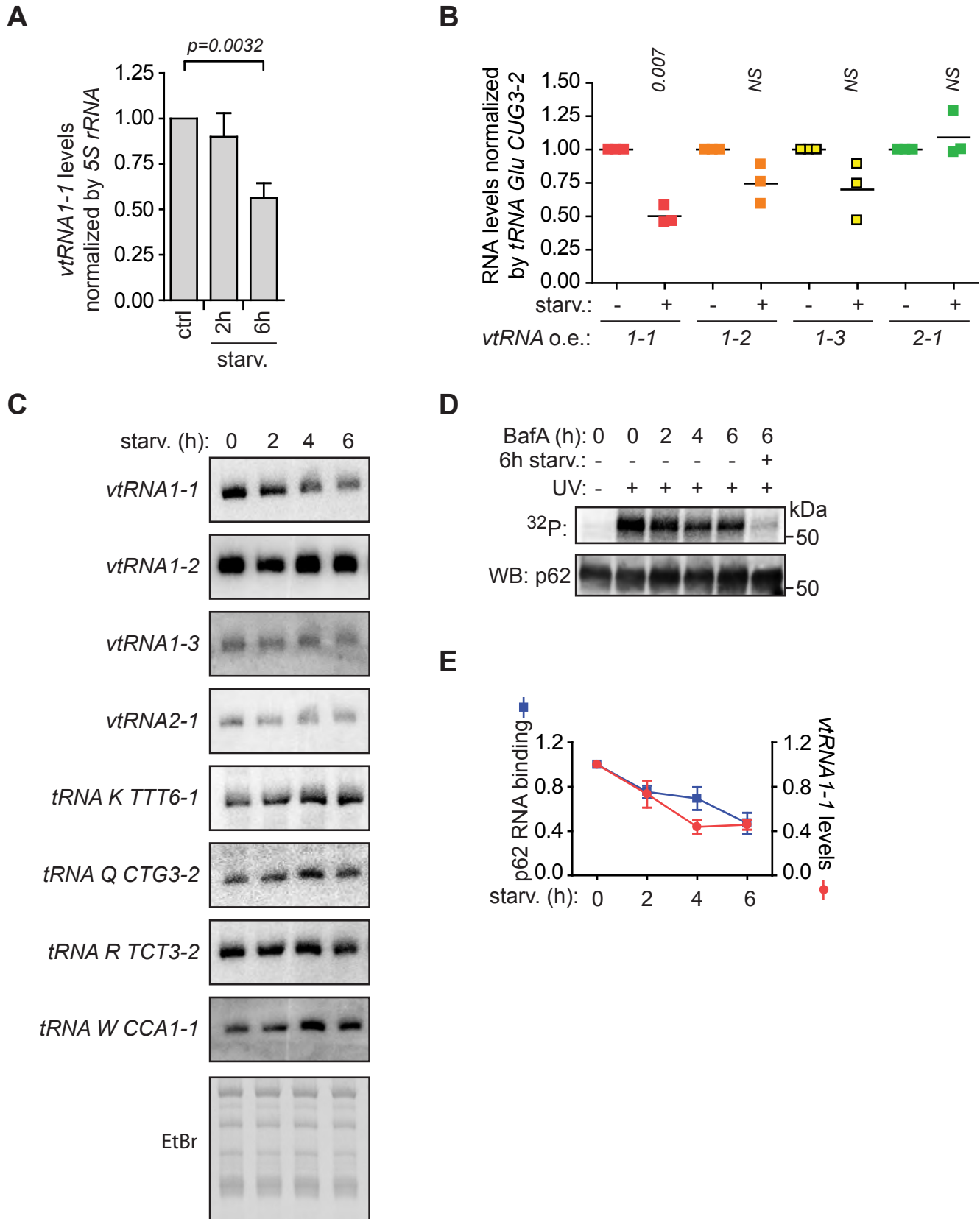
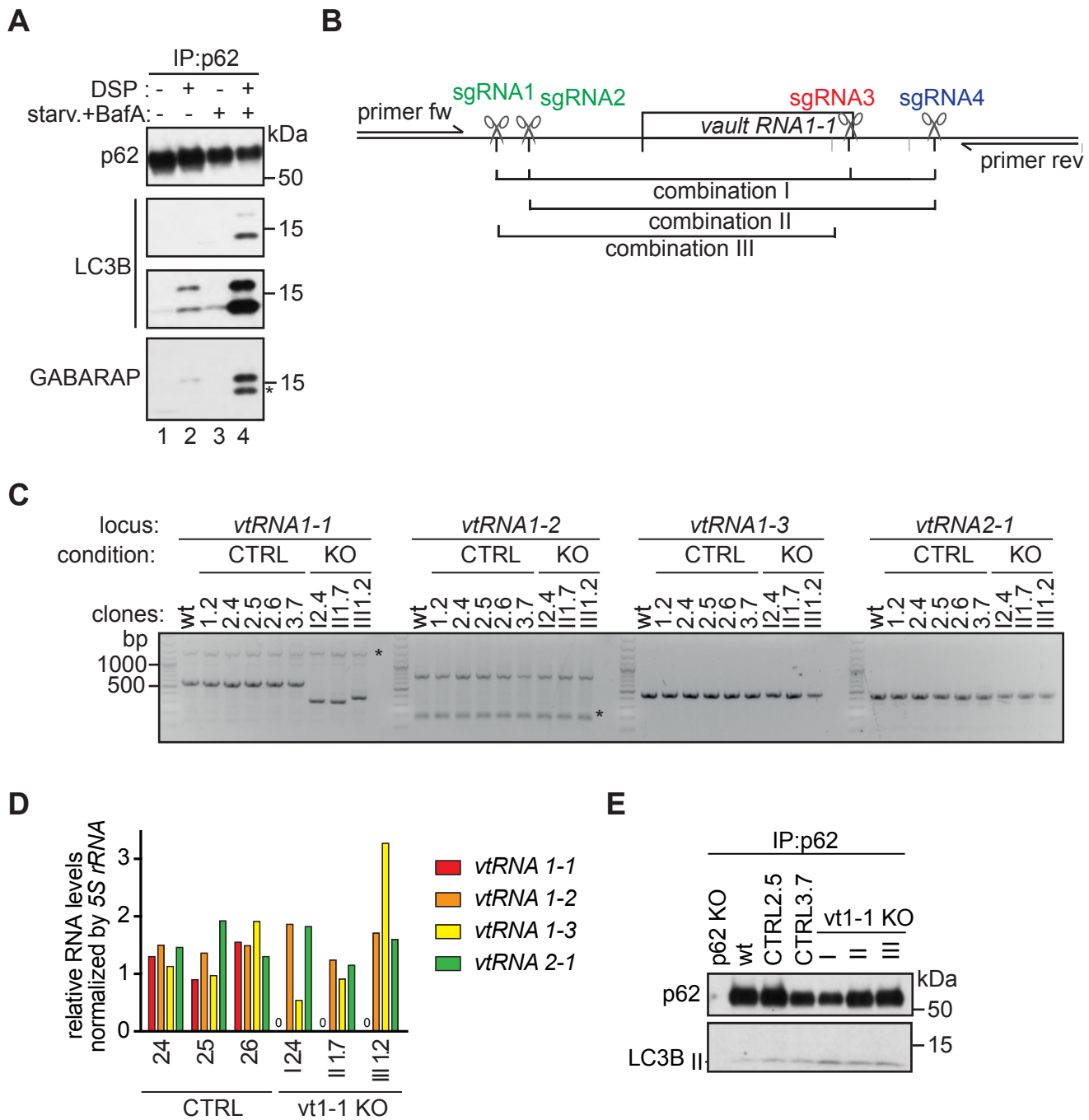


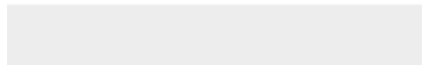
Figure S6





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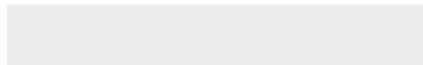
Supplemental Videos and Spreadsheets
181217_Horos et al_Table S1.xlsx





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