Abstract: Analysis of in vivo consequences of disease-relevant point mutations in p97 (VCP) in *Dictyostelium discoideum*

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p97 (VCP or valosin containing protein in mammals and Cdc48p in yeast) is a ubiquitously expressed and evolutionarily highly conserved hexameric member of the magnesium-dependent Walker P-loop AAA-ATPases. p97 has been associated with various essential cellular processes including ubiquitin-proteasome protein degradation and autophagy. Heterozygous mutations in the human VCP (p97) gene on chromosome 9p13-p12 cause a late-onset autosomal-dominant IBMPFD (inclusion body myopathy associated with early-onset Paget's disease of bone and frontotemporal dementia), ALS (amyotrophic lateral sclerosis) and HSP (hereditary spastic paraplegia). Up to now, more than 20 unique p97 missense mutations have been identified with codon 155 being a mutation hot spot. We studied the function of p97 and p97-R155C point mutation in *Dictyostelium discoideum*, and have generated strains that ectopically express p97-RFP or $p97^{R155C}$-RFP in AX2 wild-type and autophagy 9 (ATG9) knock-out cells. Co-immunoprecipitation studies using an anti-RFP antibody showed that endogenous p97 and $p97^{R155C}$-RFP form heteromers. Pull-down assays demonstrated that the R155C mutation disrupts the interaction of p97 with its specific adaptor UbxA. We also showed that UbxA promotes p97 hexamer disassembly into monomers. This activity of UbxA was abolished by the R155C point mutation. The mutant strains displayed changes in cell growth, phototaxis, development, proteasomal activity, ubiquitinated proteins, and ATG8 (LC3) indicating misregulation of multiple essential cellular processes. Immunofluorescence analysis revealed an increase of protein aggregates in $ATG9^{KO}$/$p97^{R155C}$-RFP and $ATG9^{KO}$ cells. They were positive for ubiquitin in both strains, however, immunoreactive for p97 only in the $ATG9^{KO}$ mutant. Immunoblotting showed an increase of ubiquitinated proteins and of the autophagy marker ATG8 (LC3). In a luminescence-based assay we found that proteasomal activity was slightly reduced in AX2/$p97^{R155C}$-RFP cells, but nearly completely inhibited in the $ATG9^{KO}$ mutant and partially rescued in the $ATG9^{KO}$/$p97^{R155C}$-RFP double mutant. In general, expression of $p97^{R155C}$-RFP in the $ATG9^{KO}$ strain partially or fully rescued the pleiotropic phenotype. We also observed dose-dependent effects of p97 on several cellular processes. Based on these findings in the $ATG9^{KO}$ mutant versus the $ATG9^{KO}$/$p97^{R155C}$-RFP double mutant we propose a novel mode of p97 interaction with the autophagy protein ATG9 which is based on mutual inhibition.