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Role of CHIP E3 ligase in neurodegenerative diseases and cancer

PhD Thesis

by

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Declaration

I hereby declare that I am the author of this thesis. The work herein is entirely my own unless otherwise clearly indicated and acknowledged. I can confirm that this thesis has been submitted for the degree of Doctor of Philosophy and no part of this work has been submitted for any other degree or professional qualification.

In loving memory of Halina Milewska

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Abstract in English

The C-terminus of Hsc70-interacting protein (CHIP), encoded by STUB1, is an E3 ubiquitin ligase and co-chaperone that safeguards neuronal proteostasis by targeting misfolded or unstable proteins for refolding or degradation. While CHIP's neuroprotective role is well recognised, the specific mechanisms by which it maintains neuronal structure and signalling, particularly in disorders such as Alzheimer's, Parkinson's, and Huntington's disease, have remained incompletely understood.

This thesis demonstrates that loss of CHIP disrupts axonal homeostasis, destabilises neurofilament architecture, and impairs key signalling pathways in a neuronal context. Using CRISPR/Cas9-engineered CHIP-knockout SH-SY5Y neuroblastoma cells, deep quantitative proteomics identified a core network of CHIP-sensitive proteins, including NEFL, NEFM, INA, TAU, DPYSL2 (CRMP2), ELAVL3, VGF, NPY, and netrin-1, that support axonal scaffolds, cytoskeletal dynamics, and synaptic transmission. In the absence of CHIP, this network was markedly down-regulated, leading to reduced expression of neurofilament proteins, impaired axon-guidance signals (notably the netrin–CRMP2 axis), and broad suppression of G-protein– and PKC-dependent neurotransmitter pathways. Ingenuity Pathway Analysis revealed widespread inhibition of GPCR cascades, glutamatergic signalling, and vesicular transport, alongside a modest but reproducible activation of the Ras/MAPK axis.

These proteomic changes recapitulate molecular signatures seen in multiple neurodegenerative diseases, offering mechanistic insight into how pathogenic STUB1 variants cause conditions such as spinocerebellar ataxia and early-onset dementia. Simultaneously, the up-regulation of Ras/MAPK signalling and dysregulation of proteostasis observed here mirror oncogenic processes, highlighting CHIP's dual role as both tumour suppressor and tumour facilitator in a context-dependent manner. This work thus establishes CHIP as a molecular bridge between neurodegeneration and cancer; two biological domains traditionally viewed as distinct.

Building on these findings, this thesis proposes CHIP as a potential early-life biomarker for neurodegenerative susceptibility. Newborn genomic screening for deleterious STUB1 variants could enable identification of at-risk individuals, offering opportunities for targeted neuroprotective strategies. Overall, this work positions CHIP as a master

regulator of neuronal integrity and a clinically relevant node at the intersection of proteostasis, neurodegeneration, and cancer biology.

Abstract in Polish

C-końcowy fragment białka oddziałującego z Hsc70 (CHIP), kodowany przez gen STUB1, to ligaza ubikwitynowa E3 i współbiałko opiekuńcze (ko-chaperon), które strzeże neuronalnej proteostazy poprzez kierowanie źle sfałdowanych lub niestabilnych białek do ponownego fałdowania lub degradacji. Choć neuroprotekcyjna rola CHIP jest dobrze udokumentowana, szczegółowe mechanizmy, za pomocą których utrzymuje on strukturę i sygnalizację neuronalną – szczególnie w chorobach takich jak Alzheimer, Parkinson czy Huntington – pozostają nie w pełni zbadane.

Niniejsza praca doktorska wykazuje, że utrata CHIP zaburza homeostazę aksonalną, destabilizuje architekturę neurofilamentów i osłabia kluczowe szlaki sygnalizacyjne w kontekście neuronalnym. Wykorzystując linie komórkowe neuroblastomy SH-SY5Y z nokautem CHIP uzyskanym metodą CRISPR/Cas9, przeprowadzono głęboką ilościową proteomikę, która zidentyfikowała kluczową sieć białek wrażliwych na obecność CHIP, obejmującą m.in. NEFL, NEFM, INA, TAU, DPYSL2 (CRMP2), ELAVL3, VGF, NPY i netrynę-1. Białka te wspierają struktury aksonalne, dynamikę cytoszkieletu i transmisję synaptyczną. W warunkach braku CHIP ta sieć była wyraźnie obniżona, co prowadziło do spadku ekspresji neurofilamentów, zaburzeń sygnałów naprowadzania aksonów (szczególnie osi netryna–CRMP2) oraz szerokiego wyciszenia szlaków neurotransmisyjnych zależnych od białek G i kinazy PKC.

Analiza ścieżek biologicznych metodą Ingenuity Pathway Analysis (IPA) ujawniła powszechne zahamowanie kaskad receptorów sprzężonych z białkami G (GPCR), sygnalizacji glutaminergicznej oraz transportu pęcherzykowego, przy równoczesnej, umiarkowanej lecz powtarzalnej aktywacji osi Ras/MAPK.

Zaobserwowane zmiany proteomiczne odzwierciedlają sygnatury molekularne charakterystyczne dla wielu chorób neurodegeneracyjnych, dostarczając mechanistycznego wglądu w to, jak patogenne warianty STUB1 mogą powodować schorzenia takie jak rdzeniowo-móżdżkowa ataksja czy otępienie o wczesnym początku. Jednocześnie wzrost sygnalizacji Ras/MAPK i zaburzenia proteostazy obserwowane w

tym badaniu odzwierciedlają procesy onkogenne, co podkreśla kontekstowo zależną, podwójną rolę CHIP jako supresora lub promotora nowotworów. Praca ta ustanawia zatem CHIP jako molekularne ogniwo łączące neurodegenerację i nowotworzenie – dwa obszary biologiczne tradycyjnie postrzegane jako odrębne.

W oparciu o uzyskane wyniki, rozprawa ta proponuje CHIP jako potencjalny biomarker podatności na choroby neurodegeneracyjne we wczesnym okresie życia. Genomowe badania przesiewowe noworodków w kierunku szkodliwych wariantów STUB1 mogłyby pozwolić na identyfikację osób zagrożonych, otwierając drogę do ukierunkowanych strategii neuroprotekcyjnych. Podsumowując, praca ta pozycjonuje CHIP jako główny regulator integralności neuronalnej oraz klinicznie istotny węzeł na przecięciu proteostazy, neurodegeneracji i biologii nowotworów.

Abbreviations

α-Syn	α-Synuclein
Aβ	Amyloid-β
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
BDNF	Brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
Cas9	CRISPR associated protein 9
CHIP	C-terminus of Hsc70-interacting protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4′,6-Diamidino-2-Phenylindole
DIA	Data-dependent acquisition
DCTN1	dynactin-1
DMEM	DMEM Dulbecco's Modified Eagle Media

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPYSL2	Dihydropyrimidinase-related protein 2
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELAVL3	ELAV-like protein 3
ER	Endoplasmic reticulum
FACS	Flow cytometry
FBS	Fetal bovine serum
GFP	Green fluorescent protein
gRNA	Guide RNA
HLA-A	Human leukocyte antigen A
HLA-B	Human leukocyte antigen B
HLA-C	Human leukocyte antigen C

HPLC	High Performance Liquid Chromatography
HSP	Heat-shock protein
IF	Immunofluorescence
INA	Alpha-internexin
IP	Immunoprecipitation
IPA	Ingenuity Pathway Analysis
iPSC	Induced pluripotent stem cells
kDa	Kilo Dalton
KO	Knock-out
LC	Liquid Chromatography
LC-MS/MS	Tandem mass spectrometry
mAb	Monoclonal antibody
MAPK	Mitogen Activated Protein Kinase

MHC	Major histocompatibility complexes
MND	Motor Neuron Disease
mRNA	Messenger RNA
MS	Mass spectrometry
MW	Molecular weight
NEFL	Neurofilament light polypeptide
NEFM	Neurofilament medium polypeptide
NPY	Pro-neuropeptide Y
NQO1	NAD(P)H dehydrogenase [quinone] 1
p53	Tumour suppressor protein 53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson’s disease

PFA	paraformaldehyde
PTM	Post-translational modification
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCA	Spinocerebellar ataxia
SCAR16	Spinocerebellar ataxia autosomal recessive type 16
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
STUB1	STIP1 homology and U-box containing protein 1
SWATH-MS	Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry
TEMED	Tetramethylethylenediamine
TPR	N-terminal tetratricopeptide repeat
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol

Ub	Ubiquitin
VGF	Neurosecretory protein VGF
WB	Western blot/Western blotting
WGS	Whole genome sequencing
WT	Wild Type

Contents

Chapter 1: Introduction

In the human body, an intricate system of maintenance and regulation ensures that each cell functions optimally, removing components that are no longer needed or have become damaged (Marshall and Vierstra, 2019). Among the key players in this process is ubiquitin ligase, a type of enzyme, a specialised protein that speeds up biochemical reactions, enabling the cell to carry out essential tasks efficiently (Li et al., 2022).

Ubiquitin ligase attaches a small protein called ubiquitin to other proteins within the cell. This attachment acts as a signal, marking these proteins for degradation by the cell's disposal system, known as the proteasome (Zhao et al., 2022; Motosugi and Murata, 2019; Marshall and Vierstra, 2019). The ubiquitin tag guides unwanted or damaged proteins to the proteasome, where they are broken down and recycled into usable parts. This system of marking and removal maintains cellular health by preventing the accumulation of faulty proteins, which could otherwise disrupt cell function (Cappadocia and Lima, 2018).

Beyond its role in cellular clean-up, ubiquitin ligase is involved in regulating key processes such as cell division, DNA repair, and stress responses. As a family of enzymes, ubiquitin ligases are highly specialised, targeting specific proteins based on precise signals to ensure the orderly functioning of countless cellular processes (Cappadocia and Lima, 2018; Henneberg and Schulman, 2021).

Ubiquitin ligases are classified into three principal groups, each with a distinct function in the ubiquitination process. The E1 enzymes, or ubiquitin-activating enzymes, commence this process by activating ubiquitin, setting it in readiness for transfer. Next are the E2 enzymes, known as ubiquitin-conjugating enzymes, which accept activated ubiquitin from E1 and act as intermediaries, transporting it to the subsequent stage (Marshall and Vierstra, 2019; Henneberg and Schulman, 2021). Finally, the E3 ubiquitin ligase family takes on the critical task of selectively recognising and tagging target proteins. E3 ligases detect specific proteins based on intricate cellular signals, ensuring that only designated proteins are marked for further processes, such as degradation or relocation within the cell (Cappadocia and Lima, 2018).

Within the E3 ligase family, there are specialised subgroups with unique roles, among them the co-chaperone ubiquitin ligases. These co-chaperones operate in tandem with molecular chaperones such as HSP70 and HSP90, which support other proteins in folding correctly or recovering from structural issues (Zhao et al., 2022). A prominent co-chaperone E3 ligase is CHIP, or the C-terminus of HSP70-Interacting Protein. CHIP collaborates closely with these chaperones to assess the condition of the proteins they oversee; should a protein prove irreparable, CHIP attaches ubiquitin to mark it for removal. In this capacity, CHIP serves to maintain cellular integrity by averting the accumulation of faulty proteins, thus promoting cellular health and stability (Zhang et al., 2020; Sharma et al., 2023; Kumar et al., 2022).

1.1 Neurodegenerative Diseases and Cancer

1.1.1 The burden of neurodegenerative diseases.

When asked about their biggest worry, people's responses will vary. Among the many different concerns mentioned, one seems to recur – aging. The relentless passage of time leaves its mark on the human body. In a time when looking youthful is so desired, wrinkles are seen as rather unwelcome. However, changes to the skin are not the most pressing concern. The more significant challenge lies in the decline of brain function, a prospect widely regarded as the greatest fear. The mind starts playing tricks, the memories start fading away, everything familiar becomes strange and unknown (Watson et al., 2023).

According to the World Health Organisation, dementia is the fourth leading cause of deaths worldwide. Figure 1 below represents risk of developing dementia in different parts of the world. Populations in Europe, North America, and Asia exhibit the highest risk, exceeding 70%. In comparison, individuals in Australia and Africa face moderately elevated risks of 32% and 20%, respectively. The lowest risk levels, 4%, were identified in

Central and South America (Nichols et al., 2021).

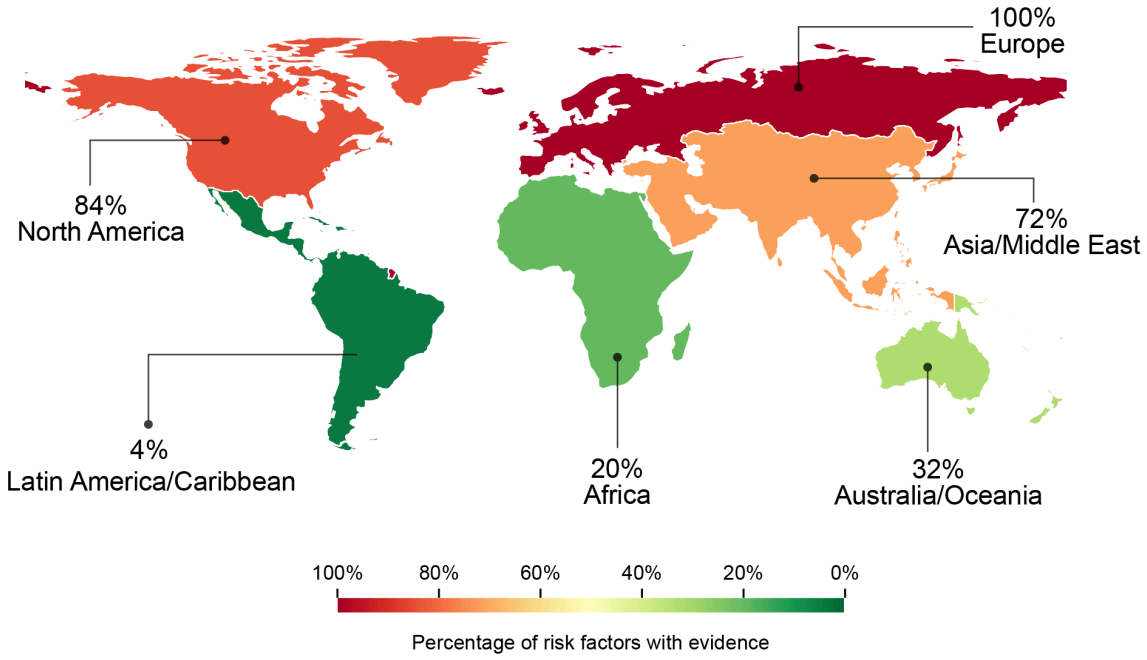

Figure 1: Risk of dementia onset across various geographical regions.

Dementia itself is not a single disease but rather a broad term encompassing a range of conditions that result from gradual death of neurons. Such diseases share common symptoms, including cognitive decline, reduced brain function, motor impairments, and behavioural changes. Some of the most recognised conditions include Alzheimer's disease, Parkinson's Disease, and Huntington's disease, collectively referred to as neurodegenerative diseases (Nichols et al., 2021). These conditions are characterised by the gradual degeneration of the brain, as illustrated in Figure 2.

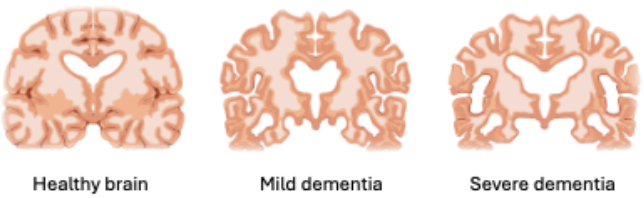

Figure 2: Comparison of brain degeneration in healthy individuals versus individuals with dementia.

Neurodegenerative diseases impact every aspect of a person's life, and despite extensive research, no cure currently exists (Pyka et al., 2024). Medications prescribed to

patients provide relief from symptoms but do not address the underlying causes. This is largely due to the complexity of these conditions (Wareham et al., 2022). While it is known that neurons are progressively lost, the exact mechanisms remain poorly understood (Tzavellas et al., 2024; Fan et al., 2015). A common feature of neurodegenerative diseases is the accumulation of proteins that form toxic clusters in the brain (Sengupta and Kayed, 2022). One such protein is **α**-synuclein, which is observed in patients with Alzheimer's disease. The role of **α**-synuclein can be compared to a car with a flat tyre. While identifying one flat tyre explains why the car isn't functioning, it does not determine whether the other tyres are also damaged or if other issues exist. Similarly, although **α**-synuclein is known to contribute to dementia, it remains uncertain whether it is the sole cause or one of several contributing factors (DeTure and Dickson, 2019). Much remains to be explored to fully understand the pathways involved in these diseases.

Another challenge lies in the lack of effective diagnostic tools. Current methods typically detect these conditions only at advanced stages, when significant neuronal loss has already occurred (Sheinerman and Umansky, 2013). In many cases, definitive diagnosis is made post-mortem, eliminating any opportunity to slow disease progression in patients (Signaevsky et al., 2022). This thesis aims to contribute to the understanding of neurodegenerative diseases and provide a foundation for the development of earlier diagnostic strategies.

1.1.2 The burden of cancer.

Cancer is the second leading cause of death globally, with nearly 20 million cases recorded in 2020. It accounted for 10 million fatalities that year, 44% of which occurred in females and 56% in males. Projections suggest that by 2040, cancer-related mortality will increase by over 64%, with an estimated one in nine men and one in twelve women expected to die from the disease. Lung cancer is the most prevalent, accounting for 12.4% of all cancers globally, followed by breast cancer in females (11.6%), colorectal cancer (9.6%), prostate cancer (7.3%), and stomach cancer (4.9%). Lung cancer also contributes to the highest number of cancer-related deaths, followed by colorectal, liver, female breast, and stomach cancers (Bray et al., 2024).

Early detection of cancer greatly improves the chances of survival. Unfortunately, around 50% of cancers are diagnosed at an advanced stage. Several critical challenges hinder progress in early detection, but addressing these could substantially improve survival rates. Firstly, there is a lack of comprehensive understanding of the biological processes that occur during the early stages of the disease. Secondly, despite extensive research efforts, knowledge about cancer risk factors remains incomplete. There is an urgent need for a deeper understanding of germline genomic susceptibility, family history, environmental exposures, demographic variables, and behavioural patterns to develop more robust risk models. Thirdly, the discovery and validation of biomarkers specific to early-stage cancers pose significant challenges. Lastly, improvements in molecular analytical technologies, advanced imaging modalities, and histopathological methods are essential to enhance the detection of tumours at earlier and more treatable stages (Crosby et al., 2022).

Cancer treatment is a complex process. Until recently, only a limited number of treatment options were available to patients, including radiation therapy, chemotherapy, and surgery. However, these conventional therapies often carry substantial risks to the patient. Radiation therapy, for instance, poses a risk of damaging healthy cells, tissues, and organs (Stone et al., 2003). Similarly, chemotherapy is known to harm healthy cells, particularly those that divide and grow rapidly. The use of drugs also introduces the risk of drug resistance, whereby cancer cells that were initially suppressed adapt and become resistant to the treatment (Zafar et al., 2025). In recent years, more advanced and safer approaches have emerged, including stem cell therapies, targeted drug therapies, and gene therapies. One of the most promising strategies is the combination of conventional and non-conventional anti-cancer treatments, which aligns with the multifaceted nature of cancer progression. This integrated approach appears to offer the most effective path forward (Liu et al., 2024; Zafar et al., 2025). This thesis seeks to contribute to broader understanding of cancer's progression within the human body, providing valuable insights into the fields of cancer detection and treatment.

1.1.3 Common factors between neurodegeneration and cancer.

At first glance, cancer and neurodegenerative diseases appear to have little in common. However, despite their obvious differences, an unexpected connection exists between these two seemingly unrelated conditions. Research has revealed that they share certain functional pathways, suggesting a potential link in their underlying mechanisms. Particularly, studies have shown that genes up-regulated in cancer are frequently down-regulated in neurodegenerative disorders, and vice versa, highlighting a complex interplay in their etiopathogenesis (Klus et al., 2015).

Conversely, studies indicate that certain neurodegenerative diseases are linked to a higher risk of developing specific tumours. For instance, patients diagnosed with Parkinson's disease have been found to exhibit an increased risk of malignant melanoma (Bertoni, 2010). Furthermore, there is evidence suggesting a potential association between melanoma and amyotrophic lateral sclerosis (ALS), a form of motor neuron disease (MND) (Fang et al., 2013). Interestingly, current data highlights that a diagnosis of Alzheimer's disease is associated with a 60% reduction in cancer risk, while a history of cancer is linked to a 30% reduced likelihood of developing Alzheimer's disease (Roe et al., 2010).

A striking example of how two seemingly unrelated diseases can be connected through shared molecular properties is the tumour suppressor protein p53. This protein exhibits physico-chemical characteristics typical of misfolded protein aggregates, a pathological conformation commonly associated with neurodegenerative diseases (Klus et al., 2015).

Another compelling example of a genetic link between neurodegeneration and cancer is the ATM gene. Ataxia-telangiectasia, a rare neurodegenerative condition, is characterised by chromosomal instability, immunodeficiency, and a heightened predisposition to cancer. This condition is caused by mutations in the ATM gene, which result in a complete loss of the ATM protein kinase (Thompson et al., 2005). The kinase plays a vital role in regulating cell division and DNA repair. Mutations in genes responsible for DNA repair have been implicated in both cancer and neurodegeneration. In particular, the ATM-encoded kinase is a critical activator of the p53 protein, further highlighting the intricate connections between these pathologies (Karakostis et al., 2024).

Another connection between neurodegeneration and cancer can be observed in post-translational modifications. Changes to proteins that make cells more prone to cell death simultaneously decrease the risk of cancer while increasing the likelihood of neurodegeneration. Conversely, conditions that promote cell growth are associated with a higher risk of cancer and a reduced risk of neurodegenerative diseases. These outcomes are highly dependent on the stage of the cell cycle, as cellular responses to these molecular and pathway modifications may vary. Nevertheless, such modifications ultimately contribute to the development of either cancer or neurodegeneration.

Drugs commonly used in the treatment of neurodegenerative diseases, such as thioridazine, have also been shown to exhibit anti-tumour properties. Similarly, certain anti-tumour agents, including cyclin-dependent kinase inhibitors and mithramycin, have demonstrated neuroprotective effects, highlighting a potential overlap in the molecular pathways targeted by these therapeutic compounds (Migliore and Coppedè, 2002).

Both cancer and neurodegeneration are thought to be the result of the interaction between genetic and environmental factors. Despite many different studies and data obtained, the relationship between cancer and neurodegeneration remains unclear. Further work to explore the link between them needs to be carried out in order to understand the connection between these two biological processes better. This thesis aims to explore the similarities and differences between the two diseases, being a stepping stone to better understand the link.

1.2 C-terminus of Hsc70-interacting protein (CHIP)

1.2.1 Structure

The C-terminus of Hsc70-interacting protein (CHIP) is a 35 kDa protein encoded by the STUB1 gene (Joshi et al., 2016). Structurally, CHIP consists of three primary domains: an N-terminal tetratricopeptide repeat (TPR) domain, a central helical domain, and a C-terminal U-box domain (Gu et al., 2025). The TPR domain facilitates interactions with heat shock proteins, while the U-box domain provides E3 ubiquitin ligase activity. The central helical domain plays a critical role in maintaining the overall stability and

conformation of the protein. This modular architecture underpins CHIP's dual role as a co-chaperone and an E3 ubiquitin ligase, which is essential in maintaining protein quality control (Joshi et al., 2016).

1.2.2 Canonical functions of CHIP

CHIP possesses three canonical functions. Firstly, it acts as a chaperone, with the ability to induce structural changes in proteins to either maintain their solubility or enhance their specific activity (Schisler et al., 2013). Secondly, CHIP serves as a co-chaperone by directly interacting with heat shock proteins, assisting in the stabilisation and refolding of their bound substrates. Thirdly, CHIP functions as a ubiquitin ligase, targeting terminally defective proteins by ubiquitinating them and directing their degradation via the ubiquitin-proteasome system.

CHIP is classified within the E3 ubiquitin protein ligase family. Members of this family facilitate the transfer of ubiquitin molecules to the epsilon-amino group of lysine residues on substrate proteins. This process results in the formation of an isopeptide bond between the ubiquitin molecule and the substrate protein, marking the latter for degradation or other cellular processes (Joshi et al., 2016).

1.2.3 Non-canonical functions of CHIP

Apart from its well-established functions as a ubiquitin ligase and chaperone, CHIP has been shown to perform various non-canonical roles that influence several cellular processes.

One such role is the regulation of signalling pathways. CHIP interacts with various kinases, modulating their stability and activity. A prominent example is its ability to regulate the stability of c-Raf protein kinase, which in turn affects cell motility and migration. Above all, c-Raf is a critical component of the mitogen-activated protein kinase (MAPK) pathway and plays a significant role in oncogenesis (Dogan et al., 2008).
CHIP is also involved in the modulation of transcription factors. For instance, it promotes

the degradation of the transcription factor Runx2, thereby acting as a negative regulator of osteoblast differentiation (Joshi et al., 2016).

Furthermore, CHIP plays a critical role in immune regulation by modulating immune pathways such as those mediated by Toll-like receptors (TLRs). It targets proteins involved in inflammatory responses for degradation (Yang et al., 2011).
Finally, CHIP contributes to the maintenance of mitochondrial integrity. It controls the turnover of proteins involved in energy metabolism and apoptosis, particularly under conditions of cellular stress (Dogan et al., 2008; Joshi et al., 2016).

1.2.4 Regulation of CHIP

The expression of CHIP is regulated through multiple mechanisms. One such mechanism involves the Wnt/β-catenin signalling pathway. β-catenin, in conjunction with its co-activator p68, binds to the promoter region of the STUB1 gene, enhancing its transcription. This regulatory mechanism has been observed in colorectal cancer, where elevated CHIP levels were found to contribute to tumour progression.
MicroRNAs (miRNAs) also play a role in modulating CHIP expression at the post-transcriptional level (Kal et al., 2022). For example, miR-764-5p targets the 3' untranslated region (UTR) of STUB1 mRNA, leading to a downregulation of CHIP. Increased levels of miR-764-5p have been shown to inhibit CHIP expression, thereby promoting osteoblast differentiation (Guo et al., 2012).

CHIP activity and stability are further influenced by post-translational modifications, including phosphorylation and ubiquitination, which modulate its function and interactions (Ranek et al., 2020). Moreover, proteins such as CHIC2 have been shown to impact CHIP levels. CHIC2 facilitates the recruitment of CHIP to the plasma membrane, thereby influencing the internalisation and degradation of cell surface receptors. This indicates that CHIC2 plays a significant role in regulating CHIP's cellular localisation and activity (Koochaki et al., 2022; Shankar et al., 2024).

1.3 Protein homeostasis

1.3.1 Regulation of protein homeostasis

The human body functions as an intricate and highly coordinated machine, relying on numerous quality control systems to maintain proper functionality. One of these essential systems is protein homeostasis, also known as proteostasis, which ensures that proteins are synthesised, maintained, and replaced when necessary. Proteins are vital to cellular function, playing roles such as structural support, waste removal, and communication within and between cells. However, they are susceptible to damage, misfolding, or degradation. Proteins are composed of amino acid building blocks, which must fold into precise three-dimensional structures to function correctly. Proteostasis safeguards these processes, ensuring that proteins are accurately produced, retain their functional structure, and are removed or replaced when damaged (Jayaraj et al., 2020).

Proteostasis involves three main steps. The first step ensures the synthesis of new proteins in the correct quantities. The second step involves repairing misfolded proteins with the assistance of molecular chaperones, such as Hsp70, which act as cellular repair workers (Mauthe et al., 2025). The third step focuses on recycling damaged or superfluous proteins by degrading them into amino acids for reuse. This balance is critical to cellular health; excessive protein misfolding or degradation can disrupt cellular processes, causing stress and potentially contributing to disease.

A complex network of systems ensures the maintenance of proteostasis. Molecular chaperones play a key role in recognising and refolding misfolded proteins, while proteasomes identify and degrade damaged or unnecessary proteins, facilitating amino acid recycling. Misfolded proteins are recognised by quality control systems in both the cytoplasm and the endoplasmic reticulum (ER). The ER-associated degradation (ERAD) pathway is crucial for identifying misfolded proteins, exporting them to the cytoplasm, and directing them to proteasomes for destruction (Luo et al., 2023; Mauthe et al., 2025).

When proteasomes become overwhelmed, cells initiate the process of autophagy, which engulfs and degrades large aggregates of damaged proteins or entire cellular structures. Stressors such as heat, toxins, or oxidative damage can exacerbate protein

misfolding, leading to harmful protein aggregates. These aggregates are a hallmark of neurodegenerative diseases such as Alzheimer's and Parkinson's (Mauthe et al., 2025).

The process of proteostasis is also influenced by key signalling pathways. For instance, the mTOR pathway regulates protein synthesis, while heat shock factor 1 (HSF1) modulates the expression of chaperones during periods of cellular stress. Together, these interconnected mechanisms maintain the balance of protein homeostasis, preserving cellular function and preventing the development of disease (Lazaro-Pena et al., 2022).

1.3.2 Regulation of neuronal proteostasis

Similar to protein homeostasis, neuronal homeostasis is essential for maintaining the balance and proper functioning of neurons. Neurons act as the brain and body's communication network, transmitting electrical and chemical signals to coordinate various physiological processes. To fulfil their role effectively, neurons require stable energy levels, which depend on a constant supply of glucose and oxygen. Without this steady influx, neuronal function and survival are compromised (Li et al., 2020).

Neurons communicate via neurotransmitters, which must be carefully balanced to ensure proper signalling. Excessive neurotransmitter levels can lead to overexcited neurons, while insufficient levels result in underactive or silent neurons (Mark et al., 2001). Furthermore, neurotransmitters must be recycled to avoid overstimulation or depletion, which can disrupt neural communication.

The excitatory/inhibitory (E/I) balance is another critical aspect of neuronal homeostasis. This equilibrium is primarily mediated by the neurotransmitters glutamate (excitatory) and gamma-aminobutyric acid (GABA, inhibitory). Disruptions in this balance have been implicated in neurological disorders such as epilepsy, autism, and schizophrenia (Turrigiano, 2008).

Synaptic connections between neurons, known as synapses, are vital for neural communication and adaptability. Synapses must remain strong and flexible to accommodate the transmission of new information. Neurons adjust the strength and

number of their synaptic connections to maintain efficient communication. When neurons receive excessive signals, synapses may weaken through a process called synaptic scaling, which prevents neuronal overload and maintains network stability.
Neuronal function also relies on maintaining the proper balance of ions, such as sodium, potassium, and calcium. These ions are actively pumped in and out of neurons to regulate electrical stability and signal transmission. Dysregulated calcium signalling, in particular, can lead to excitotoxicity and cell death, contributing to neurodegenerative processes (Turrigiano, 2008; Mark et al., 2001).

Mitochondria are critical for neuronal energy demands, producing adenosine triphosphate (ATP) to power essential functions like ion pumping and synaptic transmission. The maintenance of mitochondrial quality through dynamic processes such as fission, fusion, and mitophagy is crucial for neuronal survival and resilience under stress (Martinez-Vicente, 2017).

Similar to proteins, neurons utilise autophagy to clear damaged components, toxic aggregates, or dysfunctional organelles. This is especially important because neurons cannot regenerate like other cell types. Deficient autophagy can lead to the accumulation of harmful protein aggregates, impairing neuronal function and survival (Martinez-Vicente, 2017).

Supporting cells such as microglia and astrocytes play a pivotal role in neuronal homeostasis. Microglia monitor the neuronal environment, clear debris, and respond to injury or infection, while astrocytes provide metabolic support, recycle neurotransmitters, and regulate the blood-brain barrier. Dysfunction in these glial cells can severely impact neuronal health, leading to neuroinflammation and impaired synaptic function (Gao et al., 2023).

Due to their long lifespan and complex roles, neurons face significant proteostasis demands. Disruptions in neuronal proteostasis can result in the accumulation of misfolded proteins such as beta-amyloid or α-synuclein, which are hallmarks of neurodegenerative diseases like Alzheimer's and Parkinson's (Zhang et al., 2023; Martinez-Vicente, 2017). These disruptions underscore the importance of maintaining neuronal homeostasis to preserve cognitive and motor functions throughout life.

1.3.3 Dysregulation of proteostasis

Dysregulation of homeostasis often arises from the inability of the brain to adapt to stress or damage, leading to a cascade of detrimental effects. Several key issues contribute to this dysregulation.

Firstly, the loss of synaptic balance, resulting from shifts in the excitatory/inhibitory (E/I) balance in the brain, plays a critical role. Excessive excitation can overstimulate neurons, leading to neurotoxicity (Wu and Tymianski, 2018). Overactivation of glutamate receptors, such as N-methyl-D-aspartate (NMDA) receptors, results in an uncontrolled influx of calcium ions. This triggers apoptotic or necrotic pathways, mechanisms linked to conditions such as ischemic stroke and traumatic brain injuries (Hoffe and Holahan, 2022). Conversely, excessive inhibition can dampen brain activity, impairing learning, memory, and motor functions, thereby disrupting normal cognitive and physical performance (Duchon et al., 2020).

Secondly, calcium overload further exacerbates neuronal dysfunction. Dysregulated calcium ion levels overwhelm neurons, activating harmful enzymes that degrade the cell's structural integrity. This impairs synaptic plasticity, a key feature of learning and memory, and ultimately results in neuronal death. Calcium dysregulation has been implicated in numerous neurological disorders, underscoring its significance in maintaining neuronal health (Verma et al., 2022; Wu and Tymianski, 2018).

Thirdly, mitochondrial dysfunction contributes significantly to the dysregulation of homeostasis. Mitochondria, as the powerhouses of the cell, are critical for energy production. However, when mitochondrial function declines, it disrupts the balance between reactive oxygen species (ROS) production and antioxidant defences, causing oxidative stress (Choi et al., 2024). This oxidative damage impairs cellular components, accelerates ageing, and drives neurodegenerative processes. Mitochondrial failure is strongly associated with diseases such as Parkinson's disease, highlighting its central role in neuronal survival and function (Henrich et al., 2023).

Fourthly, chronic inflammation presents another major challenge. Overactivation of microglia, the brain's resident immune cells, can result in the release of toxic substances

that damage neurons. This phenomenon has been observed in conditions such as multiple sclerosis and brain injuries. The hyperactivation of both microglia and astrocytes leads to the secretion of cytokines, chemokines, and reactive nitrogen species, creating a toxic environment for neurons. Prolonged inflammation contributes to the progression of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Huntington's disease (Blusch and Björkqvist, 2025).

The consequences of dysregulated homeostasis can vary depending on the conditions and severity of the disruption. Acute outcomes may include seizures or ischemic damage following a stroke. Chronic outcomes, on the other hand, are often characterised by the gradual progression of neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Furthermore, the impact of neuronal dysregulation is not confined to the brain. Systemic effects can occur, as disrupted neuronal function can impair autonomic processes, including heart rate regulation, digestion, and immune responses (Choi et al., 2024; Henrich et al., 2023). These systemic dysregulations highlight the far-reaching implications of neuronal imbalance on overall health.

1.4 CHIP in neurodegeneration

1.4.1 CHIP in dementia

There is a scarcity of studies addressing the specific role of CHIP in dementia or individual neurodegenerative diseases. However, emerging evidence suggests that CHIP may play a critical role in the pathogenesis of certain neurodegenerative conditions. While the current body of research remains limited, a number of studies have explored the potential connections between CHIP and neurodegeneration. Below is a comprehensive summary of existing studies investigating the involvement of CHIP in neurodegenerative processes (Hayer et al., 2017).

1.4.2 Pathogenic CHIP mutations associated with disorders.

Pathogenic mutations in CHIP have been associated with several disorders, underscoring its critical role in maintaining cellular homeostasis. The most well-documented mutation is in the STUB1 gene, which is one of the genetic causes of Gordon Holmes syndrome. This rare, autosomal recessive hereditary condition combines cerebellar ataxia with hypogonadotropic hypogonadism. Patients with Gordon Holmes syndrome present with a range of neurological symptoms, including speech difficulties, impaired balance and coordination, memory loss, and dementia, reflecting the diverse functional roles of CHIP in neuronal health (Hayer et al., 2017; Sharma et al., 2025).

Another example of CHIP's involvement in neurodegenerative disease comes from a mouse model of Machado-Joseph disease, also known as spinocerebellar ataxia type 3 (SCA3). These SCA3 mice exhibit reduced levels of CHIP, which is speculated to result from an increased affinity of the mutant protein ataxin-3 for CHIP. This heightened interaction likely depletes CHIP levels throughout the brain, exacerbating disease progression and highlighting the potential role of CHIP dysfunction in ataxia pathogenesis (Durcan and Fon, 2013).

On top of neurodegenerative diseases, mutations in STUB1 have also been implicated in intracranial aneurysm (IA). In this context, CHIP mutations have been shown to drive vascular remodelling, a hallmark feature of the disorder. This further illustrates the wide-ranging impact of CHIP dysregulation on both neural and vascular systems (Su et al., 2013).

Collectively, these findings demonstrate the diverse roles of CHIP mutations in human disease, from neurodegenerative conditions like Gordon Holmes syndrome and Machado-Joseph disease to vascular disorders such as intracranial aneurysm, emphasising its importance as a target for further research.

1.4.3 CHIP in spinocerebellar ataxia

CHIP is encoded by the STUB1 gene (STIP1 homology and U-box containing protein 1). Recessive mutations in STUB1 have been linked to spinocerebellar ataxia, autosomal

recessive 16 (SCAR16) in certain families, as demonstrated in previous studies. To date, the Human Gene Mutation Database has identified 19 pathogenic STUB1 variants associated with SCAR16. Despite this progress, the effects of these variants on the protein folding, structural stability, and functional properties of CHIP remain poorly understood (Pakdaman et al., 2017).

Six specific STUB1 variants, E28K, N65S, K145Q, M211I, S236T, and T246M, have been identified as pathogenic in individuals with SCAR16. Research has shown that some of these mutations can disrupt protein structure and stability, as well as impair CHIP's ability to dimerize in vitro. It is hypothesised that certain STUB1 mutations mediate disease by interfering with CHIP's E3 ubiquitin ligase function and its interactions with substrates. These mutations are believed to alter CHIP's oligomeric states and compromise its structural integrity, leading to a failure in protein quality control.

As a result, the cellular machinery is unable to efficiently eliminate damaged or misfolded proteins, causing their accumulation and subsequent toxicity. This protein aggregation ultimately triggers cellular dysfunction and cell death, contributing to the pathogenesis of SCAR16 (Hayer et al., 2017; Pakdaman et al., 2017; Sharma et al., 2025). These findings underscore the need for further investigation into the molecular mechanisms underlying CHIP mutations and their impact on protein homeostasis and neuronal survival.

1.4.4 CHIP regulation of PINK!

Mutations in the gene encoding the serine/threonine protein kinase PTEN-induced putative kinase 1 (PINK1) represent the second most common cause of autosomal recessive Parkinson's disease. PINK1 is critical for neuronal cell survival and mitochondrial quality control, primarily through its kinase activity. Ubiquitin E3 ligase CHIP has been shown to promote the ubiquitination of PINK1, leading to a reduction in its steady-state levels (Yoo and Chung, 2018). This regulatory interaction has raised the question of whether the CHIP-mediated pathway contributes to the pathogenesis of Parkinson's disease.

CHIP catalyses the Hsp70-dependent ubiquitination of substrate proteins, acting as a central regulator of protein fate. Within this system, Hsp70 and Hsp90 play opposing roles in determining the outcomes for their substrate proteins. A target protein bound to Hsp70 interacts with CHIP and is directed towards ubiquitin/proteasome-dependent degradation. In contrast, when a target protein forms a complex with Hsp90, it binds to co-chaperones such as p23, leading to protein refolding instead of degradation.

Inhibition of Hsp90 by compounds such as geldanamycin (GA) or 17-allylamino-17-demethoxygeldanamycin (17-AAG) increases the cellular concentration of the Hsp70-substrate complex. This results in the rapid degradation of target proteins, including PINK1, through the proteasome pathway. Studies have shown that treatment with GA or 17-AAG accelerates the proteasomal degradation of PINK1, highlighting the significance of this pathway in regulating its stability (Quintana-Gallardo et al., 2019).

The BAG family of proteins, which contain the BAG domain, also function as co-chaperones of Hsp70, influencing the Hsp70/CHIP-mediated substrate degradation pathway (Qu et al., 2015). Specifically, BAG2 and BAG5 act as inhibitors within this pathway and prevent the ubiquitin/proteasome-dependent degradation of PINK1. These findings further emphasise the complexity of the regulatory network involving CHIP and its role in modulating PINK1 stability.

Collectively, these studies suggest that CHIP functions as a key regulator of PINK1, with significant implications for the understanding of Parkinson's disease pathogenesis (Wang et al., 2014). By influencing PINK1 degradation, CHIP and its associated pathways may represent potential therapeutic targets for mitigating mitochondrial dysfunction and neuronal degeneration in Parkinson's disease.

1.4.5 CHIP in other neurodegenerative diseases.

CHIP has been loosely associated with several neurodegenerative diseases, particularly those characterised by uncontrolled protein misfolding and the formation of toxic protein aggregates. CHIP plays a crucial role in the degradation of key proteins implicated in neurodegeneration, such as tau and amyloid precursor protein in Alzheimer's disease, **α**-synuclein and leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease, and

superoxide dismutase 1 (SOD1) in amyotrophic lateral sclerosis (ALS) (Palubinsky et al., 2015).

Interestingly, emerging evidence suggests that CHIP may also contribute to the transmission of toxic signals in neurodegenerative conditions (Quintana-Gallardo et al., 2019). For instance, CHIP has been identified as a component of Lewy bodies in the brains of Parkinson's disease patients, where it colocalises with α-synuclein and Hsp70. In cell culture models, endogenous CHIP has similarly been shown to colocalise with α-synuclein and Hsp70 within intracellular inclusions. Besides, studies have observed that in certain neurodegenerative diseases, inclusions formed in affected neurons contain CHIP along with ubiquitin, Hsp70, E1 and E2 ubiquitinating enzymes, and proteasomes (Shin et al., 2005).

These findings suggest that CHIP-containing inclusions may exert neurotoxic effects similar to those of other Lewy body components, such as α-synuclein, during the progression of neurodegenerative diseases. Conversely, the formation of CHIP-containing inclusions could also indicate a disruption of its neuroprotective functions. It is hypothesised that if the ubiquitin-proteasome system remains at least partially functional within these inclusions, albeit at a reduced rate, some degree of turnover of toxic proteins could occur. This may lead to a stabilisation of pre-existing toxicity over time as the generation of oligomers and aggregates is mitigated (McNaught et al., 2002). However, this remains speculative, and further research is required to determine whether CHIP-containing inclusions are primarily neurotoxic or represent a compensatory mechanism aimed at preserving neuronal function.

1.4.6 Neuroprotective role of CHIP

CHIP is well established for its role in cancer biology, but its implications in neurodegenerative diseases remain poorly understood. During preliminary research on CHIP's role in cancer, I encountered evidence suggesting its involvement in ataxia. Ataxia is a neurological disorder characterised by impaired coordination of voluntary muscle movements. This condition arises from dysfunction in the cerebellum, its associated neural circuits, or sensory pathways involved in proprioception. The cerebellum plays a

critical role in integrating sensory feedback with motor output to maintain balance, posture, and voluntary movement. Dysfunction in this region results in symptoms such as dysmetria, defined as inaccurate movement amplitude, and dysdiadochokinesia, the inability to perform rapid, alternating movements. Damage to Purkinje cells, granule cells, or other cerebellar neurons disrupts the fine-tuning of motor signals, leading to uncoordinated and imprecise movements. Furthermore, dysregulation of GABAergic, glutamatergic, or dopaminergic signalling within cerebellar or associated circuits exacerbates ataxic symptoms by impairing synaptic plasticity and neuronal communication. These findings led me to suspect that CHIP might have a significant connection to neuronal health and function (Dias et al., 2021; Hu et al., 2021; Pakdaman et al., 2017; Schuster et al., 2020).

Supporting evidence for this hypothesis emerged from a study reporting that CHIP-deficient mice exhibit cardiovascular stress and motor dysfunction prior to premature death. This study proposed that cellular and biochemical events in CHIP knockout (KO) mice contribute to neurodegeneration and premature ageing, although the precise mechanisms remain unclear. Observations from electron and fluorescent microscopy revealed that CHIP deficiency is associated with an increased number of mitochondria; however, these mitochondria appeared swollen and misshapen. This mitochondrial dysfunction is accompanied by acute bioenergetic stress, which induces CHIP expression and its relocalisation to mitochondria. CHIP's role in clearing damaged organelles was shown to be essential for protecting neurons following low-level bioenergetic stress (Schuster et al., 2020).

Further insights into CHIP's role in neuronal processes were provided by a study demonstrating that USP47 and the C-terminus of CHIP antagonistically regulate the stability of katanin-p60, a microtubule-severing enzyme critical for axonal growth. Katanin disassembles microtubules, a process essential for proper axonal development and repair. Another study highlighted CHIP's involvement in mitochondrial quality control through its regulation of PTEN-induced putative kinase 1 (PINK1). CHIP promotes the ubiquitination of PINK1, reducing its steady-state levels. As PINK1 is vital for neuronal survival and mitochondrial maintenance, its dysregulation has been linked to autosomal recessive Parkinson's disease, where PINK1 mutations are a common cause (Palubinsky et al., 2015; Yoo and Chung, 2018).

These findings collectively suggest that CHIP plays a neuroprotective role, particularly in maintaining mitochondrial function, regulating neuronal survival, and supporting axonal growth. Inspired by this evidence, I decided to focus my research on further elucidating the role of CHIP in neuronal health and its potential implications in neurodegenerative diseases.

1.5 CHIP in cancer

1.5.1 Dual role of CHIP in cancer.

CHIP plays a multifaceted role in cancer biology, exhibiting both tumour-suppressive and oncogenic properties depending on the cellular context.
The tumour-suppressive functions of CHIP are primarily mediated by its E3 ubiquitin ligase activity, which targets several oncogenic proteins for proteasomal degradation. By regulating protein turnover, CHIP helps to maintain cellular homeostasis and prevent uncontrolled cell proliferation, a key hallmark of cancer (Kumar et al., 2022).

Conversely, CHIP can exhibit oncogenic properties under certain conditions. Overexpression of CHIP has been linked to the degradation of tumour suppressor proteins, which disrupts cellular homeostasis and facilitates enhanced cell survival and proliferation. This aberrant activity suggests that CHIP's role in cancer is highly context-dependent (Cao et al., 2016). Furthermore, CHIP's interactions with specific signalling pathways can contribute to tumourigenesis, particularly when these pathways are dysregulated. Such findings highlight the dualistic nature of CHIP, acting as either a tumour suppressor or an oncogene depending on the cellular and molecular context (Dai et al., 2019).

1.5.2 CHIP in breast cancer.

In the context of breast cancer, the most common malignancy among women globally, CHIP has been shown to regulate tumour cell growth via the ubiquitination-proteasome pathway. Elevated CHIP expression has been associated with favourable prognostic markers, including tumour grade and tumour-node-metastasis (TNM) stage, and has

been identified as a significant predictor of overall survival over a ten-year follow-up period in patients with breast cancer.

A critical driver of breast cancer progression is oestrogen receptor alpha (ERα), and CHIP has been implicated in the regulation of its degradation. As an E3 ligase, CHIP mediates geldanamycin (GA)-induced degradation of ERα. Furthermore, in MCF-7 breast cancer cells, CHIP facilitates the ubiquitination and proteasomal degradation of ERα in response to suberoylanilide hydroxamic acid (SAHA). Overexpression of CHIP in the presence of SAHA enhances ERα ubiquitination, leading to an additive reduction in ERα levels and decreased transcriptional activity (Mokbel et al., 2010).

The receptor tyrosine kinase ErbB2, overexpressed in approximately 30% of primary breast cancers, is associated with aggressive tumour behaviour, poor prognosis, and chemotherapy resistance. CHIP has been shown to mediate ErbB2 degradation in breast cancer cell lines in response to the flavonoid quercetin, highlighting its potential as a therapeutic target in ErbB2-positive breast cancer (Jeong et al., 2008).

Besides its role in degrading oncogenic proteins, CHIP influences signalling pathways involved in breast cancer progression. Nuclear factor-κB (NF-κB) is a transcription factor that promotes cancer cell invasion and metastasis. Its activation is regulated by the tumour necrosis factor receptor-associated factor (TRAF) protein family. TRAF2, in particular, serves as an E3 ubiquitin ligase, mediating lysine-63 (K63)-linked ubiquitination of target proteins required for NF-κB activation. CHIP has been shown to interact with TRAF2 and mediate its degradation in breast cancer cells. Suppression of CHIP expression using siRNA in MCF-7 cells increases TRAF2 levels, which correlates with enhanced invasiveness of these cancer cells (Jang et al., 2011).

Collectively, these findings underscore CHIP's complex role in breast cancer biology. Its tumour-suppressive functions, mediated through the degradation of key oncogenic proteins such as ERα and ErbB2, are complemented by its ability to regulate critical signalling pathways, such as NF-κB activation via TRAF2 degradation. These mechanisms highlight CHIP's dual role in breast cancer and its potential as a therapeutic target in the management of this disease.

1.5.3 CHIP in gastric cancer.

Gastric cancer ranks as the fourth most common malignancy globally and is the second leading cause of cancer-related mortality worldwide (Liu et al., 2015). The expression of CHIP has been inversely correlated with the aggressiveness of gastric cancer. Decreased CHIP expression has been associated with a clinically aggressive phenotype, and it is almost undetectable in patients with highly lymph node-invasive gastric cancer (Dai et al., 2019). These observations suggest that CHIP expression may serve as an important prognostic indicator for gastric cancer, providing valuable insights into disease progression and potential therapeutic targets.

Angiogenesis, the formation of new blood vessels, is a critical factor in tumour growth and metastasis and is considered a key predictor of survival in gastric cancer patients. Reduced CHIP levels have been linked to enhanced tumour angiogenesis, which drives tumour invasion and metastasis, ultimately leading to a worse prognosis for gastric cancer patients. CHIP inhibits angiogenesis by interacting with the NF-κB/p65 signalling pathway, promoting the ubiquitination and proteasomal degradation of NF-κB/p65, thereby terminating NF-κB activity (Liu et al., 2015).

NF-κB is a pivotal transcription factor in cancer biology, regulating a wide range of cellular processes, including cell survival, proliferation, angiogenesis, and metastasis. While NF-κB activity is essential for normal cellular functions, its excessive and prolonged activation contributes to chronic inflammation and carcinogenesis (Mao et al., 2025). The p65 protein, a subunit of the NF-κB family, plays a critical role in this pathway. CHIP has been shown to specifically interact with p65 in the nucleus, targeting it for degradation and, in turn, terminating NF-κB activation. Notably, CHIP does not interact with p65 in the cytoplasm, highlighting a nucleus-specific regulatory mechanism (Cao et al., 2016).

These findings underscore the importance of CHIP as a negative regulator of NF-κB activity in gastric cancer. By modulating the degradation of NF-κB/p65, CHIP inhibits angiogenesis and tumour progression, suggesting its potential as both a prognostic biomarker and a therapeutic target in the management of gastric cancer.

1.5.4 CHIP in prostate cancer

Prostate cancer is the second leading cause of cancer-related mortality among men in the United States. Signalling through the androgen receptor (AR) is considered a pivotal driver of prostate cancer progression. AR is expressed in all forms of prostate cancer in humans, and androgen-AR signalling is essential for prostate cancer cell proliferation and maintenance. Consequently, reducing AR levels has emerged as a promising therapeutic strategy for managing the disease (Rawat et al., 2023).

Studies have demonstrated that AR is degraded through the activation of CHIP, highlighting its potential as a regulatory mechanism in prostate cancer treatment. Further research has revealed that AR degradation requires the association of CHIP with heat shock protein 70 (HSP70). Specifically, silencing HSP70 partially inhibits AR degradation, indicating its critical role in the process. In contrast, silencing heat shock protein 90 (HSP90) has been shown to have no significant effect on AR degradation, underscoring the specificity of the HSP70-CHIP interaction in this pathway.

Given its role in promoting AR degradation, activating E3 ubiquitin ligases such as CHIP represents a promising therapeutic approach for prostate cancer. Targeting this pathway offers the potential to disrupt AR signalling, thereby inhibiting tumour growth and progression (Cao et al., 2016). These findings suggest that CHIP-mediated pathways warrant further investigation as a novel strategy for prostate cancer treatment.

1.5.5 CHIP and glioma.

Malignant gliomas are among the most common and aggressive brain tumours, characterised by their highly invasive nature. The prognosis for patients remains poor, with an average survival time of 2–5 years for anaplastic gliomas and 12–15 months for glioblastomas, even with standard treatment protocols. Emerging evidence suggests that CHIP may play a significant role in the tumourigenesis of human gliomas, as demonstrated through both in vitro and in vivo studies.

Increased CHIP expression has been correlated with the histological grade of gliomas, indicating a potential role in tumour progression. Experimental findings reveal that CHIP

overexpression enhances proliferation and colony formation in glioma cell lines such as U251 and U87 in vitro. Furthermore, in xenograft models, tumour growth was significantly inhibited following intratumoral injection of lentivirus containing CHIP shRNA, which reduces CHIP expression. In contrast, an enhancement in tumour growth was observed after injections of lentivirus designed to overexpress CHIP (Xu et al., 2011).

It is noteworthy that these results contrast sharply with those observed in breast cancer, where CHIP typically exerts a tumour-suppressive role. These opposing effects highlight the complexity of CHIP's function, which appears to be highly context-dependent and influenced by tumour type. Further research is required to elucidate the molecular mechanisms underlying CHIP's dual role in tumour biology and to explore its potential as a therapeutic target in gliomas (Cao et al., 2016).

1.5.6 CHIP and gallbladder carcinoma.

Gallbladder carcinoma is a highly lethal disease, primarily due to its late detection and diagnosis at advanced stages when treatment options are limited. Although certain molecular biomarkers, such as cyclooxygenase-2 (COX-2) and hypoxia-inducible factor (HIF), have been identified, they are not specifically or consistently associated with gallbladder carcinoma, limiting their diagnostic and prognostic utility.

To explore potential biomarkers, tumour specimens from patients who underwent surgical treatment for gallbladder carcinoma were analysed for CHIP expression. The findings revealed an association between CHIP expression levels and certain clinicopathological features, as well as overall patient survival. Specifically, patients with high CHIP expression exhibited significantly better cancer-specific survival compared to those in the low-expression group. Interestingly, no correlation was observed between CHIP expression and other clinicopathological prognostic parameters, suggesting that CHIP may function as an independent prognostic marker (Cao et al., 2016).

It is important to acknowledge that the study was limited by its small sample size and retrospective design, which may have constrained the generalisability of the findings. Further large-scale, prospective studies are required to confirm the clinical relevance of

CHIP in gallbladder carcinoma and to fully elucidate its role in tumour progression and patient outcomes.

1.6 Objectives of this thesis

The primary objective of this thesis is to elucidate the role of CHIP in the pathways underlying neurodegenerative diseases and cancer. Current evidence strongly supports the notion that CHIP plays a critical role in the pathogenesis of both conditions. This research aims to investigate CHIP's function in neuronal growth and degeneration, providing new insights into the molecular mechanisms driving these poorly understood diseases.

A deeper understanding of CHIP's involvement could have significant implications, particularly in the context of neurodegeneration. Given its apparent importance, CHIP may serve as a potential biomarker for both neurodegenerative diseases and cancer. This is especially crucial for neurodegenerative conditions, as there are currently no biomarkers available that enable the detection of disease at an early stage or even before the onset of clinical symptoms. Identifying CHIP as a biomarker could represent a transformative step forward in the study of dementia and other neurodegenerative diseases, offering the possibility of developing screening tools for early detection. Such advancements would not only improve our understanding of these conditions but could also provide hope for earlier interventions and better patient outcomes.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals and solvents were obtained from commercial sources. All of them are analytical reagent or molecular biology grade. All the buffers had been prepared with deionized water.

2.1.2 Buffers

Laemmli Buffer 5x 0.5 M Tris-HCl pH 6.8 1.75 mL, Glycerol 4.5mL, SDS (0.25g dissolved in 1 mL of Tris-HCl) 2 mL, Bromophenol Blue (25 mg in 10 mL H_2O), β-mercaptoethanol 1.25 mL

Lysis Buffer 0.1 M Tris/HCl pH 8.5

Non-fat dry milk (NFDM) blocking buffer 5% solution containing 2.5 g of NFDM and 50 mL of TBST.

Phosphate-buffered saline (PBS) 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4

Phosphate-buffered saline with Tween® (PBST) 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, Tween® 20 detergent: 0.1%, pH 7.4

Running Buffer

Transfer Buffer x 10 30g Tris Base, 144g glycine, 1000 mL high grade H_2

2.1.3 Cell lines

Table 2.1 Cell lines and culturing conditions

Cell line	**Edited gene**	**Description**	**Cell culture environment**
SH-SY5Y	CRISPR control (CHIP WT)	Human neuroblastoma cell line	DMEM (Gibco™ 11965084), 10% fetal bovine serum (Gibco™ A3840101), 37° C, 5% CO2, humidified atmosphere
	CHIP KO		
iPSC	Unedited	iPSC cell line derived from Parkinson Disease Female Patient	
		iPSC cell line derived from Parkinson Disease Male Patient	
		iPSC cell line derived from Huntington Disease Female Patient	
		iPSC cell line derived from Huntington Disease Male Patient	

2.1.4 Antibodies

Table 2.2 Primary Antibodies

Target	Class	Name/ Reference	Source
CHIP	Mouse mAb	3.1	Gift by K. Ball
HLA-A	Rabbit mAb	TA421517	OriGene
HLA-B	Rabbit pAb	ab193415	Abcam
HLA-C	Rabbit mAb	ab126722	Abcam
VGF	Rabbit pAb	MBS151354	My Bio Source
INA	Rabbit mAb	STJ11101907	St John's Laboratory
NEFL	Mouse mAb	MA1-2010	Invitrogen
ELAVL3	Mouse mAb	CBFYH-0148	Creative Biolabs
NEFM	Mouse mAb	RMO-44	Invitrogen
NPY	Rabbit mAb	11976S	Lab Jot

IFNγ	Mouse mAb	PHC4031	Gibco
HMGA1	Rabbit mAb	A4343	Abclonal
β-actin	Mouse mAb	A5441	Sigma
DPYL2	Mouse mAb	H00001808-M01	Thermofisher
NQO1	Mouse mAb	sc-376023	Santa Cruz

Table 2.3 Secondary Antibodies

Target	Class	Name/Reference	Source
Rabbit	Goat pAb	A32731	Invitrogen
Mouse	Goat pAb	A-21422	Invitrogen

2.2 Biochemical techniques

2.2.1 Protein quantification

Implen N60/N50 Spectrophotometer was used to quantify protein concentration and assess the purity of samples before they were prepared for MS-based assays. Concentration was calculated from the absorbance at 280 nm.

2.2.2 SDS-PAGE

a. In-house polyacrylamide gels

Polyacrylamide gels were prepared using the Mini-Protean 3 Cell gel casting system (Bio-Rad) with either 1.0 mm or 1.5 mm spacer glass plates, depending on the required sample volume. The selection of combs, either 10-well or 15-well, was based on the specific assay requirements and the number of samples to be analysed. Stacking and resolving gel solutions were prepared as outlined in Table 2.4, with higher percentage resolving gels employed for the separation of lower molecular weight proteins. The resolving gel was poured first and overlaid with isopropanol to ensure uniform polymerisation and prevent the formation of air bubbles. Once polymerisation was complete, the isopropanol was carefully removed, and the stacking gel was subsequently prepared and poured into the casting assembly.

Table 2.4 Resolving and stacking gels for SDS-PAGE.

Reagents	**Resolving gel (15 mL)**			**Stacking gel (3 mL)**
	10%	12%	15%	5%
H2O	5.9 mL	4.9 mL	3.4 mL	2.1 mL
30% acrylamide mix	5.0 mL	6.0 mL	7.5 mL	0.5 mL
1.5M Tris (pH 8.8)	3.8 mL	3.8 mL	3.8 mL	-
1.5 Tris (pH 6.8)	-	-	-	0.38 mL
10% SDS	0.15 mL	0.15 mL	0.15 mL	0.03 mL
10% ammonium persulfate	0.15 mL	0.15 mL	0.15 mL	0.03 mL
TEMED	0.006 mL	0.006 mL	0.006 mL	0.002 mL

Each time around 30 µl of cell lysate was loaded onto the gel. The proteins were diluted in Laemmli Buffer 5x. The mix was heated for 10 min at 96.C (unless stated otherwise). Samples were loaded onto the gel and pre-stained protein ladders were loaded as size markers. Gels were run in 1x Tris-Glycine-SDS running buffer and at 90 V for about 50 min then 120 V for another 1h, until the dye has reached the bottom of the gel.

Table 2.5 Laemmli Buffer 5x (10 mL)

Reagent	**Volume**
0.5M Tris-HCl (pH 6.8)	1.75 mL
Glycerol	4.50 mL
SDS (0.25 g dissolved in 1 mL of Tris HCl)	2 mL
Bromophenol Blue (25 mg in 10 mL H_2O)	0.5 mL

β-mercaptoethanol	1.15 mL

Table 2.6 Laemmli Buffer 1x (2.5 mL)

Reagent	**Volume**
Laemmli Buffer 5x	0.5 mL
RIPA	2 mL

Table 2.7 Preparation of pellets for SDS-PAGE

Reagent	**Volume (per pellet)**
Laemmli Buffer 5x	100 µl
RIPA	400 µl
β-mercaptoethanol	5 µl

Table 2.8 Tris-Glycine-SDS running buffer

Reagents	**Concentration**
Glycine	192 mM
Tris base	25 mM
SDS	0.1 %

2.2.3 Western Blotting (WB)

Following the completion of SDS-PAGE, proteins were transferred onto a nitrocellulose blotting membrane (Amersham Protran®) using the Mini Trans-Blot® wet electroblotting system (Bio-Rad). The transfer was performed in tanks filled with a 1× transfer buffer, either for 1 hour and 30 minutes at 300 mA or overnight at 30 mA. To prevent overheating, overnight transfers were conducted at 4°C.

Membranes were subsequently blocked in a solution of 5% non-fat milk in Tris-buffered saline at room temperature for 30 minutes. Following blocking, they were washed three times in phosphate-buffered saline (PBS), each wash lasting 5 minutes, while continuously agitated on a nutating mixer (Fisher Scientific). The membranes were then incubated in PBS-Tween solution (0.1% (v/v) Tween-20) containing 5% bovine serum albumin (BSA, Sigma, A9647) for 1 hour at room temperature to reduce non-specific binding.

For protein detection, membranes were incubated overnight at 4°C with the appropriate primary antibodies diluted in blocking buffer. The following day, they were washed three times in PBS-Tween solution for 5 minutes each and subsequently incubated for 1 hour with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch) at a 1:3000 dilution. After a final series of washes in PBS-Tween solution, protein bands were visualised using a chemiluminescent substrate (Clarity Max™ Western ECL Substrate, Bio-Rad).

Table 2.9 Transfer Buffer (1x)

Reagents	**Concentration**
Tris	24 mM
Glycine	191 mM
Methanol	20% (v/v)

Table 2.10 PBS-Tween

Reagents	**Concentration**
1x PBS	
Tween-20	0.1 % (v/v)

Table 2.11 Blocking Buffer

Reagents	**Concentration**
PBS (1x)	
Tween-20	0.1% (v/v) Tween-20
Dried skimmed milk	5% (w/v)

2.3 Cell culture

2.3.1 Culturing of cell lines

All tissue culture disposables were sourced from VWR unless specified otherwise. Cells were routinely monitored every two days using a bright-field inverted phase-contrast microscope to assess their morphology and overall health. To ensure contamination-free conditions, periodic mycoplasma testing was conducted using the luciferase-based MycoAlert™ detection kit (Lonza), following the manufacturer's instructions. All tested cultures were confirmed to be mycoplasma negative.

a. SH-SY5Y

SH-SY5Y cells were maintained in an appropriate culture medium, which was replaced every 2–3 days or as required. Once the cells reached approximately 75% confluency, the culture medium was discarded, and the cells were washed three times with phosphate-buffered saline (PBS) to remove residual serum and debris.

For passaging, dry trypsinisation was performed. Following the final PBS wash, 0.05% trypsin with 0.02% EDTA (Gibco, 15090) was added to the cells, evenly distributed across the surface, and immediately removed. The cells were then incubated for approximately 3 minutes to facilitate detachment. To neutralise any remaining trypsin, fresh culture medium was added before cell resuspension. Cells were subsequently either plated at a 1:10 ratio for routine culturing or counted and seeded at a specific density as required for individual assays.

b. iPSC

The cells were seeded on coverslips on 6 well plates. Sterile glass coverslips were coated with Poly-L-ornithine. The 0.01% poly-L-ornithine solution was prepared by diluting it in dPBS. Then, 500 µl - 1000 µl were added per well and incubated 1 hour at room

temperature. Each well was washed 3 times with sterile dPBS to remove excess poly-l-ornithine. Prior to seeding cells, in order to promote attachment and survival, plates were coated with Laminin-521 (100 µg/mL, Biolamina). The 5 µg/mL Laminin-521 solution was prepared by diluting it in dPBS with calcium and magnesium (14040083, Gibco). Each time around 1500–2000 µl of medium was added to each well (12 well plate). Plates were covered with parafilm to prevent media evaporation and incubated overnight at 4°C.

When culturing Axol iPSC-derived neural stem cells, coverslips were coated using SureBond-XF (ax0053, Axol Bioscience). Sterile coverslips were coated with 300–500 µL of SureBond-XF per well to completely cover the coverslip. The plate with coverslips was then incubated at 37°C for at least 1 hour. The SureBond-XF was removed just before seeding the plates.

iPSC were cultured in Neural Plating - XF Medium (ax0033, Axol Bioscience) immediately after thawing to support cell attachment and initial recovery. During short-term expansion, they were cultured in Neural Expansion- XF Medium (ax0030–500, Axol Bioscience). The medium was changed daily or every two days. Once cells would reach 75% confluency or when presented early morphological signs of differentiation. iPSC were passaged by rinsing them with 1 mL of dPBS and adding 500 µL of Unlock-XF per well. They were then incubated for 3 minutes at 37°C. Once loosened, they were gently transferred to 15 mL conical tube. Next, 1 mL of Neural Expansion-XF Medium with EGF and FGF2 was added to neutralise Unlock-XF and the cells were washed. After washing the cells, they were resuspended in fresh Neural Expansion–XF Medium + EGF (20 ng/mL) + FGF2 (20 ng/mL). The cells were counted, in order to maintain desired confluency and split if necessary. Plates were covered in parafilm and kept in the incubator at 37°C, 5% CO_2. The medium was changed every other day with fresh Neural Expansion–XF Medium and growth factors.

2.3.2 Cryopreservation

For long-term storage, cell stocks were frozen in a suitable cryoprotective mixture and initially held at −80°C for 24 hours before being transferred into liquid nitrogen for

continued storage. For those cell stocks intended for short-term use, they were suspended in a cryogenic solution and temporarily stored at −80°C.

a. SH-SY5Y

To prepare the cryopreservation solution, a mixture comprising 50% foetal bovine serum (FBS; 10270–106, Gibco), 10% dimethyl sulfoxide (DMSO; 276855, Sigma), and 40% Dulbecco's Modified Eagle Medium (DMEM) was used. SH-SY5Y cells were cryopreserved upon reaching 75–80% confluency in a 10 cm culture dish or equivalent vessel. Cells were detached using dry trypsinisation, as previously described, and resuspended in fresh culture medium. Following gentle centrifugation, the supernatant was discarded, and the pellet was resuspended in 1.8 mL of the cryopreservation solution. The cell suspension was then aliquoted into cryovials and immediately placed in freezing conditions for storage.

b. iPSC

To enhance cell viability, the culturing medium (iPS-Brew XF, StemCell Technologies) was supplemented with the ROCK inhibitor Y-27632 dichloride, in accordance with the manufacturer's recommendations. Induced pluripotent stem cells (iPSCs) were cryopreserved upon reaching 75–85% confluency in a well of a 6-well plate. Cells were detached using the previously described method and transferred to medium containing Y-27632 dichloride. Following gentle centrifugation (3 minutes at 800 rpm), the supernatant was carefully removed. The resulting cell pellet was resuspended in 1.5 mL of Stem Cell Banker (GMP grade, 11890, AMSBIO). The suspension was then aliquoted into cryovials and placed in freezing conditions.

2.3.3 Cell recovery

a. SH-SY5Y

To revive cryopreserved cells, cryovials were gently thawed in a 37 °C water bath until partially liquefied. Immediately thereafter, pre-warmed culture medium (DMEM supplemented with FBS) was added to dilute the cell suspension, which was subsequently transferred to a 15 mL conical centrifuge tube. An additional 9 mL of medium was included to ensure effective dilution and removal of residual DMSO. Cells were centrifuged at 1000 rpm for 5 minutes, and the supernatant was carefully discarded. The resulting pellet was resuspended in fresh culture medium and transferred into T25 cell culture flasks for recovery and further propagation.

b. iPSC

To revive iPSCs, cryovials were gently thawed in a 37 °C water bath for approximately one minute, just until partial thawing occurred. Subsequently, 8 mL of pre-warmed iPS-Brew XF medium supplemented with 10 µM Y27632 dichloride was added. The cell suspension was centrifuged at 800 rpm for 3 minutes to remove residual cryoprotectant. After discarding the supernatant, the pellet was gently resuspended in fresh supplemented medium and plated onto 6-well culture plates for recovery and expansion.

2.3.4 Cell counting

To ensure consistency across the experiments and to allow comparison between different conditions during them, cell plates were seeded with the same number of cells. To do so, the adherent live cells were detached and an aliquot was prepared. It was then triturated to obtain single cells. They were counted using a haemocytometer or an automated cell counter. To count the cells with automated cell counter, 15 µl aliquot of cells was diluted 1:1 with 0.4% Trypan Blue stain (TC20, BioRad) and added to a cell counting slide (TC20, BioRad). This was then analysed in an automated cell counter.

2.3.5 Coverslip preparation

a. Sterilisation

Prior to use in immunofluorescence-based assays, round glass coverslips measuring either 13 mm (631–0149, VWR International) or 25 mm (631–0171, VWR International) in diameter were subjected to a sterilisation protocol. Coverslips were first washed with 70% ethanol and subsequently autoclaved at 180 °C for 30 minutes to ensure aseptic conditions.

2.4 Cell-based assays

2.4.1 Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing technology.

CRISPR/Cas9-mediated gene editing was initially planned to generate CHIP-deficient neuronal models derived from human induced pluripotent stem cells (iPSCs). The experimental strategy involved nucleofection of iPSCs with a plasmid encoding Cas9 and a guide RNA (gRNA) targeting the CHIP coding sequence. In selected conditions, this plasmid was to be co-transfected with a GFP-expressing vector, thereby allowing for the enrichment of edited cells via puromycin-based selection. For the transfection procedure, the Human Stem Cell Nucleofector™ system (Lonza) was chosen due to its efficiency in delivering nucleic acids to pluripotent cells. iPSCs were to be seeded at low density onto culture plates pre-coated with recombinant human Laminin-521, a chemically defined, xeno-free extracellular matrix protein known to enhance cell survival and promote self-renewal following single-cell dissociation.

Post-nucleofection, early survival was to be supported by the addition of the ROCK inhibitor Y-27632. Antibiotic selection would be applied to enrich for GFP-positive cells, followed by manual clonal isolation. Individual colonies were to be picked into 96-well plates, expanded progressively, and subjected to multi-tiered validation for CHIP knockout. This would include immunofluorescence staining, PCR amplification followed by direct Sanger sequencing, and Western blotting to assess CHIP protein expression. In

parallel, all validated clones were to undergo routine mycoplasma testing and be cryopreserved for downstream applications.

In practice, despite initial efforts to establish iPSC cultures suitable for this protocol, the received iPSC line was of suboptimal quality for consistent and reproducible gene editing. The colonies failed to expand adequately, and subsequent steps, such as antibiotic selection, clonal picking, and validation, could not be implemented. Consequently, this portion of the project could not be completed as originally designed. The research focus was therefore redirected toward alternative cellular models, including differentiated SH-SY5Y cells and additional neuronal assay systems, to investigate CHIP's role in proteostasis and neuronal integrity. Although ultimately unsuccessful, this experimental design is included for transparency and completeness, as it exemplifies the intended approach and informs the methodological direction of subsequent work.

2.4.3 Fixing cells

Prior to fixation, cells were washed with phosphate-buffered saline (PBS), irrespective of the fixative employed. An appropriate volume of fixative was added to ensure the coverslip was fully submerged during the procedure.

a. 4% PFA

The 16% paraformaldehyde (PFA) stock solution was prepared in-house as follows. 400 mL of double-distilled water (ddH_2O) were heated to 60 °C. Paraformaldehyde powder (160 g per 1 L of final volume) was gradually added while stirring. A few drops of 2 N NaOH were added until the solution became clear. Subsequently, 100 mL of 10× PBS were added, and the final volume was adjusted to 1 L with ddH_2O. The pH was adjusted to 7.2–7.4 using hydrochloric acid (HCl), and the solution was sterile-filtered through a 0.22 µm membrane (Stericup Durapore 0.22 µm PVDF, Millipore, 5643). The solution was aliquoted and stored at −20 °C for up to six months.

When preparing the 4% working PFA solution, the required volume was diluted from the 16% stock using pre-warmed ddH_2O (60 °C). Additional drops of 2 N NaOH were added as

necessary until the solution turned fully clear. PBS tablets (Sigma) were dissolved in ddH_2O to produce a 1× PBS solution. The pH was then adjusted to 7.2 using HCl, and the solution was again sterile-filtered using a Stericup Durapore 0.22 µm PVDF filter (Millipore). Aliquots were stored at −20 °C until use.

For fixation, coverslips were incubated with 4% PFA for 15 minutes at room temperature, protected from light. After fixation, coverslips were washed three times with PBS (5 minutes each wash) using a Thermo Scientific Digital Rocker to ensure gentle agitation.

2.4.4 Immunofluorescence (IF)

To prepare cells for immunofluorescence (IF) analysis, cells were seeded at low density onto sterile 15 mm glass coverslips placed in 12-well plates. Following incubation for a minimum of 24 hours, and once the cultures reached approximately 50% confluency, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Coverslips were then rinsed three times with phosphate-buffered saline (PBS), followed by a 5-minute permeabilisation step using 0.2% Triton X-100 in PBS. After a further three PBS washes, the cells were blocked overnight at 4 °C in PBS containing 5% bovine serum albumin (BSA) with gentle agitation on a Thermo Scientific Digital Rocker. This blocking step was performed to minimise non-specific binding of fluorophore-conjugated secondary antibodies.

The following day, cells were incubated at room temperature for 2 hours with the desired primary antibodies diluted 1:500 in blocking buffer. After staining, coverslips were washed three times in PBS supplemented with 1% BSA. Fluorescent secondary antibodies (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse; Thermo Fisher Scientific) were then applied at 1:2000 dilution for 1 hour at room temperature in the dark. Coverslips were subsequently washed three times in PBS with 1% BSA.

After the final wash, excess liquid was carefully removed, and coverslips were mounted onto microscope slides using ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific). Slides were gently blotted to remove any surplus mounting medium and sealed with clear varnish. Following 24 hours of curing at 4 °C, slides were imaged using a

Leica SP8X confocal laser scanning microscope (Leica Microsystems, Germany) equipped with a 63× oil immersion objective lens.

2.4.5 Microscopy

Cells cultured on coverslips and prepared for immunofluorescence were imaged using a Leica SP8X confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany), equipped with a 63× oil immersion objective. Image acquisition was carried out using identical laser power and detector gain settings across all experimental conditions within a given assay to ensure consistency and enable direct comparison. Acquired images were exported in TIFF format and subsequently processed using ImageJ software (National Institutes of Health, USA) for quantitative analysis and figure preparation.

2.4.6 Flow Cytometry

SH-SY5Y WT and SH-SY5Y CHIP KO cells were washed twice with phosphate-buffered saline (PBS) and resuspended in blocking buffer consisting of 5% goat serum in PBS. Cells were incubated in the blocking solution for 30 minutes at room temperature to minimise non-specific antibody binding. Following incubation, cells were centrifuged and resuspended in primary antibody solution containing anti-MHC-I antibody (1:100 dilution in blocking buffer). The cell suspension was incubated for 1 hour at room temperature, followed by three washes in PBS to remove unbound primary antibody. Subsequently, cells were incubated with a fluorophore-conjugated secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, diluted 1:200 in blocking buffer) for 45 minutes in the dark at room temperature. After incubation, cells were washed three times with PBS to eliminate excess secondary antibody. Finally, the stained cells were resuspended in 100 µL of PBS and analysed on a BD Accuri C6 flow cytometer (BD Biosciences, USA).

2.4.7 Heat shock treatment

SH-SY5Y WT and SH-SY5Y CHIP KO cells were seeded and maintained as described in Section 2.2 until reaching ~75 % confluency. For acute proteotoxic stress, culture dishes were changed to 42 °C culturing medium and transferred to a humidified incubator pre-equilibrated to 42 °C and exposed for 1 hour (heat-shock condition). Parallel control plates were kept continuously at 37 °C.

To assess post-stress recovery, an additional set of heat-shocked cultures was returned to 37 °C for 1 hour in the same growth medium (heat-shock + recovery condition). At the end of each treatment, control, immediate heat-shock, and heat-shock + recovery, cells were harvested on ice for downstream proteomic analyses.

This design allowed direct comparison of CHIP-dependent effects on the cellular proteome under baseline conditions, during acute thermal stress, and following a defined recovery period, providing a controlled model of the transient proteostasis challenges encountered in neurodegenerative disease.

2.5 Biochemical Techniques

2.5.1 Protein quantification

Protein concentrations were quantified using the Qubit™ Protein Broad Range (BR) Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions. For each sample, 20 µL of cell lysate was transferred into assay tubes containing 200 µL of working solution, prepared by diluting the Qubit reagent in the supplied Protein BR Assay Buffer. A standard curve was generated in parallel using the protein standards provided with the kit. After gentle mixing and brief centrifugation to collect contents, all tubes were incubated at room temperature for 10 minutes in the dark. Fluorescence was subsequently measured using a Qubit 4 fluorometer (Thermo Fisher Scientific), and protein concentrations were calculated automatically by the instrument software, with results expressed in µg/µL.

2.6 Mass spectrometry (MS)-based assays

2.6.1 Protein Extraction and Digestion

Pellets of 1 × 10^6 SH-SY5Y WT or CHIP KO cells were washed in phosphate-buffered saline (PBS), snap-frozen on dry ice, and stored at –80 °C until further processing. Upon thawing, cell pellets were lysed in 100 µL of extraction buffer consisting of 8 M urea, 100 mM ammonium bicarbonate (NH_4HCO_3), and 857 µL acetonitrile (ACN). The lysates were incubated at 37 °C for 30 minutes in a heating block. Protein reduction was carried out by adding 3 µL of dithiothreitol (DTT; dissolved in NH_4HCO_3) and incubating for 30 minutes at 37 °C. Alkylation was performed by adding 9.2 µL of iodoacetamide (IAA; dissolved in NH_4HCO_3), followed by a further 30-minute incubation at 37 °C in the dark.

To optimise enzymatic digestion, samples were diluted with NH_4HCO_3 to achieve a final concentration of 100 mM. Sequencing-grade trypsin (Promega) was added at a ratio of 2 µg per 1 × 10^6 cells. Digestion was performed overnight (~17 hours) at 37 °C with gentle shaking. The reaction was quenched by the addition of 50 µL of 5% trifluoroacetic acid (TFA), and the digested peptides were then diluted to a final volume of 1.5 mL with 0.1% TFA.

Peptides were desalted using C18 solid-phase extraction columns. Columns were conditioned with 3 mL of buffer containing 90% methanol and 0.1% TFA, followed by equilibration with 2 mL of 0.1% TFA in 2% ACN. Peptide samples were loaded in full, washed with 0.1% TFA, and eluted in 1 mL of 50% ACN containing 0.1% TFA. The eluates were dried in a centrifugal vacuum concentrator (SpeedVac) for approximately 3 hours and stored at –80 °C until further analysis. Prior to LC-MS, peptides were reconstituted in 0.1% formic acid.

Sample preparation was performed in collaboration with Dr Jakub Faktor and Dr Artur Piróg.

2.6.2 LC-MS/MS Acquisition

Peptide samples were analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an RSLCnano 3000 system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Samples were initially loaded onto a cartridge trap column, followed by separation on a reverse-phase C18 analytical column. Peptides were eluted using a linear gradient of increasing ACN in 0.1% formic acid at a flow rate of 300 nL/min.

2.6.3 Data Processing and Statistical Analysis

Raw mass spectrometry data were processed in MaxQuant using the integrated Andromeda search engine, with searches conducted against the UniProt human FASTA database. The false discovery rate (FDR) was controlled at 1% at both the peptide and protein levels. Relative protein abundances were estimated using label-free quantification (LFQ) intensities. Downstream data analysis was performed using Skyline and Perseus software packages. Statistical significance was determined using Student's t-test with a 5% FDR threshold.

Data processing and interpretation were carried out in close collaboration with Dr Artur Piróg and Dr Jakub Faktor, with computational analysis and bioinformatics support provided by Dr Georges Bedran.

2.7 Software and statistics

2.7.3 Imaging analysis

Confocal images were exported in TIFF format and analysed using ImageJ/Fiji (NIH). To visualise co-localisation signals, individual fluorescence channels were separated, thresholded, and merged. Maximal intensity projections were generated from z-stack images when required. For quantitative analysis, regions of interest (ROIs) were defined on the DAPI channel to delineate nuclei, and mean fluorescence intensities were measured in the corresponding protein channels. In cases where the signal of interest

extended into the cytoplasm or plasma membrane, rather than being restricted to the nucleus, measurements were normalised to the DAPI signal for each field of view to account for cell number variation.

Brightness and contrast adjustments were applied uniformly across all images and experimental replicates to optimise signal-to-background ratio. Background subtraction was performed consistently for all quantifications. Where dual-labelling was employed, co-localisation analysis was carried out using the Coloc2 plug-in in Fiji to compute Pearson's correlation coefficients between the green and red fluorescence channels. All image quantifications were performed using TIFF-format files.

2.7.5 Quantification of immunoblots and polyacrylamide gels analysed by SDS-PAGE

Immunoblots were developed using the ChemiDoc Imaging System (Bio-Rad), with both XRS+ and MP models employed over the course of the project. Developed blots were exported as high-resolution TIFF files and analysed using ImageJ (NIH). Rectangular regions of interest (ROIs) were drawn around bands of interest, and signal intensity was quantified as the integrated density (defined as the product of mean grey value and band area) following local background subtraction using an adjacent area of the membrane. Protein expression levels were normalised to β-actin as a loading control to correct for differences in total protein input.

Polyacrylamide gels prepared under denaturing conditions with Laemmli buffer were examined to verify the presence or absence of protein bands prior to immunoblotting. Coomassie blue staining was used as a qualitative indicator of total protein content but was not subjected to densitometric analysis.

2.7.6 Statistical analysis

Normality testing and statistical analyses were conducted using GraphPad Prism (GraphPad Software). For datasets such as immunoblot band intensities, fluorescence

quantifications, or proteomic measurements, the D'Agostino–Pearson omnibus normality test ($\alpha = 0.05$) was used to assess Gaussian distribution. When data passed this test, parametric analyses were performed using Student's t-test or one-way/two-way ANOVA, followed by Holm–Sidak's post-hoc correction where applicable. For datasets that did not meet the assumptions of normality, non-parametric alternatives were used, specifically the Kruskal–Wallis test with Dunn's multiple comparisons post-test. All data are reported as mean ± standard error of the mean (SEM), unless otherwise indicated. Statistical significance was defined as $p < 0.05$.

Chapter 3: Effect of CHIP on the proteome

3.1 Introduction

3.1.1 Proteome studies

Proteomics is a high-throughput approach to studying the proteome, the complete set of proteins expressed by a cell or tissue at a specific time. Its objectives extend beyond mere identification, encompassing the quantification of protein abundance, post-translational modifications (PTMs), interactions, and spatial distribution. Proteins orchestrate cellular physiology and, collectively, underpin the function and health of the entire organism. Variations in protein levels or modifications can serve as indicators of cellular stress, contribute to the onset and progression of disease, or unveil potential therapeutic targets. As such, proteomic analyses offer a direct window into cellular function under both physiological and pathological conditions.

The complexity of the proteome remains one of the principal challenges in proteomics. Due to processes such as alternative splicing and diverse PTMs, approximately 22,000 protein-coding genes in the human genome give rise to an estimated 400,000 distinct protein species. The proteome is highly active and responsive to changes in intra- and extracellular environments, resulting in a broad spectrum of qualitative and quantitative protein alterations. Comparative proteomic analyses, such as between healthy and diseased states, can elucidate the molecular mechanisms that drive these biological transitions. In particular, PTMs regulate protein folding, stability, subcellular localisation, and functional activity, and are frequently implicated in pathological processes.

Despite advances in mass spectrometry (MS) and computational tools, proteomic data typically represent a single time point, capturing a snapshot of the proteome within its continuously shifting landscape. The overarching aim is to achieve temporally resolved characterisation of the proteome and to reconstruct the signalling pathways underpinning the observed changes. Although numerous human proteins have been catalogued, it is estimated that only 5–10% of the pairwise protein–protein interactions comprising the human interactome have been characterised to date.

Proteomics continues to evolve rapidly. Early work relied on two-dimensional electrophoresis (2DE), a foundational technique for protein separation. The subsequent adoption of MS-based technologies revolutionised the field by enabling highly sensitive and specific protein identification. Parallel advances in large-scale genomics expanded the content of publicly available sequence databases, which in turn laid the groundwork for bioinformatic pipelines capable of interrogating these datasets to facilitate comprehensive proteome identification.

3.1.2 Two-dimensional electrophoresis (2DE) technology

Two-dimensional electrophoresis (2DE), introduced in the 1970s, was among the first methodologies to allow large-scale separation of proteins within complex biological mixtures. In this technique, proteins are initially separated based on their isoelectric point (pI) via isoelectric focusing, and subsequently resolved by molecular weight using SDS–PAGE in the second dimension, generating a two-dimensional protein map. This map can be visualised using various staining techniques, enabling the simultaneous resolution of thousands of proteins and offering insights into isoforms and post-translational modifications. While 2DE is relatively cost-effective and requires minimal specialised instrumentation, it is labour-intensive, biased towards highly abundant proteins, and limited in its ability to resolve extremely complex proteomes or membrane proteins. Its utility was significantly expanded by coupling with mass spectrometry for downstream protein identification; however, it has since been largely supplanted by high-throughput liquid chromatography–tandem mass spectrometry (LC–MS/MS)–based approaches, which offer improved sensitivity, dynamic range, and reproducibility.

3.1.3 Mass Spectrometry (MS)

Mass spectrometry (MS) is widely regarded as a leading technology for high-throughput proteomic analysis and comprehensive proteome coverage. Despite its utility, the method is constrained by high technical complexity, the significant cost of instrumentation, and the still-incomplete nature of proteomic databases. MS-based

proteomics can be implemented either through labelling techniques or via label-free approaches. Labelling typically involves the incorporation of stable isotopes and allows for more precise and accurate quantification of proteins. However, this approach is associated with several drawbacks, including experimental complexity, high cost, bias towards proteins with higher turnover rates, and constraints on sample type and throughput. In contrast, label-free methods offer greater flexibility in sample selection and are comparatively straightforward to perform, but they tend to suffer from lower precision and accuracy in quantification.

Mass spectrometry–based proteomics is commonly categorised into two primary strategies: bottom-up and top-down approaches. Bottom-up proteomics, the more widely adopted methodology, involves enzymatic digestion of proteins into smaller peptides, which are then analysed to infer protein identity and abundance. Top-down proteomics, by contrast, investigates intact, undigested proteins and provides information on isoforms and post-translational modifications at the full-protein level. In bottom-up workflows, proteins are typically digested using trypsin, which cleaves at lysine and arginine residues. Peptides are initially separated by liquid chromatography (LC) based on their hydrophobicity, with each species eluting at a distinct retention time (RT). Following separation, peptides are ionised and introduced into the mass spectrometer. The first MS scan generates a peptide mass fingerprint, representing the initial dimension of analysis used for peptide identification.

a. Tandem Mass Spectrometry (LC-MS/MS)

The first stage of mass spectrometry, known as MS1, provides an initial overview of peptide masses within a sample but is not sufficient on its own to achieve accurate protein identification. This limitation arises because different proteins may contain peptides with identical mass-to-charge (m/z) values, particularly given the redundancy and repetition inherent in amino acid sequences. As a result, peptide mass alone cannot uniquely determine protein identity. To overcome this challenge, tandem mass spectrometry (MS/MS or MS2) is employed. This approach enhances specificity by subjecting selected precursor ions to fragmentation, generating a unique pattern of product ions that serves as a molecular fingerprint for each peptide. These

fragmentation spectra provide both structural and sequence-level information that significantly improve the confidence of identification (Figure 3).

During tandem MS analysis, the quadrupole initially selects precursor ions from the peptide mixture. These ions are directed into a collision cell, where they undergo fragmentation upon collision with inert gases such as nitrogen. This typically results in the formation of b- and y-type ions, which are then detected by a mass analyser such as a time-of-flight (TOF) system or a third quadrupole. The resulting MS2 spectra form the basis for reliable peptide sequencing and accurate protein identification.

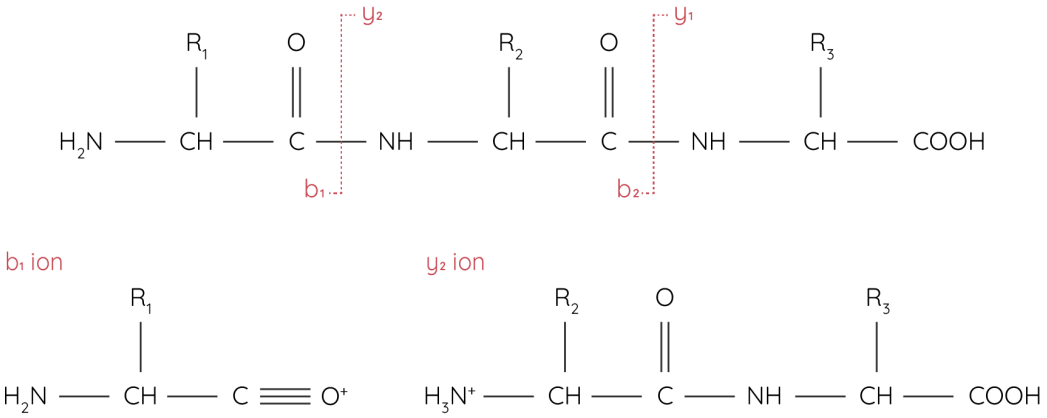

Figure 3: Peptide fragmentation into b and y ions.

The dotted lines mark the fragmentation pattern within the precursor ion, producing smaller product ions.

b. Data-dependent acquisition mass spectrometry

Data-dependent acquisition (DDA) mass spectrometry, also referred to as shotgun proteomics, preferentially identifies proteins of higher relative abundance. This occurs through the selective isolation and fragmentation of the most intense precursor ions at a given chromatographic elution time, which inherently biases the analysis toward more abundant peptides. As an alternative, selected acquisition monitoring can be employed, in which user-defined transitions (specific combinations of precursor peptides and corresponding fragment ions) are targeted for individual analysis. This targeted

approach enables the consecutive detection and quantification of multiple fragment ions originating from one or more selected precursor peptides (Figure 4).

Shotgun proteomics has significantly advanced the ability to profile complex proteomes. However, its application was historically limited by the relatively low number of proteins (typically between 50 and 100) that could be identified and quantified within a single LC–MS/MS injection. This limitation can be addressed by reducing sample complexity through fractionation strategies, such as strong anion exchange chromatography or off-gel electrophoresis, prior to mass spectrometric analysis. Nevertheless, targeted acquisition approaches also carry important constraints, including the requirement for prior knowledge of proteins of interest in order to define transitions, which may limit the discovery potential and proteomic depth in exploratory studies.

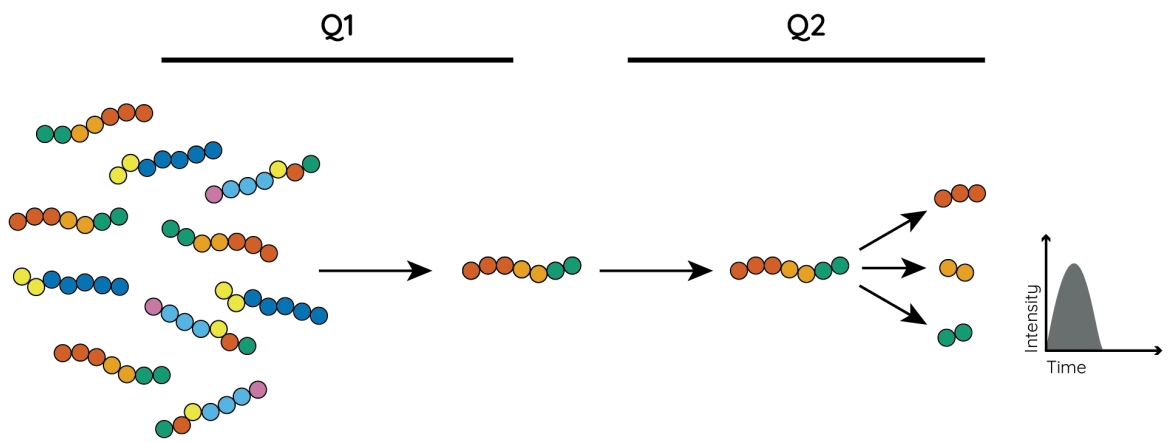

Figure 4: Schematic overview of the data-dependent acquisition (DDA) workflow.
Following peptide ionisation, ions enter the mass spectrometer and are filtered by the first quadrupole based on their mass-to-charge (m/z) ratio. The instrument selects the most intense precursor ions in real time for fragmentation, typically via collision-induced dissociation. The resulting product ions are separated and detected, generating MS/MS spectra for peptide identification.

c. Data-independent acquisition mass spectrometry

The advancement of data-independent acquisition (DIA) mass spectrometry, also referred to as untargeted proteomics, has markedly improved the reproducibility, sensitivity, accuracy, and dynamic range of proteomic analyses. Unlike data-dependent acquisition (DDA), which focuses on the most abundant peptides, DIA enables broader data capture from a sample by systematically fragmenting all ions within defined mass-to-charge (m/z) windows for tandem MS/MS analysis. A key innovation in this field is SWATH-MS (sequential window acquisition of all theoretical mass spectra), which revolutionised large-scale proteomic profiling by allowing the concurrent monitoring of thousands of peptides. This approach combines DIA with targeted signal extraction, requiring high-resolution instrumentation and comparison against a reference spectral library (Figure 5).

In SWATH-MS, all precursor ions falling within consecutive m/z windows are fragmented in a systematic fashion, producing comprehensive peptide fragment profiles. These fragment ions are analysed using a time-of-flight (TOF) mass analyser, where their transit time through a known distance under constant voltage is measured to calculate their m/z values. The result is a three-dimensional dataset, capturing fragment ion intensities across time and m/z for every detectable peptide. This data can be represented as chromatograms (precursor ion intensity over retention time) or spectra (fragment ion intensity over m/z). Because peptides originating from the same protein exhibit similar retention times, their signals can be aligned, normalised, and interpreted collectively. Protein identification is achieved by comparing observed fragmentation patterns and retention times against a spectral library, traditionally built from fractionated or enriched samples analysed via DDA, but now also increasingly constructed in silico.

One of the principal advantages of SWATH-MS is its ability to record all fragment ions across a designated mass range, ensuring that no peptide signal is excluded during acquisition. This enables wide proteome coverage in a single run and allows retrospective interrogation of datasets using targeted extraction strategies. Quantification is typically label-free and often relies on spectral counting, where the frequency of detected fragment spectra correlates with protein abundance. Although this method offers high accuracy for moderate expression changes, it may

underestimate large fold differences due to limitations in ionisation efficiency or detector response. Nevertheless, for many biological investigations (particularly those involving molecular chaperones or E3 ligases with extensive interactomes) small changes in protein abundance can have meaningful functional implications.

SWATH-MS also supports absolute quantification by integrating chromatographic peak intensities of peptide signals. While the historical requirement for empirical spectral libraries posed a constraint, large-scale proteomic initiatives have since generated extensive reference datasets for many organisms, including humans. Despite these advances, challenges remain in achieving full proteome coverage, especially given the vast dynamic range of protein expression and the difficulty in detecting low-abundance proteins or post-translational modifications. Sensitivity can be improved through sample fractionation; however, complete profiling of the human proteome continues to be a major analytical hurdle.

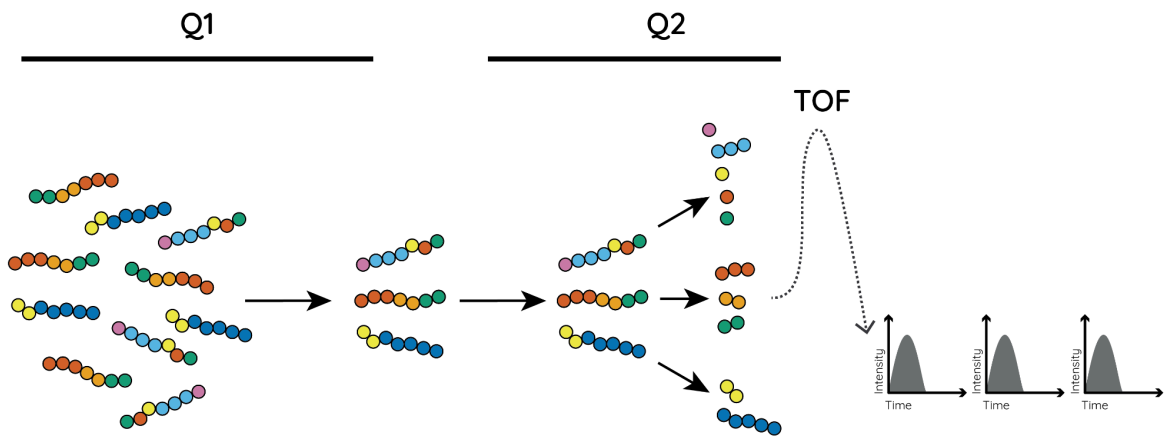

Figure 5: Principle of data-independent acquisition (DIA) mass spectrometry.
Peptides are first ionised to generate precursor ions, which are grouped into defined m/z windows. These ion populations are transmitted through the first quadrupole, fragmented in the second quadrupole, and the resulting product ions are analysed in a time-of-flight (TOF) detector. This process yields mass spectra covering all fragment ions generated from the precursors within each selected window.

3.2 The potential impact of CHIP on maintaining neuronal homeostasis.

3.2.1 General effects of CHIP deletion in proteome

The first stage of the study aimed to determine whether deletion of the STUB1 gene, resulting in loss of CHIP protein expression, led to widespread changes in the proteome. To address this, an undifferentiated SH-SY5Y cell line was used, originally generated by Erisa Nita (University of Edinburgh) via CRISPR/Cas9-mediated gene editing. This particular model was selected for its neuroblast-like phenotype, characterised by non-polarised cell bodies and the expression of immature neuronal markers (Figure S1), as well as its low cost, ease of maintenance, human origin, and capacity for differentiation. The SH-SY5Y cell line was initially derived from a metastatic bone tumour and remains a widely used model for studying neuronal processes.

Once cultured to confluence, cells were harvested and processed for proteomic analysis in collaboration with Dr Artur Piróg and Dr Irena Dapic. Protein extraction and label-free LC–MS/MS analysis were performed to profile protein identities and their relative abundances across conditions. Each sample was derived from a single well, and three independent biological replicates were generated per condition. Samples were processed and analysed separately. Comparative analysis was conducted between SH-SY5Y WT and SH-SY5Y CHIP KO cells.

The results of this comparative proteomics experiment are visualised as a volcano plot (Figure 6). The majority of detected proteins exhibited minimal fold change between conditions (grey dots), whereas outlier proteins (highlighted in red) represent candidates of potential biological interest. Over-represented proteins demonstrated increased ion product intensities in CHIP KO cells relative to WT, while under-represented proteins exhibited the opposite trend. The most significantly altered proteins in WT versus KO samples are summarised in Table S1. These findings provide an initial indication that CHIP deficiency impacts protein expression at the global level, prompting further investigation into the functional implications of the observed changes.

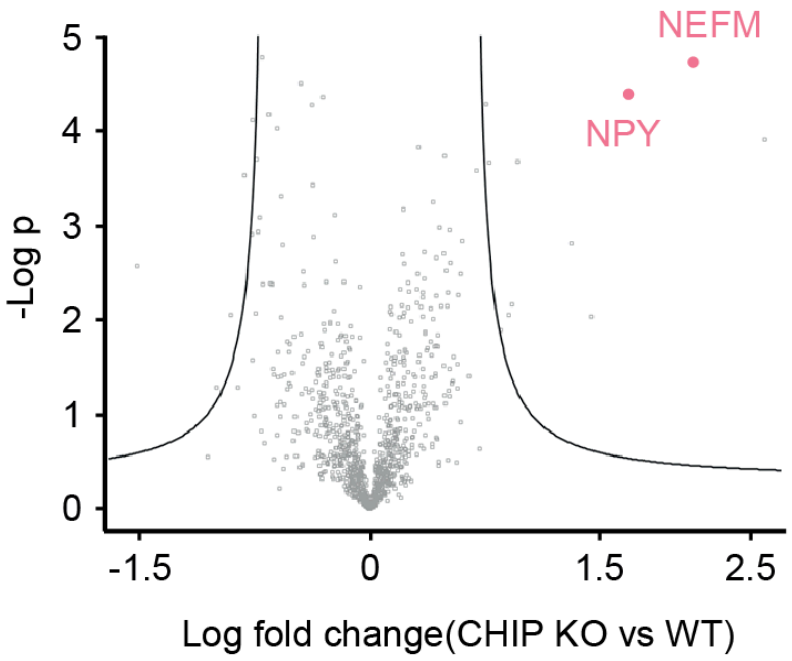

Figure 6: Comparative proteomic analysis of SH-SY5Y WT and CHIP KO cell lines.

Label-free LC-MS/MS analysis comparing protein expression profiles between SH-SY5Y WT and CHIP KO cells. Proteins showing higher abundance in the CHIP-KO samples are plotted on the right (positive $\log_2$ fold change), whereas proteins reduced in the KO samples appear on the left (negative $\log_2$ fold change). Boundary lines indicate the criteria used to define differential expression. Proteins of particular interest are highlighted in red, and the two most strongly over-represented proteins are annotated.

3.2.2 Proteins of interest

a. Neuropeptide Y

Neuropeptide Y (NPY) is a 36–amino acid neuromodulator broadly distributed in the central nervous system, particularly within GABAergic interneurons of the hippocampus and cortex. It plays a crucial role in suppressing excessive excitatory neurotransmission and contributes to the regulation of diverse physiological functions, including stress responses, circadian rhythms, and appetite control. Beyond neurotransmission, NPY provides trophic support, exposure to NPY has been shown to elevate levels of brain-derived neurotrophic factor (BDNF), thereby promoting neurogenesis and

enhancing neuronal survival. Basal NPY activity is essential for neuronal viability, as it mitigates hyperexcitability and modulates synaptic strength.

Upregulation of NPY is generally neuroprotective. Elevated NPY levels have been associated with reduced excitotoxic stress, exemplified in experimental models where NPY prevents kainate-induced apoptosis in hippocampal neurons. However, excessive NPY-mediated inhibition could potentially interfere with normal synaptic transmission and impair cognitive processes such as learning. Dysregulation of NPY expression is implicated in multiple neurodegenerative conditions. In Parkinson's disease (PD) and Huntington's disease (HD), NPY is often upregulated in specific brain regions. For example, increased NPY mRNA levels have been reported in the striatum, including the caudate, putamen, and nucleus accumbens, of PD patients, while HD brains exhibit elevated NPY levels in the basal ganglia and cortex. This upregulation is thought to represent a compensatory mechanism, as NPY-expressing interneurons are relatively preserved and may enhance NPY production to stabilise overactive neural circuits and support cell survival. In line with this hypothesis, intraventricular infusion of NPY or its analogues in rodent models of PD and HD has demonstrated neuroprotective effects on vulnerable neuronal populations (Li et al., 2019).

In contrast, NPY levels are consistently reduced in Alzheimer's disease (AD). Post-mortem analyses of AD brains reveal significant decreases in NPY concentrations within the hippocampus and cerebral cortex. Similarly, circulating NPY levels in AD patient plasma are markedly lower than in healthy controls. This decline in NPY may contribute to heightened excitotoxicity and neuroinflammation characteristic of AD pathology (Koide et al., 1995). Conversely, exogenous NPY administration has shown beneficial effects in experimental AD models. NPY and its fragments can attenuate Aβ-induced neurotoxicity both in vitro and in vivo, reducing amyloid-related neuronal death and restoring neurotrophin levels (Santos-Carvalho et al., 2012).

In summary, NPY functions as an endogenous neuroprotective factor. Its downregulation, as observed in AD, is associated with increased neurodegenerative burden, whereas its upregulation, seen in PD, HD, or therapeutic interventions, tends to preserve neuronal integrity by limiting excitotoxicity, restoring calcium homeostasis, and suppressing inflammatory responses.

b. Neurofilament medium polypeptide

NEFM encodes the medium-weight subunit of the neuronal intermediate filament protein family known as neurofilaments (NFs), which are essential structural components of the neuronal cytoskeleton. Together with the light (NEFL) and heavy (NEFH) subunits, NEFM polymerises to form heteropolymers that are most abundantly expressed in axons. The C-terminal tail domain of NEFM is particularly rich in phosphorylation sites and extends radially from the filament core, helping to maintain proper spacing between individual filaments. This lateral spacing is critical for determining axonal diameter, which in turn governs conduction velocity and long-range neuronal connectivity (Barry et al., 2012).

Beyond providing structural integrity, NEFM also interacts with signalling molecules. For instance, it modulates the trafficking and surface localisation of the dopamine D1 receptor, implicating NEFM in synaptic plasticity and receptor regulation. However, overexpression of NEFM disrupts the delicate stoichiometry of neurofilament subunits and promotes the formation of pathological protein aggregates. Experimental studies in transgenic mice overexpressing NEFM (at least two-fold above physiological levels) have shown extensive accumulation of filamentous material in neuronal soma and axons, axonal degeneration, and progressive motor deficits such as hind-limb paralysis. These pathological changes are believed to stem from impaired axonal transport, cytoskeletal disorganisation, and disruption of intracellular trafficking. To date, no neuroprotective role has been associated with NEFM upregulation; rather, its excess appears consistently deleterious to neuronal health.

Dysregulation of neurofilaments is a recurring feature in many neurodegenerative diseases, often referred to as neurofilamentopathy. Aggregated NF proteins are found in the pathological inclusions of Alzheimer's disease, where they frequently co-localise with hyperphosphorylated tau. Similarly, neurofilament proteins have been detected within Lewy bodies in Parkinson's disease (Didonna and Opal, 2019). While it remains unclear whether NEFM misregulation is a primary driver or secondary consequence of neurodegeneration, it is increasingly recognised as a contributor to axonal dysfunction, impaired connectivity, and progressive neuronal loss.

c. Paraneoplastic antigen Ma2

PNMA2 (Paraneoplastic Ma antigen 2) is a protein predominantly expressed in neurons within the central nervous system under normal physiological conditions. It belongs to the “onconeural” antigen family, a group of neuronal proteins that, when aberrantly expressed by tumours, can trigger autoimmune responses. Although the precise neuronal functions of PNMA2 remain incompletely characterised, in vitro studies suggest that it may participate in cell survival pathways, possibly through interactions with apoptosis-regulating proteins. However, its direct roles in synaptic transmission, plasticity, or circuit maintenance remain to be elucidated.

Abnormal upregulation of PNMA2 can provoke a pathogenic immune response under specific conditions. Certain tumours, most remarkably testicular germ-cell tumours, ectopically express and secrete PNMA2, which has been shown to self-assemble into virus-like capsid structures. These aggregates mimic viral particles and are recognised as immunogenic by the host immune system, triggering a potent response. This leads to the generation of PNMA2-specific autoantibodies and cytotoxic immune cells that subsequently cross-react with PNMA2-expressing neurons in the brain, resulting in immune-mediated neuronal damage. Experimental models support this mechanism: administration of PNMA2 capsid-like complexes in mice has been shown to induce a robust immune response, leading to cognitive impairments and memory deficits akin to those observed in patients with paraneoplastic neurological syndromes (Xu et al., 2023).

In humans, PNMA2 autoimmunity is a hallmark of paraneoplastic neurological syndromes (PNS), which are rare but devastating disorders characterised by subacute neurological decline. Clinical manifestations typically include rapid-onset cognitive impairment, memory loss, psychiatric disturbances, seizures, and ataxia. These symptoms are primarily driven by immune-mediated injury to the limbic system and cerebellum, resulting in limbic encephalitis and cerebellar degeneration. In particular, patients with high PNMA2 (also referred to as Ma2) autoantibody titres often present with inflammation in hippocampal and brainstem regions, highlighting the neuroanatomical specificity of this autoimmune response (Yamahara et al., 2024).

Although PNMA2 is not currently implicated as a causal factor in idiopathic neurodegenerative diseases such as Alzheimer's or Parkinson's disease, its aberrant expression in cancer and associated immune response clearly demonstrate its capacity to mediate severe neuronal dysfunction and dementia-like syndromes (Guo et al., 2021). This highlights a unique, cancer-associated autoimmune mechanism linking PNMA2 dysregulation to neurodegeneration.

d. Alpha Internexin

Alpha-internexin (INA) is a 66-kDa neuronal intermediate filament protein and a critical structural component of the neuronal cytoskeleton. It is the first intermediate filament expressed in postmitotic neurons during development and remains abundantly expressed throughout adulthood in neurons of the central nervous system (CNS). In mature neurons, alpha-internexin co-assembles with the three classical neurofilament subunits (NEFL, NEFM, and NEFH) serving effectively as a "fourth subunit" of the neurofilament network. Through this incorporation, it contributes to the structural integrity of axons, helping to regulate axonal diameter and support efficient axonal transport. Although it is not directly involved in synaptic transmission, alpha-internexin plays a fundamental role in maintaining neuronal architecture, which is essential for synaptic connectivity and signal propagation.

However, its overexpression can be deleterious to neuronal health. Transgenic mouse models with modest increases in alpha-internexin levels, approximately two- to threefold, develop pathological accumulations of intermediate filament proteins within neurons. These accumulations give rise to cerebellar "torpedoes," which are swollen and degenerating Purkinje cell axons, a hallmark of cerebellar neurodegeneration. Behaviourally, these mice exhibit progressive motor incoordination beginning within the first few months of life, and the severity of neurodegeneration correlates with the level of transgene expression. These findings provide direct experimental evidence that upregulation of alpha-internexin can drive axonal pathology, neuronal dysfunction, and ultimately neurodegeneration (Yuan et al., 2006).

Alpha-internexin is also the hallmark protein of neuronal intermediate filament inclusion disease (NIFID), a rare, early-onset neurodegenerative disorder that typically presents with a combination of frontotemporal dementia and motor system involvement. NIFID is defined by selective neuronal loss and the presence of distinctive cytoplasmic inclusions composed of abnormal intermediate filament aggregates. Alpha-internexin has been identified as a major constituent of these inclusions, frequently co-localising with the three neurofilament subunits, implicating it directly in disease pathogenesis. In addition, alpha-internexin has been observed (albeit at lower levels) in pathological structures of more prevalent neurodegenerative diseases. For instance, a subset of neurofibrillary tangles in Alzheimer's disease and certain Lewy bodies in Parkinson's disease and dementia with Lewy bodies contain alpha-internexin immunoreactivity. These findings suggest that while not ubiquitous, alpha-internexin dysregulation may contribute to broader neurodegenerative mechanisms beyond NIFID (Cairns et al., 2004).

3.2.3 Validation of proteomic analysis

It is crucial to factor in that the intensity of peptide signals detected by LC-MS/MS is influenced by a complex interplay of factors. Post-translational modifications and intermolecular interactions, including the extent of protein aggregation, can significantly affect enzymatic cleavage efficiency during sample preparation, particularly by enzymes such as trypsin. Furthermore, individual peptides exhibit varying degrees of compatibility with LC-MS/MS detection, due to differences in their ionisation efficiency and fragmentation potential. As a result, apparent differences in abundance may not always reflect true biological variation. Therefore, careful interpretation of the data is essential, and independent validation remains a critical component of powerful proteomic analysis.

Preliminary validation of the mass spectrometry (MS) data was carried out by examining a subset of the most strongly over-expressed proteins identified in the comparative proteomic analysis, specifically those implicated in neuronal health. These proteins were selected because they had not previously been directly linked to CHIP, thereby offering the opportunity to uncover novel associations. As high-quality monoclonal antibodies

are costly, only the two top-ranked candidates were chosen to keep the validation process economically feasible.

The abundance of neuropeptide Y (NPY) and neurofilament medium chain (NEFM) in SH-SY5Y WT and CHIP KO cells was assessed by western blotting (Figure 7a). Both proteins showed clear over-representation in the CHIP KO line, consistent with the LC–MS/MS results. To verify these findings and to investigate their cellular localisation, immunofluorescence imaging was performed on SH-SY5Y WT and CHIP KO cells (Figure 7 b,c). Imaging confirmed significantly elevated NPY levels in the KO cells relative to WT controls. NPY immunoreactivity was confined to the cytoplasm with pronounced enrichment in the perinuclear region, a distribution pattern typical of a secretory neuropeptide synthesised in the soma and stored in dense-core vesicles of the Golgi–vesicular pathway before release.

In contrast, NEFM immunostaining failed to produce a detectable signal above background (Figure 7c). This absence could reflect either intrinsically low expression of NEFM in both cell lines or suboptimal antibody performance. To address this limitation in future experiments, the closely related neurofilament light chain (NEFL) antibody was used as a surrogate marker (see Section 4.2.6). NEFL forms copolymers with NEFM and NEFH, therefore NEFL immunostaining provides a reliable proxy for neurofilament expression and organisation when NEFM detection is problematic.

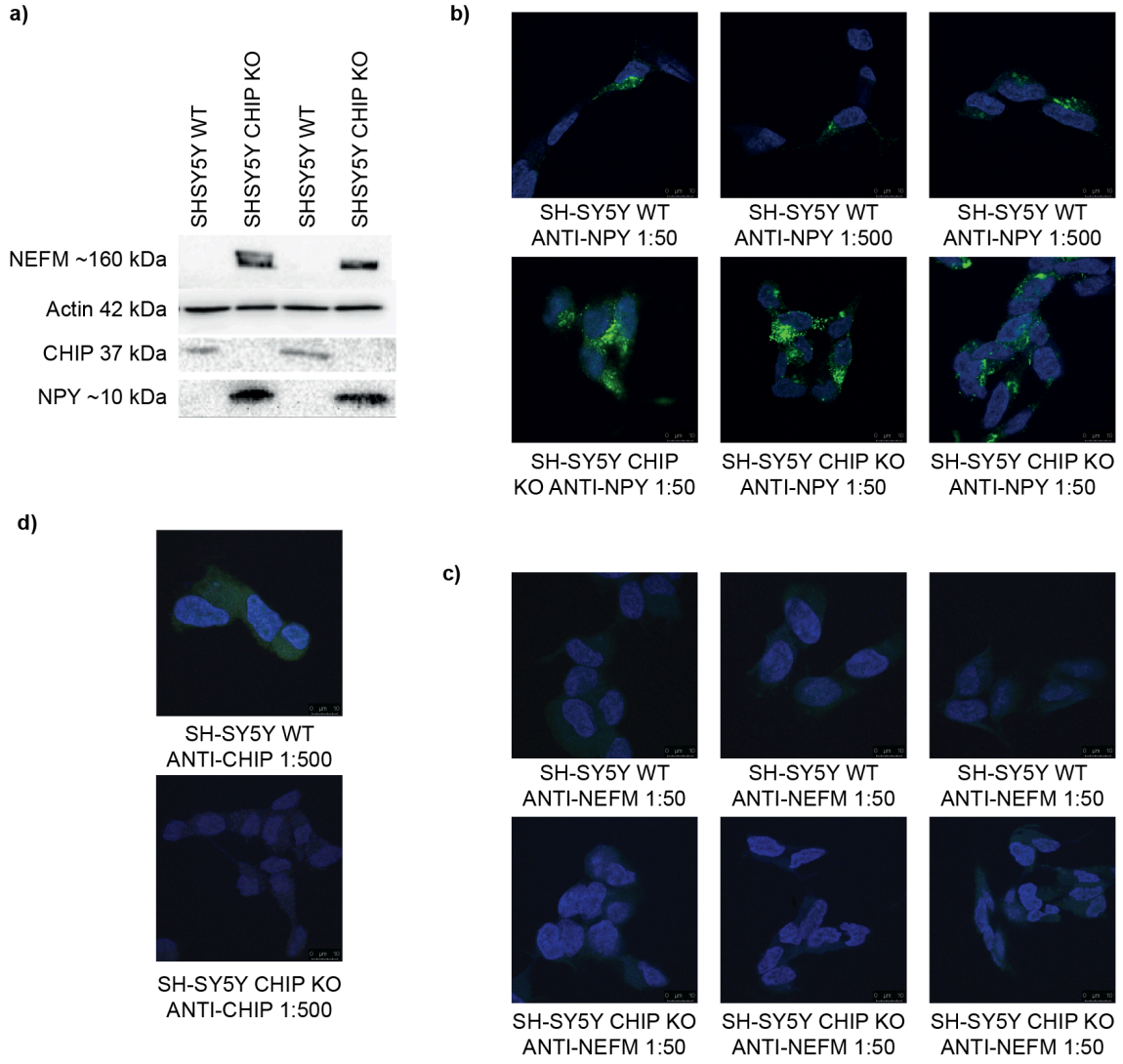

Figure 7: Validation of proteomic hits by Western Blot and immunofluorescence.

(a) Western blot analysis of neuropeptide Y (NPY) and neurofilament medium chain (NEFM) in SH-SY5Y WT and CHIP-KO cells. (b) Immunofluorescence showing elevated cytoplasmic NPY in CHIP-KO cells. (c) NEFM immunostaining was undetectable. (d) Immunofluorescence for CHIP in WT and KO cells confirming strong CHIP signal in WT and the expected absence of staining in the KO line.

3.2.4 CHIP-mediated regulation of axonal structure and neuronal health

The knockout cells display a coordinated response suggestive of neuron-like stress and structural remodelling. Marked up-regulation of neuropeptide Y points to activation of neuroprotective, anti-excitotoxic signalling, while the parallel rise in neurofilament medium polypeptide and alpha-internexin indicates cytoskeletal and axonal changes typically seen during injury or repair. The unexpected increase in paraneoplastic antigen Ma2 adds an immune-related element, suggesting altered neuronal antigen expression rather than a direct degenerative process. Together, these findings suggest that loss of CHIP triggers a programme of axonal reorganisation and switch on protective signals, with possible exposure of normally silent neuronal antigens.

3.2.5 Culture density of SH-SY5Y cells does not account for proteomic differences between WT and CHIP KO lines.

Cell confluency is a key experimental variable that influences cellular physiology and protein expression in cultured lines, including SH-SY5Y neuroblastoma cells. Published studies report that cultures maintained at low confluency (< 60 %) are in an active proliferative phase with proteomes enriched for cell-cycle and metabolic proteins, whereas cultures at high confluency (> 90 %) display proteomic shifts associated with increased cell–cell contacts, nutrient limitation, and stress responses (Lobo et al., 2024). These confluency-dependent differences highlight the need for rigorous control of culture density to ensure reproducibility in SH-SY5Y cell experiments.

Although all experiments in this project were performed at a consistent density of approximately 75 % confluency, it was essential to determine whether deviations from this level could influence the proteome of the experimental cell lines. To address this, a comparative LC–MS/MS analysis was performed on SH-SY5Y WT and CHIP KO cells cultured either at the standard 75 % confluency or at high confluency of roughly 95 %. For each condition, samples were collected with the same absolute number of cells to eliminate loading bias. Cells were harvested and processed for proteomic analysis in collaboration with Dr Artur Piróg and Dr Jakub Faktor. Protein extraction and label-free LC–MS/MS were carried out exactly as described for the primary proteomic screen,

although this time Thermo Scientific Orbitrap Exploris 480 high resolution mass spectrometer was used. Three independent biological replicates were generated per condition and processed separately.

Comparative analyses were performed across four groups: WT cells at 75 % confluency, WT cells at 95 % confluency, CHIP KO cells at 75 % confluency, and CHIP KO cells at 95 % confluency. The results are presented as volcano plots (Figure 8a–c). When comparing regular versus high confluency within each cell line, all detected proteins clustered below the significance threshold ($p > 0.5$), indicating no measurable proteomic impact of increased confluency alone. Particularly, the WT-versus-KO comparison reproduced, and indeed expanded upon, the findings of the initial LC–MS/MS screen, yielding a larger number of significantly altered proteins (Table S3). Among these, four previously identified hits, neuropeptide Y (NPY), neurofilament medium chain (NEFM), Paraneoplastic Ma antigen 2 (PNMA2) and alpha internexin (INA) were again validated as significantly over-expressed in the CHIP KO samples.

These results reinforce the conclusion that CHIP deficiency drives broad alterations in protein expression, while also confirming that moderate variations in culture confluency do not confound the observed proteomic differences between WT and CHIP KO SH-SY5Y cells.

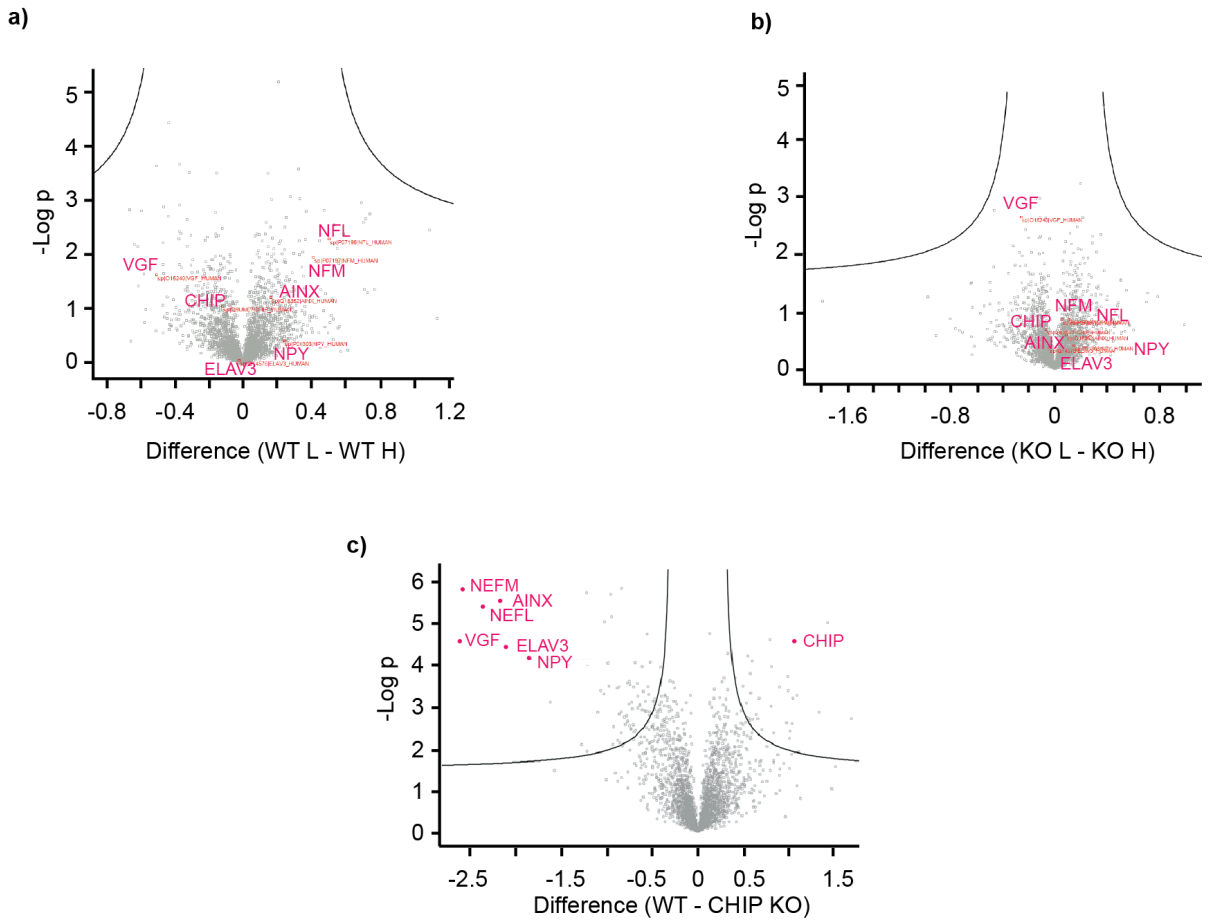

Figure 8: Comparative proteomic analysis of SH-SY5Y WT and CHIP-KO cells cultured at different confluencies

(a) Volcano plot comparing SH-SY5Y WT cells grown at standard (≈75 %) versus high (≈95 %) confluency. (b) Volcano plot comparing SH-SY5Y CHIP-KO cells grown at standard versus high confluency. (c) Volcano plot comparing SH-SY5Y WT and CHIP-KO cells (standard confluency), highlighting proteins significantly altered by CHIP deletion.

3.2.6 Possible effect of CHIP on functioning of neurons

Because the expanded proteomic analysis revealed an even greater number of up-regulated proteins in the CHIP KO line compared with the WT cells, the findings provided compelling evidence for CHIP involvement in neuronal function. Consequently, five additional proteins of interest were selected for follow-up validation. These

candidates were drawn from those meeting the significance threshold for p-value and represented the five most strongly up-regulated proteins in the CHIP KO samples.

a. Neurofilament Light Polypeptide

Neurofilament light polypeptide (NEFL) is the smallest subunit of the neurofilament triplet and forms a fundamental building block of neuronal intermediate filaments. It is the most abundant subunit in healthy neurons and largely determines the total number of assembled filaments. NEFL plays a critical role in maintaining axonal calibre, providing structural support, and organising intracellular transport pathways, thereby sustaining neuronal connectivity. Experimental evidence underscores its importance: mice lacking NEFL exhibit markedly reduced filament assembly together with thinner axons, confirming its essential structural function (Didonna and Opal, 2019).

Elevating NEFL levels beyond their normal physiological range can be deleterious, particularly if the increase is not matched by proportional changes in the medium (NEFM) and heavy (NEFH) neurofilament subunits. Transgenic mice overexpressing NEFL by approximately four-fold develop abnormal neurofilament accumulations, giant axonal swellings, and progressive axonal degeneration. These animals display severe motor deficits, including muscle atrophy, demonstrating that excessive NEFL disrupts axonal architecture and neuronal function. The underlying mechanism parallels that observed with NEFM: surplus NEFL upsets the stoichiometric balance required for proper filament assembly, leading to misassembled aggregates that obstruct axonal transport. Interestingly, increasing NEFL can in some contexts mitigate pathology caused by imbalance of other subunits. For example, in models where NEFH overexpression produced toxic aggregates, co-overexpression of NEFL helped restore correct filament organisation and reduced toxicity.

NEFL is also widely recognised as a biomarker of neurodegeneration. When neurons are damaged or axons degenerate, NEFL is released into cerebrospinal fluid (CSF) and blood, where its concentration often reflects disease severity and progression. Elevated CSF or plasma NEFL levels have been reported in numerous neurodegenerative disorders, including Alzheimer's disease and frontotemporal dementia. Pathologically,

NEFL together with NEFM and NEFH is frequently detected in the characteristic inclusions of degenerating neurons: neurofibrillary tangles in Alzheimer's disease often contain neurofilament proteins, and Lewy bodies in Parkinson's disease also harbour neurofilament components. Besides, genetic evidence directly implicates NEFL in human neurodegeneration (Campos-Melo et al., 2018). More than twenty autosomal-dominant mutations in NEFL have been identified as causes of Charcot–Marie–Tooth disease type 2E, an inherited axonal neuropathy. These mutations disrupt filament assembly and impair axonal transport, leading to progressive peripheral nerve degeneration (Didonna and Opal, 2019). The fact that pathogenic variants of NEFL alone can drive such a neuronopathy highlights its pivotal role in maintaining axonal integrity and demonstrates that NEFL dysfunction can directly initiate neurodegenerative processes.

b. ELAV like protein 3

ELAV-like protein 3 (ELAVL3) is a neuronal RNA-binding protein (RBP) that regulates RNA stability, alternative splicing, and translation within the central nervous system. It is highly expressed in mature neurons, where it binds preferentially to AU- and U-rich elements in the 3′ untranslated regions of target mRNAs, stabilising these transcripts and frequently enhancing their translation. Many ELAVL3 targets encode proteins critical for synaptic signalling, neuronal metabolism, and neurite outgrowth. ELAVL3 is indispensable for axonal integrity: knockout of Elavl3 in mice results in Purkinje-cell axonopathy characterised by swollen axons, disrupted synaptic terminals, and progressive ataxia, demonstrating that ELAVL3 maintains axonal transport and polarity, most likely by stabilising mRNAs essential for axonal structure and function (Ogawa et al., 2018).

The consequences of ELAVL3 upregulation are context dependent. Physiological increases occur during development and regeneration; for example, neurons upregulate ELAVL proteins after axonal injury to promote repair. Pathological or excessive elevation, however, can be harmful. In amyotrophic lateral sclerosis (ALS) models, overexpression of the closely related ELAVL4 protein is sufficient to cause synaptic dysfunction and neurodegeneration. Elevated ELAVL levels in motor neurons impair neuromuscular junctions and trigger apoptosis, a "gain-of-function" toxicity thought to arise when

surplus ELAVL proteins misprocess mRNAs and disturb the finely tuned neuronal proteome (Rosa et al., 2023).

The ELAVL family has been implicated in several neurodegenerative disorders, often indirectly. In Alzheimer's and Parkinson's diseases, ELAVL4 binds and stabilises mRNAs encoding proteins such as tau and amyloid-β precursor protein (APP), potentially influencing their expression and aggregation. Altered ELAVL expression or localisation has been reported in brains from patients with these disorders, although findings remain mixed. Furthermore, the Parkinson's-associated kinase LRRK2 can directly phosphorylate ELAVL4, and hyperactive LRRK2 may disrupt its RNA-binding function, leading to defects in neuronal mRNA regulation (Silvestri et al., 2022). Collectively, these observations highlight ELAVL3 and its relatives as important regulators of neuronal RNA metabolism whose dysregulation can contribute to neurodegenerative disease.

c. Dihydropyrimidinase-related protein 1

Dihydropyrimidinase-like 1 (DPYSL1), also known as collapsin response mediator protein 1 (CRMP1), is a cytosolic phosphoprotein expressed abundantly in neurons. In the adult brain, DPYSL1 and other CRMP family members remain present in axons and dendrites, indicating an ongoing role in maintaining synaptic connections and neuronal polarity (Xu et al., 2019). DPYSL1 is essential for normal synaptic plasticity- mice lacking this protein display deficits in hippocampal-dependent memory, underlining its importance for learning and higher cognitive function (Su et al., 2007).

Under conditions of neural injury or cellular stress, DPYSL1 signalling often becomes hyperactive, frequently through increased phosphorylation. This hyperactivation has marked consequences for neuronal architecture. Experimental work demonstrates that a phospho-mimicking DPYSL1 mutant (Thr509Asp) induces growth-cone collapse and neurite retraction, showing that excessive DPYSL1 activity destabilises axons and restricts their regenerative capacity. Thus, while basal DPYSL1 expression supports neuronal structure and connectivity, overactivation or overexpression can promote cytoskeletal instability and increase neuronal stress (Kawamoto et al., 2022).

Dysregulation of DPYSL1 has been implicated in multiple neurodegenerative disorders. Proteomic network analyses of Alzheimer's disease brain tissue identify DPYSL1 as a key regulator of disease-associated protein changes, consistent with its role in axonal and dendritic maintenance and its potential contribution to the early synaptic loss characteristic of Alzheimer's disease (Xu et al., 2019). Although direct evidence for DPYSL1 in Parkinson's disease remains limited, the semaphorin–DPYSL signalling axis is relevant to dopaminergic neuron connectivity and may influence Parkinson's disease pathology. Huntington's disease studies further suggest that mutant huntingtin disrupts DPYSL1 pathways, contributing to axonal transport defects and progressive neuronal degeneration (Kawamoto et al., 2022).

Collectively, these findings position DPYSL1 as a critical regulator of neuronal morphology and plasticity whose altered activity (whether through upregulation, aberrant phosphorylation, or mislocalisation) can drive or impair neurodegenerative processes in conditions such as Alzheimer's, Parkinson's, and Huntington's disease.

d. VGF

VGF is a secreted protein precursor highly expressed in the brain and regulated by neuronal activity as well as neurotrophic factors. It gives rise to a range of bioactive peptides that support synaptic plasticity, metabolism, and neurogenesis. Among these, TLQP-62 enhances long-term potentiation and activates the TrkB receptor (the primary receptor for brain-derived neurotrophic factor), thereby promoting downstream survival signalling. Other peptides derived from VGF, such as TLQP-21 and AQEE-30, have been implicated in regulating energy expenditure, modulating hippocampal synaptic strength, and facilitating β-amyloid clearance by microglia (Yu et al., 2023). VGF thus plays a dual role in coordinating both central and peripheral responses: centrally, it contributes to learning, memory, and neuroprotection, while peripherally, it modulates metabolic functions, including appetite regulation and circadian adaptation.

Elevated VGF expression appears beneficial for neuronal resilience, particularly under stress or pathological conditions. In transgenic models of Alzheimer's disease, VGF overexpression has been shown to enhance neurodegenerative phenotypes, improving

memory performance and reducing β-amyloid pathology (Beckmann et al., 2020). Some VGF-derived peptides also exhibit anti-inflammatory properties, such as dampening microglial activation in response to amyloid, suggesting that VGF may mitigate neuroinflammatory processes. Its expression is upregulated in models of nerve injury and Huntington's disease, supporting the idea that VGF induction constitutes an adaptive neuroprotective response to cellular stress (Yu et al., 2023).

However, in contrast to this protective upregulation observed in experimental models, human neurodegenerative diseases frequently show a consistent downregulation of VGF. Post-mortem analyses of brain tissue from patients with Alzheimer's disease and Parkinson's disease reveal reduced VGF mRNA and protein levels relative to age-matched controls. In Alzheimer's disease, diminished VGF expression in the cerebral cortex correlates with accelerated cognitive decline, as reported in quantitative proteomic studies. Similarly, in Parkinson's disease, significantly lower levels of VGF-derived peptides have been detected in cerebrospinal fluid and blood samples from affected individuals (Alqarni and Alsebai, 2022). This pervasive reduction suggests that the loss of VGF expression may be a shared pathological feature across neurodegenerative conditions, contributing to synaptic dysfunction and increased neuronal vulnerability (Beckmann et al., 2020). Overall, VGF emerges as a neuropeptide with protective roles in the brain, whose depletion in human disease states may exacerbate neurodegeneration.

e. Netrin-1

Netrin-1 is a secreted protein belonging to the netrin family and was originally identified as a canonical chemotropic cue essential for axon guidance (Cai et al., 2024). During embryonic neural development, netrin-1 plays a crucial role in directing neuronal migration and axonal pathfinding by establishing concentration gradients that attract or repel axonal growth cones, thereby contributing to the formation of precise neural circuits. Beyond its classical guidance role, netrin-1 exerts diverse functions in the nervous system, including modulation of neuronal adhesion, differentiation, and synaptogenesis. Critically, it also acts as a survival factor by engaging with so-called "dependence receptors." In the absence of netrin-1, these receptors activate

pro-apoptotic signalling pathways; however, when netrin-1 is present, they inhibit apoptosis and promote neuronal survival. This mechanism positions netrin-1 as a key regulator of neuronal longevity through suppression of cell death signalling cascades.

Importantly, netrin-1 expression is not restricted to development. In the adult human brain, it remains expressed across multiple regions, with particularly high levels in midbrain dopaminergic nuclei, suggesting a role in maintaining adult neuronal integrity and synaptic architecture. Experimental models support it- the exogenous netrin-1 administration has been shown to restore synaptic function and memory in otherwise impaired rodents. For instance, repeated delivery of netrin-1 in mice with amyloid β-induced cognitive deficits successfully restored long-term potentiation and improved memory performance, highlighting its ability to counteract synaptic stress and enhance resilience (Zamani et al., 2020).

Nevertheless, netrin-1 upregulation in neurodegenerative contexts may produce complex, context-dependent effects. In Alzheimer's disease, netrin-1 expression is increased, and it interacts with both amyloid β (Aβ) peptides and the amyloid precursor protein (APP). On one hand, this interaction may be beneficial: netrin-1 has been shown to shift APP processing towards non-amyloidogenic pathways, therefore reducing Aβ production. On the other hand, netrin-1's direct binding to Aβ may facilitate its aggregation and incorporation into amyloid plaques. Indeed, studies in Alzheimer's models demonstrate that Aβ accumulation induces netrin-1 expression, which in turn promotes Aβ clustering through direct molecular interactions (Cai et al., 2024). This suggests a potentially self-perpetuating cycle, where netrin-1 upregulation, initially a protective response, becomes co-opted into disease pathology by exacerbating plaque formation.

In summary, netrin-1 upregulation is generally associated with neuroprotective effects, including suppression of apoptosis and support for synaptic function. However, in chronic neurodegenerative diseases such as Alzheimer's, the biochemical context in which netrin-1 is elevated may determine whether its actions are protective or inadvertently pathogenic. This duality highlights the nuanced role of netrin-1 in disease

progression and suggests that its therapeutic modulation must be approached with precision.

3.2.7 Validation of proteomic analysis

As this proteomic analysis identified an even greater number of potentially relevant proteins upregulated in the absence of CHIP, several were selected for further validation. These candidates were among the top seven most significantly upregulated proteins in the CHIP KO cells compared with WT controls. The expanded list of differentially expressed proteins may be attributed to the use of a more advanced mass spectrometer (Orbitrap Exploris) known for its enhanced sensitivity, resolution, and dynamic range, which facilitates deeper proteome coverage and more precise quantification.

The proteins selected for further validation included VGF, NEFL, INA, and ELAVL3. In particular, both VGF and NEFL had previously been reported to show increased abundance in SH-SY5Y CHIP KO cells under both differentiated and undifferentiated conditions, based on quantitative proteomic analyses documented in the doctoral thesis of Dr Catarina Dias (Dias, 2019). These earlier findings added further confidence to their selection. As with earlier validation efforts, cost considerations necessitated a focused approach, and only a subset of promising candidates was pursued. Nevertheless, the selection prioritised biologically relevant proteins with established links to neuronal function and neurodegeneration, thus strengthening the rationale for their further investigation.

SH-SY5Y WT and CHIP KO cells were cultured and harvested, with each sample derived from an individual well. In line with standard practice for validation experiments, three biological replicates per condition were prepared, each with corresponding technical replicates to ensure reproducibility and statistical robustness. Validation of the selected proteins of interest was performed via immunofluorescence microscopy, following previously described protocols. Cells were stained using specific antibodies targeting the candidate proteins, and representative images of SH-SY5Y WT and CHIP KO cells were acquired (Figure 9a–d).

The loss of CHIP resulted in a pronounced increase in the expression and altered subcellular localisation of key neuronal proteins in SH-SY5Y cells. VGF exhibited a distinct punctate cytoplasmic staining pattern, indicative of its accumulation within vesicular or secretory granules, consistent with its known role as a neuropeptide precursor. ELAVL3 and alpha-internexin (INA) both showed strong cytoplasmic and perinuclear signals with regions of co-localisation, aligning with their respective roles in RNA-binding and intermediate filament assembly within the neuronal cytoskeleton. NEFL displayed intense filamentous labelling extending along neurite-like projections, characteristic of mature neurofilament network organisation.

Taken together, these staining patterns suggest that CHIP deletion promotes not only the upregulation but also the spatial redistribution of neuronal cytoskeletal and RNA-regulatory proteins. This is consistent with enhanced structural remodelling reminiscent of axonal differentiation or injury responses, supporting the hypothesis that CHIP plays a critical role in maintaining neuronal architecture under basal conditions.

To provide independent validation of the proteomic findings, the same primary antibodies used in the immunofluorescence assays were sent to Dr Janette Popławski in the Proteostasis and Immune Signalling in Cancer and Dementia group, led by Professor Kathryn Ball at the University of Edinburgh. These antibodies were applied to a second SH-SY5Y CHIP KO cell line (an independently generated CRISPR-edited model of CHIP deficiency), together with its isogenic wild-type control. Western blot analysis confirmed elevated expression of alpha-internexin (INA), VGF, neurofilament medium chain (NEFM), and neurofilament light chain (NEFL) (Figure S2).

This independent replication in a parallel knockout model, conducted in a separate laboratory setting, provides strong orthogonal support for the mass spectrometry results. The consistent upregulation of neuronal structural proteins across two distinct CHIP-deficient cell lines, and the use of the same antibody panel under different experimental conditions, strengthens the conclusion that these proteomic changes are a robust and reproducible consequence of CHIP loss. These findings reduce the likelihood that the observed expression patterns arose from clonal variation, antibody artefacts, or laboratory-specific variables, and instead support the interpretation that CHIP plays a fundamental role in maintaining neuronal proteome homeostasis.

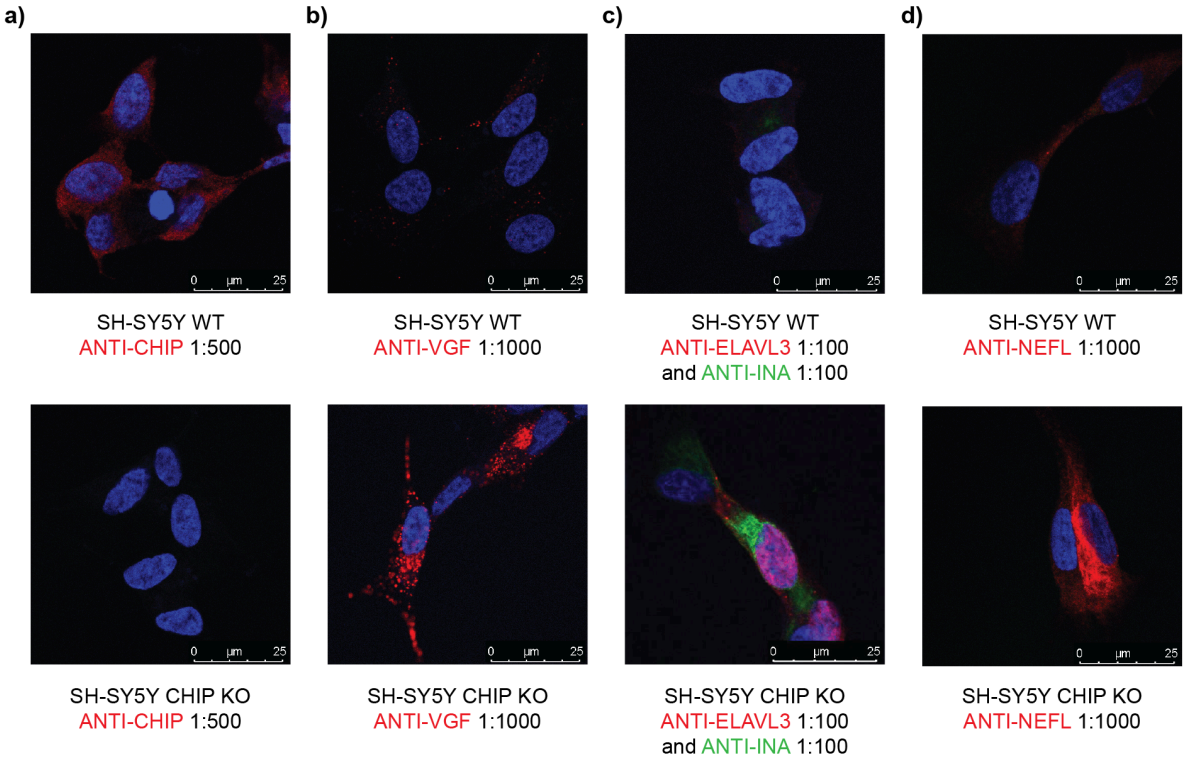

Figure 9: CHIP deletion induces cytoplasmic accumulation and filamentous localisation of neuronal proteins in SH-SY5Y cells

Representative confocal micrographs of wild-type (WT, top row) and CHIP-knockout (KO, bottom row) SH-SY5Y neuroblastoma cells. (a) CHIP immunostaining (red) used to verify loss of the protein in KO cells. (b) VGF immunostaining (red). (c) Dual staining for ELAVL3 (red) and INA (green). (d) NEFL immunostaining (red). Cell nuclei are counterstained with DAPI (blue) in all panels.

4.3.1 Overview of changes in the proteome of cells under heat shock conditions.

A hallmark feature of neurodegenerative diseases is proteotoxic stress, characterised by the accumulation of misfolded or aggregated proteins that overwhelm the cellular protein quality control machinery. In vitro, one common method of modelling proteotoxicity involves subjecting cells to heat shock (Wallace et al., 2015). Acute thermal stress, such as exposure to 42 °C for one hour, disrupts protein homeostasis by inducing protein unfolding and misfolding, which in turn triggers the formation of aggregates and activates the cellular heat shock response (HSR). The HSR is an evolutionarily conserved pro-survival pathway that upregulates heat shock proteins (HSPs) to re-establish proteostasis. These chaperones assist in the refolding of denatured proteins, prevent irreversible aggregation, and facilitate degradation of irreparable proteins. Many HSR components are considered neuroprotective, and heat shock proteins have been found co-localised with pathological aggregates in Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis, implying that the HSR is engaged under disease conditions (San Gil et al., 2017). Consequently, the heat shock model provides a valuable experimental framework to simulate proteotoxic stress and to study cellular coping mechanisms, showing similarities with the protein-folding challenges encountered in neurodegenerative disorders.

To evaluate the role of CHIP in shaping the proteomic response to proteotoxic stress, SH-SY5Y human neuroblastoma cells were subjected to heat shock at 42 °C for one hour, with or without an additional one-hour recovery phase at physiological temperature (37 °C). Both WT and CHIP-KO SH-SY5Y cell lines were subjected to the same experimental conditions (including untreated control, heat shock alone, and heat shock followed by recovery), enabling direct comparisons of proteomic adaptation in the presence or absence of CHIP. Incorporating a post-stress recovery interval was critical, as this period allows cells to prepare proteostasis mechanisms and attempt functional restoration. During recovery, aggregates formed during heat shock may be disassembled and refolded by molecular chaperones. Indeed, previous studies have demonstrated that non-lethal heat-induced protein aggregates are fully reversible upon

return to regular conditions, with proteins regaining solubility and functionality (Xu et al., 2012). This design allowed assessment of both immediate proteotoxic damage and subsequent proteome recovery, including the efficiency of chaperone-mediated repair processes.

At the mechanistic level, CHIP plays a crucial role in activating the HSR. It facilitates the transcriptional activation of heat shock factor 1 (HSF1), which is essential for the induction of key chaperones such as HSP70. Consistent with this role, previous studies have shown that depletion of CHIP impairs HSP70 induction during heat stress, thereby reducing the cell's capacity to buffer misfolded proteins and resolve aggregates. Therefore, it was anticipated that CHIP-deficient cells would display a distinct proteomic trajectory under heat stress compared with their WT counterparts (Dias et al., 2021).

Results from the label-free quantitative proteomic analysis are presented in Figure 10a–c and Supplementary Figure S3. The most pronounced differences in protein expression were observed between CHIP KO and WT under control (unstressed) conditions (Figure 10a and S3e), yielding several newly deregulated candidates of potential interest. Interestingly, this contrast was greater than that observed when comparing CHIP KO versus WT cells under heat shock (Figure 10b and S3f), suggesting that baseline proteostatic differences between the two genotypes were more prominent than their acute stress responses. A similarly marked difference was seen between the CHIP KO and WT cells following heat shock and subsequent recovery (Figure 10c and S3c). Moderate proteomic changes were observed when comparing post-recovery samples with their respective untreated controls for both WT and KO lines (Figure S3a and S3h).

In contrast, the lowest levels of differential protein expression were observed across several within-cell line comparisons: CHIP KO cells following heat shock versus post-recovery (Figure S3b), CHIP KO control versus heat shock (Figure S3d), WT control versus WT heat shock (Figure S3g), and WT heat shock versus post-recovery (Figure S3i). The most significantly upregulated and downregulated proteins in each condition are summarised in Supplementary Tables S4–S9. Remarkably, several proteins that were highly dysregulated in control conditions between CHIP KO and WT also appeared among the most altered proteins under heat shock or recovery conditions. These overlapping hits were considered of particular interest and are discussed in the following

section, alongside a selection of additional candidates that exhibited marked dysregulation under specific experimental conditions.

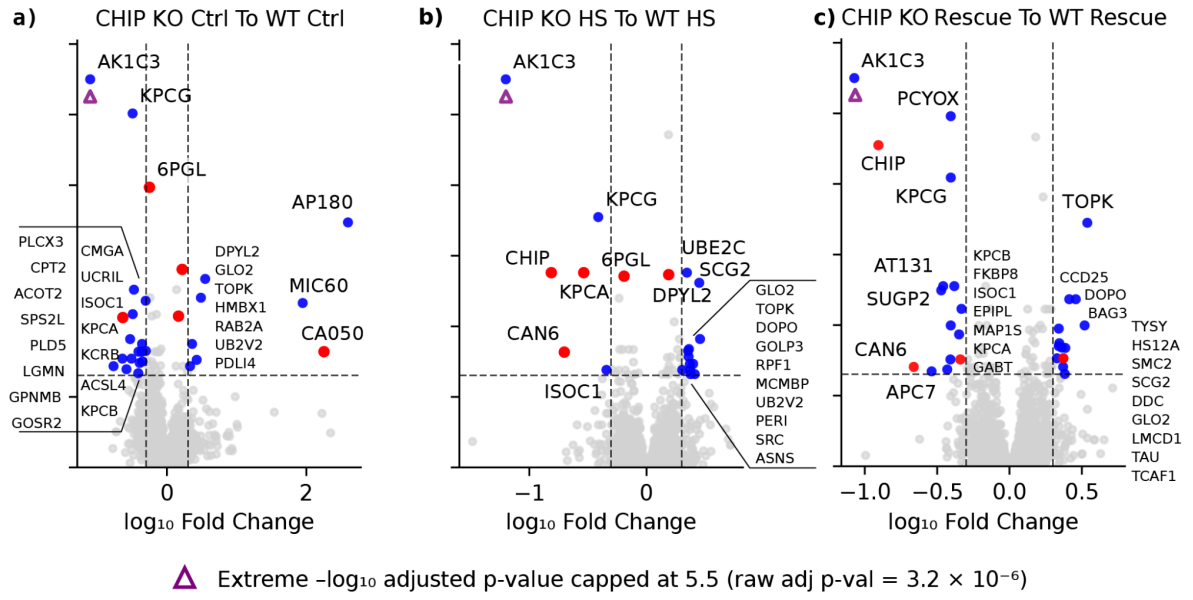

Figure 10: Chosen comparative proteomic analysis of SH-SY5Y WT and CHIP-KO cells cultured under heat shock conditions.

Volcano plots summarising label-free LC–MS/MS comparisons of SH-SY5Y CHIP-knockout (KO) and wild-type (WT) cells. (a) Baseline: differential protein expression between CHIP-KO and WT cells under standard culture conditions. (b) Heat shock: proteomic changes between CHIP-KO and WT cells after a 1 h heat shock at 42 °C. (c) Recovery: proteomic differences between CHIP-KO and WT cells after 1 h heat shock followed by a 1 h recovery at 37 °C. For each plot, the x-axis shows signed $\log_2$ fold change and the y-axis shows $-\log_{10}$ adjusted p-value. Proteins exceeding $|\log_2 FC| > 1$ (fold change > 2) with adjusted $p \leq 0.05$ are deemed significant. To prevent axis compression, $-\log_{10}$ p-values above 5.5 are capped, with higher outliers indicated by open purple triangles at their true x-positions. Grey points represent non-significant proteins, blue points indicate significant hits, and key candidate proteins are highlighted in red. Horizontal and vertical dashed lines mark the significance ($p = 0.05$) and $\pm\log_2(2)$ fold-change thresholds, respectively.

4.3.2 Proteins of interest

a. 6-phosphogluconolactonase

6-phosphogluconolactonase (PGLS) is a key enzyme within the pentose phosphate pathway (PPP), a metabolic route critical in neurons for the production of nicotinamide adenine dinucleotide phosphate (NADPH). In the central nervous system, NADPH plays a vital role in maintaining redox balance by fuelling antioxidant defences, including the regeneration of reduced glutathione. Under physiological conditions, the PPP is particularly important in neurons due to their high metabolic demands and susceptibility to oxidative stress. Downregulation of PGLS impairs the efficiency of the PPP, leading to decreased NADPH availability. This reduction compromises neuronal resilience to oxidative insult, thereby increasing the risk of reactive oxygen species-induced damage, dysfunction, and ultimately neuronal death (Bouzier-Sore and Bolanos, 2015).

A similar disruption in redox homeostasis is observed in several neurodegenerative diseases, where oxidative stress is a well-established pathogenic feature. In both Alzheimer's disease and Parkinson's disease, impaired PPP activity has been associated with elevated oxidative burden and mitochondrial dysfunction. In particular, studies in Parkinson's disease models demonstrate that compromised PPP function contributes to selective dopaminergic neurodegeneration and exacerbates chronic neuroinflammation (Tu et al., 2019). These findings highlight the potential significance of PGLS downregulation in promoting neuronal vulnerability through impaired antioxidant defence mechanisms.

b. Calpain-6

Calpain-6 (CAPN6) is an atypical member of the calpain family, a group of calcium-dependent proteases, distinguished by its lack of catalytic activity. Unlike conventional calpains, CAPN6 lacks critical residues in its active site and is thus considered proteolytically inactive. Instead, it appears to have non-enzymatic regulatory functions, including roles in cytoskeletal dynamics and cell signalling. CAPN6 possesses a microtubule-binding domain and is known to localise to the cytoskeleton, suggesting a structural or modulatory role within neuronal cells (Chen et al., 2020).

In neurons, downregulation of CAPN6 may result in the loss of these protective or stabilising functions. One proposed mechanism is that CAPN6 normally restrains pro-apoptotic pathways by inhibiting the activity of executioner proteases or by dampening apoptotic signalling cascades. Its reduction could, therefore, enable these pathways, making neurons more susceptible to programmed cell death, particularly under stress conditions. In addition, due to its association with microtubules, decreased CAPN6 levels may impair cytoskeletal stability or plasticity. Evidence from developmental studies has shown that CAPN6 downregulation correlates with increased cytoskeletal remodelling, as observed in the maturing auditory cortex (Hackett et al., 2015). In mature neurons, a similar reduction may compromise axonal architecture and disrupt the transport of organelles and synaptic components, processes that are essential for neuronal function and survival.

More broadly, dysregulation of the calpain system has been implicated in a range of neurodegenerative disorders. Overactivation of classical calpains such as calpain-1 and calpain-2, driven by calcium influx, can lead to cleavage of essential neuronal proteins, contributing to excitotoxicity in conditions such as stroke and Alzheimer's disease. CAPN6, by contrast, may serve a regulatory function that counterbalances such proteolytic damage. Its downregulation could shift this balance towards unregulated protease activity, thereby aggravating neurodegenerative processes. Indeed, bioinformatic analyses have linked CAPN6 to several neurodegenerative disease-related pathways, including amyloid precursor protein processing, Huntington's disease signalling, and ALS pathways.

In models of spinal cord injury and multiple sclerosis, where axonal degeneration is prominent, the Rho/ROCK signalling pathway has been shown to inhibit regeneration. CAPN6 downregulation in these contexts could remove an inhibitory checkpoint on apoptosis, thus compounding injury. Conversely, some developmental studies indicate that CAPN6 deficiency can promote cellular outgrowth, as observed in muscle cells, by relieving a suppressive signal (Tonami et al., 2011). These findings underline the context-dependent nature of CAPN6 function. In the setting of chronic neurodegenerative diseases, downregulation of CAPN6 may contribute to the destabilisation of neuronal cytoskeleton, loss of trophic support, and increased vulnerability to degeneration.

c. Dihydropyrimidinase-like 2

Dihydropyrimidinase-like 2 (DPYSL2) encodes collapsin response mediator protein 2 (CRMP2), a cytosolic protein abundantly expressed in neurons. DPYSL2 is essential for neuronal development and axonal guidance. It transfers signals from Semaphorin-3A to mediate growth cone collapse and plays a key role in stabilising microtubules within axons and dendrites. By binding to tubulin heterodimers, DPYSL2 promotes microtubule polymerisation, therefore facilitating axon elongation, branching, and overall cytoskeletal integrity. Besides its structural functions, DPYSL2 interacts with synaptic vesicle proteins and voltage-gated calcium channels, regulating neurotransmitter release.

Following neural injury or cellular stress, neurons often upregulate DPYSL2 in an effort to promote axonal regeneration and synaptic remodelling. Experimental models have demonstrated that DPYSL2 contributes to recovery post-nerve injury, and genetic variants that enhance its function may confer neuroprotective benefits. However, this compensatory upregulation can become maladaptive under certain conditions. DPYSL2 activity is tightly regulated by phosphorylation, and in pathological states, upregulated DPYSL2 is frequently hyperphosphorylated, rendering it inactive. This aberrant phosphorylation not only disrupts its microtubule-binding capacity but may also promote its aggregation. Furthermore, excessive DPYSL2 can disturb calcium channel function, with downstream effects on synaptic signalling (Feuer et al., 2023).

DPYSL2 dysregulation has been implicated in multiple neurodegenerative disorders. In Alzheimer's disease, it is a known substrate of glycogen synthase kinase-3β (GSK-3β), the same kinase responsible for tau hyperphosphorylation. When phosphorylated by GSK-3β, DPYSL2 loses its ability to bind microtubules, which may compromise axonal transport and contribute to synaptic degeneration. Post-mortem studies of Alzheimer's disease brains have identified truncated CRMP2 fragments and phosphorylated DPYSL2 co-localised with tau in neurofibrillary tangles, implicating DPYSL2 in the structural pathology of the disease. Genetic studies have further linked DPYSL2 variants with increased susceptibility to late-onset Alzheimer's disease and other dementias (Mohallem et al., 2024). Beyond neurodegeneration, DPYSL2 is also a prominent candidate gene in neurodevelopmental and psychiatric disorders, including

schizophrenia, underscoring its broad role in neuronal connectivity and function (Feuer et al., 2023).

In this context, DPYSL2 upregulation may represent a double-edged sword. While initially a neuroprotective response aimed at structural repair, it risks contributing to pathology if dysregulated or made dysfunctional by upstream signalling abnormalities such as kinase overactivation.

d. Protein kinase C alpha

Protein kinase C alpha (PKCα) is a serine/threonine kinase that plays a critical role in neuronal signalling and synaptic function. It phosphorylates a wide range of substrate proteins involved in neurotransmitter release, receptor sensitivity, and ion channel regulation. In dopaminergic neurons, PKCα modulates both dopamine release and receptor signalling, contributing to the neuronal activity. Under physiological conditions, PKCα shows broadly neuroprotective effects by phosphorylating proteins that inhibit apoptotic cascades. However, under pathological conditions, such as ischaemic injury, excessive PKC activation has been implicated in excitotoxic damage, illustrating its context-dependent effects.

Downregulation of PKCα disrupts multiple signalling pathways essential for neuronal health. A primary consequence is reduced synaptic efficacy, as PKCα activity is necessary for optimal synaptic vesicle release and post-synaptic receptor modulation. This can impair synaptic plasticity, contributing to memory dysfunction or motor impairment depending on the neuronal circuits involved. Moreover, diminished PKCα activity shifts the phosphorylation balance of several key proteins. In models of Alzheimer's disease, reduced PKCα function has been associated with increased production of β-amyloid and hyperphosphorylation of tau. This is partly due to reduced inhibition of glycogen synthase kinase-3β (GSK-3β), a major tau kinase, thereby promoting neurofibrillary tangle formation, a hallmark of Alzheimer's pathology (Singh et al., 2024).

PKCα has been extensively investigated in the context of Alzheimer's disease. Post-mortem studies have revealed alterations in both total levels and membrane-bound active PKCα in affected brain regions, with some reports noting a marked reduction in its activity. Furthermore, PKCα is implicated in the pathophysiology of Parkinson's disease through its role in dopamine signalling. It modulates the sensitivity of dopamine receptors and regulates dopamine release within the striatum, a region essential for motor control. Disruptions in PKCα signalling may therefore contribute to the motor deficits characteristic of Parkinson's disease and may also indirectly influence α-synuclein aggregation. Furthermore, aberrant PKC signalling has been observed in tauopathies and frontotemporal dementias, suggesting a broader relevance across neurodegenerative conditions (Wang et al., 1994).

In summary, PKCα downregulation removes a key modulatory layer that maintains synaptic integrity and regulates the phosphorylation state of proteins involved in neurodegeneration. Its loss may aggravate toxic protein accumulation and contribute to the progression of neurodegenerative diseases, particularly Alzheimer's and Parkinson's disease.

e. HMBX1

HMBOX1 (Homeobox-containing 1) is a transcription factor belonging to the homeobox gene family, expressed across a variety of tissues, including the brain, although its abundance varies depending on cellular context. It is especially elevated in certain immune cell types (Jiang et al., 2023). As a transcriptional regulator, HMBOX1 influences gene expression in a tissue-specific manner. In endothelial cells, for instance, it has been shown to interact with the metallothionein MT2A to modulate intracellular zinc homeostasis. This interaction leads to the suppression of apoptotic signalling and the activation of autophagic pathways, thereby supporting cell survival under stress conditions. In addition, HMBOX1 plays a role in regulating immune cell dynamics, particularly in modulating the function and differentiation of natural killer (NK) cells (Ma et al., 2015).

In contrast to other proteins discussed in this thesis, HMBOX1 has not yet been extensively characterised in the context of classical neurodegenerative disorders. It does not appear prominently in the literature relating to Alzheimer's disease or Parkinson's disease. Nonetheless, emerging evidence suggests it may still hold relevance in neurodegenerative contexts. For example, systematic analyses of gene expression following neuronal injury have identified HMBOX1 among genes implicated in promoting neuronal survival. Its capacity to enhance autophagy provides a possible link to neurodegenerative diseases such as Alzheimer's, where impaired autophagic clearance of misfolded proteins like amyloid-beta and hyperphosphorylated tau is a well-documented pathological hallmark (Wu et al., 2011).

Although direct functional studies remain limited, it is possible that HMBOX1 upregulation in neurodegenerative states may reflect an intrinsic cellular attempt to restore proteostatic balance and prevent neurodegeneration. If so, its role may be compensatory, contributing to neuronal resilience by enhancing autophagic clearance mechanisms. Further studies will be required to establish whether HMBOX1 represents a novel neuroprotective factor or a marker of cellular stress in the diseased brain.

f. Tau

Tau is a microtubule-associated protein highly enriched in neuronal axons, where it binds to tubulin and facilitates the organisation and stabilisation of microtubule (MT) tracks. It modulates MT dynamics and plays a crucial role in neurite outgrowth and the efficient transport of cargo along axons. Beyond its role in axonal architecture, tau influences interactions between the microtubule and actin cytoskeletons, supports motor-driven organelle trafficking, and contributes to the structural integrity of dendritic spines, thereby affecting synaptic function (Barbier et al., 2019).

When tau is abnormally elevated, such as through MAPT gene duplication, or undergoes excessive phosphorylation, it loses its affinity for microtubules, detaches, and misfolds. Hyperphosphorylated tau progressively accumulates as soluble oligomers and caspase-cleaved fragments before aggregating into insoluble paired helical filaments, which ultimately form neurofibrillary tangles. Pre-tangle tau species have been shown to

exhibit direct neurotoxicity in mouse models, impairing synaptic and cellular functions even before overt tangle formation (Dickey et al., 2006).

The pathological accumulation of tau underlies several neurodegenerative disorders collectively referred to as tauopathies. In Alzheimer's disease, hyperphosphorylated tau aggregates predominantly in the hippocampus and cortex, forming neurofibrillary tangles that spread in a stereotypical pattern associated with disease progression. The extent of tau pathology correlates more strongly with synaptic loss and cognitive decline than does amyloid burden, positioning tau as a central driver of neurodegeneration in Alzheimer's disease (Barbier et al., 2019).

Cellular clearance of pathological tau relies heavily on the heat shock protein (HSP)-mediated proteostasis machinery. In particular, HSP70-bound phospho-tau is recognised by the E3 ubiquitin ligase CHIP (C-terminus of HSC70-Interacting Protein, also known as STUB1), which catalyses its polyubiquitination and directs it toward proteasomal degradation. Experimental evidence demonstrates that CHIP deficiency impairs this quality-control mechanism: CHIP KO mice accumulate elevated levels of hyperphosphorylated and caspase-cleaved tau, accompanied by signs of neuronal stress. In particular, these mice display increased levels of soluble tau species without necessarily forming insoluble tangles, underscoring the role of CHIP in managing early-stage tauopathy (Dickey et al., 2006).

CHIP therefore serves as a critical tau surveillance factor, selectively targeting aberrant, phosphorylated tau species for degradation. Impairment of this regulatory axis, whether through genetic deletion, functional inhibition, or overload of the chaperone system, permits toxic tau species to accumulate, exacerbating proteotoxic stress and contributing to the progression of neurodegenerative disease. The disruption of this pathway in CHIP-deficient contexts highlights the essential role of CHIP in preserving neuronal proteostasis and maintaining tau homeostasis under both physiological and pathological conditions.

4.3.3 Cross-platform verification of proteomic findings by SWATH-MS

To further substantiate the proteomic differences identified in my Orbitrap Exploris 480 LC–MS/MS experiments, I examined an independently generated SWATH-MS dataset produced by a collaborating laboratory at University of Edinburgh Prof Kathryn Ball Research Group. This dataset was acquired for an unrelated project using the same SH-SY5Y WT and CHIP KO cell models but analysed by data-independent acquisition (DIA/SWATH) rather than data-dependent acquisition (DDA). Although I was not involved in generating these samples, I obtained permission to review the processed results. Remarkably, the SWATH analysis reproduced the key protein changes revealed in my own DDA-based proteomics, including the up-regulation of VGF, DPYSL2, NEFM, NEFL and ELAVL3 and other top candidates (Table S4). Because SWATH provides comprehensive, quantitative, label-free coverage of virtually all detectable peptides in a sample without pre-selection, this constitutes a powerful orthogonal validation of my findings. Independent confirmation of the same CHIP-dependent proteomic signature by a distinct acquisition strategy, performed on different instrumentation and by an external group, greatly strengthens the robustness and generalisability of the conclusions presented in this thesis.

4.3.4 Predicted CHIP interaction network with proteins implicated in neurodegenerative pathways.

Given the observation that loss of CHIP influences the abundance of multiple proteins implicated in neuronal homeostasis, the next step involved exploring potential regulatory pathways that might support these effects. This was approached through a combination of protein–protein interaction network analysis using the STRING database and an extensive review of relevant literature.

One proposed pathway suggests that CHIP exerts its regulatory influence via modulation of HSPA7 (a member of the HSP70 family), which in turn regulates ELAVL3 -a neuron specific RNA binding protein known to stabilise mRNA transcripts encoding neuronal cytoskeletal components. According to STRING-based interaction models, this cascade may extend to impact key structural proteins such as DPYSL2, NEFM, NEFL, and

INA (Figure 11a). Mechanistically, CHIP is known to physically associate with HSP70 family chaperones to facilitate client protein folding or target misfolded proteins for proteasomal degradation. ELAVL3 (also known as HuC), a member of the neuronal ELAV-like family of RNA-binding proteins, binds AU- and U-rich sequences in the 3′ untranslated regions of neuronal mRNAs, thereby modulating their stability and splicing (Ogawa et al., 2018). Experimental evidence from mice models has demonstrated that loss of ELAVL3 disrupts neuronal polarity and results in Purkinje cell axon degeneration, attributed to mis-splicing of critical neural transcripts. Taken together, these findings support a model in which CHIP–HSP70 complexes modulate the expression and function of key axonal proteins indirectly through the ELAVL3-mediated regulation of mRNA targets, aligning with CHIP's established role in proteostasis and HuC's function in neuronal RNA metabolism (Liu et al., 2018; Ogawa et al., 2018).

Along with the HSPA7–ELAVL3 axis, further network analysis points to several alternative routes by which CHIP may influence the expression of neuropeptides such as NPY. These are illustrated in Figure 11b and suggest three putative regulatory pathways. First, CHIP may act via the transforming growth factor-beta (TGF-β) signalling axis, particularly through SMAD3. The SMAD3 protein, a transcriptional effector of TGF-β, has been shown to recruit the histone acetyltransferase EP300 (also known as p300), enhancing transcriptional activity through chromatin remodelling. Particularly, EP300 directly binds the NPY gene promoter and facilitates its activation via deposition of histone acetylation marks (Melas et al., 2013). Secondly, CHIP may affect NPY expression via the glucocorticoid receptor NR3C1, which has been shown to strongly induce NPY mRNA and protein expression. This induction is abolished by pharmacological inhibition of the receptor, suggesting a robust transcriptional regulatory role (Ma et al., 2012). Thirdly, interaction network analyses position NPY and the oestrogen receptor ESR1 within ERBB2-related signalling modules, implying a potential CHIP–ERBB2–ESR1 axis. Given that ERBB2 signalling intersects with pathways regulating synaptic plasticity and stress responses, it is plausible that CHIP-mediated modulation of this axis could influence neuropeptide gene expression, including that of VGF and NPY (Jin et al., 2022).

While these models remain hypothetical, they provide a framework for testable mechanistic hypotheses. The logical next step in validating these proposed pathways would involve targeted immunoprecipitation (IP) assays to determine whether CHIP

physically associates with HSPA7, ELAVL3, SMAD3, EP300, NR3C1, or ERBB2 in SH-SY5Y cells. Confirming such interactions would strengthen the case for CHIP functioning as an upstream modulator of neuronal RNA-binding proteins and transcriptional regulators involved in neuropeptide expression and structural remodelling.

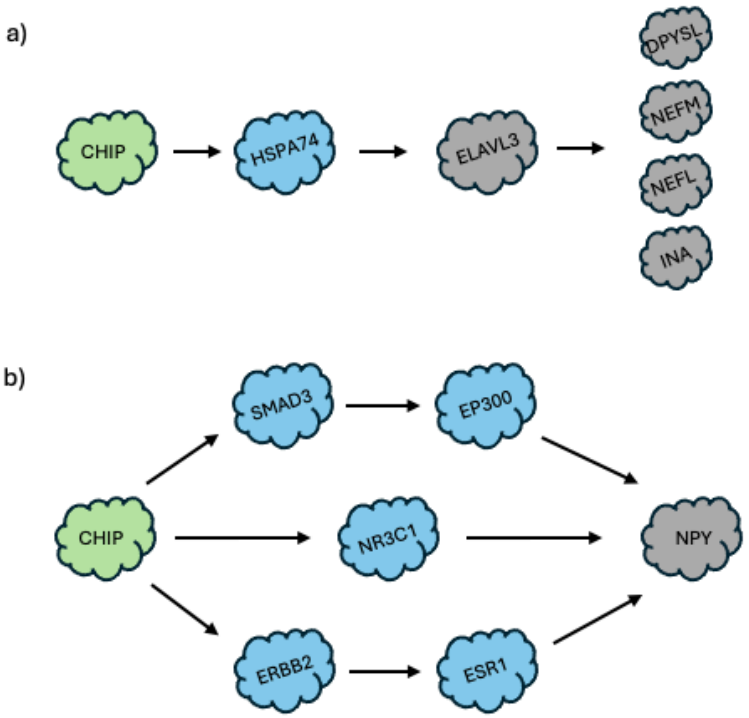

Figure 11. Proposed CHIP dependent regulatory network linking chaperon pathways to neuronal cytoskeletal and neuropeptide proteins.

(a) Proposed CHIP-HSP74-ELAVL3 axis controlling DPYSL2, NEFM, NEFL and INA expression. (b) Proposed CHIP signalling through SMAD3/EP300, NR3C1, or ERBB2/ESR1 pathways regulating NPY expression.

4.3.5 In-Silico Investigation of STUB1 Variants Using Genome-Wide Association Studies.

To assess whether genetic variation in STUB1 contributes to neurodegenerative disease susceptibility, an in-silico analysis of publicly available genome-wide association study (GWAS) datasets was conducted in collaboration with Dr Georges Bedran. The analysis encompassed eight Parkinson's disease cohorts (8,849 cases and 11,161 controls) and three Alzheimer's disease cohorts (89,641 cases and 421,268 controls). GWAS have been instrumental in uncovering common genetic risk factors for complex diseases. By

comparing allele frequencies of millions of single nucleotide polymorphisms (SNPs) between well-matched cases and controls, these studies can pinpoint genomic loci associated with disease predisposition in an unbiased manner.

In this investigation, an intronic variant in STUB1 (rs141948106) showed a nominal association with Alzheimer's disease ($p = 0.01$). While this does not meet the stringent genome-wide significance threshold typically applied in GWAS ($p < 5 \times 10^{-8}$), it suggests a potential link between STUB1 and Alzheimer's pathology that warrants further exploration. Caution is necessary when interpreting these results, given the recognised limitations of GWAS, including their dependency on predefined common variants, limited power to detect rare alleles, and incomplete capture of genetic architecture contributing to disease heritability.

Future studies incorporating rare variant analysis, functional validation, and multi-omics integration may better delineate the contribution of STUB1 to neurodegenerative disease risk.

Chapter 5: CHIP as a potential marker of neurodegeneration.

5.1 Signalling pathways shaped by CHIP

CHIP is increasingly recognised for its involvement in neurodegenerative processes, although the extent of its role remains incompletely defined. While several reports have highlighted its neuroprotective properties, systematic investigation of its function in neuronal systems has been limited. At the time this doctoral project was conceived, the precise role of CHIP in neurons, particularly at the proteome level, was poorly characterised. The present thesis was therefore designed to address this gap by defining how CHIP loss alters the neuronal proteomic landscape and evaluating whether these perturbations intersect with pathways implicated in neurodegeneration. The data presented here provide compelling evidence that CHIP significantly influences neuronal integrity, particularly through its regulation of proteins involved in neurofilament organisation and cytoskeletal maintenance.

To understand CHIP's impact on cellular signalling networks, a quantitative proteomic comparison was undertaken between CRISPR/Cas9-engineered CHIP KO SH-SY5Y neuroblastoma cells and their isogenic WT counterparts. A total of ~4,235 proteins were quantified, and differential expression analysis (false discovery rate ≤ 0.05) was followed by Ingenuity Pathway Analysis (IPA) to identify significantly enriched canonical pathways. Pathways with IPA z-scores ≤ –1 or ≥ 1 were prioritised, corresponding to predicted pathway inhibition or activation, respectively. The resulting dataset revealed a broad suppression of signalling pathways in CHIP KO cells, with one prominent exception: a modest up-regulation of the NF1/Ras-MAPK cascade (Figure 12). The following sections dissect these pathway alterations into functional themes, including G-protein–coupled receptor (GPCR) and G-protein signalling, cytoskeletal and mitotic control, immune and inflammatory processes, metabolic and secretory pathways, and neuron-specific or axon-guidance modules.

Within these categories, recurring molecular disruptions were observed. These include the downregulation of PRKCA, PRKCB, and PRKCG (encoding isoforms of protein kinase C), GNG2 (encoding a G-protein **γ**-subunit), and DPYSL2(encoding CRMP2, a cytoskeletal regulatory protein). These changes are interpreted in the context of CHIP's E3 ubiquitin ligase function, which is known to coordinate proteostasis by targeting misfolded or

excess proteins for proteasomal degradation. The selective loss of CHIP is hypothesised to destabilise multiple signalling axes, leading to reduced activity in pathways critical for neuronal structure, stress response, and metabolic adaptation.

Limitations of the current study are acknowledged, particularly regarding the observational nature of pathway inference and the lack of functional validation for certain nodes. Nonetheless, this analysis provides a strong rationale for future mechanistic studies, including CHIP interactome mapping, phospho-proteomic profiling, and loss-of-function rescue assays in neuronal contexts.

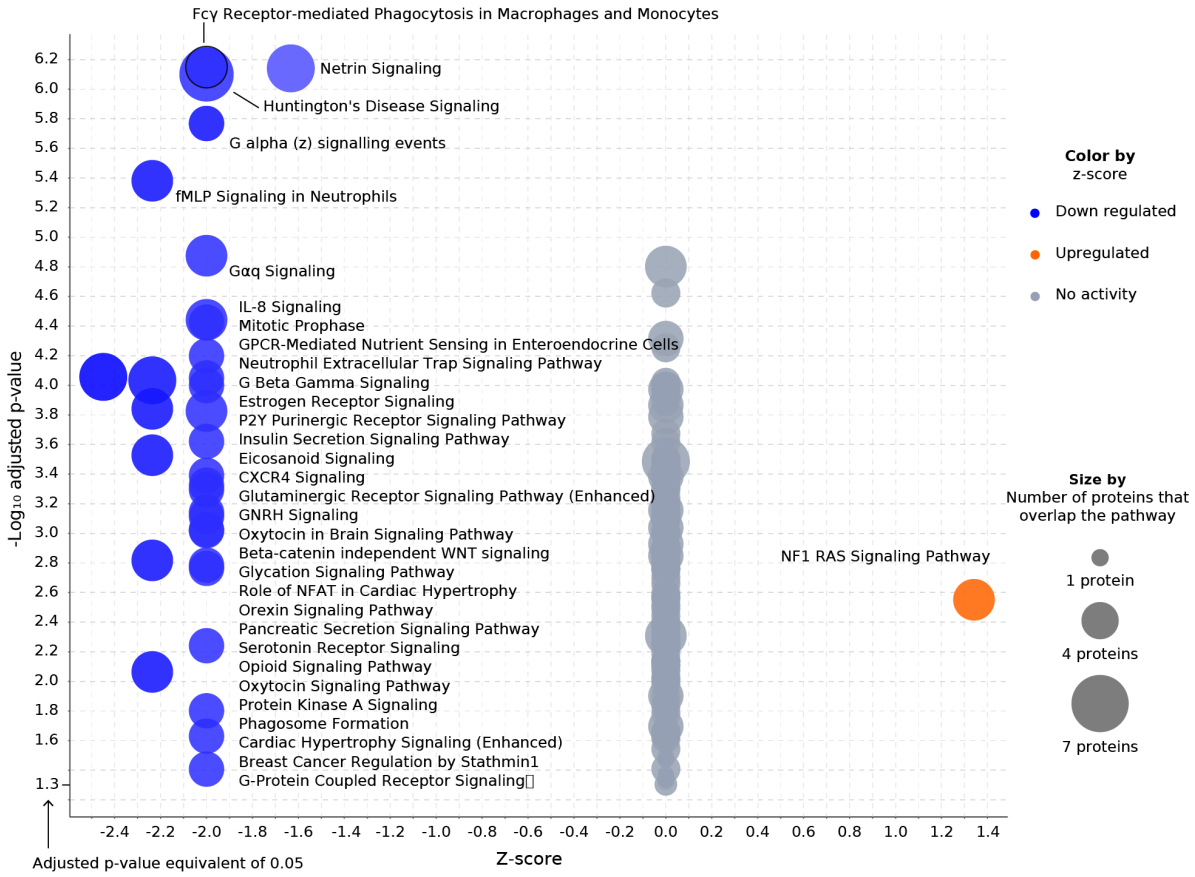

Figure 12: Global proteomic impact of CHIP deletion in SH-SY5Y neurons

Volcano-plot and pathway-enrichment summary of quantitative LC–MS/MS analysis comparing CRISPR/Cas9-engineered CHIP-knockout (KO) SH-SY5Y cells with isogenic wild-type controls. Approximately 4,200 proteins were quantified (false-discovery rate ≤ 0.05). Ingenuity Pathway Analysis highlighted widespread suppression of signalling networks. Most strikingly G-protein/PKC cascades, cytoskeletal and mitotic regulators, immune-inflammatory modules, metabolic and secretory pathways, and neuron-specific axon-guidance routes, accompanied by a modest activation of the NF1/Ras–MAPK pathway.

5.1.1 Loss of CHIP Suppresses GPCR–PKC Signalling

A prominent finding from the pathway-level analysis was the broad suppression of G-protein–coupled receptor (GPCR) signalling in CHIP-deficient cells. Multiple signalling cascades, including canonical Gαq and Gαz/Gβγ modules, general GPCR pathways, and ligand-specific axes such as serotonin, opioid, orexin, oxytocin, gonadotropin-releasing hormone (GnRH), CXCR4, interleukin-8 (IL-8), and P2Y purinergic signalling, exhibited negative IPA z-scores ranging from approximately –1 to –2. Several of these pathways shared down-regulated components in the proteomic dataset, most strikingly the G-protein subunit GNG2 and multiple isoforms of Protein Kinase C (PRKCA, PRKCB, PRKCG), suggesting a global attenuation of GPCR-mediated second-messenger signalling in the absence of CHIP (Joshi et al., 2016; Zhang et al., 2020).

CHIP's E3 ubiquitin ligase activity plays a key role in modulating the folding, turnover, and degradation of a range of client proteins, including those that are not misfolded. This regulatory mechanism affects both the intensity and temporal resolution of downstream signalling. For instance, CHIP ubiquitinates the catalytic subunit of Protein Kinase A (PKA) following GPCR–cAMP activation, promoting its degradation as part of a negative-feedback loop (Rinaldi et al., 2019). Consistent with this, deletion of CHIP is known to prolong PKA signalling under acute conditions. However, in the current analysis, IPA predicted inhibition of PKA signalling ($z \approx -$), a finding that initially appears contradictory. This apparent paradox is best explained by a broader reduction in upstream GPCR components, including Gα and Gβγ subunits, and a likely adaptive decrease in intracellular cAMP production as part of a compensatory mechanism under chronic CHIP deficiency (Ranek et al., 2020).

Additional layers of pathway crosstalk reinforce this interpretation. In particular, the proteomic data revealed upregulation of PDE9A, a cGMP-specific phosphodiesterase that depletes cyclic nucleotide levels and disrupts kinase homeostasis. This change has previously been linked to neuronal vulnerability and cognitive dysfunction in neurodegenerative models. These converging lines of evidence suggest that the observed down-regulation of GPCR pathways arises from two distinct but complementary mechanisms: (1) impaired proteostatic maintenance and reduced

stability of key signalling proteins, and (2) the absence of CHIP-mediated fine-tuning of signal termination, exemplified by the dysregulated PKA feedback loop (Ranek et al., 2020; Rinaldi et al., 2019).

CHIP's role in GPCR signalling is further supported by its capacity to regulate receptor stability and surface expression. For example, CHIP has been shown to ubiquitinate interferon-γ receptor 1 and interleukin-4 receptor α, thereby reducing their membrane localisation and modulating downstream immune responses. In neuronal contexts, CHIP (in conjunction with VCP) facilitates the ER-associated degradation of misfolded nicotinic acetylcholine receptor subunits. Loss of CHIP therefore risks the intracellular accumulation of dysfunctional GPCR subunits, diminishing the overall signalling capacity by preventing proper receptor assembly, trafficking, or degradation (Ng et al., 2022).

Beyond receptor regulation, CHIP also modulates downstream signalling complexes. It can recruit kinases such as atypical PKCζ and Src to Toll-like receptor complexes, facilitating signal propagation, before later targeting these same kinases for ubiquitin-dependent degradation. Thus, CHIP acts as both an enabler and terminator of kinase-driven signalling events (Yang et al., 2011). In CHIP-deficient cells, this duality is lost, resulting in the impaired formation of receptor–effector complexes and the failure to inactivate others. The net outcome, as reflected in the proteomic and IPA data, is a broad suppression of GPCR- and PKC-dependent signalling. This may represent a protective adaptation to proteostatic stress, or it may stem from a genuine depletion of functional receptor and kinase pools due to misfolding or aggregation.

This interpretation is reinforced by the observed down-regulation of phospholipase C (PLC) and Gαq/11 signalling, both of which rely on upstream GPCR activity and PKC activation. Reduced abundance of multiple PKC isoforms (α, β, and γ) further supports this conclusion. PKC family members are known clients of HSP90, and CHIP is required for their proper triage: without CHIP, unstable PKCs may be targeted for degradation through alternative pathways or become sequestered in inactive conformations. Normally, CHIP helps regulate the turnover of chaperone-bound kinases, such as Akt, LKB1, MLK3, and MEKK2, by ubiquitinating activated or misfolded forms (Joshi et al., 2016). In CHIP-deficient cells, these kinases may escape degradation but fail to achieve a functional conformation, leading to persistent inactivity or aberrant signalling.
Taken together, these findings indicate that CHIP deficiency compromises the integrity of

proteostasis networks that support GPCR signalling. This includes destabilisation of receptors, G-proteins, and effector kinases, culminating in a cellular state characterised by impaired responsiveness to extracellular stimuli. In neuronal systems, such disruption could attenuate neurotransmitter responses and synaptic plasticity. In oncogenic contexts, it may impair growth-factor sensing and chemokine-driven signalling. These results highlight the far-reaching consequences of CHIP loss for transmembrane signal transduction and point to its broader relevance in both neurodegenerative and cancer biology.

5.1.2 CHIP deficiency impairs axonal microtubule dynamics and cell-division machinery.

Loss of CHIP disrupts multiple pathways integral to cytoskeletal organisation, mitotic progression, and intracellular trafficking. Several pathways were predicted as inhibited in CHIP-deficient cells, including Mitotic Prophase, Breast Cancer Regulation by STATHMIN1 (a microtubule-destabilising protein), Phagosome Formation, Fcγ Receptor–mediated Phagocytosis, and Neutrophil Extracellular Trap Signalling. While some of these pathways originate from immune-cell contexts, they converge mechanistically on the requirement for effective remodelling of actin filaments and microtubule networks. Key differentially expressed proteins from this dataset, ACTR3 (Arp3, a component of the Arp2/3 complex essential for actin nucleation), LMNA (lamin A/C, a nuclear envelope structural component), and DPYSL2 (CRMP2, a microtubule-regulating protein involved in axon guidance), were all significantly down-regulated in CHIP KO cells, in alignment with negative IPA z-scores across cytoskeleton-dependent pathways.

CHIP's role as an E3 ubiquitin ligase is well documented in the regulation of cytoskeletal integrity. It facilitates the degradation of microtubule-associated proteins such as tau, thereby preventing pathological aggregation and supporting cytoskeletal homeostasis (Dias et al., 2021; Joshi et al., 2016). In keeping with this, the dataset identified inhibition of Huntington's disease Signalling, and reduced abundance of DCTN1 (dynactin-1), a motor-protein component essential for microtubule-based transport. This suggests impaired axonal trafficking and diminished cellular capacity to handle aggregation-prone substrates. Furthermore, CHIP modulates cellular motility by

targeting profilin-1, a key actin-binding protein required for filament extension at the leading edge of migrating cells; consistent with this, the proteomic data revealed broad suppression of cytoskeletal assembly programmes. Beyond its role in cytoplasmic structural maintenance, CHIP also targets centrosomal and mitotic-spindle regulators, including MKKS (a protein associated with Bardet–Biedl syndrome), to ensure fidelity in mitosis. Loss of CHIP could therefore result in the abnormal accumulation or persistence of centrosomal proteins, contributing to the observed inhibition of mitotic-phase pathways.

A particularly noteworthy protein within this context is CRMP2 (DPYSL2), which serves as a critical modulator of neuronal cytoskeletal dynamics. CRMP2 stabilises microtubules within axons and is subject to inhibitory phosphorylation by kinases such as GSK3β and CDK5. In neurons, this phosphorylation impairs CRMP2 function and collapses growth cones. The Neurofibromin 1 (NF1) Ras Signalling Pathway, which was modestly up-regulated in CHIP KO cells, is known to antagonise these kinases and thereby promote CRMP2-mediated stabilisation of axonal microtubules (Joshi et al., 2016; Tan et al., 2014). However, despite NF1 activation, CRMP2 abundance was reduced, suggesting that its functional depletion may override compensatory signalling inputs. Moreover, LMNA down-regulation points to compromised nuclear structure during cell division. Lamin A/C plays essential roles in nuclear envelope integrity and mitotic progression; its destabilisation is known to trigger chromosomal missegregation and genomic instability.

Taken together, these findings position CHIP as a central guardian of cytoskeletal maintenance across multiple cellular contexts. In neurons, the absence of CHIP compromises the stability of structural proteins (e.g. tau, CRMP2) and fails to clear misfolded or aggregated components, impairing axonal transport and neurite extension. In proliferating cells, CHIP loss disrupts centrosome and spindle function, delays mitosis, and potentially contributes to chromosomal instability. These dual consequences underscore the proteostatic importance of CHIP in both post-mitotic and dividing cellular populations, with implications for neurodegeneration and oncogenesis alike.

5.1.3 CHIP supports mitochondrial metabolism and vesicular secretion.

IPA revealed pronounced suppression of multiple metabolic and secretory pathways in CHIP-KO SH-SY5Y cells. Pathways with negative z-scores (approximately –2.4 to –1.4) included Insulin Secretion, Pancreatic β-cell Signalling, Eicosanoid Signalling, and Advanced Glycation End-product (AGE) Signalling. These biological systems are critically dependent on efficient mitochondrial energy production and coordinated vesicular trafficking. For example, insulin release requires mitochondrial ATP synthesis and calcium-dependent exocytosis; eicosanoid signalling depends on lipid metabolism and production of inflammatory mediators; and glycation pathways function to detoxify reactive carbonyl species. Several proteins within these pathways-PDHB (the E2 component of the pyruvate dehydrogenase complex), GOSR2 (a Golgi-localised SNARE essential for vesicle fusion), AKR1C3 (an aldo-keto reductase involved in prostaglandin metabolism), and multiple PKC isoforms, were found to be down-regulated, indicating impaired metabolic throughput and reduced secretory competence.

A possible mechanistic explanation is mitochondrial dysfunction. CHIP is a critical regulator of mitochondrial proteostasis, where it facilitates the removal of oxidatively damaged proteins and promotes mitophagy. CHIP-deficient mouse models have demonstrated defective mitophagy, diminished antioxidant responses, and accumulation of damaged mitochondrial proteins, culminating in reduced ATP production (Joshi et al., 2016). The observed reduction in PDHB abundance within this dataset is consistent with a bottleneck in the tricarboxylic acid (TCA) cycle and aligns with impaired ATP generation and weakened support for ATP-dependent cellular functions such as exocytosis.

Besides its mitochondrial role, CHIP modulates metabolic signalling pathways more directly. It ubiquitinates the insulin receptor (INSR) and associated effectors, modulating downstream insulin and IGF1 signalling. Furthermore, CHIP interacts with TFEB, a key transcription factor governing autophagy and lysosomal biogenesis, suggesting that CHIP loss could impair both anabolic processes (e.g. insulin-mediated growth and secretion) and catabolic responses (e.g. autophagy and the unfolded protein response). Supporting this interpretation, the XBP1(S)-activated chaperone pathway was found to

be inhibited (z ≈ –2), and reduced abundance of HYOU1, an endoplasmic reticulum (ER) chaperone involved in hypoxic stress responses, was detected, indicative of an impaired capacity to manage proteotoxic stress despite accumulating misfolded proteins (Zhang et al., 2020).

The suppression of Eicosanoid Signalling via down regulation of AKR1C3 and PKC isoforms and AGE Signalling through reduced expression of HAGH, a glyoxalase involved in carbonyl detoxification further underscores systemic disruptions in lipid and glucose metabolism (Ullah et al., 2020). Collectively, these findings indicate that CHIP deficient neuroblastoma cells are metabolically compromised: mitochondrial ATP production is diminished, secretory vesicle trafficking is attenuated, and cellular defences against oxidative and glycation stress are weakened. These deficits would be expected to impair neurotransmitter release and exacerbate susceptibility to toxic metabolic by-products, thereby linking CHIP-mediated proteostasis failure to broader metabolic dysfunction with potential relevance to both neurodegenerative and oncogenic processes.

5.1.4 CHIP as a master regulator of axonal integrity and synaptic homeostasis.

In undifferentiated SH-SY5Y cells lacking CHIP, multiple neuronal pathways were predicted to be suppressed, including Netrin Signalling, Axonal Guidance, Glutamatergic Receptor Signalling, Oxytocin in Brain Signalling, and Huntington's disease Signalling. A central node across these pathways is DPYSL2, a cytosolic protein that regulates axon elongation, branching, and pathfinding. DPYSL2 abundance was markedly reduced in CHIP-KO cells, and given its established role in stabilising microtubules downstream of netrin-1 guidance suggests, its loss is likely to impair cytoskeletal dynamics essential for neurite extension (Feuer et al., 2023). The strong inhibition of the netrin pathway (z ≈ –2.4) therefore supports a model in which CHIP deficiency disrupts axonal navigation and neuronal differentiation.

In parallel, several synaptic-signalling modules exhibited reduced activation. Oxytocin and Serotonin Receptor Signalling, both mediated by GPCRs, showed negative enrichment scores, consistent with broader suppression of GPCR networks in

CHIP-deficient cells. These pathways rely on second messengers such as calcium ions (Ca^{2+}), inositol triphosphate (IP_3), and cyclic AMP (cAMP), and connect downstream kinases, including protein kinase C (PKC) isoforms. All three conventional PKCs (α, β, γ) were down-regulated, likely weakening neuropeptide responses across the oxytocin, orexin, and opioid systems. Glutamatergic Signalling was similarly impaired ($z \approx -2.2$), with reduced expression of PLD5 (a phospholipase involved in glutamate receptor recycling), and further depletion of PKC isoforms, potentially blunting excitatory neurotransmission.

CHIP's established role in receptor quality control provides a mechanistic explanation for these observations. As an E3 ubiquitin ligase that partners with molecular chaperones, CHIP facilitates proper folding and degradation of synaptic receptors, including nicotinic acetylcholine and GABA_B receptors (Joshi et al., 2016). In the absence of CHIP, misfolded or incompletely assembled receptors may accumulate intracellularly and fail to traffic to the membrane, therefore reducing receptor density and impairing synaptic efficacy.

The Huntington's disease Signalling pathway emerged as another pathway significantly affected in CHIP-KO cells. This IPA category encompasses defects in axonal transport, mitochondrial energetics, and proteostasis-all hallmarks of Huntington's disease pathology. CHIP has been shown to mitigate the toxic effects of aggregation-prone proteins, including mutant huntingtin, by promoting their clearance through ubiquitination. Overexpression of CHIP reduces huntingtin inclusions and slows neurodegeneration, whereas CHIP deficiency accelerates pathology. In line with this, the current proteomic data revealed reduced levels of DCTN1 (dynactin-1), a critical component of the dynein-dynactin motor complex, suggesting impaired axonal cargo transport and partial phenocopying of Huntington-like neuronal deficits (Joshi et al., 2016; Zhang et al., 2020).

Taken together, these findings reinforce the view that CHIP is an essential neuroprotective regulator. By maintaining the proteostatic integrity of receptors, kinases, and cytoskeletal scaffolds, CHIP supports both synaptic signalling and axonal structure. Its loss leads to impaired axon-guidance pathways, reduced responsiveness to

neurotransmitters, and a weakened ability to clear pathogenic proteins. Molecular deficits that mirror features of several neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Huntington's disease.

5.1.5 Compensatory activation of the NF1/Ras–MAPK pathway in CHIP deficient cells

Among all pathways examined, the NF1/Ras signalling cascade emerged as the only one with a positive IPA z-score (~ +1.3) in CHIP KO cells, indicating a modest upregulation of Ras/MAPK activity. This pathway, anchored by neurofibromin 1 (NF1) and Ras GTPase, governs the canonical Ras–Raf–MEK–ERK axis, which plays a key role in cell survival, proliferation, and differentiation. It also intersects with neuronal regulators such as CRMP2, whose loss may compound Ras dysregulation (Joshi et al., 2016).

Several mechanisms may underlie this compensatory activation. Firstly, the broad suppression of GPCR- and PKC-mediated signalling observed in CHIP-deficient cells could induce a shift towards Ras-dependent pathways as an adaptive survival mechanism. CHIP is known to ubiquitinate and degrade multiple Ras/MAPK components, including BRAF, MEKK2, and MLK3, and its absence may therefore prolong the half-life and activity of these kinases, subtly increasing downstream ERK or JNK activation.

Secondly, CHIP loss may indirectly impair NF1 function or its regulatory interactions. NF1 normally accelerates the hydrolysis of Ras-GTP to Ras-GDP, thereby attenuating Ras activity. CRMP2 (also down regulated in CHIP KO cells) interacts with NF1 to stabilise axonal growth (Tan et al., 2014). Loss of CRMP2 may therefore weaken this cooperative axis, prolonging Ras-GTP signalling and contributing to axon-guidance defects.

A third possible contributor is proteotoxic stress. Accumulation of misfolded proteins in CHIP-deficient cells is known to activate stress-response pathways, including MEK/ERK, which can confer transient survival benefits (Lim et al., 2020). Although the z-score indicates only mild pathway activation, this may reflect an early-stage adjustment or a low-level chronic stress response, rather than overt hyperactivation.

Further indirect effects may stem from CHIP's established role in degrading oncogenic substrates such as HER2/ErbB2, mutant p53, and c-MYC. Stabilisation of these oncoproteins in CHIP KO cells would be expected to enhance Ras/MAPK signalling. In the context of neuroblastoma, receptor tyrosine kinases such as Trk are potent Ras activators, and their turnover may likewise be regulated by CHIP (Joshi et al., 2016).

Taken together, these observations suggest that the NF1/Ras signalling axis acts as a compensatory node in CHIP-deficient cells, potentially buffering the loss of upstream signalling networks and supporting survival under proteotoxic stress. In neurons, this may influence differentiation and axonal elongation; in cancer cells, it could provide a proliferative advantage. These findings further underscore CHIP's dual role as a proteostatic guardian and context-dependent tumour suppressor, and point to Ras/MAPK modulation as a viable therapeutic avenue in CHIP-related disorders.

5.2 Translational implications: CHIP as a neurodegenerative disease biomarker

The data presented in this thesis demonstrate that loss of the co-chaperone and E3 ubiquitin ligase CHIP disrupts axonal transport, axon-guidance signalling, and neurite development, underscoring the essential role of CHIP in maintaining neuronal integrity. These findings are consistent with emerging evidence that CHIP deficiency contributes to neurodegenerative pathology. In this context, CHIP may hold potential as a biomarker for neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease. Although CHIP is expressed in numerous human tissues, its particularly high abundance in metabolically active organs (including the brain, heart, skeletal muscle, pancreas, and placenta) underscores its importance within the central nervous system.

Given the severity and often late-stage diagnosis of neurodegenerative conditions, early identification of at-risk individuals could provide an important window for intervention. Whole-genome sequencing (WGS) in newborns offers a theoretical route for early detection of pathogenic STUB1 variants. However, the incorporation of WGS into routine newborn screening programmes must be approached with caution. Many STUB1 mutations exhibit variable expressivity and incomplete penetrance, such that carriage of

a deleterious variant does not necessarily predict disease manifestation. In addition, the broader ethical and logistical implications of genomic screening in neonates, including uncertainty in clinical significance, psychological burden, and the need for follow-up infrastructure, require cautious implementation and robust genetic counselling frameworks.

Nevertheless, in cases where high-risk STUB1 variants are confidently identified, the possibility of pre-symptomatic intervention becomes tangible. Lifestyle-based neuroprotective strategies could be adopted early in life to strengthen neuronal resilience and delay or prevent the onset of pathological changes. Several non-pharmacological measures have demonstrated efficacy in supporting brain health:

- Sustained, High-Quality Sleep: Sleep plays a crucial role in the clearance of metabolic waste products, synaptic plasticity, and memory consolidation. Chronic sleep disruption has been linked to cognitive decline and increased vulnerability to neurodegenerative processes (Bishir et al., 2020; Reiter et al., 2023).
- Regular Physical Exercise: Both aerobic and resistance-based training have been shown to enhance neurogenesis, reduce oxidative stress, and improve cognitive performance. Clinical and preclinical studies have reported slowed progression of neurodegenerative disease in individuals engaging in consistent physical activity (Raichlen and Alexander, 2017; Vecchio et al., 2018).
- Genistein-Enriched Diet: Genistein, a naturally occurring isoflavone abundant in soy products and other legumes, is capable of crossing the blood–brain barrier and modulating multiple neuroprotective pathways. Experimental studies have demonstrated that dietary genistein reduces oxidative stress, suppresses neuroinflammation, and promotes the clearance of aggregated proteins, with consequent improvements in behavioural and cognitive performance (Pierzynowska et al., 2024, 2025).

Implementation of such lifestyle interventions from an early age may carry long-term neurological benefit in genetically predisposed individuals. While these measures cannot replace disease modifying therapies, they offer a pragmatic and low-risk approach to delaying neurodegeneration. Together with continued research into CHIP's function in neuronal homeostasis and pathology, these findings introduce a translational dimension to the present thesis highlighting the possibility of leveraging CHIP not only as a

mechanistic target but also as a tool for early diagnosis and preventative care in neurodegenerative disease.

Chapter 6: The role of CHIP in MHC expression.

6.1 Introduction

6.1.1 Immune recognition studies

CHIP is already recognised for its roles in oncogenic signalling and tumour suppression, and several proteins differentially expressed between the WT and CHIP KO SH-SY5Y cell lines in this proteomic dataset (such as aldo-keto reductase family member AKR1C3), are known to be associated with cancer biology. Against this backdrop, it was considered pertinent to explore whether CHIP loss influences antigen presentation pathways, specifically via modulation of major histocompatibility complex class I (MHC I) surface expression. MHC I molecules display intracellularly derived peptides to cytotoxic T cells and are essential components of immune surveillance. Changes in their abundance on the cell surface can significantly alter immune recognition; for example, reduced MHC I expression is a common immune evasion strategy employed by tumour cells to escape cytotoxic detection.

6.2 Results and discussion

6.1.1 Basal MHC I levels in SH-SY5Y WT vs SH-SY5Y CHIP KO cells.

To investigate whether CHIP deficiency affects MHC I presentation, flow cytometry (FACS) analysis was performed on both WT and CHIP KO SH-SY5Y cells under basal conditions. The results (Figure 13a) showed no significant difference in MHC I surface levels between the two genotypes. To further sensitise the system and increase detection sensitivity, cells were treated with interferon-gamma (IFN-**γ**), a cytokine known to robustly enhance MHC I expression by inducing components of the antigen processing and presentation machinery. As expected, IFN-**γ** stimulation resulted in a marked increase in MHC I surface levels in both WT and CHIP KO cells compared to untreated controls (Figure 13b). However, even under these induced conditions, MHC I surface expression remained widely comparable between genotypes.

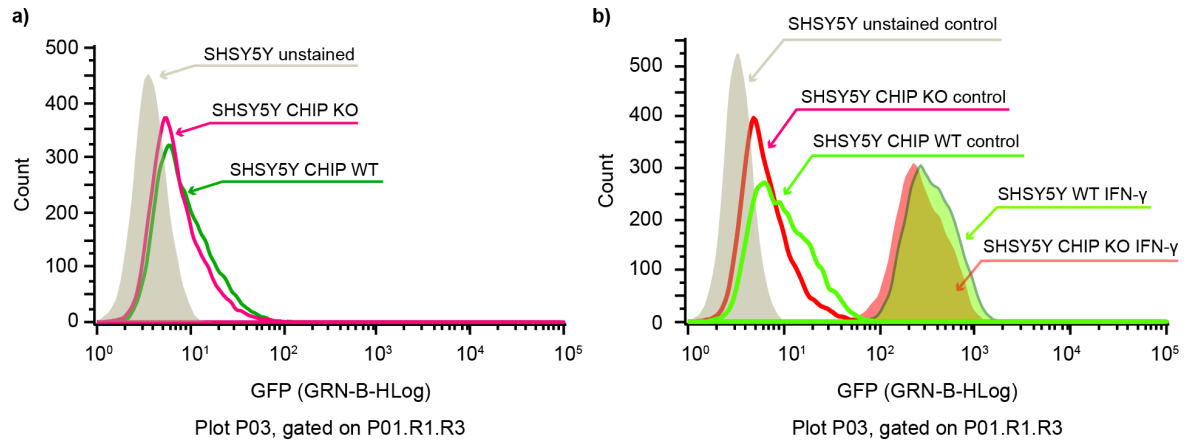

Figure 13. CHIP deletion does not alter MHC I surface abundance in SH-SY5Y cells.

(a) Basal condition: Flow-cytometric analysis of major histocompatibility complex class I (MHC I) surface expression in WT and CHIP-KO SH-SY5Y cells under unstimulated culture conditions shows no significant difference between genotypes. (b) IFN-γ stimulation: WT and CHIP-KO cells treated for 24 h with interferon-γ exhibit the expected global up-regulation of MHC I surface levels, but the magnitude of induction remains comparable between WT and KO lines.

These findings suggest that, under the tested conditions, CHIP deficiency does not significantly affect MHC I surface abundance in SH-SY5Y cells. While CHIP may modulate the expression of various cancer-associated proteins, its absence does not appear to compromise classical antigen presentation via surface MHC I. Nevertheless, the implications for antigen presentation extend beyond the mere quantity of MHC I molecules. Given CHIP's role in targeting proteins for proteasomal degradation, its loss may substantially alter the repertoire of intracellular peptides available for loading onto MHC I molecules, thus reshaping the immunopeptidome. Although MHC I surface levels are unchanged, the qualitative nature of presented peptides may differ profoundly between WT and CHIP KO cells. Investigating these potential differences in the immunopeptidome was beyond the scope of this doctoral work, but represents an interesting avenue for future research, particularly in the context of immune recognition and tumour immunology.

Chapter 7: Final discussion and future perspective.

7.1 Summary

When this project was initiated, the functional consequences of CHIP (STUB1) loss in neurons, particularly its contribution to neurodegenerative disease, were poorly understood. Although CHIP had been implicated in protein quality control, its specific proteostatic and signalling functions in neuronal systems remained largely unexplored.

This thesis demonstrates that CHIP deficiency triggers a unified cellular stress phenotype with mechanistic implications for both neurodegeneration and cancer. In the absence of CHIP's E3 ligase activity, misfolded proteins and unstable signalling components accumulate, disrupting proteostasis and broadly suppressing G-protein/PKC signalling, cytoskeletal regulation, metabolic flux, and vesicular trafficking. A modest compensatory activation of Ras/MAPK signalling emerges, suggesting partial cellular adaptation.

Neurons are particularly susceptible to these disruptions. Their reliance on sustained axonal transport, structurally stable neurofilament scaffolds, and finely tuned neurotransmitter responses renders them vulnerable to proteostatic imbalance. The data presented herein show that CHIP deficiency attenuates axon-guidance signalling (specially the netrin–CRMP2 axis), down-regulates glutamatergic and GPCR-mediated pathways, and destabilises core neurofilament proteins. Together, these perturbations create a cellular environment primed for synaptic dysfunction, impaired neurite outgrowth, and progressive neurodegeneration.

In dividing cells, however, the impact of CHIP loss diverges. CHIP is known to regulate oncogenic signalling through interactions with HER2, mutant p53, and other effectors, yet it also targets tumour suppressors, giving rise to context-dependent roles in cancer. In this study, complete CHIP ablation suppressed many pro-proliferative pathways and imposed a proteotoxic burden likely to promote dormancy or apoptosis. The observed Ras/MAPK activation may represent a survival mechanism in this context, offering a potential escape from proteostasis-induced stress. These findings help integrate conflicting literature on CHIP's tumour-suppressive versus oncogenic functions.

Through quantitative LC–MS/MS proteomics, orthogonal validation using SWATH-MS, and follow-up experiments via western blotting and immunofluorescence, this work identified a coherent network of fourteen CHIP-sensitive proteins involved in neurofilament assembly and axonal signalling. This constitutes the first detailed proteomic characterisation of CHIP's role in maintaining neuronal structural integrity.

These findings support the proposition that CHIP expression, or STUB1 mutation status, could serve as a predictive biomarker of vulnerability to neurodegenerative disease. Early-life screening for pathogenic STUB1 variants may enable identification of at-risk individuals prior to symptom onset, facilitating future preventative or neuroprotective strategies, even in the absence of currently available disease-modifying therapies.

In contrast to previous studies that concentrated on individual CHIP substrates, this thesis provides an integrated proteomic view of CHIP-dependent signalling and structural regulation in neurons. It also offers a mechanistic explanation for CHIP's apparently paradoxical roles in cancer biology. Taken together, the data establish CHIP as a central guardian of neuronal homeostasis and a context-sensitive regulator of tumour progression. Loss of CHIP function leads to proteostatic collapse and widespread signalling rewiring, offering a unifying molecular rationale for both the neurodegeneration observed in STUB1-related ataxias and the dual tumour-suppressive or tumour-promoting phenotypes reported in malignancy. These insights highlight proteostasis-supporting or Ras-targeting interventions as promising diagnostic and therapeutic strategies.

7.2 Future directions and experiments

To build upon the findings presented in this thesis, the following experimental strategies are proposed to further elucidate CHIP's biological roles and evaluate its potential as a biomarker for neurodegenerative disease:

7.2.1 Rescue of CHIP Function:

A critical next step is to reintroduce WT CHIP into the CHIP KO cell line to determine whether the proteomic and pathway-level alterations observed in this study are

reversible. This would provide strong evidence for a causal relationship between CHIP loss and the dysregulated pathways. Restoration of CHIP function is expected to increase the levels of down-regulated proteins such as PKC isoforms, CRMP2, and Arp3, and to normalise z-scores of affected signalling pathways. Rescue experiments could involve transient or stable expression of STUB1 or, if available, pharmacological activation using small molecules that enhance CHIP expression or activity. Phenotypic rescue could be evaluated through restoration of cytoskeletal organisation (e.g., via phalloidin staining for F-actin or microtubule assays) and normalisation of signalling responses (e.g., calcium flux or ERK phosphorylation following GPCR stimulation).

7.2.2 Pharmacological Modulation of Dysregulated Pathways.

Given the significant changes observed in GPCR/PKC signalling, functional consequences of CHIP deficiency should be explored using pathway-specific modulators. For example, treatment with PKC activators (e.g., phorbol esters) or cAMP analogues could reveal whether CHIP KO cells exhibit attenuated downstream responses, such as reduced phosphorylation of PKA or PKC substrates. Conversely, inhibitors of the Ras/MAPK pathway (e.g., MEK inhibitors) could be used to test whether CHIP KO cells display increased dependency on compensatory Ras signalling for survival. A heightened sensitivity to Ras inhibition in KO cells would support the hypothesis that Ras/MAPK activation serves as a survival mechanism in the absence of CHIP. In parallel, increasing intracellular cAMP using phosphodiesterase inhibitors or forskolin may induce toxicity in CHIP KO cells if they are unable to properly attenuate PKA activity, an effect consistent with observations in CHIP-mutant fibroblasts from SCAR16 patients (Rinaldi et al., 2019).

7.2.3 Differentiation and Neuronal Functional Assays:

While this study utilised undifferentiated SH-SY5Y neuroblastoma cells, inducing neuronal differentiation (e.g., with retinoic acid or brain-derived neurotrophic factor) may better reveal CHIP's role in neuronal development and function. It is hypothesised that CHIP KO cells will exhibit impaired neurite outgrowth, reduced neurite length, or growth cone collapse, due to deficits in CRMP2 and microtubule stabilisation. These phenotypes can

be quantified using standard morphometric analyses. In addition, functional assays such as calcium imaging or electrophysiological recordings in differentiated cells could be used to assess synaptic responsiveness, e.g., whether CHIP KO-derived neurons display diminished calcium transients in response to depolarisation or GPCR agonists, consistent with the observed suppression of GPCR-related pathways. These experiments would provide a more direct link between proteomic alterations and neuronal functionality.

7.2.4 In Vivo and In Vivo-Like Models.

Translating the findings from cell culture to more physiologically relevant systems will be critical. Neuron-specific CRISPR-mediated knockout of Stub1 in mice could be used to assess neurodegenerative phenotypes in vivo, including deficits in motor or cognitive behaviour, altered neuronal signalling, and protein aggregation. Previous studies show that global CHIP KO in mice causes severe systemic effects such as muscle wasting, cataracts, and early death, but nervous-system–specific models may better delineate the neuronal consequences of CHIP loss (Min et al., 2008). Measurement of key proteins (e.g., PKC isoforms, CRMP2) and activation markers (e.g., phospho-ERK, phospho-JNK) in brain tissue could validate the signalling deficits identified in this thesis. Parallel investigations using patient-derived models, such as fibroblasts or induced pluripotent stem cell (iPSC)-derived neurons from individuals with STUB1 mutations, could assess whether the CHIP-regulated proteomic signature described here recurs in clinically relevant systems. Indeed, the dysregulation of PKA signalling in CHIP-mutant fibroblasts reported by Rinaldi et al. (2019) aligns with the current findings (Rinaldi et al., 2019).

Exploration of CHIP's role in tumour biology also warrants further investigation. Knocking out STUB1 in cancer cell lines or xenograft models may reveal whether loss of CHIP suppresses tumour growth by inducing proteotoxic stress, or alternatively, promotes proliferation through oncoprotein stabilisation. The proteomic data presented here suggest an initial suppressive effect; however, long-term culture or in vivo selection may favour adaptive responses, such as upregulation of Ras signalling or acquisition of secondary mutations. Such adaptations could be exploited therapeutically, e.g., by testing sensitivity to HSP90 inhibitors, which may be selectively toxic to CHIP-deficient tumour cells with compromised proteostasis.

7.3 Project limitations

This project was initially designed to include studies using iPSC-derived neurons to enhance physiological relevance. Although training in iPSC culture was completed and appropriate cell lines acquired, logistical and administrative constraints during the course of the doctoral programme prevented full implementation of this experimental arm. As a result, undifferentiated SH-SY5Y neuroblastoma cells were used as an alternative. While this model provides a useful platform for exploring CHIP-dependent signalling and proteostasis, it does not fully recapitulate the complexity or maturity of post-mitotic neurons. Future studies incorporating iPSC-derived neuronal systems will be essential to validate the findings reported here and to extend their relevance to human neurodegenerative disease.

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Appendix I

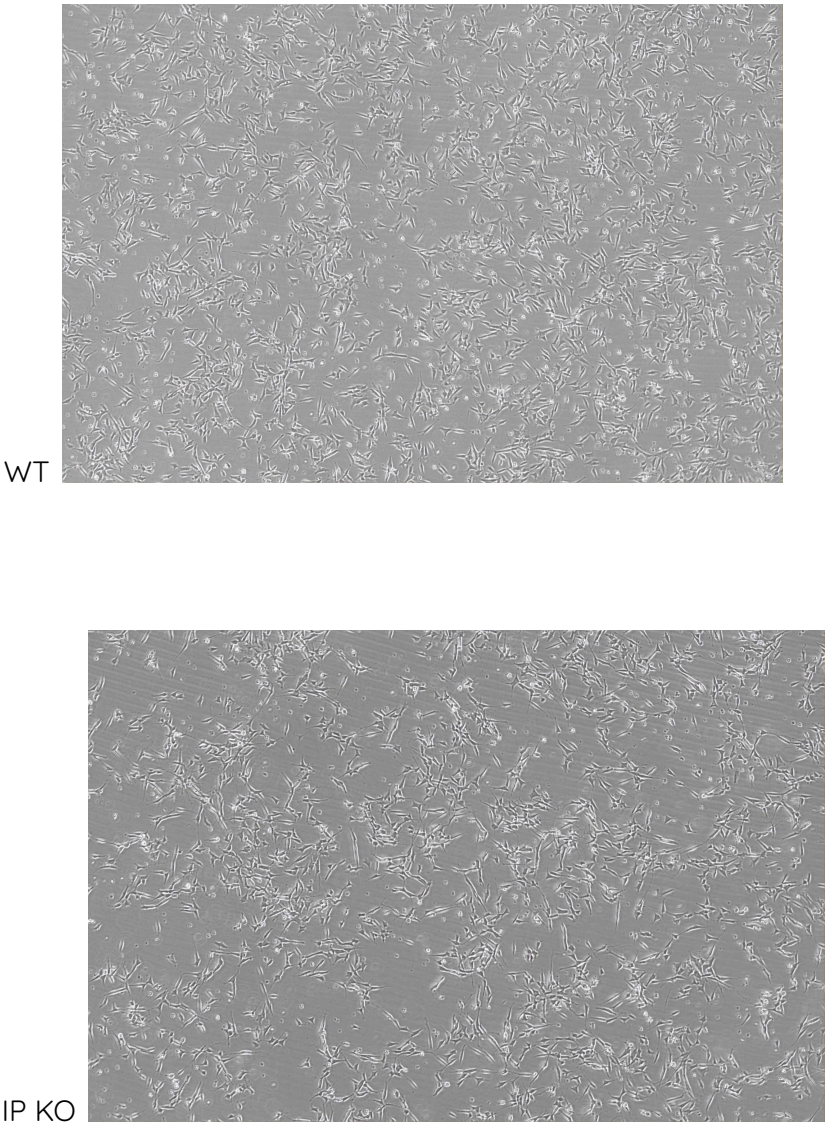

Figure S1. Morphology of undifferentiated SH-SY5Y CHIP model

Phase-contrast micrographs illustrating the morphology of SH-SY5Y wild-type (WT) and CHIP knockout (KO) cells in their undifferentiated state. Scale bar: 200 µm.

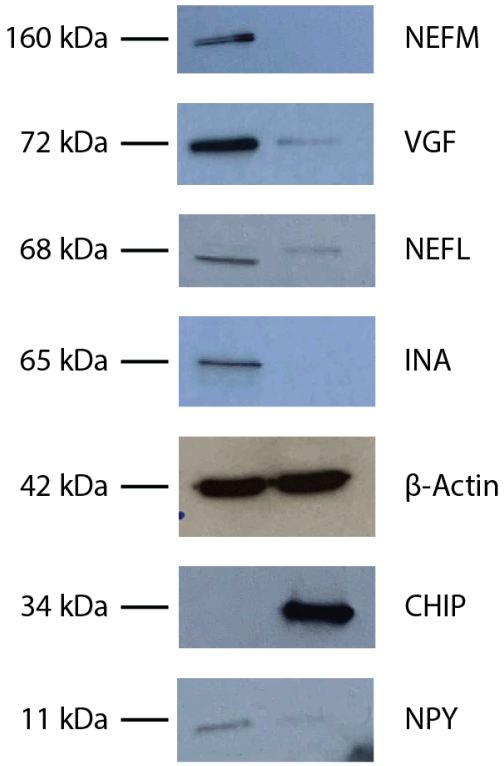

Figure S2. Independent Western-blot confirmation of CHIP-dependent protein changes in a second SH-SY5Y CHIP-KO model.

Western blot analysis performed by Dr Janette Popławski (University of Edinburgh) demonstrates elevated levels of INA, VGF, NEFM and NEFL in an independently generated SH-SY5Y CHIP-KO cell line compared with its wild-type control.

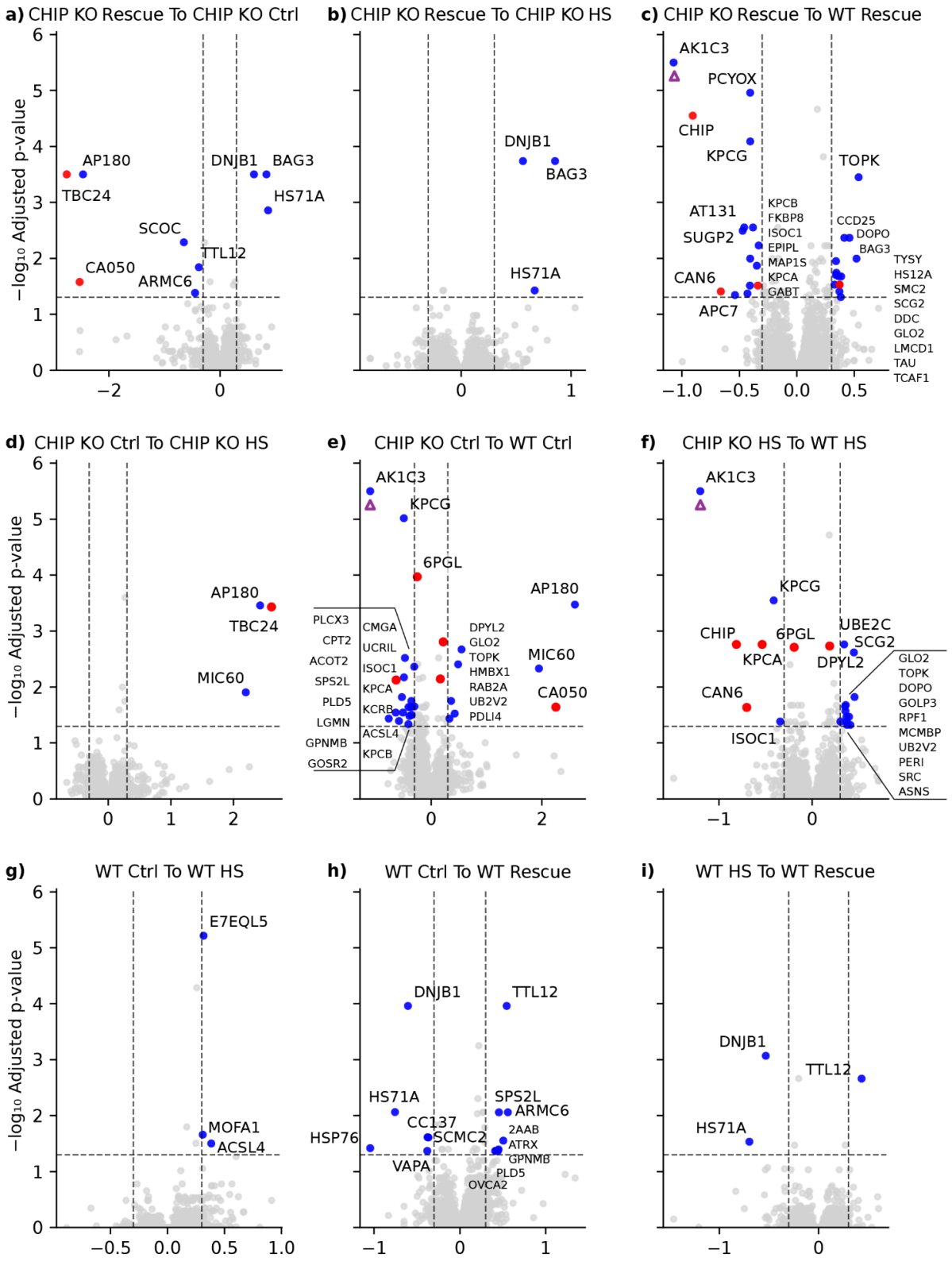
a) CHIP KO Rescue To CHIP KO Ctrl
b) CHIP KO Rescue To CHIP KO HS
c) CHIP KO Rescue To WT Rescue
d) CHIP KO Ctrl To CHIP KO HS
e) CHIP KO Ctrl To WT Ctrl
f) CHIP KO HS To WT HS
g) WT Ctrl To WT HS
h) WT Ctrl To WT Rescue
i) WT HS To WT Rescue
−log10 Adjusted p-value
AP180
TBC24
DNJB1
BAG3
HS71A
SCOC
TTL12
CA050
ARMC6
AK1C3
PCYOX
CHIP
KPCG
TOPK
AT131
SUGP2
CAN6
APC7
KPCB
FKBP8
ISOC1
EPIPL
MAP1S
KPCA
GABT
CCD25
DOPO
TYSY
HS12A
SMC2
SCG2
DDC
GLO2
LMCD1
TAU
TCAF1
6PGL
MIC60
PLCX3
CPT2
ACOT2
SPS2L
PLD5
LGMN
GPNMB
GOSR2
CMGA
UCRIL
ISOC1
KCRB
ACSL4
DPYL2
HMBX1
RAB2A
UB2V2
PDLI4
UBE2C
SCG2
DPYL2
ISOC1
RPF1
MCMBP
PERI
SRC
ASNS
E7EQL5
MOFA1
ACSL4
HSP76
CC137
SCMC2
VAPA
SPS2L
ARMC6
2AAB
ATRX
GPNMB
PLD5
OVCA2
X axis: log10 Fold Change
Extreme −log10 adjusted p-value capped at 5.5 (raw adj p-val = 3.2 × 10−6)

Figure S3. Comparative proteomic analysis of SH-SY5Y WT and CHIP-KO cells cultured under heat shock conditions.

Volcano plots display the proteome-wide comparison for each experimental contrast (nine pairwise conditions indicated by panel titles). The x-axis shows the signed $\log_{10}$ fold change derived from $\log_2$ fold change values, and the y-axis shows $-\log_{10}$ of the adjusted p-value for differential abundance. Proteins were considered significantly altered when the absolute $\log_2$ fold change exceeded 1 (equivalent to a raw fold change > 2) and the multiple-testing adjusted p-value was ≤ 0.05. To prevent compression of the vertical scale by extreme outliers, $-\log_{10}$ p-values were capped at 5.5; any proteins exceeding this threshold are marked with open purple triangles and plotted at their true x-coordinate. Non-significant proteins are shown as light-grey points, significant proteins that meet the fold-change and p-value criteria are shown in blue, and a predefined subset of key proteins (6PGL, KPCA, DPYL2, CHIP, CAN6, TAU, 6PGL, KPCA, DPYL2, HMBX1, CA050, TBC24) of particular biological interest is highlighted in red. Horizontal dashed lines indicate the significance threshold (adjusted p = 0.05) and vertical dashed lines mark the $\pm\log_{10}(2)$ fold-change boundaries. Together, the plots summarise global proteomic shifts induced by CHIP loss and heat-shock treatment, highlighting both broad patterns of differential expression and specific proteins of interest.

Table S1. Most over-represented proteins in SH-SY5Y CHIP KO cell line compared to SH-SY5Y WT cell line.

Protein	p-value	Fold Change	-Log (p-value)	Log Fold Change
Neuropeptide Y	2,72E-04	96,03175846	3,565942883	1,982414881
Neurofilament Medium Polypeptide	2,90E-05	83,91315156	4,536862844	1,923830032
Paraneoplastic antigen Ma2	2,13E-03	28,91415022	2,671560062	1,461110433
Histone H2A family member B	2,16E-02	8,80742623	1,665207796	0,944849014
Beta 2 Adaptin	3,19E-03	8,663726265	2,495945112	0,937704722
Nestin	2,72E-04	8,216247012	3,565790218	0,914673487
Alpha Internexin	5,24E-04	6,549170081	3,280964899	0,816186269

Table S2. Most downregulated proteins in SH-SY5Y CHIP KO cell line compared to SH-SY5Y WT cell line.

Protein	p-value	Fold Change	-Log (p-value)	Log Fold Change
Galectin-3-binding protein	1,31E-02	0,033783426	1,882574596	-1,47129631
Phospholipase D family member 3	1,41E-04	0,088584775	3,85168851	-1,052640915
Urotensin-2	2,66E-03	0,137414611	2,575724641	-0,861967087
Prelamin-A/C	2,79E-04	0,154241962	3,553751679	-0,81179746
Endoplasmin	1,65E-06	0,163379555	5,781400286	-0,786802292

Table S3. Nine top proteins with increased abundance in SH-SY5Y CHIP-KO cells compared with WT, as revealed by the second LC–MS/MS analysis on the Orbitrap Exploris 480, plus an additional candidate of interest.

Protein	p-value	Fold Change	-Log (p-value)	Log Fold Change
Neurosecretory protein VGF	0,00E+00	404,625973	0	-2,607053757
Neurofilament Medium Polypeptide	0,00E+00	385,6085657	0	-2,586146673
Paraneoplastic antigen Ma2	0,00E+00	358,7619628	0	-2,554806391
Neurofilament Light Polypeptide	0,00E+00	222,6010385	0	-2,347527186
Alpha Internexin	0,00E+00	146,1123207	0	-2,164686839
ELAV-like protein 3	0,00E+00	125,3539933	0	-2,098138173
Neuropeptide Y	0,00E+00	70,39201081	0	-1,847523371
Dihydropyrimidinase-related protein 1	0,00E+00	16,75027385	0	-1,224021912
Netrin	0,00E+00	7,649504346	0	-0,883633296

Table S3. Nine most downregulated proteins in SH-SY5Y CHIP-KO cells compared with WT, as revealed by the second LC–MS/MS analysis on the Orbitrap Exploris 480.

Protein	p-value	Fold Change	-Log (p-value)	Log Fold Change
Protein kinase C alpha	0,00E+00	0,037088231	0	1,43076388
Malonate and methylmalonate semialdehyde dehydrogenase	3,51E-04	0,077301182	0	1,111813863
Acyl-coenzyme A thioesterase 2	0,00E+00	0,081335873	0	1,089717865
Integrin Subunit Alpha 9	0,00E+00	0,089243408	0	1,049423854
Ras GTPase-activating-like protein	0,00E+00	0,090637081	0	1,042694092
Glycosylated lysosomal membrane protein	0,00E+00	0,097196484	0	1,012349447
HIG1 domain-containing protein	3,44E-04	0,097947533	0	1,0090065
Voltage-dependent calcium channel subunit alpha-2/delta-1	0,00E+00	0,101662211	0	0,992840449
Serine/threonine-protein kinase 10	0,00E+00	0,101676053	0	0,992781321

Table S4. Orthogonal SWATH-MS validation of CHIP-dependent proteomic changes.

Selected proteins showing significant up- or down-regulation in SH-SY5Y CHIP-knockout (KO) cells relative to wild-type (WT) controls, as identified by an independent data-independent acquisition (DIA/SWATH) LC–MS/MS analysis performed by the University of Edinburgh Prof Kathryn Ball research group.

Protein	p-value	Fold Change	-Log (p-value)	Log Fold Change
Carboxyl terminus of Hsp70-interacting Protein	1.80E-04	0.19	3.74	-0.71
Dihydropyrimidinase-related protein 2	2.60E-05	1.67	4.59	0.22
Neurofilament Light Polypeptide	5.25E-07	5.11	6.28	0.71
Neurofilament Medium Polypeptide	1.51E-06	5.22	5.82	0.72
ELAV-like protein 3	4.90E-04	7.44	3.31	0.87
Neurosecretory protein VGF	4.08E-09	10.83	8.39	1.03

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Scientific accomplishments

Awards

- ★ Best Poster Audience Award by CANVAS Spring School March 2023
- ★ 2nd place winner of the 2023 Women in STEM - contest by Polish - United States Fulbright Program
- ★ EMBO Travel Grant for "Neural Stem Cells: From Basic Understanding to Translational Applications" June 2022
- ★ Winner of King's College London Student Opportunity Fund June 2018

Publications

In progress: **Bedran D.***, Faktor J, Piróg A, Popławski J, Nita E, Bedran G, Kote S, Sznarkowska A, Dias C, Goodlet D, Węgrzyn G, Ball K, Hupp T.* CHIP as a potential biomarker for neurodegenerative diseases. (*co-corresponding authors: Bedran D, Hupp T).

Bedran D, Bedran G, Kote S. A Comprehensive Review of Neurodegenerative Manifestations of SARS-CoV-2. Vaccines (Basel). 2024 Feb 21;12(3):222. doi: 10.3390/vaccines12030222. PMID: 38543856; PMCID: PMC10974019.

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Olszewska, Anna, **Dominika Pankanin** "Searching for vitamin D receptor splice in human keratinocytes", Acta Biochemical, vol. 65, suppl. 2, 2018 p. 166,