

Targeting p62 Palmitoylation as a Potential Therapeutic Strategy towards Huntington Disease

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Huntington's Disease (HD) is a neurodegenerative disorder caused by a mutation in the huntingtin gene leading to the accumulation of mutant huntingtin (mHTT) protein and disruption of many cellular pathways causing neuronal cell death. Lowering mHTT levels has become a primary therapeutic strategy. Autophagy, an intracellular clearance pathway is disrupted in HD at various stages including the cargo-loading step. mHTT aggregation in HD is believed to stem, partly, from the cargo-loading defect during autophagy resulting in empty autophagosomes. p62 is an autophagy receptor responsible for delivering misfolded proteins and aggregates to autophagosomes and eventually lysosomes for degradation. We have identified that p62 is palmitoylated. Palmitoylation is a critical post-translational modification required for protein trafficking and membrane associations. The dynamic nature of palmitoylation may allow for the re-localization of toxic proteins to the autophagosomes.

Unpublished data by Dr. Martin and colleagues has shown that palmitoylation of p62 is significantly reduced in the cortex of HD patients and the YAC128 HD mouse models and may be the cause of the cargo-loading defect of autophagosomes in HD. We predict that rescuing p62 palmitoylation will improve cargo loading and promote the clearance of toxic aggregates. A high-throughput screen of FDA-approved drugs previously identified Vorinostat, a histone deacetylase inhibitor, that can penetrate the blood-brain barrier and may increase palmitoylation.

The goal of this project was to Investigate the effect of Vorinostat on the palmitoylation of p62 as well as its localization to the autophagosomes and lysosomes. Through the work described in this thesis, I have confirmed that palmitoylation of p62 increases in response to

Vorinostat treatment with a concomitant increase in localization to the autophagosomes and the lysosomes. Additionally, Vorinostat improves the localization of HTT to the lysosomes. This study demonstrates that an increase in palmitoylation of p62 results in the inhibition of its acetylation providing insights into a potential mechanism of p62 regulation by Vorinostat. Understanding the underlying mechanism of this regulation will be integral in identifying new therapeutic targets for HD. Subsequently, the enzymes that affect protein palmitoylation were explored leading to the identification of a few candidates that may be regulating p62 palmitoylation. Identification of these enzymes may provide a therapeutic target for rescuing p62 palmitoylation. The investigations thus far suggest that Vorinostat is capable of rescuing p62 palmitoylation as well as improving the targeting of p62 and HTT to the lysosomes which may improve autophagy and have a therapeutic potential in HD.

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List of Abbreviations

2BP	2-bromopalmitate
ABE	Acyl Biotin Exchange
ABHD	α/β hydrolase domain serine hydrolases
APT	Acyl protein thioesterase
ATG	Autophagy-related proteins
BafA1	Bafilomycin A1
BTAA	2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid
CHX	Cycloheximide
Co-IP	Co-immunoprecipitation
DC	Detergent compatible
DMSO	Dimethyl sulfoxide
FVB	Friend leukemia Virus B
HAM	Hydroxylamine
HD	Huntington Disease
HDAC	Histone Deacetylase
HTT	Huntingtin
IP	Immunoprecipitation
LC3 (MAP1-LC3)	Microtubule associated proteins 1A/AB light chains 3
mHTT	Mutant huntingtin
MMTS	Methylmethanethiosulfate
mTORC1	mechanistic target of rapamycin complex 1
p62/SQSTM1	Sequestosome 1
PalmB	Palmostatin B
PAT	Palmitoyl acyl transferase
PCC	Pearson's Correlation Coefficient
PolyQ	Polyglutamine
PTM	Post-translational Modification
Pyrox	Pyroxamide
SH	Serine Hydrolase
TBTA	Tris((1-benzyl-4-triazolyl)methyl)amine
THPTA	(tris-hydroxypropyltriazolylmethylamine)
TSA	Trichostatin A
UBA	Ubiquitin-binding domain
UPS	Ubiquitin Proteasome System
WT	Wild-type

CHAPTER 1

INTRODUCTION

1.1 – Huntington Disease (HD):

Huntington Disease (HD) is an adult-onset progressive neurodegenerative disorder that is caused by selective striatal degradation which leads to loss of motor control and cognitive ability (Ross & Tabrizi, 2011; McColgan & Tabrizi, 2018). The average age of onset can range from 30-50 years. Patients ultimately die between 10-20 years post-onset (Ross & Tabrizi, 2011).

At the molecular level, HD is caused by a mutation in the huntingtin (*HTT*) gene. It is an autosomal dominant disease caused by a CAG trinucleotide expansion in the *HTT* gene resulting in a polyglutamine (polyQ) extension at the N-terminal of the HTT protein (Tabrizi et al., 2020). HTT is widely expressed in the body with the highest levels in the brain and the nervous system. Typically, the normal HTT protein has less than 35 CAG repeats however, mutant HTT (mHTT) has greater than 35 CAG repeats (Ross & Tabrizi, 2011; McColgan & Tabrizi, 2018). If there are more than 39 repeats, a person is almost guaranteed to develop HD. However, the likelihood of developing the disease is reduced for those with 36 to 39 repetitions. The risk of earlier onset of HD increases with the number of tandem repeats (McColgan & Tabrizi, 2018; Ferguson et al., 2022). As an autosomal dominant disease, a person will develop HD if they inherit one *mHTT* copy from either of their parents. Most of the patients are heterozygous for the disease genotype. So far there is no approved disease-modifying therapy. Nonetheless, research and development have focused on lowering mHTT levels as a therapeutic strategy towards HD (Ferguson et al., 2022).

The mutation is linked to the accumulation of mHTT aggregates within the cell. The mHTT aggregates are prone to sequestering other proteins and therefore the accumulation of mHTT and toxic molecules can disrupt various cellular pathways including autophagy. Like many neuropathies, the accumulation of aggregates in HD is linked to dysregulated autophagy (Martin et al., 2015; Menzies et al., 2017).

1.2 – Autophagy

Many neurodegenerative diseases are characterized by the accumulation of proteins due to protein turnover deficiencies (Menzies et al., 2017; Stavoe & Holzbaur, 2019; Ma et al., 2019). The cell has two major mechanisms to remove misfolded proteins: the Ubiquitin Proteasome System (UPS) and autophagy (Ning & Wang, 2019). Dysregulation in either of these mechanisms can contribute to aggregation in neurodegenerative diseases.

Autophagy is an intracellular pathway responsible for the clearance of damaged organelles and toxic or aggregated proteins. It plays a critical role in maintaining cellular homeostasis, adapting to stress conditions, and ensuring proper cellular function. Maintaining neuronal health is crucial, and autophagy plays a vital role in achieving this (Stavoe & Holzbaur, 2019). With age, neuronal cells tend to accumulate damaged organelles that can be toxic and harmful. Dysregulated autophagy contributes to proteostasis deficiencies in various diseases. Many neurodegenerative disorders have been found to have dysfunctional autophagy as a common pathway (Nah et al., 2015; Menzies et al., 2017; Ma et al., 2019; Aman et al., 2021). Neurons, being postmitotic and non-dividing cells, rely on autophagy to maintain cellular health by effectively eliminating toxic substances (Kulkarni & Maday, 2018; Stavoe & Holzbaur, 2019).

There are three types of autophagy pathways: microautophagy, macroautophagy, and chaperone-mediated autophagy (Aman et al., 2021). The best-characterized pathway is macroautophagy which will be the focus of this thesis, henceforth referred to as 'autophagy.'

Autophagy is a tightly regulated process and begins with the formation of a double membrane precursor structure called a phagophore which engulfs the target waste, consisting of damaged organelles and toxic protein aggregates (Aman et al., 2021). The phagophore matures and closes to form a vesicle called an autophagosome. The cargo-carrying autophagosomes fuse with lysosomes forming autolysosomes. Within the autolysosomes, the toxic cargo is exposed and degraded or recycled by the action of the lysosomal enzymes (Figure 1.1 a).

Autophagy is regulated by the conserved autophagy-related proteins (ATG) (Ohsumi, 2014). LC3 (Microtubule-associated protein 1 light chain 3), also known as ATG8, is a crucial protein involved in autophagy. LC3 exists in two forms: LC3-I and LC3-II. LC3-I is mostly cytosolic while LC3-II is predominantly associated with autophagosomes (Martin et al., 2015; Menzies et al., 2017; Yoshii & Mizushima, 2017). The C-terminus of LC3 is cleaved by a cysteine protease called ATG4 forming LC3-I. Autophagosome elongation requires the conjugation of phosphatidylethanolamine (PE) to LC3-I, resulting in the formation of LC3-II. LC3-II acts as a marker of autophagosomes. Many autophagy cargo receptors bind to LC3-II via their LC3-interacting region (LIR) thereby delivering toxic waste to the autophagosomes. This interaction facilitates selective targeting to the autophagosomes and thus degradation (Martin et al., 2015; Menzies et al., 2017; Ma et al., 2019).

A key autophagy receptor is p62, also known as Sequestosome1 (SQSTM1) (Ma et al., 2019; Kumar et al., 2022). p62 binds to the ubiquitinated proteins, marked for degradation, via the ubiquitin-binding (UBA) domain. p62 directly binds with LC3-II, through its LC3 interacting region located at its C-terminus. The interaction of p62 and LC3-II results in the formation of autophagosomes, carrying damaged proteins, that later fuse with lysosomes for the removal of cellular waste (Pankiv et al., 2007; Ma et al., 2019). During autophagy, p62 gets actively degraded (Bjorkoy et al., 2005). LC3-II on the other hand is present on the outer surface as well as inside the autophagosomes (Kabeya et al., 2000). LC3-II inside the autophagosomes gets degraded in the lysosomes. While LC3-II on the outer surface of the autophagosomes is recycled back to LC3-I which can be reused for autophagosome formation (Tanida et al., 2005; Yoshii & Mizushima, 2017).

1.3 – Autophagy Dysregulation in HD

HTT is a scaffold protein that has a role in various cellular processes including autophagy (Ochaba et al., 2014; Gelman et al., 2015; Rui et al., 2015; Ashkenazi et al., 2017). HTT interacts with p62 and facilitates its association with LC3 thereby playing an integral role in autophagosome formation (Rui et al., 2015). The mHTT aggregates are prone to sequestering other proteins and, therefore, the accumulation of mHTT and toxic molecules can disrupt the cellular pathways. In HD, autophagy is disrupted at various stages including the cargo-recognition step (Martin et al., 2015; Figure 1.1 b). An increased abundance of autophagosomes in HD cells has been reported, however, these vesicles lacked cytosolic cargo (Martinez-Vicente et al., 2010). Thus, despite the induction of autophagy, the toxic aggregates fail to get targeted

in the autophagosomes. This cargo-loading defect in HD leads to the accumulation of empty autophagosomes and toxic proteins in the cells (Figure 1.1 b). The exact mechanism of this defect remains unknown but may be associated with p62 which selectively facilitates the clearance of mHTT aggregates (Bjørkøy et al., 2005).

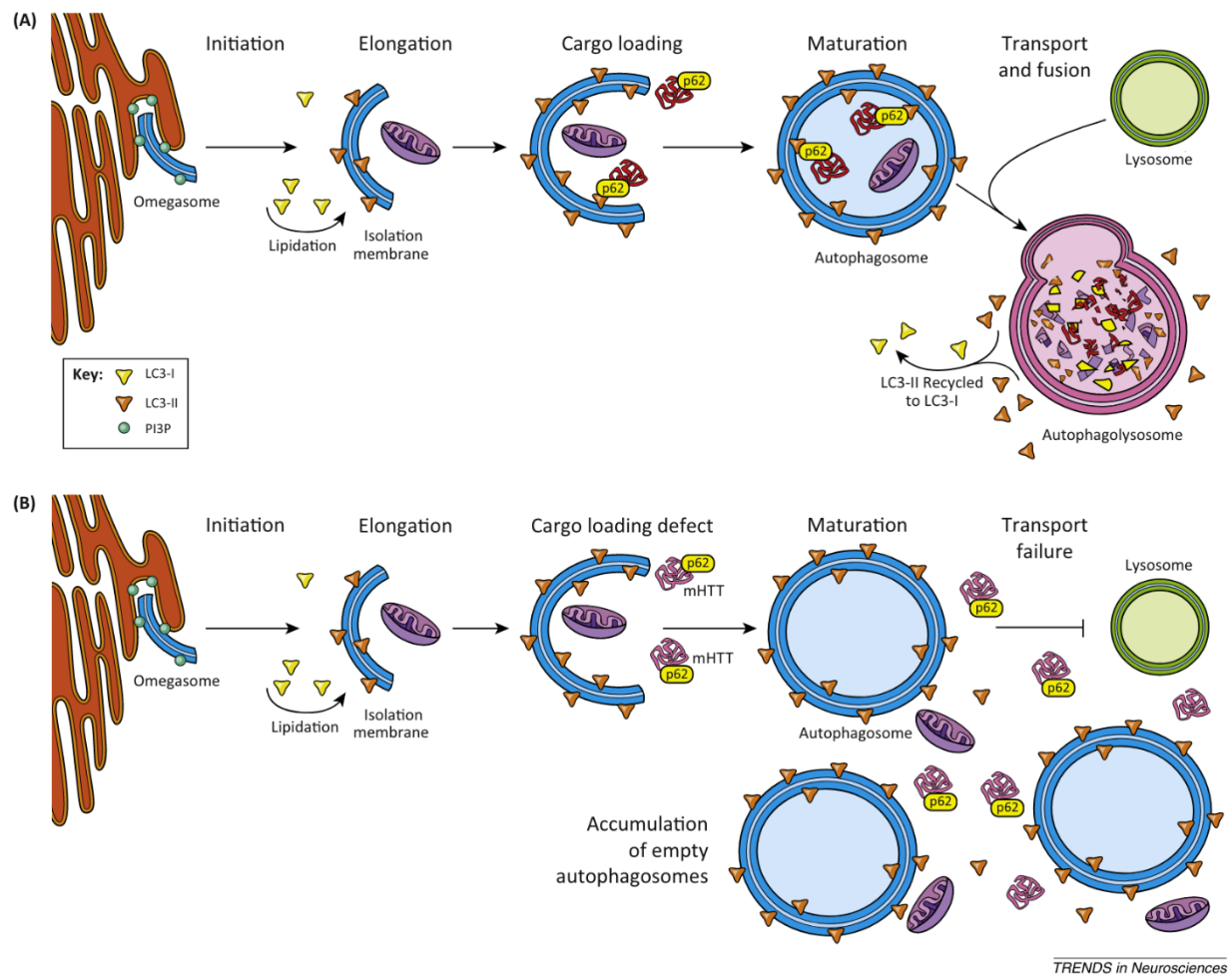


Figure 1.1. Autophagy dysregulation in HD. (A) The normal autophagic pathway involves the formation of autophagosomes through LC3 and cargo-loading through receptors like p62. **(B)** In HD, there is a defect at the cargo-loading step contributing to the accumulation of toxic aggregates and empty autophagosomes. (Martin et al., 2015).

1.4 – Autophagy and Palmitoylation

Autophagy is a highly membrane-dependent process that requires quick re-localization and targeting of the proteins onto membranes. An emerging mechanism that may govern the protein localization to the membranes is palmitoylation. Several autophagy-related proteins are predicted to be palmitoylated (Ren et al., 2013), while a few have already been validated (Blaustein et al., 2021; Sanders et al., 2015). A study reported that diseases with proteostasis deficiencies were enriched in palmitoylated proteins (Sanders et al., 2015). S-acylation or palmitoylation is a form of lipidation that is critical for protein trafficking and membrane associations (Zaballa & van der Goot, 2018; Main & Fuller, 2022). The process of palmitoylation is crucial in attaching proteins to membranes, which enables protein localization to be dynamically regulated (Aicart-Ramos et al., 2011; Zaballa & van der Goot, 2018). Palmitoylation is enriched in neuronal processes including synaptic vesicle fusion and recycling, implicating an important role of palmitoylation in the brain and neurodegenerative diseases (Sanders et al., 2015). Moreover, I did a comparison of the known genes of proteins from mouse, rat, and humans available on the palmitoyl-proteome database, SwissPalm (Blanc et al., 2015) and autophagy core and regulatory proteins from the autophagy regulatory network (Türei et al., 2015) (Figure 1.2). Based on the analysis, it was discovered that more than 60% of autophagy regulatory genes have a proteoform that could potentially be palmitoylated (Ramzan et al., 2023). Together, these findings implicate the role of palmitoylation in autophagy.



Figure 1.2. Autophagy is enriched in Palmitoylation. A comparison of autophagy-related genes and palmitoylated protein genes shows that about 60% of proteins in autophagy are palmitoylated, represented by the overlap between genes in the Venn diagram. The genes that encode palmitoylated proteins are from the SwissPalm database (Blanc et al. 2015) and the core as well as genes encoding regulatory autophagy proteins from the Autophagy Regulatory Network (Türei et al. 2015). (Ramzan et al., 2023).

1.5 – Palmitoylation

Palmitoylation is a reversible post-translational modification (PTM) that involves the addition of the saturated 16-carbon fatty acid palmitate onto a cysteine residue via a liable thioester bond (Zaballa & van der Goot, 2018; Main & Fuller, 2022). The enzymes that mediate this mechanism are called palmitoyl acyltransferases (PATs) (Fukata & Fukata, 2010; Ohno et al., 2006). There are 23 known PATs in mammals. This family of enzymes has a highly conserved Zn^{2+} -binding motif and the DHHC (Aspartate-Histidine-Histidine-Cysteine) motif within their active site and thus are also known as the ‘ZDHHC’ enzymes (Fukata & Fukata, 2010)

The removal of palmitate, or depalmitoylation, is catalyzed by acyl protein thioesterases (APTs) APT1 and APT2, which are members of the metabolic serine hydrolase superfamily (Won et al., 2018). More recently, some of the α/β hydrolase domain (ABHD) serine hydrolases including ABHD17A, B, C and ABHD16A were identified as novel depalmitoylation enzymes (Lin & Conibear, 2015; Shi et al., 2022).

1.6 – Methods for Detecting Palmitoylation

Detection of palmitoylation requires specialized assays (Wan et al., 2007; Yap et al., 2010; Forrester et al., 2011; Main & Fuller, 2022). A group of assays exploit the reversibility of palmitoylation and replace the endogenous palmitate on the proteins with biotin or thiol-reactive beads, which allow for affinity purification of palmitoylated proteins from protein lysates (Sanders et al., 2015; Edmonds et al., 2017). Acyl biotin exchange (ABE) assay is a biotin-based approach. Free cysteines of the proteins are first blocked followed by the selective cleavage of the thioester bonds resulting in newly available free cysteines which are linked with biotin. The biotinylated proteins can then be isolated using streptavidin or neutravidin affinity purification and subjected to various downstream analyses, such as mass spectrometry or Western blotting, enabling a comprehensive understanding of the dynamic nature and functional implications of protein palmitoylation in cellular processes.

Another approach is metabolic labelling with click chemistry using bioorthogonal compounds which has emerged as a powerful technique for the precise detection and study of palmitoylation. Bioorthogonal compounds do not occur naturally, but the reaction can take place in a biological environment without interfering with the biochemical processes (Saxon &

Bertozzi, 2000; Agard et al., 2006; Bird et al., 2021; Scinto et al., 2021). Click chemistry reaction involves the copper-catalyzed azide-alkyne cyclo-addition (Agard et al., 2006; Figure 1.3 b). For palmitoylation detection, cells are metabolically labelled with 16-carbon alkynyl-palmitate followed by a click reaction between the fatty acid analogues and azide-tagged biotin probes (Yap et al., 2010; Liao et al., 2021). This reaction covalently links the alkyne-palmitate with biotin allowing downstream analyses. These assays led to the discovery of many candidate proteins that need to be validated through low-throughput methods (Sanders et al., 2015).

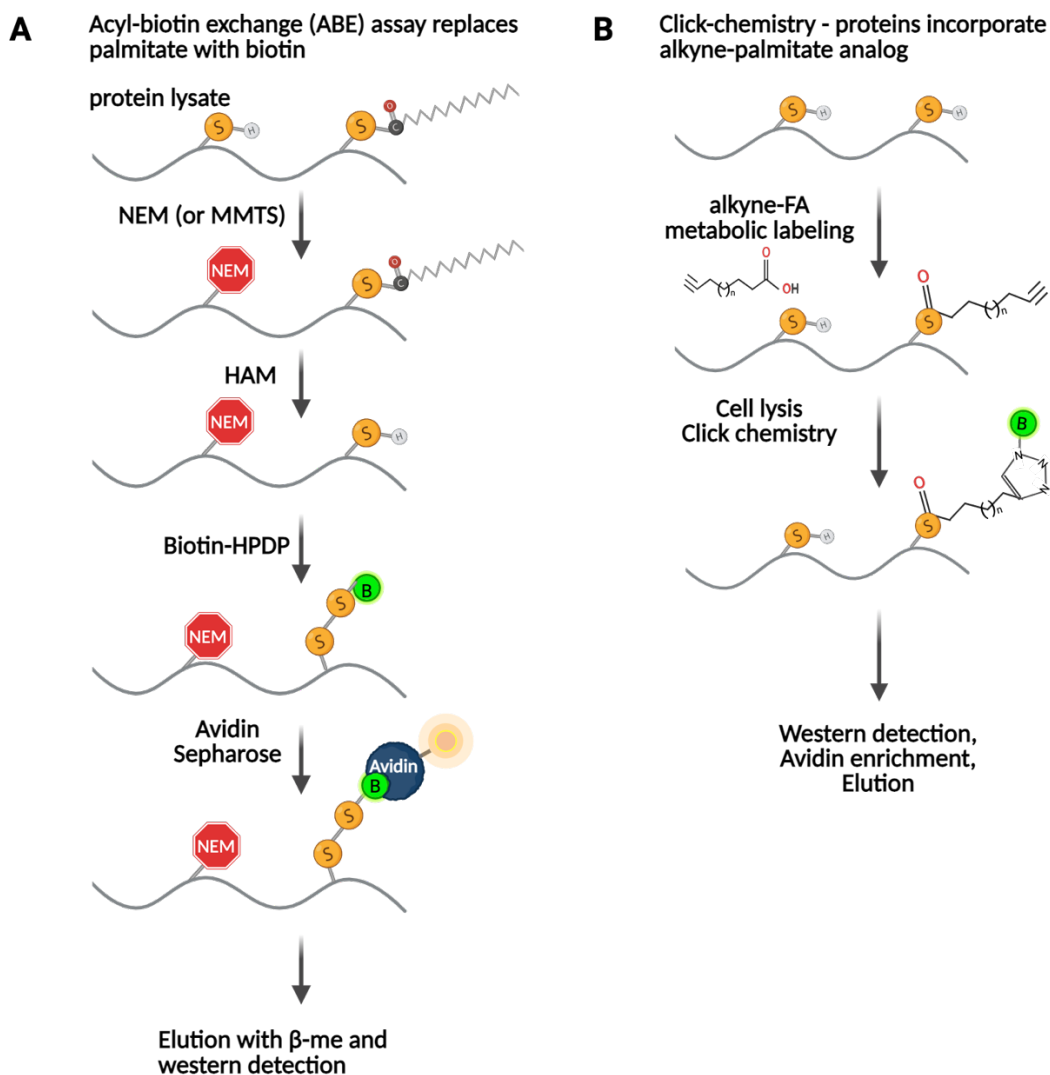


Figure 1.3. Palmitoylation Detection Methods. (A) The ABE assay uses N-ethylmaleimide (NEM) or methylmethanethiosulfonate (MMTS) to block the free cysteines followed by the removal of endogenous palmitate using hydroxylamine (HAM). ABE uses thiol-reactive biotin (HPDP) to replace palmitate with biotin. (B) The click chemistry approach involves metabolically labelling cells with alkyne-palmitate analogues followed by a click reaction to covalently link palmitate to biotin. (Figure generated on BioRender, provided by Dr. Martin).

1.7 – Palmitoylation in HD

Even though HD primarily results from a polyQ expansion at the N-terminus of HTT, there is enrichment of palmitoylated proteins related to HD pathogenesis (Sanders et al., 2015). This indicates that this PTM could be a crucial factor in HD. HTT is palmitoylated at C105, C214, C433, C3134 and C3144 (Yanai et al., 2006; Lemarié et al., 2023). The palmitoylation of this protein is mediated by ZDHHC17 and ZDHHC13 (Yanai et al., 2006; Butland et al., 2014) while the removal of palmitate is catalyzed by APT1 and APT2 (Lin & Conibear, 2015; Lemarié et al., 2021).

Lemarié and colleagues reported reduced palmitoylation of mHTT in HD mouse models which becomes more pronounced with increasing age (Lemarié et al., 2021). The mHTT palmitoylation levels normalize upon treatment with APT inhibitors in mHTT-expressing cells. Furthermore, the improved palmitoylation levels reduced mHTT aggregation and cytotoxicity in COS-7 cells and cultured YAC128 mouse neurons (Lemarié et al., 2021). Thus, their results suggest that inhibiting depalmitoylation, and therefore increasing palmitoylation, improves the clearance of mHTT aggregates. Another study reported that APT1 inhibitor ML348 treatment restored neuropathology in CAG140 knock-in HD mouse models (Virlogeux et al., 2021). Although this study did not investigate mHTT palmitoylation specifically, their results suggest that restoration of overall brain palmitoylation improves HD phenotype in HD mice. Collectively, these studies suggest a potential therapeutic effect of improved palmitoylation in HD models.

Unpublished data by Martin and colleagues has confirmed the palmitoylation of p62. They also found that the palmitoylation of p62 is significantly reduced in age-matched HD patient brains and YAC128 HD mice. This was accompanied by a noticeable overall increase in the p62 protein levels in patient brains. Thus, suggesting that non-palmitoylated p62 is not getting

degraded. We believe that reduced palmitoylation of p62 in HD may be linked to dysregulated autophagy at the cargo-loading step. A drug capable of rescuing the palmitoylation of p62 may have therapeutic potential in HD by restoring palmitoylation of mHTT and p62.

1.8 – Vorinostat is a Putative Drug Candidate for Increasing Palmitoylation

In a previous study, Dr. Martin performed a high-throughput screen of FDA-approved drugs and identified Vorinostat as a potential drug capable of increasing palmitoylation (Martin et al., 2019). Suberoylanilide hydroxamic acid (SAHA) or Vorinostat is an FDA-approved drug that inhibits histone deacetylases (HDAC) (Li & Seto, 2016). Vorinostat inhibits class I and class II HDAC enzymes which include HDACs 1, 2, and 3 from class I, and HDAC 6 from class II. It can easily cross the blood-brain barrier and is currently being used as a weak chemotherapeutic agent (Athira et al., 2021). Vorinostat is being tested in neurodegenerative disorders and has demonstrated neuroprotective effects in models of different diseases including HD, Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease and Parkinson's Disease (Shukla & Tekwani, 2020; Athira et al., 2021). In an accelerated R6/2 HD mouse model, that only expresses the polyQ expanded N-terminus, Vorinostat decreased mHTT insoluble aggregates in the cortex and brain stem, thereby reducing disease progression (Hockly et al., 2003).

HDACs mediate the deacetylation of proteins which is the reverse process of acetylation. Acetylation is a PTM that is one of the major regulators of protein function (Narita et al., 2019). It involves the reversible addition of an acetyl group onto a lysine residue. The acetylation of the proteins is mediated by lysine acetyltransferases (KATs). Deacetylation is mediated by lysine deacetylases (KDACs) or more commonly referred to as HDACs since acetylation was first

discovered in histones (Allis et al., 2007). Acetylation is the most common modification of non-histone proteins and is involved in diverse cellular mechanisms (Choudhary et al., 2014; Narita et al., 2019).

In 2019, the Liu group confirmed the acetylation of p62 at the K420 and K435 sites within its UBA domain (You et al., 2019). Acetylation of p62 is mediated by TIP60/KAT5 (You et al., 2019) and deacetylation is catalyzed by HDAC6 (Yan et al., 2013; You et al., 2019). The acetylation of p62 facilitates the autophagic degradation of ubiquitinated substrates under starvation (You et al., 2019). p62 is prone to dimerization within the UBA; acetylation disrupts the self-dimerization of p62 enhancing its binding to the ubiquitinated substrates and thus promoting the p62 cargo assembly (You et al., 2019; Xu & Wan, 2023).

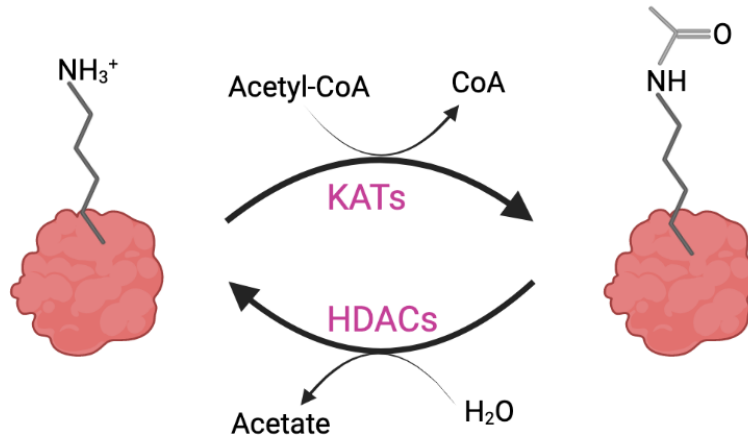


Figure 1.4. Acetylation Process. Acetylation is mediated by KDACs in the forward direction. It involves the reversible addition of an acetyl group onto a lysine residue. The deacetylation of proteins is catalyzed by HDACs (or KDACs). (*Generated in BioRender*).

1.9 – Research Objective

Rescuing p62 palmitoylation may be a therapeutic strategy in HD as this may re-direct p62 to the autophagosomes and promote degradation of toxic aggregates hence, improving the cargo-loading defect in autophagy. The goal of this project was to investigate the effect of Vorinostat on p62 palmitoylation. This study aims to determine whether a potential increase in p62 palmitoylation influences the localization of p62 to the autophagosomes and the lysosomes. Additionally, the enzymes that may be regulating p62 palmitoylation were explored as identifying these enzymes may provide potential therapeutic targets to rescue p62 palmitoylation in HD.

CHAPTER 2

Methods and Materials

2.1 Cell Culture and Transfection:

1) 2.1.1 Cell Culture

Mammalian Cell culture:

Henrietta Lacks (HeLa) carcinoma cells (Sigma, 93021013) were maintained in 10-cm petri dishes in Dulbecco's Modified Eagle Medium (DMEM) (Winstent, 319015CL) supplemented with 10% Fetal bovine serum (FBS), 1X Penicillin-Streptomycin, 2 mM L-glutamine, and 100 mM sodium pyruvate (1% vol/vol). These cells were split every 2-3 days once they reached 80-90% confluency.

Cell splitting:

The cells were split up to 30 passages. The cells were first washed with 1x phosphate buffer saline (PBS). They were then incubated with 1X EDTA-enriched trypsin solution for 5 minutes at 37°C under 5% CO₂. These detached cells were then diluted 5-fold in fresh DMEM media into new 10-cm plates. The freshly diluted HeLa cells were maintained at 37°C, 5% CO₂.

2) 2.1.2 Calcium Phosphate Transfection:

HeLa cells were plated and cultured in 6-well dishes or 6-cm plates the night before and grown at 60-80% confluency. The cells were transfected the next day with the indicated plasmids using the calcium phosphate DNA precipitation as previously

described by (Narita et al., 2019). Cells cultured in 6-well dishes were transfected with 5 μ g of DNA per well and cells in 6-cm plates with 10 μ g DNA. The DNA mix was prepared as described in Table 1 and added to the cells grown in DMEM media at 37°C, 5% CO₂. 2 hours post-transfection the media was replaced with fresh media. Drugs at their desired dosages were added to the cells at this stage for the experiments that required overnight drug treatments (Table 7). Cells were transfected for 18-20 hours prior to doing downstream biochemical assays.

Table 2.1: Transfection DNA mix

	6-well dishes / μL	6-cm plates / μL
0. 25 μg/mL DNA	20	40
2 M CaCl₂	10.2	20.4
2X HBS*	83.3	166.6
Sterile Water	53.1	106.2

*HBS: 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) buffered saline

2.2 Immunoprecipitation and Co-immunoprecipitation

1) 2.2.1. Cell Lysis:

Post transfection, the cells were lysed and harvested with a modified Radio immunoprecipitation assay (RIPA) buffer containing 0.1% SDS, 50 mM HEPES (pH 7), 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 2 mM MgCl₂, and 1 mM EDTA. 1X Protease inhibitor cocktail (PIC) was added to the RIPA buffer for lysing cells on ice. 0.5-1 mL of buffer was added to cells. The cells were scraped and collected in sterile 1.5 mL centrifuge tubes. The samples were lysed by rotating at 4°C for about 15-20 minutes. The tubes were centrifuged at 13.2 (x1000) rpm at 4°C for 10 minutes. The supernatant

was collected into fresh centrifuge tubes and the lysates were stored at -20°C until ready for further biochemical investigations by running on a gel for western blotting.

The concentration of proteins following lysis was determined using a detergent compatible (DC) assay as per manufacturer's instructions (BioRad), bovine serum albumin (BSA) was used as the protein concentration standard which included 0, 2, 4, 6, 8, 10, 12 and 15 µg/µL of BSA.

2) *2.2.2 Immunoprecipitation (IP):*

Equal amounts of protein, between 200-600 µg diluted in RIPA buffer, were added to fresh centrifuge tubes and immunoprecipitated end-over-end at 4°C overnight with 0.5-1 µL of antibody.

Magnetic Protein G beads were equilibrated in RIPA lysis buffer thrice and then resuspended. 15-20 µL of beads slurry was added to each sample and rotated at 4°C for 2 hours. After this incubation, the beads were washed with RIPA lysis buffer thrice. Beads were then resuspended in 60 µL of an elution buffer containing 2X Sample Loading buffer (SLB) and 5% beta-mercaptoethanol. The samples were then boiled at 95°C for 5 minutes followed by centrifugation pulsed to about 10,000 rpm. The supernatant was collected and stored at -20°C until ready to for downstream analysis via Western blotting.

2.3 Acyl Biotin Exchange (ABE) assay:

1) 2.3.1 Cell Lysis:

Cells transfected with the DNA of interest for 18-20 hours were lysed in ABE lysis buffer containing 50 mM HEPES (pH 7), 2% SDS, and 1 mM EDTA post-PBS wash. 500 μ L of ABE lysis buffer was added to the cells with freshly added 1X PIC and 1% methyl methanthiosulfate (MMTS). The cells were then scraped and collected in fresh 1.5 mL centrifuge tubes. These lysates were sonicated for 12s at continuous power with 25% amplitude, with 6s ON and 2s OFF.

2) 2.3.2 Blocking of Free Thiols and Acetone Precipitation:

The sonicated lysates were transferred to 15 mL falcon tubes. An equal amount (i.e., 500 μ L) of ABE lysis buffer containing 1X PIC and 1% MMTS was added to the lysates. The samples were incubated at 50°C for 30 minutes with brief vortexing in between. 4 mL of 100% chilled acetone was added to the samples to an 80% final concentration. The samples were mixed by inversion and stored at -20°C overnight.

3) 2.3.3. Removal of Endogenous Palmitate and Acetone Precipitation:

Samples were mixed by inversion to dissolve the acetone-precipitated protein. Samples were washed thrice with 6 mL of 80% chilled acetone by centrifuging at 2200 rpm, 4°C for 5 minutes. The pellet was resuspended in 1 mL of 4X Suspension buffer (4SB) containing 50 mM Tris (pH 7.5), 5 mM EDTA, 4% SDS, and 1X freshly added PIC. The resuspended proteins were transferred to the new 15 mL tubes. 30 μ L of the samples were aliquoted into separate centrifuge tubes to prepare “no HPDP” input samples. 18 μ L of 4SB containing 1X PIC, and 12 μ L of 5X SLB with 5% beta-mercaptoethanol were

added to each sample. 12 μ L of 5X SLB containing 5% beta-mercaptoethanol was added and the samples were boiled at 95°C for 5 minutes to denature the proteins. The samples were stored at -20°C until ready to be run on a gel.

25 mM HPDP-biotin (Soltech) was prepared in N, N-Dimethylformamide and diluted to a stock concentration of 4 mM in DMSO. 1 M hydroxylamine (HAM) stock was prepared, and pH adjusted between 7.3 -7.5 with aqueous sodium hydroxide solution. Samples were incubated with 1ml of HAM master mix (1 mM HPDP-biotin, 0.7 M HAM, 50 mM Tris pH 7.5, 0.2% Triton X-100, 1X PIC) and rotated end-over-end at room temperature for 1 hour in the dark.

Post incubation, 8 mL of 100% chilled acetone was added to each sample at an 80% final concentration to acetone precipitate proteins. Samples were stored at -20°C overnight.

4) 2.3.4 Affinity Purification of Palmitoylated Proteins with Neutravidin beads

Protein samples were washed with 6 mL of 80% chilled acetone by centrifugation at 2200 rpm, 4°C for 5 minutes. The pellets were resuspended in 1 mL of ABE lysis buffer (with 1X PIC, no MMTS). Using the DC assay the concentrations of proteins were determined in each sample. About 15-20 μ g of protein was diluted in ABE lysis buffer to a final volume of 48 μ L to prepare “HPDP” input samples. To denature proteins, 12 μ L of 5X SLB without beta-mercaptoethanol was added to these samples and boiled at 95°C for 5 minutes.

30 μ L of Neutravidin agarose resin beads (Thermofisher Scientific, 29202) per sample were equilibrated and resuspended in dilution buffer (50 mM Tris pH 7.0, 1% TX-

100, 1 mM EDTA, 1 mM EGTA) containing 150 mM NaCl and freshly added protease inhibitors, 10 µg/mL Pepstatin A and 1 mM phenylmethylsulfonyl fluoride (PMSF). Based on protein quantification via DC assay, 400-500 µg of protein was diluted in ABE lysis buffer to a volume of 500 µl. 30 µl of beads slurry was added to each sample to pulldown palmitoylated proteins. The samples were incubated in 9.5 mL of dilution buffer containing 150 mM NaCl for 3 hours with end-over-end rotation at 4°C.

The protein-beads samples were pelleted down by centrifugation at 2000 rpm, 4°C for 3 minutes and resuspended in 1 mL of dilution buffer containing 0.5M NaCl and freshly added 10 µg/mL Pepstatin A and 1 mM PMSF. Proteins were transferred to new 1.5 mL centrifuge tubes and washed twice more with 0.5 M NaCl dilution buffer. The pellet was further washed twice with dilution buffer (without NaCl). The pellet was then resuspended in 50 µL of elution buffer (0.2% SDS, 250 mM NaCl, 1% beta-mercaptoethanol – diluted in dilution buffer). The beads were incubated at 37°C for 10 minutes with flick-mixing. Following elution, beads were spun down by quick centrifugation, and 40 µL of the supernatant was collected into fresh 1.5 mL centrifuge tubes. To these eluted protein samples, 10 µL of 5X SLB containing 5% beta-mercaptoethanol was added and samples were boiled at 95°C for 5 minutes to denature the proteins. Both HPDP input and pulldown samples were stored at -20°C until ready to be run on a gel for further analysis.

2.4. Click Chemistry Assay:

1) 2.4.1. Lipid Deprivation:

Click assay requires cells to be metabolically labelled with alkyne-fatty acid.

About 170,000 cells were plated in 6 cm plates. Following overnight transfection, media was replaced with deprivation media containing DMEM supplemented with delipidated FBS (5% Dextran Charcoal Stripped Fetal Bovine Serum (DCS-FBS)) instead of 10% FBS, as described previously (Liao et al., 2021; Yap et al., 2010). The cells were incubated for 1 hour at 37°C, 5% CO₂. For investigations requiring drug treatments using click chemistry, the drugs were added 30 minutes before the addition of fatty acids.

2) 2.4.2. Preparation of Fatty Acids and Saponification:

The alkyne-labelled fatty acid analog, alkyne palmitate, was saponified in potassium hydroxide (KOH). The saponified fatty acid was incubated with 20% fatty acid-free BSA (FAFBSA), as described in Table 2 (Liao et al., 2021), for 5 minutes at 70°C. The cells were metabolically labelled with saponified alkyne palmitate for at least 4 hours before lysis by adding 200 µL of the fatty acid label to the lipid-deprived cells.

Table 2.2: Concentration of Reagents for Labelling

Reagents	Working conc	Volume per plate (4 mL)
100 mM alkyne-palmitate	100 µM	4 µL
120 mM KOH	120 µM	4 µL
20% FAFBSA	1%	200 µL

3) *2.4.3. Cell Lysis:*

Following the metabolic labelling of HeLa cells, the cells were washed with PBS once. The cells were lysed in 1 mL of RIPA buffer without EDTA containing freshly added 10 µg/mL Pepstatin A and 1 mM PMSF. The samples were incubated at 4°C and rotated for 15-20 minutes. Next, the samples were centrifuged at 13.2 (x1000) rpm, 4°C for 10 minutes. Lysates were collected and stored at -20°C.

4) *2.4.4. DC Assay and Input Sample Preparation:*

Proteins were quantified using the DC assay. About 20 µg of protein was diluted in RIPA buffer (without EDTA) to a final volume of 60 µL. To these input samples, 12 µL of 5X SLB containing 5% beta-mercaptoethanol was added and the samples were boiled at 95°C for 5 minutes. Input samples were stored at -20°C until ready to be run on a gel.

5) *2.4.5. Click Reaction:*

Equal amounts of proteins (about 500 µg) were diluted in RIPA buffer (without EDTA) for click reaction. Click reaction was set up as shown in Table 3. The composition of components varied depending on the ligand being used. To equal amounts of protein samples, click reaction components were added in the following order: ligand, copper sulfate, biotin azide plus, followed by sodium-L-ascorbate to a final volume of 500 µL (as shown in Table 3).

Table 2.3: Concentrations of Click Reagents

	TBTA reaction		THPTA reaction		BTAA reaction	
	Working concentration	Volume/ reaction	Working concentration	Volume/ reaction	Working concentration	Volume/ reaction
TBTA	0.1 mM	5 μ L	-	-	-	-
THPTA	-	-	5 mM	25 μ L	-	-
BTAA	-	-	-	-	5 mM	25 μ L
Copper sulfate	1 mM	25 μ L	5 mM	25 μ L	2.4 mM	25 μ L
Biotin azide plus	100 μ M	10 μ L	100 μ M	10 μ L	100 μ M	10 μ L
Sodium-L-Ascorbate	1 mM	5 μ L	15 mM	25 μ L	12.45 mM	25 μ L

After the addition of click reaction reagents, the samples were rotated at room temperature in the dark for 1 hour. 10 mM EDTA (pH 8.0) was used to stop the click reaction. 2 mL of 100% acetone was added to each sample (final concentration 80%) and the samples were frozen overnight.

6) *2.4.6. Affinity Purification of clicked proteins:*

Samples were mixed by inversion and centrifuged at 2000 rpm at 4°C for 5 minutes. The pellet was washed thrice with 6 mL of chilled 80% acetone. The pellet was then resuspended in 250 μ L of ABE lysis buffer with 1X PIC. About 4.75 mL of dilution

buffer (50 mM HEPES pH 7.0, 1% TX-100, 1 mM EDTA, 1 mM EGTA) containing 150 mM NaCl and freshly added protease inhibitors, 10 µg/mL Pepstatin A and 1 mM PMSF, was added to resuspended protein sample. 30 µL of Neutravidin agarose resin beads equilibrated in the same dilution buffer were added to each sample. Pulldown with beads was set up for 3 hours at 4°C. The protein-bead samples were washed thrice with dilution buffer containing 0.5 M NaCl and once with dilution buffer without NaCl. The proteins were eluted from the beads by 1-hour incubation at room temperature with 40 µL of elution buffer (1 M HAM, 50 mM HEPES pH 7.0, 150 mM NaCl, 0.1% SDS, 10 µg/mL Pepstatin A and 1 mM PMSF) at constant shaking ~250 rpm. Samples were centrifuged by pulsing to 9000 rpm, and the supernatant was collected. To these pulldown samples, 10 µL of 5X SLB containing 5% beta-mercaptoethanol was added. The samples were further boiled at 95°C.

2.5. SDS-PAGE and Western Blotting:

1) 2.5.1. Electrophoresis and Transfer:

Samples were subjected to electrophoresis on 10% Bis-Tris gels in 1x XT MOPS running buffer (BioRad, 1610788) at 140 V post-denaturation of proteins by beta-mercaptoethanol and boiling. The proteins were then transferred onto the nitrocellulose membrane via semi-dry transfer using Transfer Buffer (BioRad) or Transfer buffer (192 mM Glycine, 25 mM Tris-Base, 20% v/v ethanol, and 0.05% SDS) at 25 V limit, 1 A constant for 25 minutes. Membranes were rinsed and washed in PBS-T (1X PBS, 0.1%

Tween-20) and incubated in 5% skim milk (diluted in 1X PBS-T) blocking buffer overnight at 4°C with gentle agitation.

2) *2.5.2. Immunodetection/Immunoblotting:*

Primary antibodies were diluted in 5% skim milk in 1X PBS-T or 5% bovine serum albumin (BSA) in 1X PBS-T in accordance with vendor recommendation (Table 4). The membranes were incubated with primary antibody overnight at 4°C with gentle agitation. Blots were washed with 1X PBS-T five times (5 minutes per wash) before the addition of fluorescently tagged secondary antibody. Secondary antibodies were used to probe for the protein of interest while rhodamine-tagged antibodies for housekeeping proteins were used as loading control for protein normalization (Tables 5 and 6). These antibodies were diluted in 5% BSA in 1X PBS-T containing 0.01% SDS. Blots were incubated with the secondary antibody for 1 hour at room temperature with gentle agitation and protection from light. The blots were washed five times with 1X PBS-T. Following the washes, blots were imaged, and protein bands were visualized using ChemiDoc (BioRad).

2.6. Fluorescence Microscopy and Immunofluorescence:

1) *2.6.1 Cell Fixation:*

75,000 HeLa cells were seeded per well in a 6-well plate onto 0.01 µg/mL poly-D-lysine (PDL) coated coverslips (#1.5, VWR). The cells were transfected with plasmids for the protein(s) of interest and treated with drugs where required as described in 2.1.2.

Post-transfection, cells were fixed by incubation in 2 mL of 4% paraformaldehyde (PFA) for 30 minutes. The cells were washed with 1X PBS twice.

For lysosomal staining, cells were incubated in fresh DMEM containing 50 nM LysoTracker Deep Red (Thermofisher Scientific, L12492) for 1 hour at 37°C. The staining of lysosomes was done post-transfection and right before fixation.

2) 2.6.2 *Fluorescence Microscopy:*

Post PBS washes, cells were incubated in 1 µg/mL DAPI (Sigma D9542) diluted in PBS for 30 minutes. Cells overexpressing fluorescently tagged proteins were then mounted onto microscope slides using ProLong Gold antifade mountant (Invitrogen, P36934). The coverslips were sealed with nail polish on the slides. Once the nail polish dried, the slides were imaged using Nikon AXR laser scanning confocal microscope.

3) 2.6.3 *Immunofluorescence:*

Immunofluorescence microscopy was used for cells overexpressing the HA-tagged protein of interest or when looking at the localization of endogenous proteins.

Post PBS washes in 2.6.1., the cells were permeabilized in 2 mL of 0.1% Triton X-100 in PBS for 1 minute. The cells were washed in 1X PBS and blocked with 2 mL of 0.2% gelatin (in PBS) for 30 minutes. Next, cells were washed in 0.2% gelatin thrice. The cells were incubated with 1:500 primary antibody diluted in gelatin for 1 hour followed by three washes (Table 4). The cells were incubated with 1:500 secondary antibody for 1 hour followed by gelatin washes (Table 5). The cells were incubated in 1 µg/mL DAPI diluted in gelatin for 30 minutes. The coverslips were mounted onto slides and then

sealed with nail polish. The slides were imaged using the Nikon AXR scanning confocal microscope.

Table 2.4: List of Primary Antibodies

Antigen	Host Species	Method	Dilution		Vendor
anti-GFP	Rabbit	WB	1:10,000	5% skim milk	Eusera
anti-HA	Mouse	WB	1:1000	5% skim milk	
anti-HA	Rat	IF	1:500	0.2% gelatin	
anti-HA (C29F4)	Rabbit	WB	1:1000	5% BSA	Cell Signalling
anti-p62 (D1Q5S)	Rabbit	WB	1:1000	5% BSA	Cell Signalling
anti-p62	Rabbit	IF	1:500	0.2% gelatin	Invitrogen
anti-HTT (1HU-4C8)	mouse	IF	1:500	0.2% gelatin	Millipore
anti-acetylated Lysine (1C6)	mouse	WB	1:500	5% BSA	Invitrogen

Table 2.5: List of secondary Antibodies

Name	Ex/Em	Species	Against	Dilution	Method	Vendor
StarBright 520	440/520	Goat	Rabbit	1:2500	WB	BioRad
StarBright 700	470/700	Goat	Rabbit	1:2500	WB	BioRad
StarBright 520	440/520	Goat	Mouse	1:2500	WB	BioRad
StarBright 700	470/700	Goat	Mouse	1:2500	WB	BioRad
Alexa Fluor 594	591/614	Donkey	Rat	1:500	IF	BioRad
Alexa Fluor 488	488/519	Goat	Rabbit	1:2500	WB	Jackson ImmunoResearch
Alexa Fluor 555		Donkey	Mouse	1:500	IF	Invitrogen
Alexa Fluor 488		Goat	Rabbit	1:500	IF	Invitrogen

Table 2.6: List of Loading Controls for Western Blotting

Name	Ex/Em	Against	Dilution	Vendor
Anti-Tubulin hFAB rhodamine	530/820	Tubulin	1:5000	BioRad
Anti-GAPDH hFAB rhodamine	530/580	GAPDH	1:5000	BioRad
Anti-Actin hFAB rhodamine	530/580	Actin	1:5000	BioRad

2.7. Motor test on YAC128 mice

Age-matched non-transgenic FVB wild-type (WT) and YAC128 HD transgenic mice were placed 30 cm above the bottom of the home cage, on a wired cage top. The cage was inverted and the latency to fall when the mouse was released from the wire was recorded. This was repeated thrice, and the trial with the longest time was recorded. The test was done on mice at 8 months and 12 months.

2.7. Neuronal Culture and Transfection

Primary cortical neurons were derived from FVB strain mouse embryos at embryonic day 17 (E17). Papain was used to dissociate the embryonic cortices which were seeded at 180,000 cells/well for microscopy experiments on 0.1 mg/mL poly-L-lysine coated glass coverslips (#1.5, VWR) in 6-well plates. These cells were plated in plating media (Neurobasal supplemented with 2.5% FBS, 2.5% Horse serum, Penicillin-streptomycin, L-Glutamine, and B27). The following day, the media was changed to neuronal media (Neurobasal supplemented with pen-strep, L-Glutamine, and B27).

At 16 days in vitro (DIV), the cells were transfected with p62-GFP using lipofectamine 2000 (Invitrogen) and treated with drugs for 16-18 hours post-transfection (Table 7). The cells were fixed the next day using 4% PFA in 1x PBS (as described in sections 2.6.1 and 2.6.2).

Table 2.7: List of Drugs/ Inhibitors

Inhibitor	Working concentration
2-bromopalmitate	20 μ M or 50 μ M
Palmostatin B	10 μ M
Bafilomycin A1	100 nM
Vorinostat	10 μ M
Pyroxamide	10 μ M
Trichostatin A	500 nM

CHAPTER 3

Regulation of p62 by Vorinostat

p62 can polymerize around mHTT aggregates facilitating the degradation of toxic molecules via the autophagy pathway. However, autophagy is dysregulated in HD at various stages including the cargo-loading step which leads to the accumulation of toxic protein aggregates as well as empty autophagosomes (Martinez-Vicente et al., 2010; Martin et al., 2015). Autophagy is a highly membrane-dependent process that requires the re-localization of proteins to membranes therefore, changes in palmitoylation may play a role in the regulation of the autophagy mechanism (Ramzan et al., 2023).

Preliminary data from our lab has shown that p62 is palmitoylated. Moreover, the palmitoylation of p62 is significantly reduced in the cortex of HD patients and mouse models. We predict that the cargo-loading defect during autophagy in HD may be related to the loss of p62 palmitoylation. Therefore, targeting p62 palmitoylation may serve as a potential therapeutic strategy in HD.

Vorinostat was previously identified in a drug screen for modifiers of palmitoylation (Martin et al., 2019). Preliminary data from the Martin Lab suggests that it increases p62 palmitoylation. Vorinostat is an HDAC inhibitor that affects acetylation of proteins by inhibiting HDAC 1, 2, 3 and 6 (Athira et al., 2021). p62 is acetylated at two different sites within the UBA domain and this modification is important for the clearance of ubiquitinated substrates (You et al., 2019). This reversible process is catalyzed by TIP60 in the forward direction, and HDAC6 in the reverse direction (You et al., 2019). Vorinostat is an FDA-approved drug that can easily cross

the blood-brain barrier and is now being tested in neurodegenerative diseases (Athira et al., 2021).

I aimed to investigate whether Vorinostat had an impact on the palmitoylation of p62 and its potential localization to the autophagosomes. In the findings detailed in this section, I examined the influence of Vorinostat treatment on the palmitoylation of p62 and its distribution to the autophagosomes as well as the lysosomes. Additionally, I explored the changes in p62 acetylation following Vorinostat treatment. Collectively, these investigations provided better insights into the development of a plausible mechanism through which Vorinostat may modulate p62 function.

3.1 – Vorinostat Increases Palmitoylation of p62

Previous high-throughput analysis of FDA-approved drugs performed by Dr. Martin found Vorinostat as a potential drug capable of increasing palmitoylation (Martin et al., 2019). Therefore, I explored the effect of Vorinostat on the palmitoylation of p62 specifically.

An ABE assay was performed on HeLa cells transfected with HA-tagged p62 (p62-HA). When treated with 50 μ M 2-bromopalmitate (2BP), an inhibitor of palmitoylation (Draper & Smith, 2009), the acylation signal reduced as expected (Figure 3.2 a, b). In response to 10 μ M Palmostatin B (PalmB) treatment, an inhibitor of depalmitoylating enzymes (Davda & Martin, 2014), p62 palmitoylation slightly increased. I also tested Pyroxamide (Pyrox) due to its function as an HDAC inhibitor and its structural similarities to Vorinostat (as shown in Figure 3.1). Pyrox targets HDAC 1 specifically (Xu et al., 2022). Remarkably, when treated with 10 μ M Vorinostat or Pyrox, a robust increase in acylation signals was evident when compared to untreated DMSO conditions (Figure 3.2a, b).

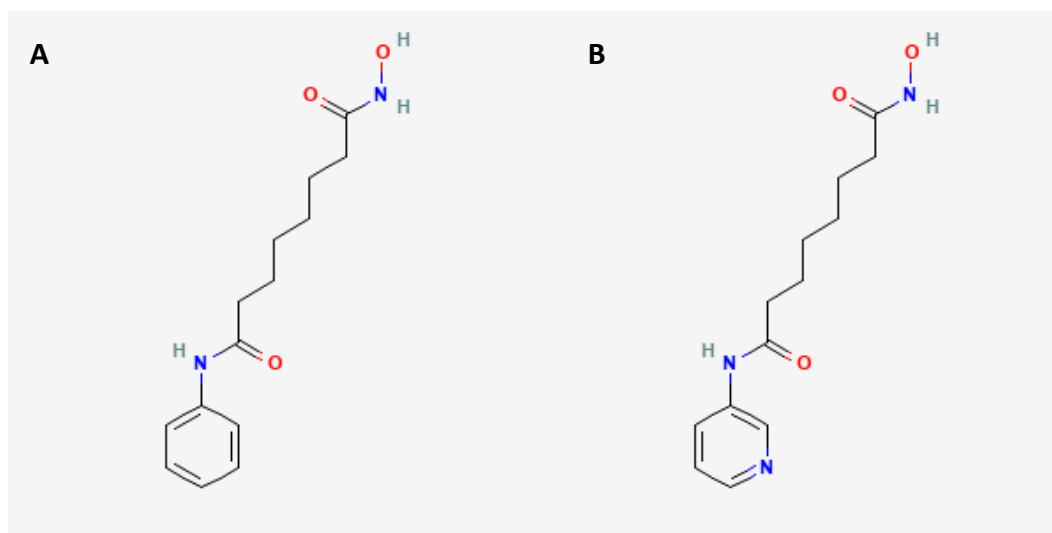


Figure 3.1. Structure of (A) Vorinostat and (B) Pyroxamide. Images retrieved from PubChem.

Comparison of Vorinostat and Pyrox treatments shows an overall increase in p62 protein levels (Figure 3.2 c). Looking closely, Pyrox elicited a more pronounced increase in the p62 protein levels compared to Vorinostat (Figure 3.2 c). As for palmitoylation, however, Vorinostat exhibited a more substantial increase in the palmitoylation of p62 in contrast to Pyrox (Figure 3.2 b). Suggesting that Vorinostat may exert a more specific effect on palmitoylation of p62 compared to Pyrox.

Although there was no statistical difference between Vorinostat treatment and the control (DMSO), Vorinostat increased palmitoylation approximately three times (Figure. 3.2 b), indicating that Vorinostat may be a novel depalmitoylation inhibitor which increases the palmitoylation of p62.

In the absence of hydroxylamine (HAM- lane in Figure 3.2 a), there was no signal since HAM is integral for the replacement of endogenous palmitate with biotin. The ABE results from the two independent experiments confirm that Vorinostat increases the palmitoylation of p62.

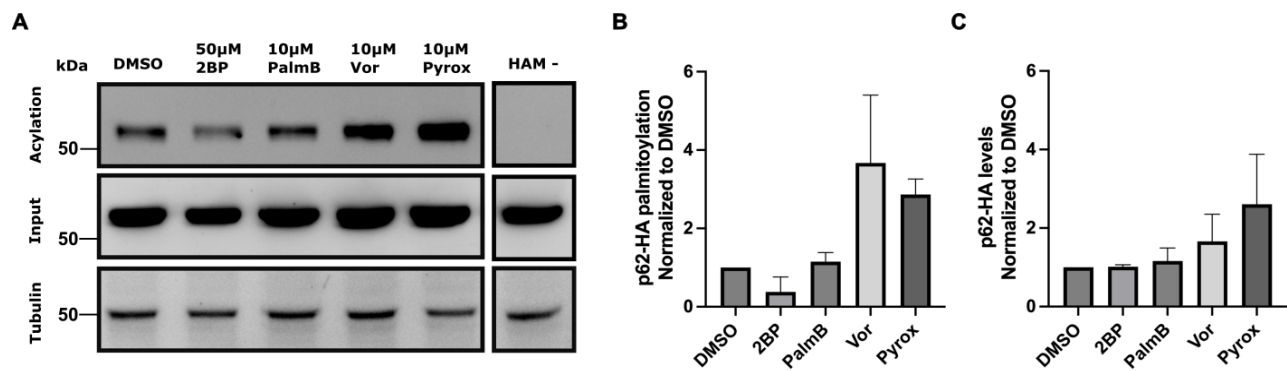


Figure 3.2. Vorinostat increases p62 palmitoylation (A) Western blot showing ABE assay results of HeLa cells transfected with p62-HA and treated with DMSO, 2-bromopalmitate (2BP), Palmostatin B (PalmB), Vorinostat (Vor), and Pyroxamide (Pyrox). The drug treatments were done at their respective mentioned dosages for 16-18hr post-transfection. The acylation row shows the palmitoylated p62 that was affinity purified using biotin. p62-HA was detected in Input and acylation blots using a mouse-anti-HA antibody. Anti-Tubulin was used as a loading control. No palmitoylation signal was detected in the absence of hydroxylamine (HAM- lane). **(B)** Palmitoylation of p62-HA increases in response to PalmB, Vorinostat, and Pyroxamide drug treatment with most increase caused by Vorinostat **(C)** p62-HA protein levels increase in response to PalmB, Vor, and Pyrox. The bars in both charts are represented as the mean $\pm$ SEM palmitoylation and protein level for each drug treatment from two independent experiments (n=2).

3.2 – Vorinostat Improves the Localization of p62 to the Autophagosomes

To investigate whether Vorinostat affected p62 localization to the autophagosomes, HeLa cells were co-transfected with p62-GFP and RFP-LC3. LC3 is a marker for autophagosomes (Klionsky et al., 2008, 2021). The cells were treated with 2BP, PalmB, Vorinostat, Pyrox, and Trichostatin A (TSA) and visualized by fixed cell microscopy. TSA is a broad-spectrum class I and class II HDAC inhibitor (Yoshida et al., 1995; Monneret, 2005).

To quantitatively assess the extent of protein colocalization, Pearson's colocalization coefficient (PCC) was measured. Overall, in response to the drugs being tested, the overlap between p62-GFP and RFP-LC3 increased when compared to the untreated DMSO condition as measured by PCC (Figure 3.3 a, b).

Strikingly, there was a statistically significant increase in colocalization between p62-GFP and RFP-LC3 when treated with PalmB and Vorinostat (Figure 3.3 b). Increased colocalization between the two proteins indicated that the localization of p62 to the autophagosomes improved. The significantly high overlap in response to PalmB and Vorinostat drug treatments suggests that Vorinostat may be regulating the change in localization in a similar way as PalmB, i.e., via increasing palmitoylation.

In addition to changes in colocalization, there were variations in the size of p62-GFP and RFP-LC3 associated puncta, suggesting alterations in the size of the autophagosome (and potentially autophagolysosomes) when treated with drugs (Figure 3.3 c, d). The presence of larger-sized p62-GFP and RFP-LC3 puncta was higher in PalmB-treated cells. Overall, the variation in the size of p62-GFP puncta was much greater than the variation in the size of RFP-LC3 in response to the drug treatments (Figure 3.3 c, d).

There was a change in the average number of puncta per cell with the treatments (Figure 3.3 e, f). Compared to DMSO, there was about a two-fold increase in the average number of p62-GFP puncta per cell for 2BP and HDAC inhibitor treatments, with a slight increase with PalmB (Figure 3.3 e). The RFP-LC3 puncta per cell increased under HDAC inhibitor treatments and slightly with 2BP and PalmB (Figure 3.3 f).

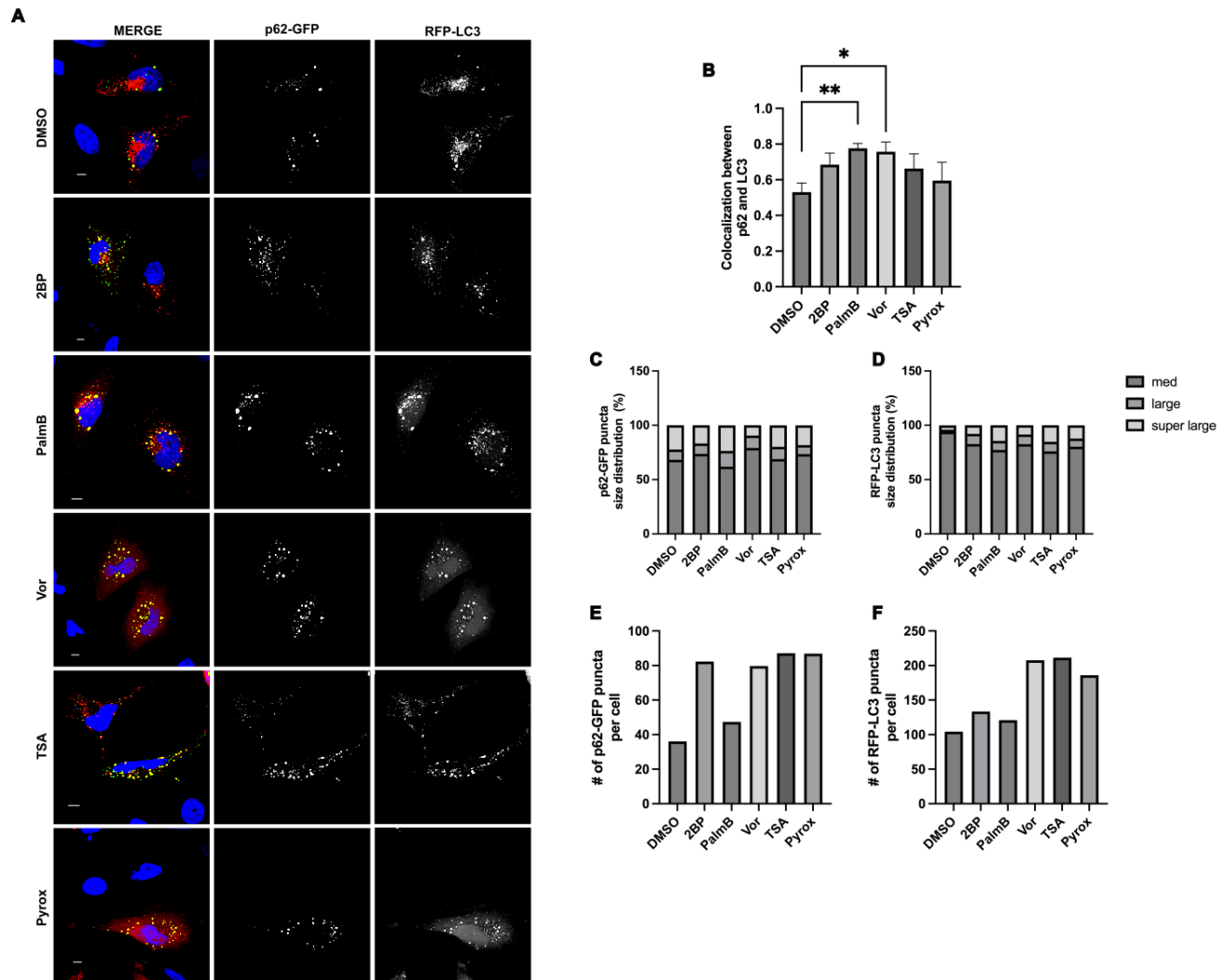


Figure 3.3. Vorinostat increases localization of p62 to autophagosomes (A) Confocal images of HeLa cells under different drug conditions co-expressed with p62-GFP RFP-LC3; Scale bar = 10µm. 0.1% DMSO, 20µM 2BP, 10µM PalmB, 10µM Vorinostat (Vor), 500nM TSA, 10µM Pyroxamide (Pyrox). (B) Co-localization between p62-GFP and RFP-LC3 under different drug treatments represented as Pearson's correlation coefficient (PCC) for 6 – 14 cells per condition; mean ± SEM (n=1). One-way ANOVA test $p < 0.05$. (C) and (D) represent the distribution of different-sized puncta for p62-GFP and RFP-LC3, respectively. (E) and (F) show the average number of puncta per cell for overexpressed p62-GFP and RFP-LC3, respectively (n=1). Puncta were detected by setting the threshold from 1000 to 65535 by using 3D object measurement on Nikon AXR. Volume range of puncta for med (medium) = 0.05 - 0.5 µm³, large = 0.5 - 1 µm³, super large = > 1 µm³

3.3 – Increase in Autophagosome Size is Specific to p62

To determine if the increased puncta size was related to p62, LC3, or both key autophagy regulators, HeLa cells were co-transfected with p62-GFP and empty mCherry vector, as well as RFP-LC3 and the empty GFP vector in two independent experiments to observe the effect of the drugs on the size of these proteins individually. The cells were then treated with the drugs and visualized by fixed cell microscopy.

As illustrated in Figure 3.4, the presence of larger-sized p62-GFP puncta increased in response to the drugs. The distribution of larger-sized puncta was more under PalmB and Vorinostat treatments (Figure 3.4 a, c). There was no effect on RFP-LC3 puncta (Figure 3.4 a, d). Thus, suggesting that the increase in autophagosome size, from the earlier results in 3.2, was more specific to p62 than LC3. In addition to puncta size change, the average number of puncta per cell varied for both proteins. The number of p62-GFP puncta reduced overall in response to the 2BP, PalmB, Vorinostat, TSA and Pyrox. In comparison to DMSO, the decrease was greater with the 2BP treatment (Figure 3.4 e). The results showed that the number of puncta was similar in the presence of PalmB and Vorinostat. For RFP-LC3, the puncta number decreased when treated with 2BP (Figure 3.4 f). PalmB, Vorinostat, and TSA had more puncta per cell.

Overlap between the p62-GFP and mCherry (Figure 3.4a) was observed. This may be due to mCherry being non-selectively incorporated into autophagosomes with cytosolic proteins.

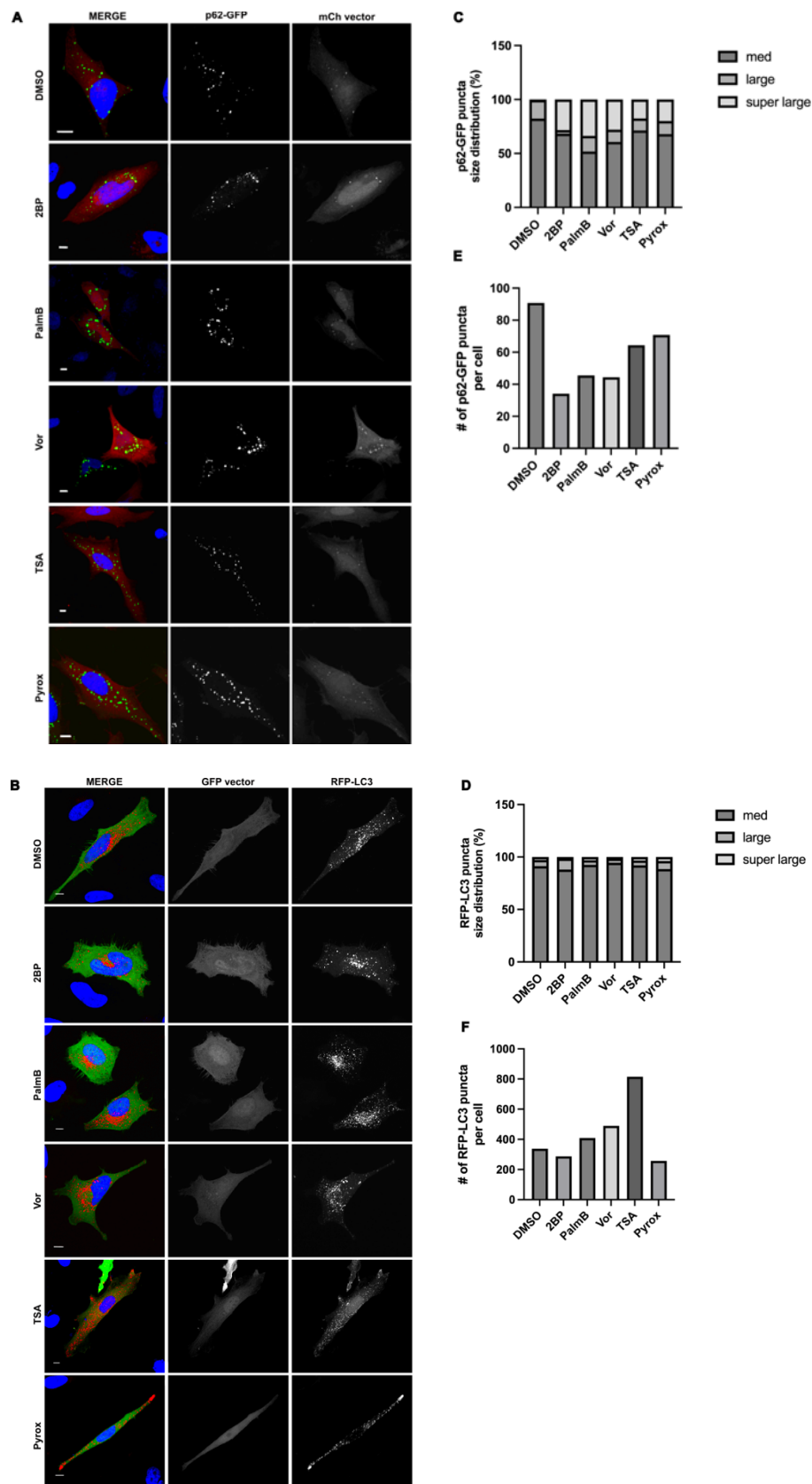


Figure 3.4. Change in puncta size is specific to p62. Confocal images of HeLa cells under different drug conditions co-expressed with **(A)** p62-GFP and m-Cherry vector, and **(B)** RFP-LC3 and GFP vector; Scale bar = 10µm. 0.1% DMSO, 20µM 2BP, 10µM PalmB, 10µM Vorinostat (Vor), 500nM TSA, 10µM Pyroxamide(Pyrox). **(C)** and **(D)** represent the proportion of different-sized puncta for p62-GFP and RFP-LC3, respectively. **(E)** and **(F)** show the average number of puncta per cell for overexpressed p62-GFP and RFP-LC3, respectively (n=1). Puncta was detected by setting the threshold from 1000 to 65535 by using 3D object measurement on Nikon AXR. Volume range of puncta for med (medium)= 0.05 - 0.5 μm^3 , large = 0.5 - 1 μm^3 , super large= > 1 μm^3

3.4 – Vorinostat Increases Puncta Size of Endogenous p62

To confirm the effect of Vorinostat was not due to overexpression of p62, I tested the effect of Vorinostat on endogenous p62 via immunofluorescence microscopy on HeLa cells. The cells were treated with DMSO, PalmB and Vorinostat and visualized by fixed cell confocal microscopy.

Consistent with overexpression of p62, larger puncta were detected in HeLa cells treated with PalmB and Vorinostat when compared to the untreated (DMSO) conditions (Figure 3.5). Thus, confirming that p62 indeed undergoes a size change for puncta when treated with PalmB and Vorinostat suggesting that this effect may be specific to palmitoylation.

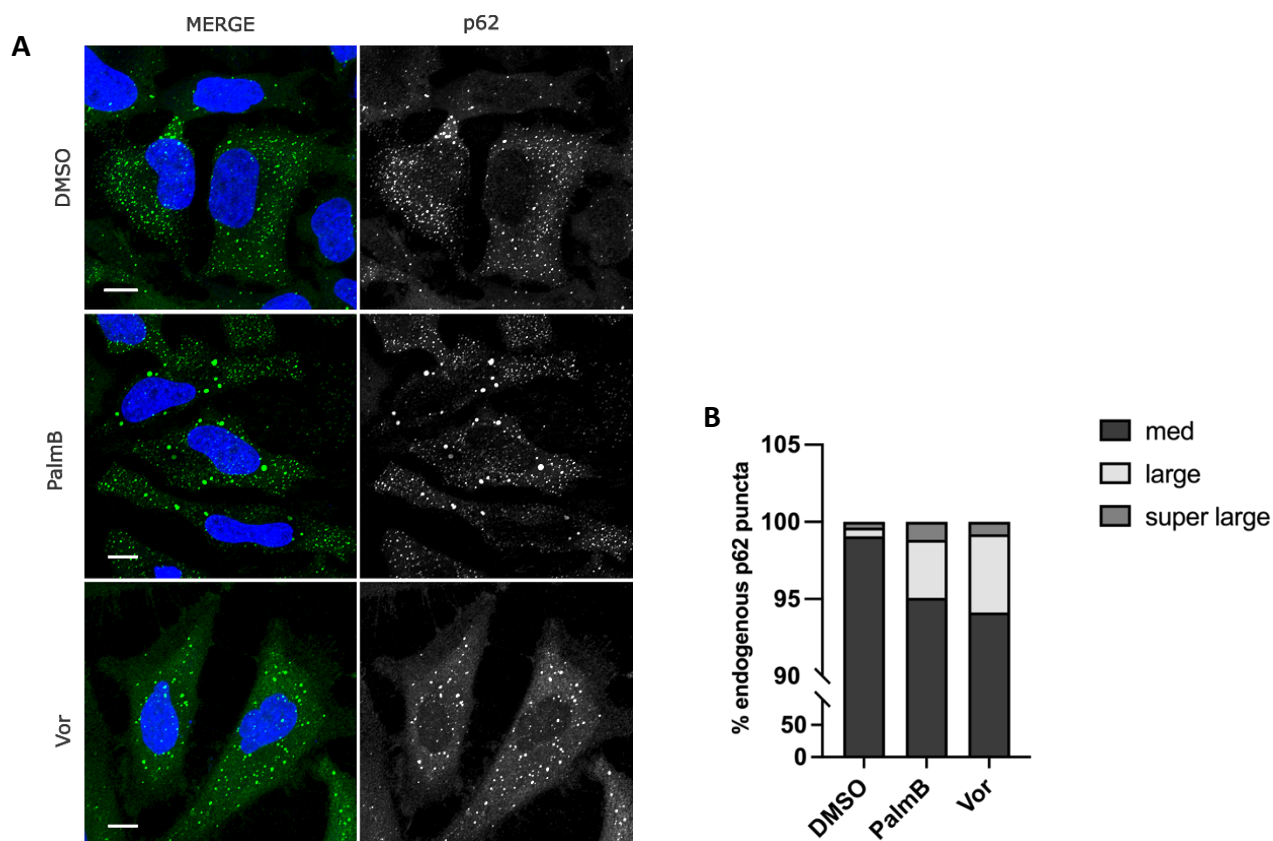


Figure 3.5. Vorinostat increases endogenous p62 puncta size (A) Confocal images of HeLa cells under different drug conditions. An immunofluorescence experiment was done to probe for endogenous p62; Scale bar = 10 μ m. Drug treatments: 0.1% DMSO, 10 μ M PalmB and 10 μ M Vorinostat (Vor) for 16-18 hours post-transfection. **(B)** The graph represents the proportion of different-sized endogenous p62 puncta (n=1). The puncta size was detected by using the 3D object measurement tool on Nikon AXR with the threshold set from 1000 – 65535 voxels.

3.5 – Vorinostat Improves Endogenous p62 and HTT Localization to the Lysosomes

Next, the effect of Vorinostat on p62 localization to the lysosomes was investigated in HeLa cells. Endogenous p62, endogenous HTT and lysosomes were visualized through fixed cell microscopy.

Overall PalmB, TSA, Vorinostat, and Pyrox all improved colocalization between p62, HTT and lysosomes as indicated by PCC measurements (Figure 3.6). There was a significant improvement in colocalization of the proteins under HDAC inhibitor treatments. Moreover, a

noticeable increase in the overlap between p62 and HTT was observed when treated with PalmB (Figure 3.6 b). PalmB treatment led to a statistically significant increase in colocalization between HTT and lysosomes (Figure 3.6 d). These results suggest that Vorinostat and PalmB improve the interaction between p62 and HTT, as well as improve their localization to the lysosomes.

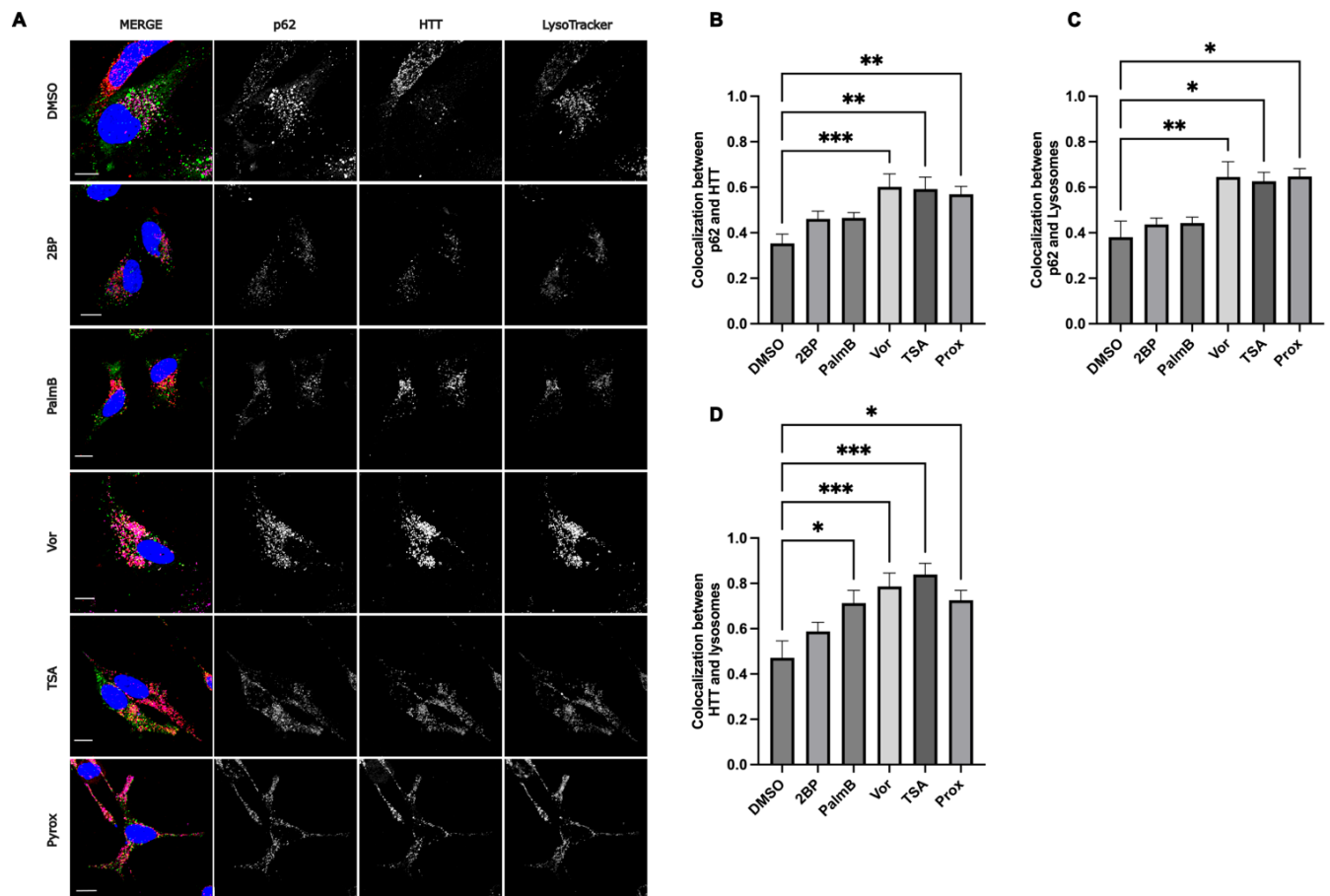


Figure 3.6. Vorinostat increases localization of p62 and HTT to lysosomes. (A) Confocal images of HeLa cells under different drug conditions treated for 16-18 hours before fixation. An immunofluorescence experiment was done to probe for endogenous p62 (1:500 rabbit-anti-p62; green) and endogenous HTT (1:500 mouse-anti-HTT; red). Lysosomes (magenta) were stained with 50 nM LysoTracker Far Red (Thermofisher Scientific, L12492); 1 hour incubation at 37°C prior to fixation. Scale bar = 10 μ m. Drug treatments: 0.1% DMSO, 20 μ M 2BP, 10 μ M PalmB, 10 μ M Vorinostat (Vor), 500 nM TSA, 10 μ M Pyroxamide (Pyrox). Co-localization between (B) p62 and HTT, (C) p62 and Lysosomes, and (D) HTT and Lysosomes under different drug treatments normalized to DMSO. PCC for 5 – 9 cells per condition; mean $\pm$ SEM (n=1). One-way ANOVA test $p < 0.05$.

3.6 – Acetylated p62 Levels Decline in Response to Vorinostat Treatment

HeLa cells were transfected with p62-GFP and p62-HA in two individual experiments. The cells were treated with 10 μ M Palmostatin B and 10 μ M Vorinostat in the presence and absence of 100 nM bafilomycin A1 (BafA1). The outcomes exhibited similar patterns for both tagged-p62.

The results show that in response to Vorinostat, total acetylated protein levels in the lysates increased (Figure 3.7 b, d), as expected since Vorinostat promotes acetylation (Athira et al., 2021). Tagged p62 was immunoprecipitated and then probed for acetylation to look at the effect of Vorinostat on p62 acetylation specifically. Interestingly, Vorinostat led to a significant decrease in acetylated p62 levels (Figure 3.7 a, c). The decline was also observed for the cells treated with the depalmitoylation inhibitor, PalmB. Suggesting that Vorinostat is affecting p62 acetylation through a different mechanism that may involve a role of palmitoylation as well.

BafA1 is a late-stage autophagy inhibitor as it blocks the fusion between autophagosomes and lysosomes hence, p62 degradation is prevented (Klionsky et al., 2008). As expected, overall, the levels of p62 increased in the presence of BafA1 (Figure 3.7 e). Acetylated p62 significantly declined when treated with BafA1 (Figure 3.7 c). Although the levels of acetylated p62 increased with BafA1 treatment with PalmB and Vorinostat compared to their individual treatment counterparts, however, this increase still led to significantly lower acetylated p62 levels compared to DMSO (Figure 3.7 c).

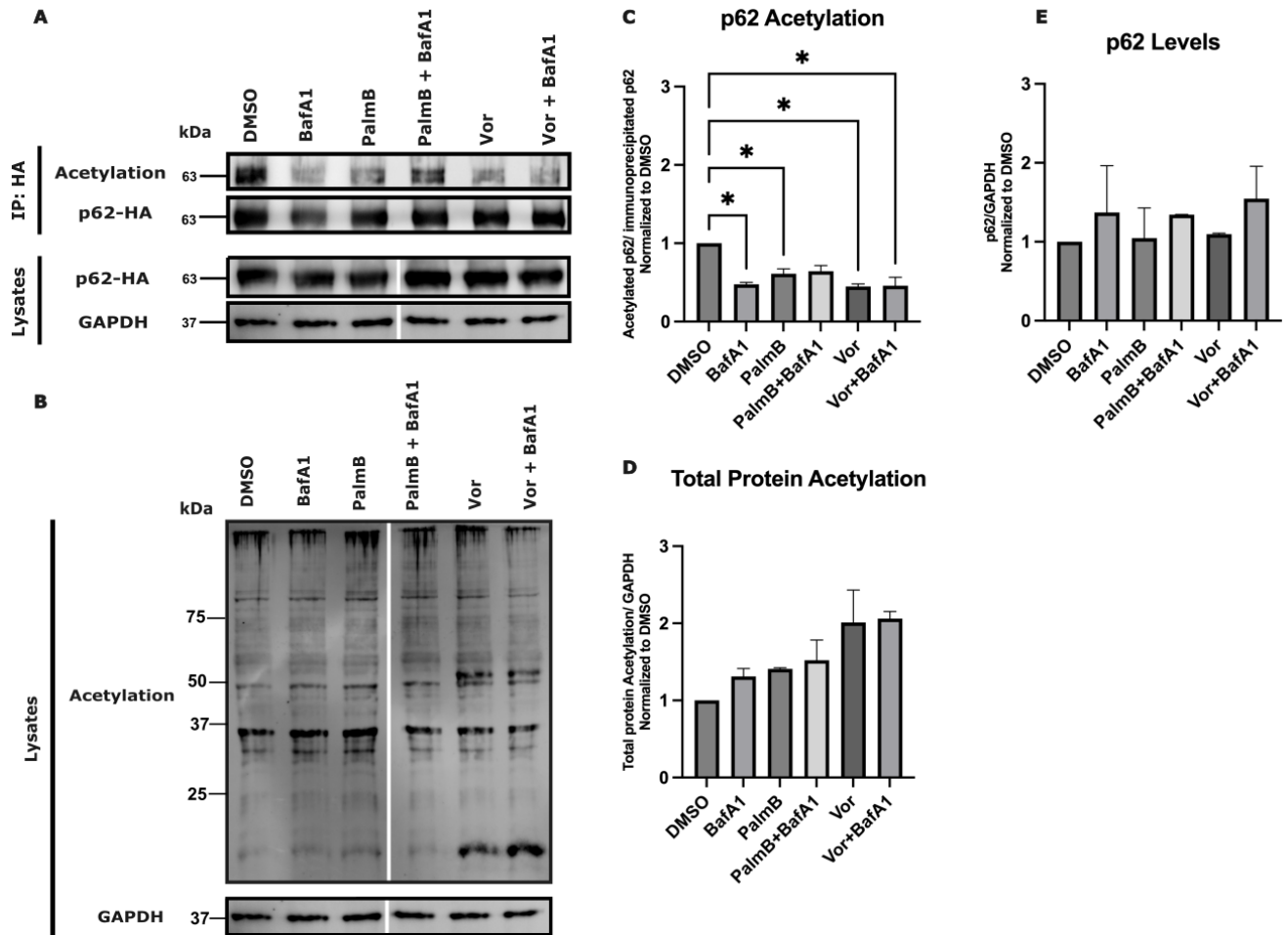


Figure 3.7. Acetylated p62 levels decline with Vorinostat treatment. Western blot showing **(A)** p62 acetylation and **(B)** total protein acetylation results of HeLa cells transfected with p62-HA and treated with 0.1% DMSO, 100 nM BafA1, 10 μ M PalmB, 10 μ M Vor, and PalmB and Vor with BafA1. The drug treatments were done for 7-8 hours pre-lysis. p62 was immunoprecipitated (IP) with rat-anti-HA antibody and then probed for acetylation. Acetylation was detected in IP and input blots with a mouse-anti-acetylated lysine). p62-HA was detected in Input and IP blots using rabbit-anti-HA antibody and anti-GAPDH was used as a loading control. **(C)** Variation in acetylation signal of p62 represented as acetylated p62/ total p62 immunoprecipitated. **(D)** Total protein acetylation variation in response to drug treatments. **(E)** Total p62-HA protein levels in lysates. p62 levels increase in response to BafA1 treatments. The bars in the charts are represented as the mean $\pm$ SEM, One-way ANOVA test $p < 0.05$ two independent experiments ($n=2$). The second experiment was done with p62-GFP and had similar trends to those of p62-HA.

3.7 – Vorinostat Stabilizes p62 Protein

Being an HDAC inhibitor, Vorinostat can increase the expression of proteins by increasing the acetylation of histone proteins however, this does not affect the transcription of plasmid DNA. Therefore, the effect of Vorinostat on the stability and turnover of p62 was investigated. A pulse-chase assay was done on HeLa cells transfected with p62-HA. The cells were treated with DMSO, 5mg/mL cycloheximide (CHX), and 10 μ M Vorinostat over a period of 0-8 hours pre-lysis. The cells were then lysed at the indicated time points.

p62-HA levels declined by 63% when treated with CHX, as expected (CHX+DMSO in Figure 3.8) while the levels of p62-HA increased by 36% in response to Vorinostat-only treatment (Vor+DMSO). However, when HeLa cells were treated with CHX and Vorinostat together, the levels declined and reached a steady level between 4 to 8 hours. Overall, there was an approximately 36% decrease in protein levels with the combined treatment. However, this decline was not as pronounced as the CHX alone condition, suggesting that the addition of Vorinostat may improve the stability of p62-HA.

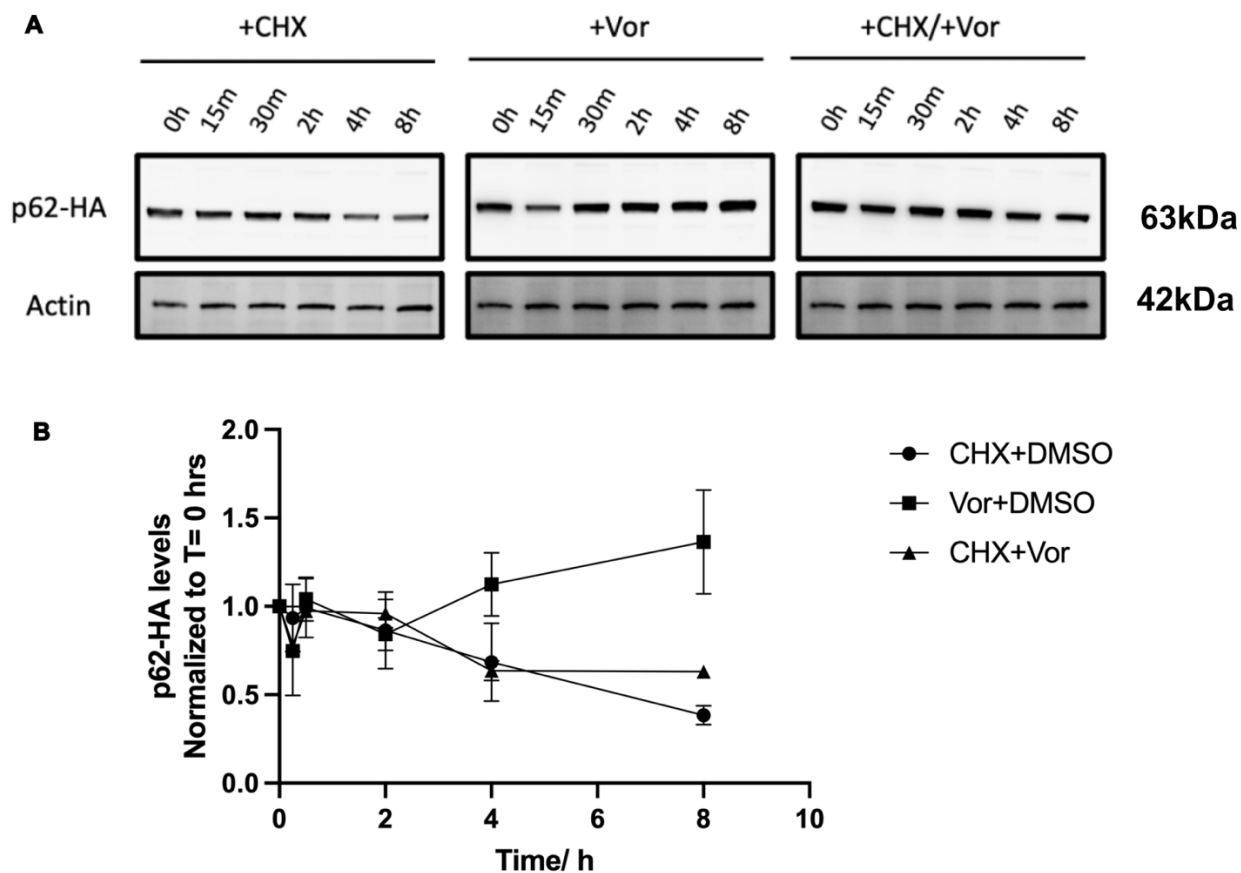


Figure 3.8. Vorinostat improves p62 stability. (A) Western blot shows the change in p62-HA levels in response to the indicated drug treatments. p62-HA was detected using mouse-anti-HA antibody and goat-anti-mouse-SB520. Rhodamine anti-actin was used as a loading control. (B) show the change in p62-HA levels over 8 hours in response to 5mg/ml cycloheximide (CHX) and 10 μ M Vorinostat (Vor). The points on the graphs are represented as the mean $\pm$ SEM from two independent experiments (n=2).

CHAPTER 4

Enzymes regulating p62 Palmitoylation

Palmitoylation plays a crucial role in the localization and trafficking of signal proteins. Palmitoylation is enriched in neuronal synapses and neurodegenerative diseases (Sanders et al., 2015). Our work has demonstrated that palmitoylation is enriched in autophagy as well (Ramzan et al., 2023).

Palmitoylation is a reversible PTM that is catalyzed by palmitoyl acyl transferases or PATs (ZDHHCs). The depalmitoylation of proteins is mediated by serine hydrolases which include APTs and ABHDs. Through our previous findings as well as the results in Chapter 3, we have confirmed that p62 is palmitoylated. However, the enzymes that regulate this mechanism remain unknown.

HTT is palmitoylated by ZDHHC17 and ZDHHC13 (Yanai et al., 2006; Butland et al., 2014) and depalmitoylated by APT1 and APT2 (Lin & Conibear, 2015; Lemarié et al., 2021). Since p62 palmitoylation may play a role in the dysregulation of autophagy in HD, identifying the enzymes that regulate p62 palmitoylation may serve as potential therapeutic targets for rescuing p62 palmitoylation in HD. My work has shown that Vorinostat increases p62 palmitoylation (Chapter 3). One way this could be regulated is via direct inhibition of the depalmitoylating enzymes. Thus, identifying the enzymes that regulate the palmitoylation of p62 would be of interest.

I carried out investigations to identify the enzymes that interact with p62 as well as the enzymes that may potentially regulate p62 palmitoylation. The results described in this chapter highlight the work done towards this goal.

4.1 – ZDHHC5 is an Interactor of p62

There are 23 palmitoyl acyl transferases (PATs) that catalyze palmitoylation in humans. These enzymes add palmitate onto a cysteine residue via a thioester bond. To identify the PATs that may affect the palmitoylation of p62, we began by performing a microscopy screen. I directed and supervised an undergraduate thesis project, where we did a qualitative wide-field microscopy screen of the 23 PATs co-expressed with p62-GFP to identify the PAT(s) that may influence the localization of p62 or the PAT(s). From the screen, we identified ZDHHC2, ZDHHC5, ZDHHC6, ZDHHC7, ZDHHC8, ZDHHC14, ZDHHC15, ZDHHC17, ZDHHC21, and ZDHHC24.

We expected to see colocalization between p62 and its potential PAT(s) however, the screen revealed no overlap between p62 and any of the PATs. The 10 PATs mentioned above showed a variation in p62 puncta size and overall distribution compared to the control p62-GFP condition.

Subsequently, I did a co-immunoprecipitation (co-IP) assay for the 10 identified PATs and p62-GFP to investigate the interactions of these PATs with p62 (Figure 4.1). p62 is a protein that gets actively degraded during autophagy (Klionsky et al., 2008; Ma et al., 2019). Therefore, I used BafA1 to block the degradation of p62 (Klionsky et al., 2008, 2021) to potentially enhance the interaction between p62 and its potential PAT(s). Upon immunoprecipitation of HA-ZDHHCs, p62-GFP co-immunoprecipitated with HA-ZDHHC5 only. Suggesting that ZDHHC5 is an interactor of p62.

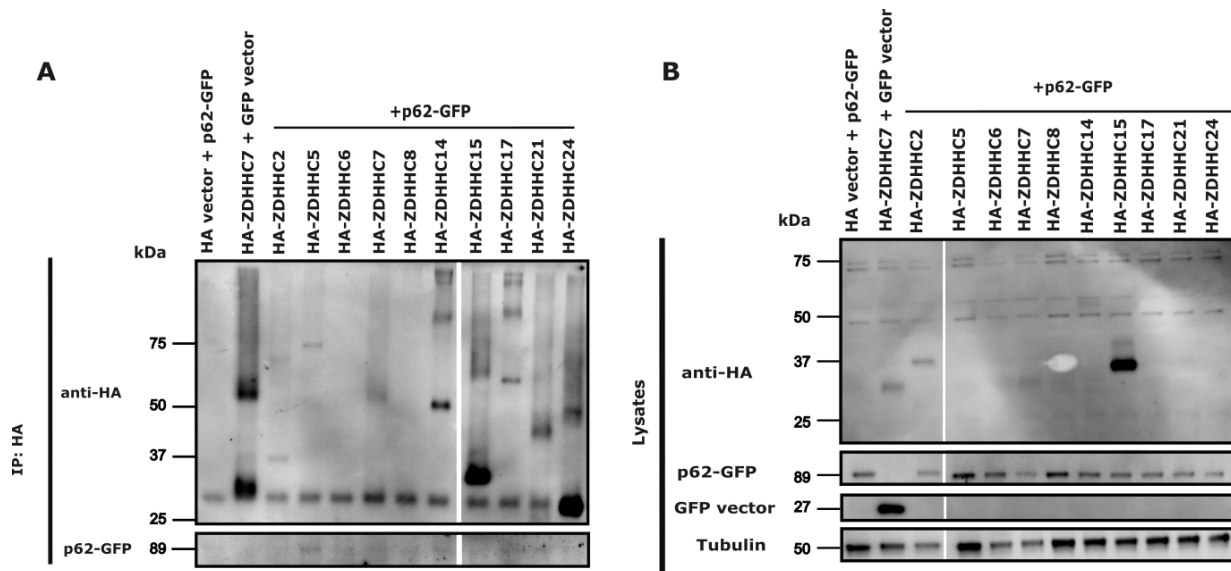


Figure 4.1. ZDHHC5 interacts with p62. Co-immunoprecipitation experiment was done in HeLa cells co-transfected with p62-GFP and the 10 identified HA-tagged PATs (HA-ZDHHCs). These cells were treated with 100 nM of BafA1 for 16-18 hours post-transfection. Anti-tubulin was used as a loading control. **(A)** The individual PATs were immunoprecipitated using the rat-anti-HA antibody. Western blot analysis revealed that p62-GFP coimmunoprecipitated with ZDHHC5-HA. **(B)** Western blot shows the individual HA-ZDHHCs and p62-GFP in the lysates

4.2 – APT1 Co-localizes and Interacts with p62

Subsequently, I proceeded to identify the depalmitoylating enzymes that may be regulating p62 palmitoylation. Initially, the impact of APTs on p62 was examined. In the presence of p62-GFP, there was a change in the localization of APT1-HA. APT1-HA colocalized with p62-GFP as puncta (Figure 4.2 a). APT2-HA on the other hand, did not co-localize with p62-GFP. The PCC measurements revealed that HA-tagged APT1 had a statistically significant higher degree of colocalization with p62-GFP compared to APT2 (Figure 4.2 b). Thus, indicating that APT1 but not APT2, colocalizes with p62.

Next, the interaction of p62 with APT1 and APT2 was investigated with a co-IP assay. HA-tagged APTs were immunoprecipitated (IP-ed) to examine whether p62-GFP interacted with

either of these thioesterases. Alternatively, p62-GFP was immunoprecipitated to look at the interaction between p62-GFP and the APTs in the other direction.

When p62-GFP was immunoprecipitated, both APT1 and APT 2 co-immunoprecipitated with p62-GFP (Figure 4.2 d). However, when HA-tagged APTs were immunoprecipitated, p62-GFP co-immunoprecipitated with APT1-HA (Fig 4.2 c). Although there was some p62-GFP that IP-ed with APT2-HA, the signal was lower than the background levels observed with the control HA vector, implying that this interaction was likely non-specific. The co-IP results indicated that APT1 interacts with p62. Together, the confocal microscopy and co-IP results (Figure 4.2) suggest that APT1 and p62 are interactors.

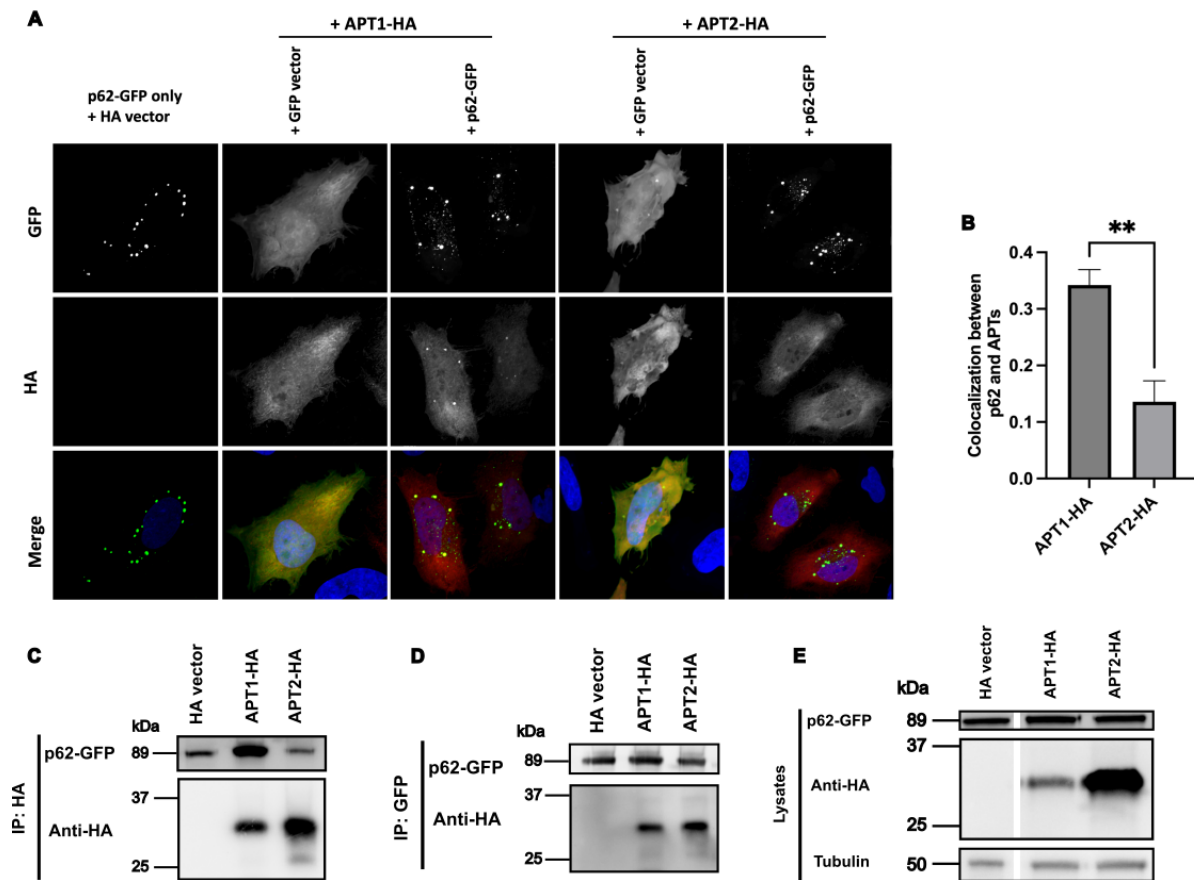


Figure 4.2. APT1 colocalizes and interacts with p62. (A) Confocal images of HeLa cells co-expressed with p62-GFP and APT1-HA or APT2-HA. The cells were subjected to 0.1% DMSO treatment. An immunofluorescence experiment was done. APT1-HA overlapped with p62-GFP in the form of puncta. (B) Co-localization between p62-GFP and APTs-HA represented as PCC for 8–9 cells per condition. The bars represent mean $\pm$ SEM from one experiment (n=1). Mann-Whitney unpaired t-test, $p < 0.05$. Later, a Co-IP experiment was done on HeLa cells co-transfected with p62-GFP and the two APTs-HA. Anti-tubulin was used as a loading control. (C) The individual APTs-HA were immunoprecipitated using rat-anti-HA antibody. Western blot analysis revealed that p62-GFP co-immunoprecipitated (co-IP 'ed) with APT1-HA. Some p62-GFP co-IP 'ed with APT2-HA which was lower than what co-IP 'ed with the control HA vector. (D) p62-GFP was immunoprecipitated using a goat-anti-GFP antibody. Both APT1-HA and APT2-HA co-IP 'ed with p62-GFP. (E) Western blot shows the expression of APT1-HA, APT2-HA, and p62-GFP in the lysates. (APT1 co-IP

4.3 – ABHD17A and ABHD17C Co-localize with p62

Serine hydrolases are a new group of depalmitoylating enzymes (Lin & Conibear, 2015), therefore, I investigated the effect the serine hydrolases may have on p62. HA-tagged ABHDs were co-expressed with p62-GFP. The HeLa cells were treated with DMSO or BafA1.

ABHD16A-HA did not colocalize with p62-GFP under DMSO or BafA1 conditions. However, ABHD17A and 17C both had a change in localization and colocalized in puncta with p62-GFP under DMSO and BafA1 conditions. Moreover, overall ABHD17C-HA had a significantly higher colocalization with p62-GFP compared to ABHD16A and ABHD17A under both DMSO and BafA1 conditions as indicated by PCC measurements (Figure 4.3 b). Both ABHD17A and ABHD17C had a significantly higher overlap with p62-GFP in contrast to ABHD16A, which had a low PCC. The results show that ABHD17A and 17C colocalize with p62 while ABHD16A does not.

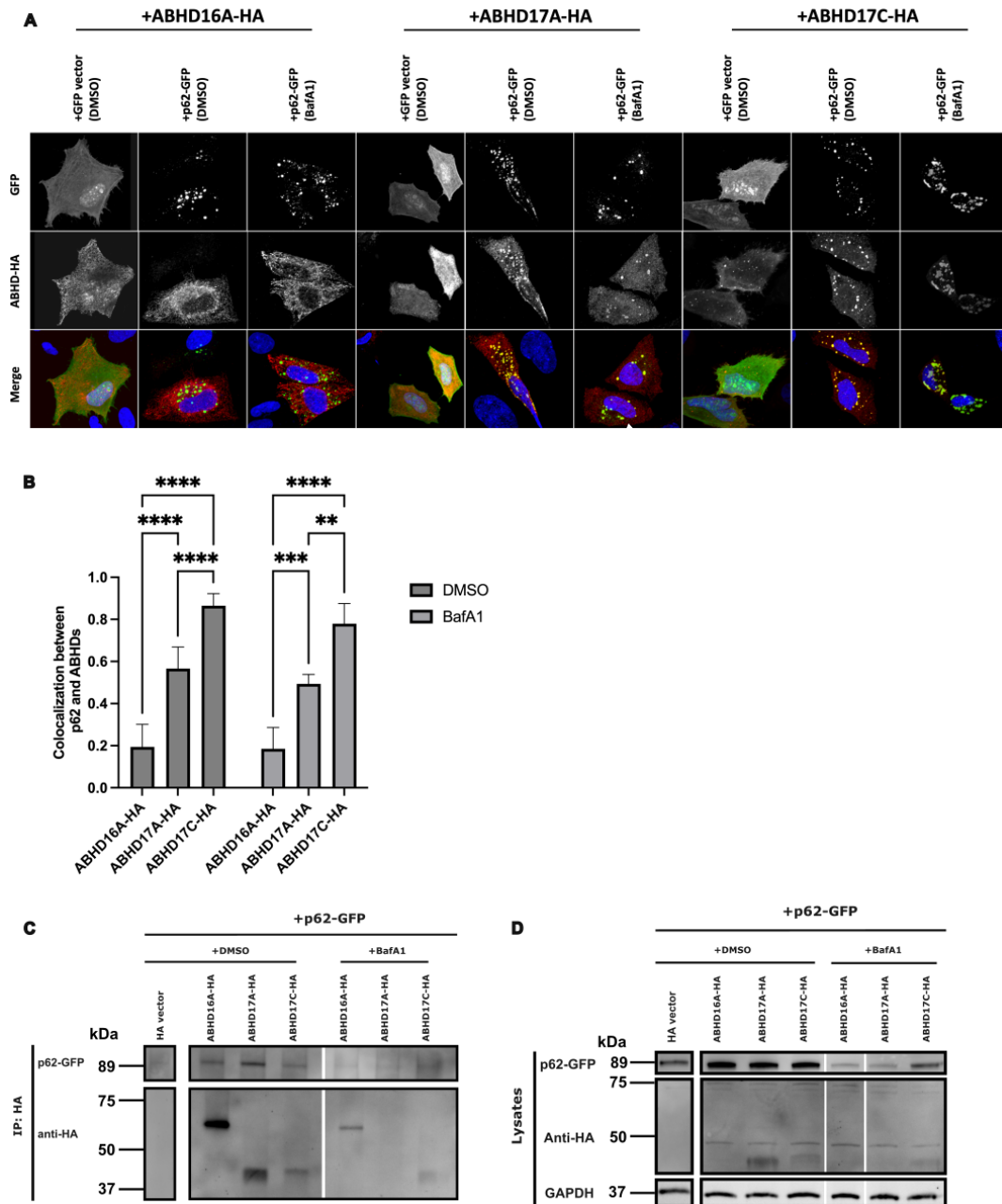


Figure 4.3. ABHD16A, 17A, and 17C interact with p62. HeLa cells were co-expressed with p62-GFP and the three ABHDs-HA. The cells were subjected to 0.1% DMSO or 100 nM BafA1 16-18hr treatment. **(A)** Confocal images for an immunofluorescence experiment. ABHD17A-HA and ABHD17C-HA overlapped with p62-GFP in the form of puncta. **(B)** Co-localization between p62-GFP and ABHDs-HA under different DMSO or BafA1 treatments represented as PCC for 3 – 10 cells per condition. The bars represent mean $\pm$ SEM (n=1). One-Way ANOVA, $p < 0.05$. **(C) & (D)** Co-immunoprecipitation experiment was done on HeLa cells co-transfected with p62-GFP and the three ABHDs-HA. Anti-GAPDH was used as a loading control. **(C)** The individual ABHDs-HA were immunoprecipitated using rat-anti-HA antibody. Western blot analysis revealed that p62-GFP coimmunoprecipitated with all three ABHDs. Higher levels of p62-GFP IP-ed with the individual ABHDs under DMSO condition than the BafA1 treatment.

4.4 – ABHD16A, ABHD17A and ABHD17A are p62 Interactors

To investigate the interaction between p62 and ABHD16A, 17A, and 17C, I completed a co-IP assay in presence or absence of BafA1. Interestingly, p62-GFP co-immunoprecipitated with all three of the serine hydrolases. These results suggest that p62 interacts with ABHD16A, 17A and 17C, even though only ABHD17A and 17C were the only ones that co-localized with p62 (as mentioned in 4.3; and shown in Figure 4.3a).

With the BafA1 treatment, however, the levels of ABHDs and p62 declined (Figure 4.4 c). This shows that BafA1 may be affecting serine hydrolases, leading to a decrease in protein levels. Regardless of the treatment, p62 interacted with ABHD16A, 17A, and 17C.

4.5 – Confirming Strongest Interactors of p62

Compared to the HA vector control, the signal of p62-GFP was stronger when it co-IP 'ed with HA-ZDHHC5, APT1-HA, and ABHD17A-HA (results from 4.1 – 4.4). Thus, to confirm the strongest interactions and effect of BafA1, HA-ZDHH5, APT1-HA, and ABHD17A-HA were co-expressed with p62-GFP in HeLa cells. BafA1 treatments were done overnight (O/N) or for 4 hours. p62-GFP co-immunoprecipitated with HA-ZDHHC5, APT1-HA, and ABHD17A-HA when IP was done for HA-tagged proteins. APT1-HA and ABHD17A-HA coimmunoprecipitated with p62-GFP when the GFP IP was performed (Figure 4.4 c).

Under untreated conditions (DMSO) the p62-GFP protein levels declined when co-expressed with ZDHHC5, APT1, and ABHD17A (Figure 4.4 d). These findings imply that p62 may be a substrate of these enzymes. These enzymes, specifically APT1 and ABHD17A may be

affecting overall autophagy which leads to reduced p62 levels. To determine the effect of these enzymes on autophagy, future studies should monitor LC3 levels.

Moreover, the BafA1 treatments decreased the p62-GFP levels compared to the control HA vector DMSO condition. p62 is involved in autophagy as well as the UPS (Ning & Wang, 2019). Thus, one possibility of this decrease could be that blocking of late-stage autophagy with BafA1 may be directing the accumulated p62 towards UPS where it is degraded and thus, we see an overall degradation. To investigate this, future studies should look at the p62 levels in the presence of UPS inhibitors as well as BafA1.

Regardless of the treatments, p62-GFP co-immunoprecipitated with ZDHHC5, APT1, and ABHD17A thus suggesting that these enzymes are interactors of p62.

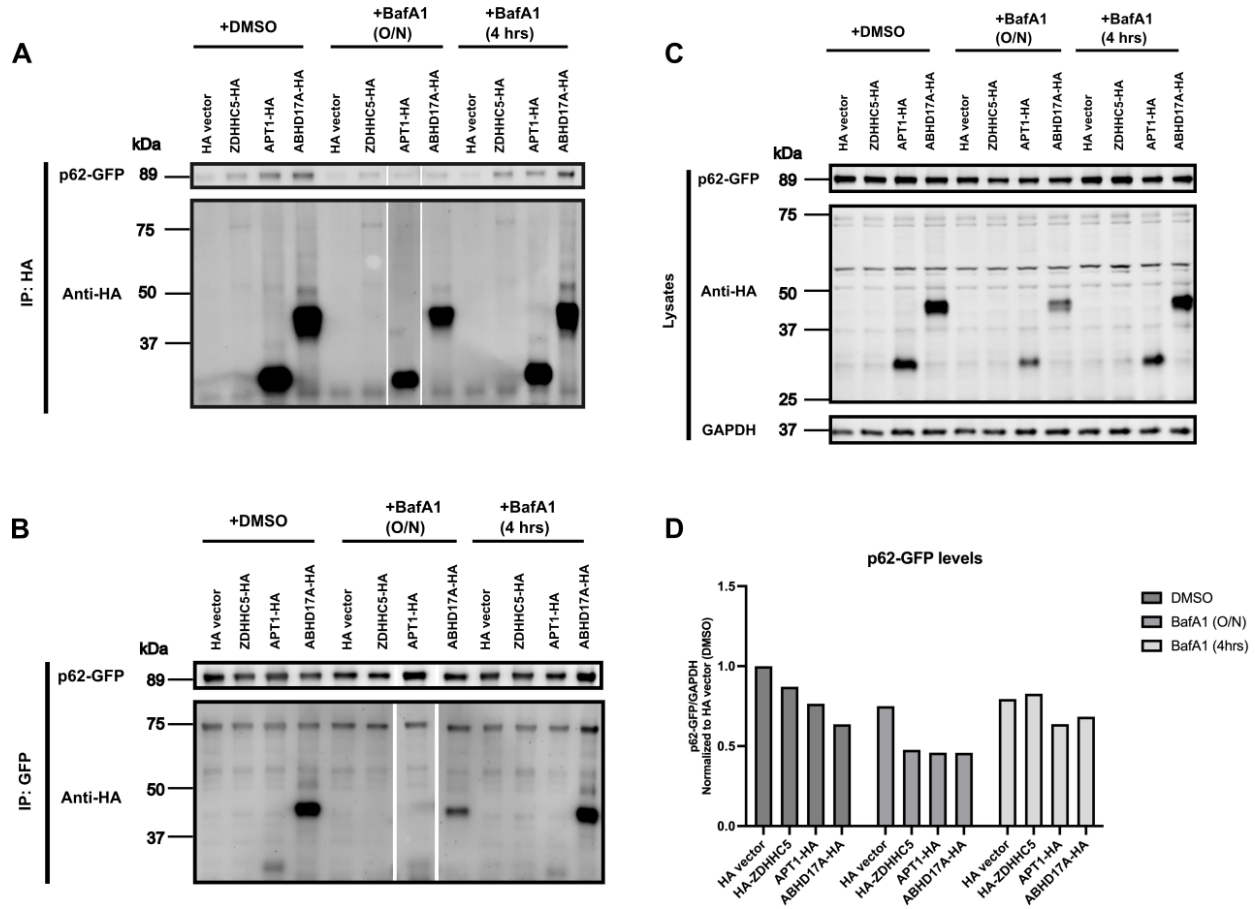


Figure 4.4. Strongest interactors of p62. HeLa cells were co-expressed with p62-GFP and HA-ZDHHCS or APT1-HA or ABHD17A-HA. The cells were subjected to 0.1% DMSO or 100 nM BafA1 O/N treatment (for 16-18 hours), or BafA1 treatment for 4 hours. **(A)** Co-IP results when individual HA-tagged enzymes were immunoprecipitated and **(B)** when p62-GFP was immunoprecipitated. **(C)** Total p62-GFP and HA-tagged enzymes in the lysates. GAPDH was used as a loading control. The WB analysis from the Co-IP assay confirmed the interaction of p62 with all three enzymes under DMSO and BafA1 treatments. APT1-HA BafA1 (O/N) and the rest of the samples from the IPs were on separate gels. The white space on the edges of APT1-HA (BafA1 O/N) lanes in (A) and (B) represents the merge of two gels imaged under the same exposure. **(D)** Quantification of p62-GFP levels/ GAPDH from the lysates in (A) normalized to HA vector under DMSO conditions shows an overall decrease in p62-GFP levels (n=1).

4.6 – Effect of Enzymes of p62 Palmitoylation

Next, I investigated whether the enzymes that interacted with p62 influenced the palmitoylation of p62. An ABE assay experiment was done on lysates from HeLa cells co-expressing p62-GFP and HA-tagged enzymes under basal conditions. Two independent experiments revealed that some of the enzymes may be regulating the palmitoylation of p62 while others may be just substrates of p62.

The PATs, APTs and ABHDs are known to be palmitoylated and thus were detected in the pulldown blots showing affinity purified palmitoylated proteins (Figure 4.5 a). ZDHHC5 did not increase palmitoylation levels. ZDHHC17 is a well-conserved PAT that is highly expressed in the brain; therefore, I tested the effect of this PAT on the palmitoylation of p62-GFP. ZDHHC17-HA led to an increase in palmitoylation levels with a decrease in p62-GFP protein levels, suggesting that palmitoylation may be increasing the degradation of p62. As for the depalmitoylating enzymes (serine hydrolases or SHs), surprisingly, all the enzymes led to an increase in palmitoylation levels as well as an increase in the p62-GFP protein levels.

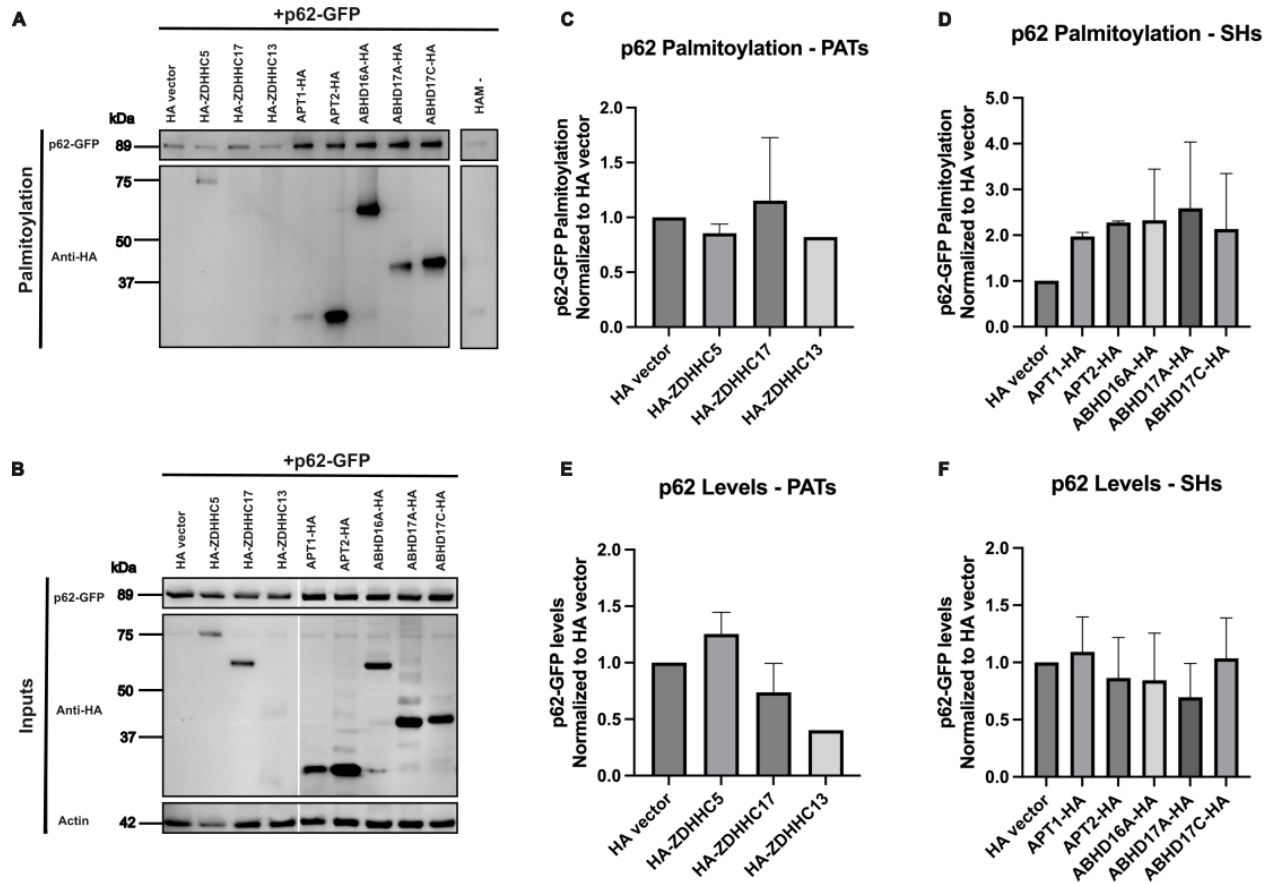


Figure 4.5. ZDHHC17 may palmitoylate p62. (A) & (B) Western blot showing ABE assay results of HeLa cells transfected with p62-GFP and the HA-tagged enzymes. **(A)** The acylation blot shows the palmitoylated p62 and enzymes that were affinity purified using biotin. A very low palmitoylation signal was detected in the absence of hydroxylamine HAM- lane. **(B)** The blots show the expression of p62-GFP and the HA-tagged enzymes in the inputs. p62-HA was detected in Input and acylation blots using mouse-anti-HA antibody and anti-actin was used as a loading control. **(C)** The levels of palmitoylation of p62-GFP, when co-expressed with the PATs **(D)** and the depalmitoylating enzymes or serine hydrolases (SHs). **(E)** The variation of p62-GFP protein levels/Actin in the presence of PATs **(F)** depalmitoylating enzymes (or SHs) normalized to HA vector control. The bars in both charts are represented as the mean $\pm$ SEM palmitoylation and protein level for each condition from two independent experiments (n=2). HA-ZDHHC13 condition is from one experiment (n=1).

CHAPTER 5

Future Directions and On-going Investigations

5.1 - Click Chemistry Reaction for p62 Palmitoylation Detection:

One approach to detect palmitoylation involves introducing alkynyl-palmitate into cells through metabolic labelling, followed by performing a click reaction (Hang et al., 2007; Yap et al., 2010; Gao & Hannoush, 2018;).

After metabolically labelling the transfected cells with alkynyl-palmitate and lysing, an IP was set up for isolating the transfected protein of interest. Then, a click reaction was performed on the IP-ed protein to look at the palmitoylation of the protein of interest, as previously described by (Liao et al., 2021). This approach, however, did not work for p62. Therefore, I tried an alternative approach for click that involves affinity purifying palmitoylated proteins after covalently linking them with biotin through click chemistry.

To identify the optimal conditions that work for p62, I tested three different click chemistry reaction ligands - TBTA, THPTA, and BTAA - in the presence of sodium ascorbate as the reducing agent.

p62 signal was detected with this alternate approach (Figure. 5.1). The use of THPTA resulted in the cleanest output with no signal in the negative control, palmitate with no alkyne group. As expected, the lane with alkynyl-palmitate under the same conditions had a p62 signal. No p62 was detected in the presence of BTAA. In the presence of TBTA, there was a non-specific signal as there was a signal present in both cold and alkynyl-palmitate lanes. Thus, the

optimal conditions for doing a click reaction on p62 are in the presence of THPTA as a ligand and sodium ascorbate as the reducing agent (Figure 5.1).

I also probed for endogenous valosin-containing protein (VCP), another palmitoylated protein we are investigating in the lab. VCP palmitoylation was detected with this method and consistent with the p62 results, the cleanest result was also in the presence of THPTA (Figure 5.1).

Now, that the conditions for click chemistry have been optimized, we have a more specific approach for the detection of palmitoylation for p62. The ABE assay is capable of detecting S-nitrosylation as well as palmitoylation. Hence, this approach will be valuable in studying palmitoylation specifically *in vitro*.

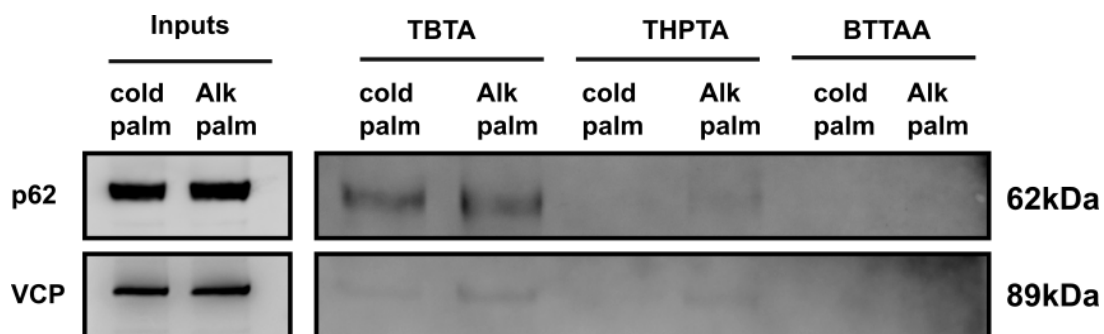


Figure 5.1. Detection of Palmitoylation via click chemistry. Western blot shows the results of a click chemistry experiment with the three chelators (TBTA, THPTA, BTAA). 15 µg of protein was separated for Input samples from cold palmitate and alkynyl palmitate samples. About 538 µg of protein was used for the click reaction for each chelator and palmitate label condition. Endogenous p62 and VCP were probed in the input and the pulled-down palmitoylated protein samples. The palmitoylation of both VCP and p62 was detected using this click chemistry approach. The cleanest results were for THPTA as the cold palmitate lane did not have any signal while the alkynyl palmitate did.

5.2 – Effect of Vorinostat on p62 in Neurons

It is necessary to conduct investigations on neuronal cultures alongside HeLa cells. This is particularly important for studying the impact of Vorinostat on p62 in neurons of the YAC128 HD mouse models. WT neurons were transfected with p62-GFP and treated with drugs (2BP, PalmB, and Vorinostat) at DIV 16 overnight (Figure 5.2 a).

Preliminary data suggests that under drug treatment the size of p62 puncta reduced while the number of puncta in the neurites increased (Figure 5.2b, c). However, this result is for 1 to 3 neurons per condition and will need further investigations and repetitions to conclude the effect Vorinostat has on p62 in neurons. The future investigations should study the effect of Vorinostat on p62 and LC3 localization in YAC128 HD mouse neurons.

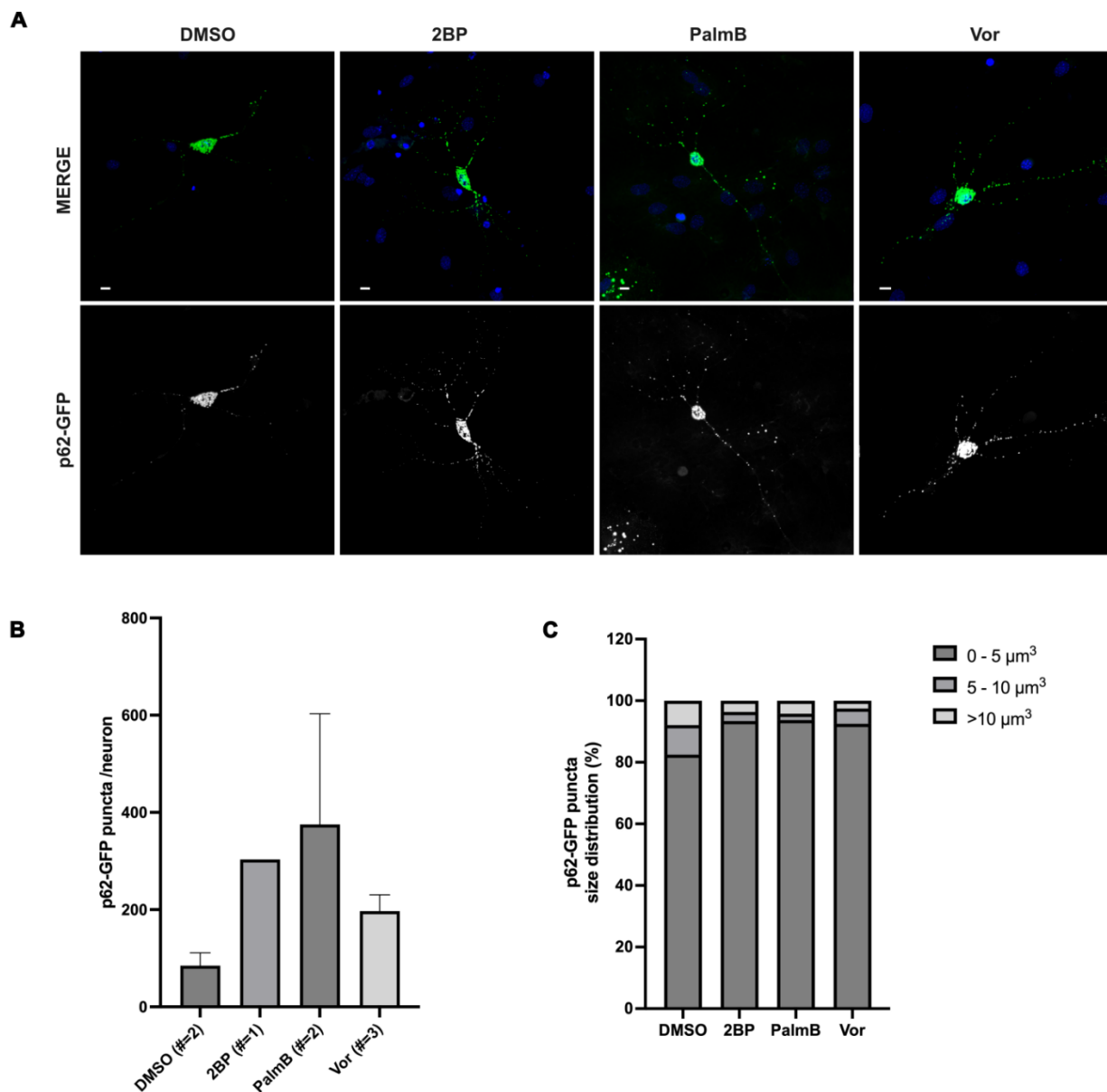


Figure 5.2. Effect of Vorinostat on p62 in Neurons. (A) Confocal images of mouse neurons from non-transgenic FVB strain expressed with p62-GFP. The neurons were subjected to 0.1% DMSO, 20 μM 2BP, 10 μM PalmB, and 10 μM Vorinostat treatments at DIV 16 for 16-18 hours. (B) p62-GFP puncta was quantified in the neurites for 1-3 neurons per condition (# = no. of neurons). (C) represents the distribution of different-sized p62-GFP puncta in the neurites under each drug condition.

5.3 – Motor Hang Test on HD mice

HD impacts motor behaviour in YAC128 mouse models. The selective striatal neuronal loss leads to motor deficits in these mice (Slow et al., 2003). Determining the effect of Vorinostat on HD is important for this project therefore, establishing baseline experiments to study the effect of the drug in HD was needed. We have optimized a protocol for the mouse hanging test on our in-house YAC128 HD mouse models and the non-transgenic FVB (WT) strain. The mice were placed on a wired cage top, 30 cm above the bottom of the home cage. The cage was inverted and the latency to fall when the mouse was released from the wire was recorded. The hang test was done on YAC128 and age-matched WT mice at 8 months and 12 months (Figure 5.3).

The initial findings indicate that the hang time is significantly reduced at 8 months and noticeably shorter at 12 months for transgenic YAC128 mice overall when compared to the WT FVB (Figure 5.3 a, b). Furthermore, there is a sex-specific difference with the male YAC128 mice at 8 months having a significantly shorter hang time than their WT male littermates (Figure 5.3 c). The hang test can be used for future pre-clinical studies with Vorinostat. Vorinostat can easily cross the blood-brain barrier thus, it can be administered to mice in water. The YAC128 mice can be monitored for motor changes which will help investigate the therapeutic potential of Vorinostat in HD mouse models.

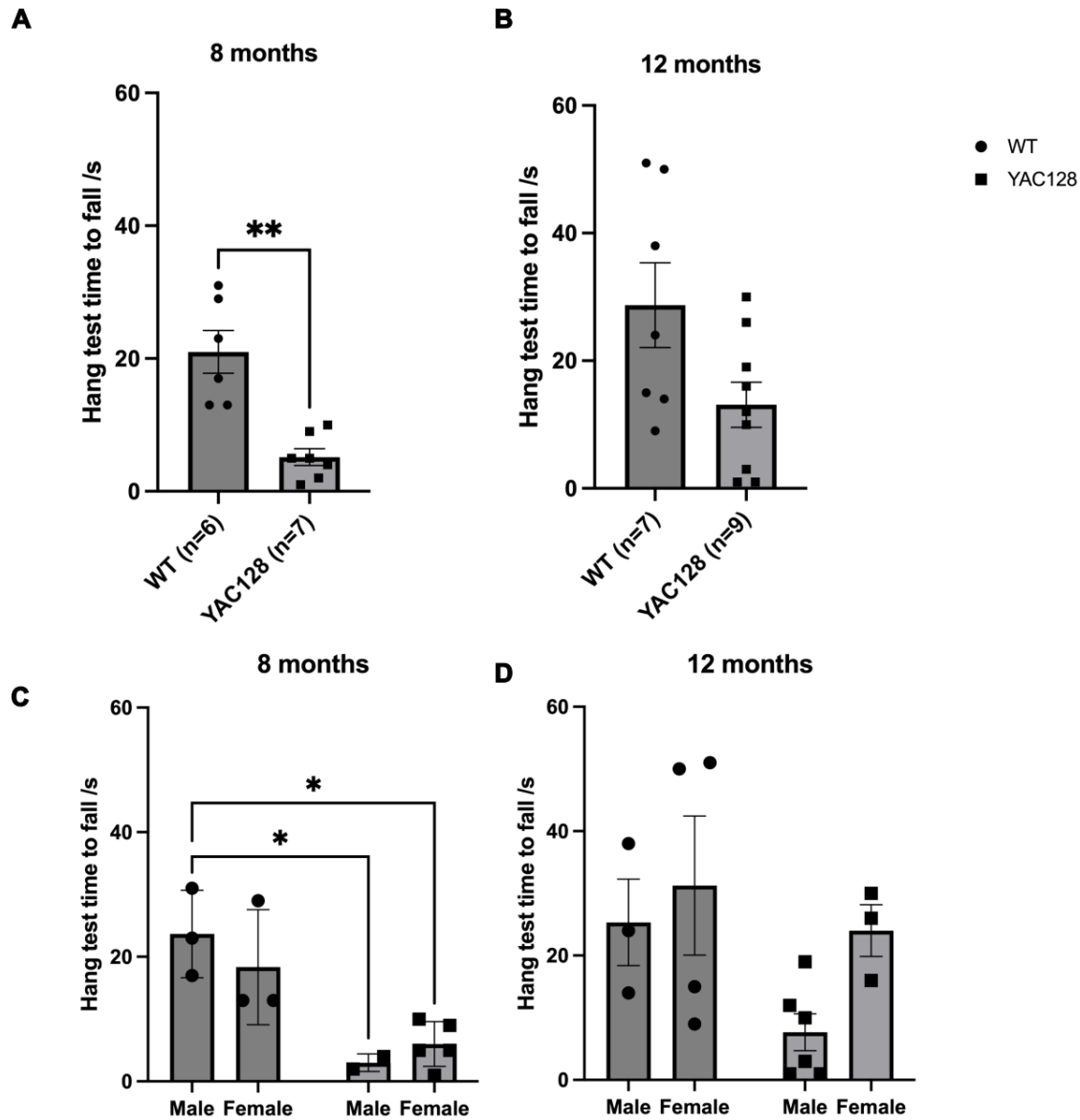


Figure 5.3. Motor Hang Test. YAC128 HD mice or WT (FVB) littermates were placed on a wire cage top and inverted from a 30cm height from the bottom of the home cage. Latency to fall was recorded for mice at **(A)** 8 months and **(B)** 12 months for WT and YAC128 mice. Three trials were performed per animal and the longest time was recorded. Error bars represent $\pm$ SEM. In **(A)** ** indicates significance $p < 0.05$. Non-parametric Mann-Whitney t-test. Time to fall was analyzed based on sex differences at **(C)** 8 months and **(D)** 12 months. * Indicates $p < 0.05$. Two-Way ANOVA test.

5.4 – PATs affecting p62 Puncta Size

The initial qualitative screen performed via wide-field microscopy provided us with a few PATs that I followed up on to look at their interaction with p62 and the potential effect on p62 palmitoylation (Chapter 4). Our lab has recently acquired a Nikon AXR laser scanning confocal microscope. We reimaged the fixed cells expressing p62-GFP and the HA-ZDHHCs on the confocal microscope and ran a quantitative analysis to determine the variation and distribution of p62 puncta. Through our quantitative screening, we have identified a slightly different list of PATs that were not caught by our initial qualitative screening process using widefield microscopy. These include ZDHHC3, ZDHHC5, ZDHHC8, ZDHHC12, ZDHHC14, ZDHHC15, and ZDHHC23 (Figure 5.4 a). We have missed ZDHHC3, ZDHHC12, and ZDHHC23 from this list which should be investigated in future studies. Overall, ZDHHC3, ZDHHC8, ZDHHC14, ZDHHC15, and especially ZDHHC23 seem to have a more robust effect on the distribution of p62 puncta size (Figure 5.4).

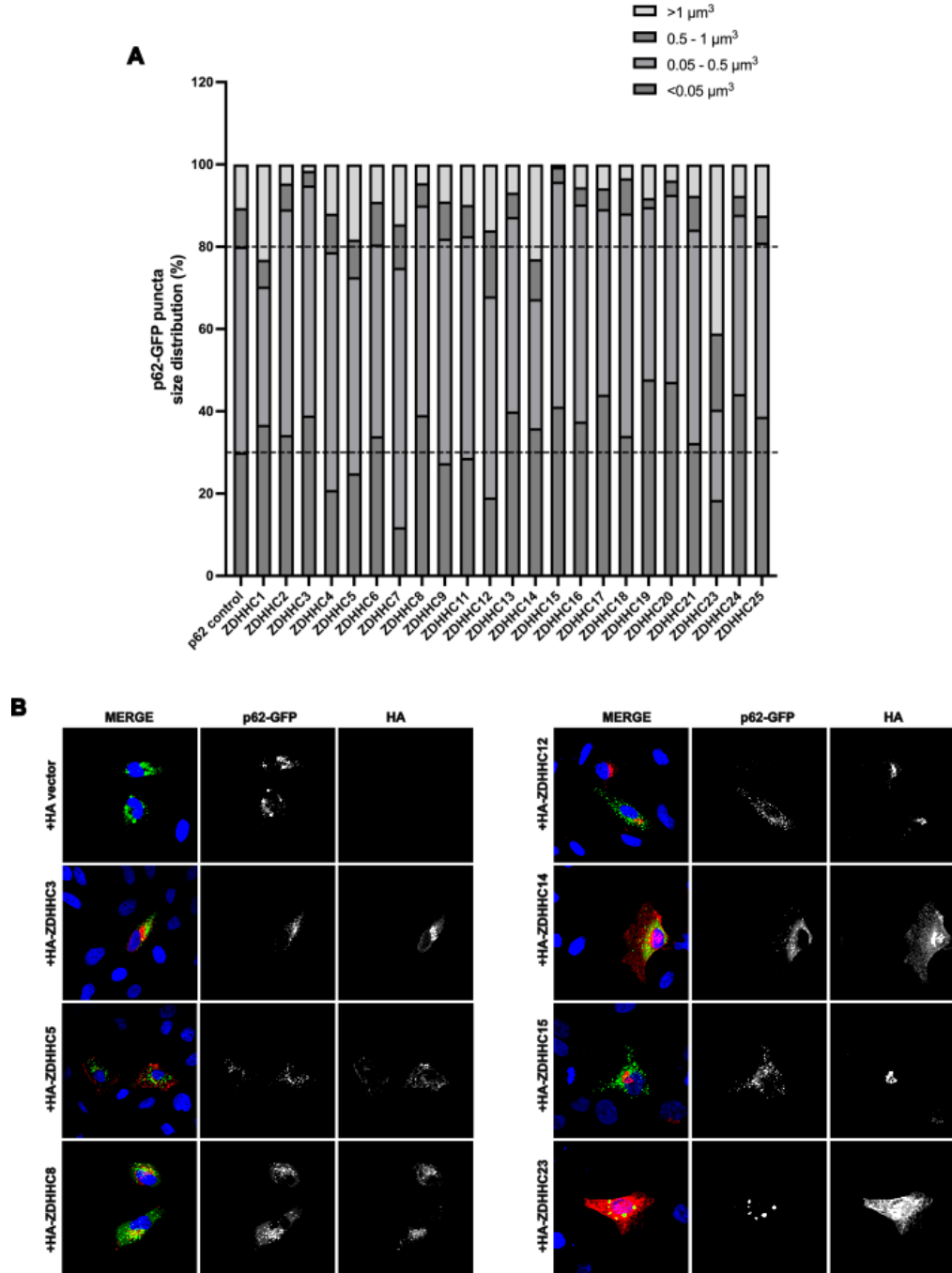


Figure 5.4. Microscopy Screen of PATs co-expressed with p62. All 23 PATs (HA-ZDHHCs) were co-expressed with p62-GFP. No colocalization was observed but a change in p62 puncta size was detected with some PATs. **(A)** Data represents variation in p62-GFP puncta size distribution compared to p62 control (co-expressed with HA vector). ZDHHC3, ZDHHC8, ZDHHC14, ZDHHC15, and ZDHHC23 seem to have a more robust effect. The dotted lines represent the percentage of puncta $<0.05\mu\text{m}^3$ and $>0.5\mu\text{m}^3$ of p62 control. **(B)** Confocal images of HeLa cells co-expressing PATs showed a more robust change in puncta distribution compared to the control as shown in the screen in (A). These images were taken on Nikon AXR.

5.5 – Effect of Vorinostat on APT1-HA and APT2-HA

Vorinostat may increase palmitoylation by directly inhibiting depalmitoylating enzymes that regulate p62 palmitoylation. Therefore, I tested the effect of Vorinostat on the localization of two well-characterized thioesterases, APT1 and APT2, with p62-GFP. As mentioned in Chapter 4, p62 interacted and colocalized with APT1-HA, but not with APT2-HA. The results show that p62 colocalized with APT1 under all drug treatments (Figure 5.5a). p62 also seemed to colocalize with APT2 under Vorinostat treatment (Figure 5.5b).

I quantified the colocalization between these enzymes and p62 (Figure 5d). There was a significant increase in colocalization with Vorinostat as suggested by PCC measurements. I compared the colocalization of APT1 and APT2 with p62 under untreated (DMSO) conditions (Figure 5.5 d). Compared to APT1, the colocalization of APT2 with p62 was significantly lower.

There was a noticeable increase in APT2 and p62 overlap with Vorinostat treatment however, this was still significantly lower than APT1 colocalization with p62 under the same treatment. These preliminary results suggest that Vorinostat may be affecting the interaction of p62 with APT2. The effect of Vorinostat on the depalmitoylating enzymes should be examined to determine how Vorinostat may be regulating these enzymes.

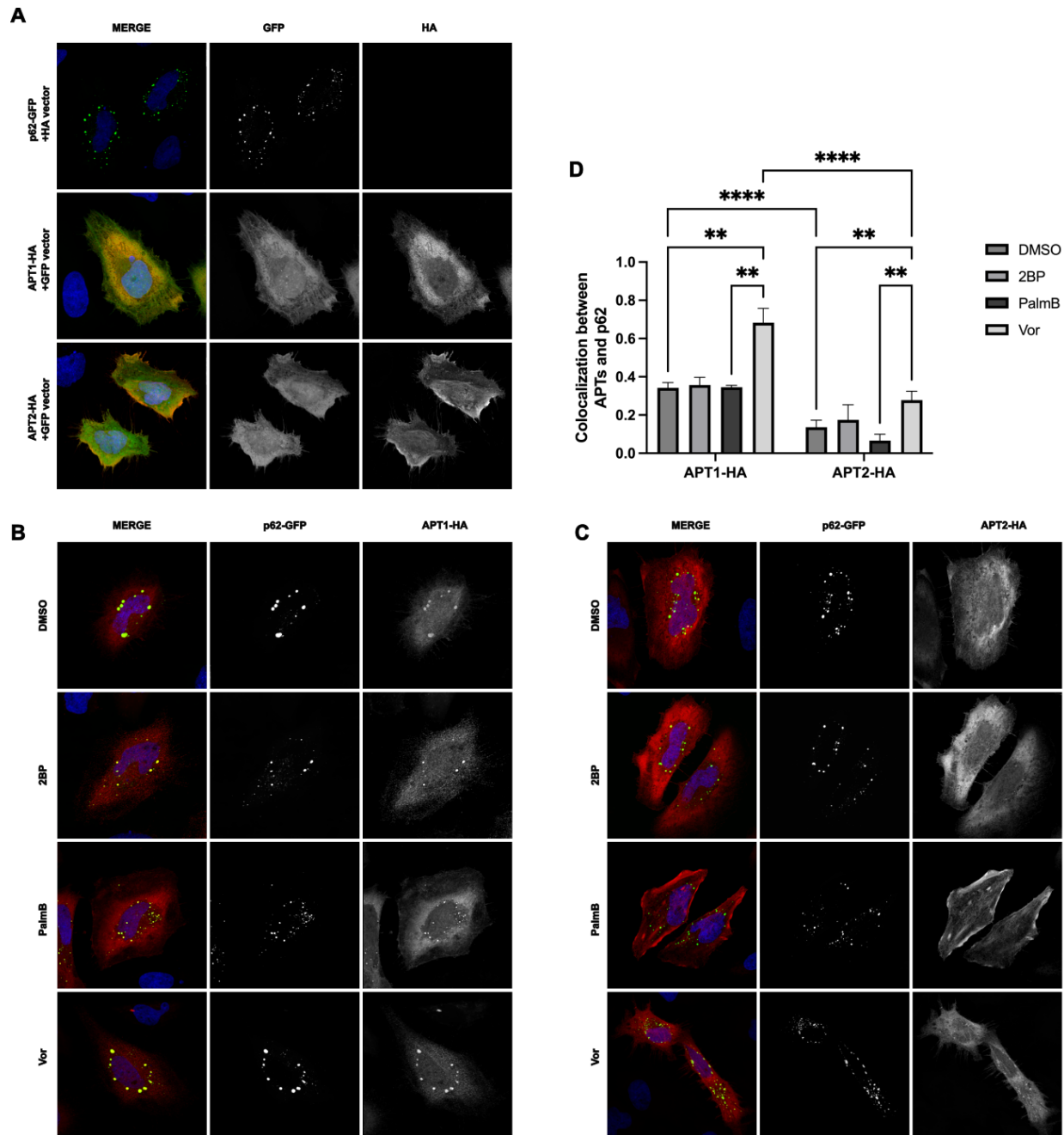


Figure 5.5. Effect of Vorinostat on co-localization between p62 and APTs. HeLa cells were co-expressed with p62-GFP and APTs-HA and subjected to 0.1% DMSO, 20 μ M 2BP, 10 μ M PalmB, 10 μ M Vorinostat (Vor). **(A)** Confocal images of p62-GFP and HA vector, and APTs-HA and p62-GFP, and treated with 0.1% DMSO. Confocal images of HeLa cells co-expressed with **(B)** APT1-HA and p62-GFP **(C)** and p62-GFP and APT2-HA treated with different drugs. **(D)** Colocalization was quantified as PCC for p62-GFP with APT1 and APT2 (3-9 cells, n=1). Error bars represent mean $\pm$ SEM. Asterisks indicate $p < 0.05$. Two-way ANOVA test.

CHAPTER 6

Discussion and Conclusion

6.1 – Vorinostat Regulation of p62

Our previous work has shown that palmitoylation of p62 is reduced in the cortex of HD patients as well as YAC128 HD mouse models. We hypothesize that the cargo-loading defect in the autophagy pathway in HD could be related to the reduced palmitoylation of p62. A drug that is capable of rescuing palmitoylation of p62 may be a potential strategy in HD. An earlier study identified Vorinostat as a potential candidate for increasing palmitoylation (Martin et al., 2019).

Therefore, we sought to investigate how Vorinostat regulated p62. Our results show that palmitoylation of p62 increases with a concomitant increase in localization to the autophagosomes in response to the Vorinostat treatment. The increase in palmitoylation was about three times stronger than PalmB, an inhibitor of depalmitoylating enzymes that increases palmitoylation (Figure 3.2 a, b). These results suggest that Vorinostat is capable of improving p62 palmitoylation and may be a novel inhibitor of depalmitoylating enzymes. Moreover, there was an increase in the localization of p62 to the autophagosomes as suggested by a significant increase in colocalization between p62-GFP and RFP-LC3 (autophagosome marker) in response to Vorinostat treatment (Figure 3.3 a, b).

There was a general increase in colocalization between LC3 and p62 in the presence of the drugs being tested. TSA and Vorinostat, both HDAC inhibitors, have been shown to induce autophagy (Gammoh et al., 2012; Bai et al., 2019). The number of LC3 puncta increased in

response to TSA treatment (Zhang et al., 2015; Bai et al., 2019), therefore, we saw an improvement in colocalization with TSA compared to DMSO (Figure 3.3 a, b). Other HDAC inhibitors, Pyrox and Vorinostat increased colocalization as well. However, our results revealed that there was a significant increase in colocalization between p62 and LC3 under PalmB and Vorinostat treatments. PalmB increases palmitoylation and since PalmB and Vorinostat showed comparable increases in the overlap, both these drugs may be regulating this change in localization through the same mechanism i.e., via palmitoylation. Thus, increasing palmitoylation of p62 may improve its localization to the autophagosomes.

An increase in colocalization between LC3 and p62 was also observed in the presence of 2BP treatment (Figure 3.3 b). 2BP is an inhibitor of palmitoylation and therefore leads to a decrease in palmitoylation of the proteins. Autophagy is regulated by many proteins that can act as either enhancers or inhibitors of the process. Therefore, alterations in protein palmitoylation has the potential to exert diverse effects on autophagy. For example, the mechanistic target of rapamycin complex 1 (mTORC1) is a key negative regulator of autophagy which suppresses autophagy by inhibiting the ULK1 complex (Deleyto-Seldas & Efeyan, 2021). Studies have shown that key components of mTORC1 including mTOR and LAMTOR1 are palmitoylated (Sanders et al., 2019). The inhibition of palmitoylation with 2BP led to a reduction in mTORC1 activation suggesting that mTORC signalling is dependent on palmitoylation (Sanders et al., 2019). Reduced activation of mTORC signalling may lead to increased autophagy. Thus, increased autophagy may explain the increased overlap between p62 and LC3 in response to 2BP (Figure 3.3 a, b).

Variations in the size of LC3 and p62 puncta were observed when these proteins were expressed together and treated with the drugs (Figure 3.2c, d). This suggested that the size of autophagosomes was changing in response to drug treatments. Therefore, we tested whether this effect was specific to p62 or LC3 or both. Individual experiments revealed a more profound change in puncta size in cells expressing p62-GFP compared to cells expressing RFP-LC3 (Figure 3.4). Thus, indicating that the change in autophagosome size was more specific to p62. Generally, more p62-GFP puncta was present under PalmB and Vorinostat treatments. Consistent with the overexpression results, the presence of larger-sized endogenous p62 puncta increased in HeLa cells under these two drugs suggesting a common mechanism of p62 regulation by PalmB and Vorinostat.

Apart from size, the number of puncta also varied in response to the drug treatments (Figure 3.2 e, f; Figure 3.3 d, e; Figure 3.4 b). Overall, there was a decrease in the number of puncta per cell with an increase in size for cells expressing p62-GFP. This phenomenon may be due to the self-dimerization of p62 at the UBA domain (Xu & Wan, 2023) or it may be caused by an accumulation of p62 in the autophagosomes.

Studies have shown that p62 is acetylated at K420 and K435 within the UBA domain (You et al., 2019). Acetylation increases p62 binding to ubiquitinated proteins and promotes their degradation (You et al., 2019). It could be possible that p62 binds to ubiquitinated proteins and therefore, the localization to the autophagosomes increases in response to PalmB and Vorinostat. Thus, more p62 accumulates in the autophagosomes, and the p62 puncta size appears to be larger. These may be autophagosomes or autolysosomes. Since p62 and lysosome

colocalization increases in response to Vorinostat (Figure 3.6 a, c), we suspect that these larger-sized vesicles may be autophagolysosomes.

HTT is a ubiquitinated protein and a substrate for p62 (Kalchman et al., 1996; Martin et al., 2015). We investigated the localization changes of endogenous HTT and p62 to the lysosomes in response to the palmitoylation and acetylation-altering drugs (Figure 3.6).

Studies have shown that Vorinostat improves motor deficits in HD mice (Hockly et al., 2003). Moreover, in 2009, a group reported that acetylation promotes mHTT clearance as well as its trafficking to the autophagosomes (Jeong et al., 2009). Acetylated-mHTT interacts with p62 and knockout of endogenous p62 led to a reduction of mHTT clearance (Jeong et al., 2009). Likewise, my findings demonstrate a significant improvement in the localization of p62 and HTT to the lysosomes in response to the HDAC inhibitors including Vorinostat.

Additionally, previous studies have reported that increasing palmitoylation of mHTT improved its solubility (Lemarié et al., 2021). It was also demonstrated that increasing overall palmitoylation in the brain improved phenotype in HD mice (Virlogeux et al., 2021). My results show a significant increase in the overlap between HTT and lysosomes and a noticeable increase between HTT and p62 with the PalmB treatment. Thus, indicating that improvement in phenotype and mHTT solubility may occur because of improved HTT localization to the lysosomes, which may promote its degradation. I report improved HTT and p62 localization to the lysosomes, as well as an increase in the overlap between p62 and HTT in response to Vorinostat treatment, which may indicate increased interaction between the two proteins. These findings imply that Vorinostat may increase clearance and degradation, thus, it may hold therapeutic potential in HD.

Since p62 is an acetylated protein, I aimed to investigate whether Vorinostat also affected p62 acetylation besides its palmitoylation. Acetylation of p62 is mediated by TIP60 and the deacetylation is regulated by HDAC6 (You et al., 2019). Vorinostat inhibits HDAC enzymes (HDAC1, 2, 3, and 6), increasing the total acetylated protein levels in the lysates (Figure 3.7 b, d). Interestingly, Vorinostat reduced the levels of acetylated p62 (Figure 3.7a, c). In 2012, a group showed that Vorinostat increased autophagic flux and therefore reduced p62 protein levels (Gammoh et al., 2012). However, while my data reveals that acetylated p62 levels decrease, I did not observe a change in p62 protein levels in the lysates (Figure 3.7 e) suggesting that Vorinostat is affecting p62 acetylation through a different mechanism.

The results show that PalmB also increased the levels of total acetylated proteins in the lysates compared to DMSO, although lower than Vorinostat treatment. Moreover, like Vorinostat, PalmB decreased the levels of acetylated p62 implying a role of palmitoylation in p62 acetylation regulation.

Interestingly, BafA1 acetylated p62 compared to DMSO (Figure 3.7a, c). BafA1 treatments overall increase the levels of total tagged-p62 protein as expected (Figure 3.7 e) since BafA1 blocks the fusion of autophagosomes and lysosomes leading to the accumulation of p62 (Klionsky et al., 2008, 2021). BafA1 treatments with PalmB and Vorinostat led to a slight increase in levels of acetylated p62 compared to their DMSO counterparts, which may be due to increased accumulation of p62. However, overall, the BafA1 treatments had less acetylated p62 compared to the control DMSO conditions.

The results from Figure 3.7 suggest that palmitoylation may be regulating acetylation as suggested by the overall increase in acetylated protein levels in the lysates. But for p62

specifically, increasing palmitoylation with PalmB seems to inhibit acetylation. The findings in this chapter demonstrate that Vorinostat increases palmitoylation and reduces acetylated p62, indicating that palmitoylation of p62 may inhibit its acetylation (Figure 6.1).

TSA was previously shown to increase acetylation and increase protein stability (Kim et al., 2010). Since Vorinostat decreases p62 acetylation, we wanted to investigate the effect of this on p62 protein stability. Treatment of p62-HA transfected HeLa cells with CHX, a translation inhibitor, decreased protein levels (Figure 3.8). The administration of Vorinostat together with CHX to these cells, stabilized protein levels to a steady state suggesting that Vorinostat improves p62 protein stability.

Overall, Vorinostat improves the localization of p62 to the autophagosomes as well as the lysosomes. Vorinostat increases the palmitoylation of p62 while potentially inhibiting its acetylation. This may be happening because of direct inhibition of p62 acetylation by Vorinostat or indirect inhibition via increasing p62 palmitoylation (Figure 6.1). Vorinostat may be inhibiting depalmitoylation enzymes thus increasing p62 palmitoylation. Future studies should investigate the effect of Vorinostat on the acetylation activity of the depalmitoylating enzymes regulating p62 palmitoylation.

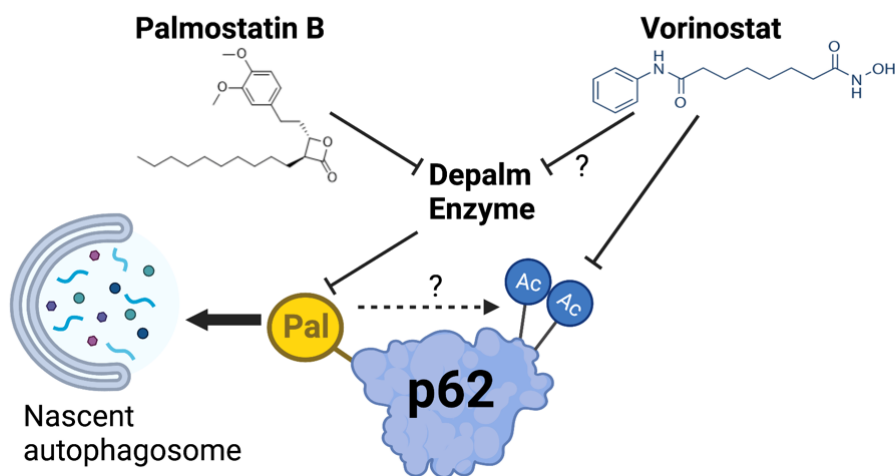


Figure 6.1. Possible mechanism for p62 regulation by Vorinostat. Schematic of the possible mechanism of Vorinostat regulation of p62 palmitoylation and acetylation. Vorinostat increases palmitoylation of p62 and decreases its acetylation. Increase in palmitoylation in response to Vorinostat may be occurring via inhibition of depalmitoylating enzymes. The decrease in p62 acetylation levels may be a downstream effect of increased palmitoylation.

6.2 – Enzymes Impacting p62 Palmitoylation

Palmitoylation is a reversible post-translational modification that is mediated by palmitoyl acyl transferases (PATs) in the forward direction. The depalmitoylation of proteins is catalyzed by acyl protein thioesterases (APT) and α/β serine hydrolases (ABHDs) (Main & Fuller, 2022). Our work has shown that p62 is palmitoylated however, the enzymes that regulate this process remain unknown.

Therefore, we sought to identify the enzymes that are regulating this pathway. We began by performing a microscopy screen of p62-GFP co-expressed with the HA-tagged 23 individual PATs (HA-ZDHHCs). There was no colocalization between p62 and the PATs however, we did observe changes in the puncta size of p62 when co-expressed with 10 of the 23 PATs. These included ZDHHC2, ZDHHC5, ZDHHC6, ZDHHC7, ZDHHC8, ZDHHC14, ZDHHC15, ZDHHC17, ZDHHC21, and ZDHHC24.

Interactions between p62 and the PATs were assessed. The co-IP assay revealed that out of the 10 PATs, only ZDHHC5 interacted with p62 (Figure 4.1). Therefore, I tested the effect of ZDHHC5 co-expression on p62 palmitoylation. Interestingly, palmitoylation of p62-GFP did not increase in response to overexpression with ZDHHC5 (Figure 4.5 a, c and e). Taken together, the Co-IP and ABE results suggest that ZDHHC5 may only be a substrate of p62 and not the PAT regulating its palmitoylation.

Another possibility of the interaction of ZDHHC5 with p62 (Figure 4.1 a) could have resulted from the effect of overexpression. ZDHHC5 is generally localized at the plasma membrane. It forms a complex with GOLGA7B which maintains it at the plasma membrane (Woodley & Collins, 2019). Overexpression of ZDHHC5 results in a lack of GOLGA7B; thus,

inducing mislocalization of ZDHHC5 to other cell compartments (Woodley & Collins, 2021). To tease apart whether ZDHHC5 is a substrate of p62, the interaction between endogenous p62 and endogenous ZDHHC5 needs to be investigated. We recently received CRISPR knockouts for the PATs that can be used to study this interaction in the future.

ZDHHC17 is the most well-conserved PAT from humans to yeast (Young et al., 2012; Butland et al., 2014). It is highly expressed and enriched in the brain (Singaraja, 2002). Moreover, it palmitoylates many synaptic proteins including PSD-95, SNAP25, HTT, and synaptotagmin I (Huang et al., 2004; Yanai et al., 2006). Palmitoylation-independent interactions of ZDHHC17 are also important in mediating the role of some of the autophagy regulators such as optineurin (OPTN) and CALCOCO1 (Butland et al., 2014; Nthiga et al., 2021). AMP-activated protein kinase (AMPK) promotes autophagy by phosphorylating mTOR1 and ULK1. Studies have demonstrated that palmitoylation of AMPK by ZDHHC17 is important for its activation (Sun and Du, 2022). Moreover, some of the proteins involved in autophagy that our lab is currently studying are also palmitoylated by ZDHHC17. Taken together, these studies implicate the importance of ZDHHC17 in the nervous system as well as in the autophagic pathway. Thus, it was of interest to investigate its potential effect on p62 palmitoylation. The ABE results showed a noticeable increase in palmitoylation of p62-GFP in tandem with decreased p62-GFP levels upon HA-ZDHHC17 overexpression (Figure 4.4 a–c, and e). Hence, this suggests that ZDHHC17 may be the PAT involved in regulating p62 palmitoylation and may be playing a role in its degradation.

Among the 23 PATs, ZDHHC17 and ZDHHC13 are the only two PATs that contain the ankyrin repeat (AR) domain at the N-terminus. The AR domain helps with substrate recruitment

and palmitoylation-independent functions (Lemonidis, Salaun, et al., 2017). The AR domain binds to proteins containing ZDHHC-AR-binding motifs (zDABM) (Lemonidis et al., 2015; Lemonidis, MacLeod, et al., 2017). ZDHHC17 and ZDHHC13 regulate the palmitoylation of HTT (Yanai et al., 2006; Butland et al., 2014). Therefore, besides ZDHHC17, I tested the effect of ZDHHC13 on the palmitoylation of p62 as well. The ABE assay revealed that ZDHHC13 did not increase p62 palmitoylation. Thus, indicating that ZDHHC13 is not involved in regulating palmitoylation of p62.

Next, I investigated the depalmitoylating enzymes that may be influencing p62 palmitoylation. APT1 and APT2 catalyze thioester hydrolysis of palmitoylated proteins (Davda & Martin, 2014). I began by performing a microscopy screen of p62 co-expressed with APT1, APT2, ABHD16A, ABHD17A, and ABHD17C, to look for changes in localization. This was done with co-IP assays to investigate the interaction of these enzymes with p62. Both APT1 and APT2 immunoprecipitated with p62-GFP when a GFP IP was done (Figure 4.2 d). However, when the APTs were immunoprecipitated, p62-GFP had a robust signal with APT1-HA and not with APT2-HA. APT2 has been reported to be ubiquitinated and undergoes degradation via the UPS (Abrami et al., 2020). p62 is involved in mediating the degradation of proteins through UPS by binding to K43-ubiquitinated proteins (Ning & Wang, 2019), this may explain the interaction of APT2 with p62 when p62-GFP is in abundance (i.e., with GFP IP) (Figure 4.2 d). Whereas, when the two APTs are in abundance, i.e., via an HA IP, p62 co-immunoprecipitated with APT1 only, suggesting that p62 interacts with APT1 (Figure 4.2 c).

Recently, some of the serine hydrolases from α/β hydrolases family which include ABHD16A, ABHD17A, B, and C have been identified as novel depalmitoylating enzymes (Lin &

Conibear, 2015; Yokoi et al., 2016; Shi et al., 2022). The microscopy screen shows that ABHD17A and ABHD17C colocalized with p62. In addition to these three depalmitoylating enzymes, ABHD16A also interacted with p62.

I did an ABE assay to investigate the effect of these enzymes on the palmitoylation of p62. Although we expected to see reduced palmitoylation in response to the overexpression of the potential depalmitoylating enzyme(s), the results revealed that all the enzymes increased palmitoylation of p62. This was contrary to what was expected.

ABE assay (using hydroxylamine as a nucleophile) can potentially detect both nitrosylation and palmitoylation. In 2022, the Lipton group showed that p62 is S-nitrosylated at C331 (Oh et al., 2022). Nitrosylation is the addition of nitric oxide onto a cysteine residue. Since both nitrosylation and palmitoylation occur on a cysteine residue, there have been reports of crosstalk between the two PTMs in regulating the protein function (Zareba-Kozioł et al., 2019; Ramzan et al., 2023). Some studies have reported an inhibitory effect of the two PTMs on each other (Hess & Stamler, 2012). A study showed increasing PSD-95 palmitoylation reduced its nitrosylation (Ho et al., 2011). Similarly, the induction of nitrosylation of neuronal proteins, SNAP-25 and GAP43, led to reduced palmitoylation of these proteins (Hess et al., 1993; Hess & Stamler, 2012). Therefore, it is possible that some of the enzymes are depalmitoylating p62, which results in reduced palmitoylation and may increase p62 nitrosylation therefore, we detect a stronger signal in the ABE assay (Figure 4.4 a).

The unpublished data from Dr. Martin and colleagues showed p62 nitrosylation was not detected in HD patient brains. However, in Parkinson's disease models, upregulation of p62

nitrosylation contributes to dysregulated autophagic flux (Oh et al., 2022). Thus, a possibility is that these depalmitoylating enzymes might be having an effect on nitrosylation directly.

Our lab is currently working on optimizing a specialized biotin exchange assay for detecting nitrosylation. This assay is referred to as SNO assay. Investigating the direct effect of depalmitoylating enzymes on nitrosylation of p62 through this approach will be of interest.

Moreover, to investigate the effect on palmitoylation, an assay that will specifically detect palmitoylation is needed. Click chemistry is a method that can be used to detect palmitoylation (Gao & Hannoush, 2018). This approach involves metabolic labelling with clickable alkynyl-palmitate probes 2023-10-18 6:34:00 PM . I have optimized click chemistry for the detection of p62 palmitoylation (described in detail in Chapter 5). In the future, this approach can be used to look at the effect of the depalmitoylating enzymes on palmitoylation specifically.

6.3 – Conclusion and Future Directions

Overall, the work presented in this thesis has demonstrated that Vorinostat increases p62 palmitoylation while reducing its acetylation. Subsequently, Vorinostat increases the localization of p62 to the autophagosomes and the lysosomes. This effect was comparable to the Palm B treatment, suggesting that palmitoylation may be involved in localization regulation and Vorinostat may be a novel depalmitoylation inhibitor. Vorinostat treatment improved HTT localization to lysosomes as well as its binding to p62. Together, these investigations indicate that Vorinostat may potentially improve the cargo-loading defect in autophagy in HD by rescuing p62 palmitoylation. Improved autophagy may increase the mHTT degradation and improve disease phenotype in HD. Hence, Vorinostat may be a novel therapeutic agent for HD. As for the p62 palmitoylation regulation, this process may be mediated by ZDHHC17 while ZDHHC5 may only be a substrate of p62. The depalmitoylating enzymes, APT1, ABHD16A, ABHD17A, and ABHD17C are all interactors of p62 and p62 depalmitoylation may be catalyzed by one or some of these serine hydrolases.

Preliminary results described in Chapter 5 should be investigated further as they are important for preclinical studies in mice. To comprehensively determine the therapeutic potential of Vorinostat in HD, future studies should investigate the effect of Vorinostat on the enzymes that depalmitoylate p62 to gain a better insight into the mechanism regulating p62 palmitoylation as well as its acetylation (Figure 6.1). Moreover, determining the effect of Vorinostat in neurons will be important before beginning a pre-clinical trial which will look into Vorinostat-treated HD mice behaviour and the overall impact of Vorinostat treatment on p62 palmitoylation in mice neurons and tissues.

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Autophagy in Huntington disease and huntingtin in autophagy

Author: Dale D.O. Martin, Safia Ladha, Dagmar E. Ehrnhoefer, Michael R. Hayden

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