Chapter 9:

Summary and Conclusion

9.1 Summary

9.1.1 Cloning, Expression and Purification of Recombinant full-length huntingtin proteins with SA or SD mutations

This study employed site-directed mutagenesis to substitute Ser13 and Ser16 in HTT with alanine (A) or aspartate (D), generating phosphoresistant and phosphomimetic variants to explore the effects of HTT phosphorylation. Using the *Sf9* insect cell-Baculovirus system, we efficiently expressed these variants and purified them employing FLAG affinity chromatography, yielding high-quality proteins for further analysis. The major findings of **Objective 1** are summarized as below:

- Generation of HttQ23 S13A, HttQ23 S13D, HttQ23 S16D, HttQ46 S13A, HttQ46 S13D and HttQ46 S16D mutagenized clones was successfully done by site-directed mutagenesis and Bacmid DNA of these recombinant clones were also generated.
- Bacmid DNA carrying wild type HttQ23, HttQ46 and HttQ78 (without any modifications) huntingtin gene were successfully generated.
- Wild type HTTQ23, HTTQ46 and HTTQ78 (without any modifications) huntingtin protein were expressed using *Sf9* insect cell-Baculovirus system and purified by affinity chromatography.
- Recombinant huntingtin proteins with SA or SD mutation (HTTQ23 S13A/S13D/S16D and Q46 S13A/S13D/S16D) were also expressed and purified using *Sf9* insect cell-Baculovirus system and affinity chromatography.

9.1.2 To Study the impact of phosphorylation status of Ser13 and Ser16 of normal and mutant huntingtin on PRC2 activity using cell-free and cell-based models

In this study, we explored the role of mutant huntingtin phosphorylation at Ser13 and Ser16 residues in influencing Polycomb Repressive Complex 2 (PRC2)-mediated epigenetic modifications associated with HD. Additionally, we investigated the induction of mHTT phosphorylation at these sites through the treatment with three compounds: Kinetin, BMS 345541, and Bay 11-7082. The major findings of **Objective 2** are summarized as below:

- Polycomb-repressive complex 2 (PRC2) was expressed and purified using FLAG Affinity chromatography. Mutant HTT (mtHTT Q78) with expanded Poly-Q repeats exhibits

greater PRC2 mediated H3K27me3 activity compared to HTT Q23 and HTT Q46 Poly-Q repeats. This confirms the already established fact that HTT interacts with PRC2 and facilitates PRC2 H3K27me3 activity in a Poly-Q length dependent manner.

- The reconstituted *in vitro* PRC2 histone H3K27-tri-methylase activity assay with purified huntingtin proteins with SA or SD mutation was successfully completed. More PRC2 mediated H3K27me3 signal is generated by mutant huntingtin (HTT Q46) with expanded Poly-Q repeats than HTT Q23. Yet, interestingly, the histone H3K27-trimethylase activity of huntingtin proteins with SD mutations (Q23 S13D, S16D, and Q46 S13D and S16D) was significantly reduced. This suggests that Ser13 and Ser16 residue replacement with phosphomimetic aspartate, which rescues the mutant huntingtin phenotype, is PRC2 dependent.
- The study assessed the effects of Kinetin, BMS 345541, and Bay 11-7082 on mHTT phosphorylation at Ser13/Ser16 in $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells. These compounds significantly increased the phosphorylation of mHTT in $STHdh^{Q111/Q111}$ cells compared to untreated controls.
- PRC2 activity, involved in histone methylation and gene regulation, was investigated alongside mHTT phosphorylation. Treatment with the compounds decreased PRC2-mediated trimethylation of histone H3 at lysine 27 (H3K27me3) in $STHdh^{Q111/Q111}$ cells. These results suggest that Kinetin, BMS 345541, and Bay 11-7082 altered the interaction between huntingtin and PRC2.

9.1.3 Deciphering the global impact of phosphorylation status of Ser13 and Ser16 on mutant huntingtin (mHTT) toxicity

In this study, we investigated the neuroprotective effects of Kinetin, BMS 345541, and Bay 11-7082 in preventing mutant huntingtin aggregation and its associated pathology in HD150Q cells. We examined the impact of these compounds on mHTT phosphorylation at Ser13 and Ser16 sites, ATP production, insoluble mHTT aggregates, restoration of soluble huntingtin, mitigation of various mitochondrial dysfunctions and ER stress, and overall cell viability. The major findings of **Objective 3** are summarized as below:

- HD150Q cells treated with Kinetin, BMS 345541, and Bay 11-7082 showed statistically significant elevated phosphorylation of Ser13/Ser16 compared to untreated cells.
- The impact of Kinetin, BMS 345541, and Bay 11-7082 treatment on cellular ATP production was also investigated using luminescent based ATP determination kit. The level of ATP was observed significantly lower in Ponasterone A treated HD 150Q cells compared to uninduced HD150Q cells. Interestingly, after treatment with different concentrations of Kinetin, BMS 345541 and Bay 11-7082 the ATP levels in Ponasterone A treated HD 150Q was significantly restored.
- Induction of mtHTT aggregates was successfully achieved using 1µM of Ponasterone A and it was characterized using fluorescence microscopy and western blotting. There was a time dependent increase in size and number of aggregates. Treatment with Kinetin, BMS 345541 and Bay 11-7082 led to prevention of mtHTT aggregate formation in a dose dependent manner.
- Kinetin, BMS 345541 and Bay 11-7082 were able to rescue some of the mitochondrial dysfunctions in HD condition.
- Kinetin, BMS 345541 and Bay 11-7082 could easily reverse the expression of some of the key genes as well as proteins involved in ER stress suggesting that these compounds could rescue ER stress successfully.
- Kinetin, BMS 345541, and Bay 11-7082 could also enhance the survival of HD150Q cells, positioning them as potential therapeutic agents for HD.

9.1.4 Identification of differentially expressed proteins associated with mHTT expression using quantitative proteomics approach

In this study, we conducted label-free quantitative proteomics to comprehensively profile protein expression levels in HD150Q control, Ponasterone A induced, Kinetin, BMS 345541, and Bay 11-7082 co-treated samples. This approach, which avoids the need for isotopic labels, enabled the identification and quantification of proteins dysregulated by mHTT expression, providing detailed insights into the molecular alterations associated with HD. The major findings of **Objective 4** are summarized as below:

- Protein quantification employed MaxQuant software utilizing maxLFQ for calculating label-free intensities, resulting in 1,598 confirmed proteins. Of these, 1,319 proteins with at least two unique peptides, with 779 proteins consistently present across all samples. Notably, 127 proteins exhibited "switch" behavior, changing their expression in response to Pon A and subsequent treatments with Kinetin, BMS 345541, and Bay 11-7082.
- Venn diagrams were used to assess common and unique proteins across treatment groups, revealing significant overlap and unique identifiers for different treatments.
- Differentially expressed proteins were analyzed using ShinyGO to identify enriched Gene Ontology terms and KEGG pathways associated with mHTT toxicity.
- Key pathways altered by treatment included the proteasome and ribosome pathways, showing downregulation in HD and upregulation post-treatment, indicating restored function.
- Treatments normalized downregulated processes (e.g., translation, peptide biosynthesis) in HD cells, evidencing functional recovery.
- Findings suggest Kinetin, BMS 345541, and Bay 11-7082 restored cellular functions and reduced mHTT-induced toxicity, offering potential therapeutic avenues in Huntington's Disease.

9.1.5 Studying the peripheral effects of mutant huntingtin (mHTT) expression in cell line models of HD

In this study, we explored the impact of Kinetin, BMS 345541, and Bay 11-7082 treatments on the expression levels of key pro-inflammatory (*IL-1β*, *IL-6*, *TNF-α*) and anti-inflammatory (*TGF-β1*, *IL-10*) marker genes in HD and control lymphoblastoid cell lines. We also examined the impact of secretome from HD150Q cells on immune cells with normal huntingtin by exposing freshly isolated human PBMCs and THP-1 cells to conditioned media from uninduced or Ponasterone A-induced HD150Q cells for different time periods. The major findings of **Objective 5** are summarized as below:

- HD lymphoblastoid cell lines (LCLs) exhibited significantly elevated pro-inflammatory (*IL-1β*, *IL-6*, *TNF-α*) and anti-inflammatory (*TGF-β1*, *IL-10*) marker genes compared to controls; treatments reduced these markers to baseline levels.

- The secretome from HD150Q cells alters gene expression in immune cells (PBMCs and THP-1), indicating mHTT's role in inducing inflammation.
- Treatment of *STHdh*$^{Q7/Q7}$ cells with HD150Q secretome affects ATP levels, highlighting metabolic impacts linked to mHTT.

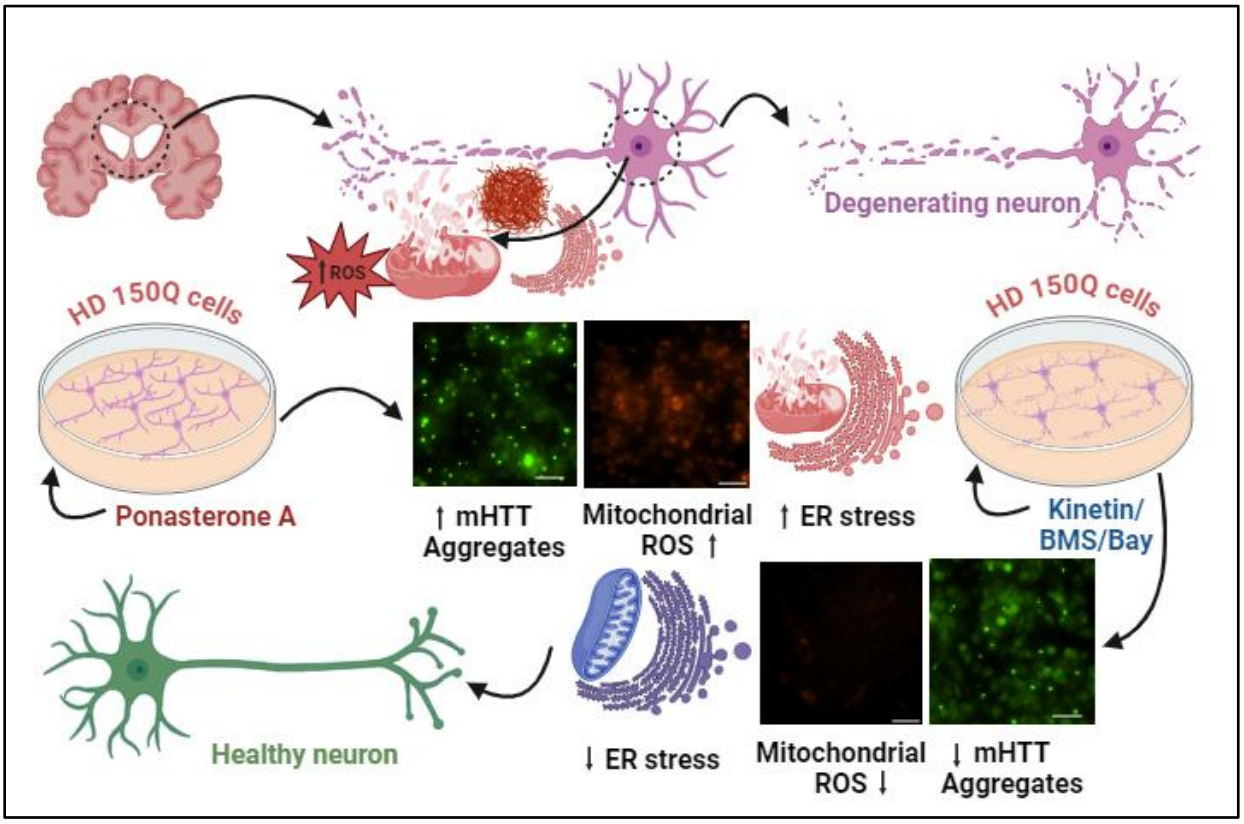

Figure 9.1: Schematic representation of the current study.

9.2 Conclusion

In this comprehensive study, we explored the molecular and cellular underpinnings of Huntington's Disease by focusing on the phosphorylation of mutant huntingtin and its interactions with various cellular components and pathways. Through a series of meticulously designed experiments, we cloned, expressed, and purified full-length huntingtin proteins with specific Ser13 and Ser16 mutations to generate phosphoresistant and phosphomimetic variants. These variants allowed us to investigate the impact of HTT phosphorylation on Polycomb Repressive Complex 2 activity and other cellular processes using cell-free and cell-based models.

Our findings revealed that mHTT with expanded poly-Q repeats enhances PRC2-mediated H3K27me3 activity, and interestingly, the phosphomimetic variants exhibited significantly reduced PRC2 activity. This suggests a potential therapeutic role for manipulating HTT phosphorylation. Furthermore, treatment with Kinetin, BMS 345541, and Bay 11-7082 significantly increased mHTT phosphorylation and altered PRC2 interactions, leading to decreased histone H3K27 trimethylation, indicating these compounds' potential to modulate epigenetic regulation in HD.

We also demonstrated that these treatments prevented mHTT aggregation, rescued mitochondrial dysfunctions, alleviated ER stress, and improved cell viability in HD150Q cells. This underscores the therapeutic potential of these compounds in mitigating HD pathology. Additionally, our label-free quantitative proteomics approach provided a detailed profile of differentially expressed proteins, highlighting key pathways and biological processes altered by HD and normalized post-treatment, including translation, peptide biosynthesis, and proteasome function.

Lastly, we examined the peripheral effects of mHTT expression in cell line models, showing that HD lymphoblastoid cells exhibit altered pro- and anti-inflammatory marker expression, which is ameliorated by our treatments. The secretome from HD cells also impacted immune cell gene expression and metabolic functions, further illustrating the widespread effects of mHTT.

In conclusion, our study provides significant insights into the molecular mechanisms underlying HD and highlights the potential of targeting mHTT phosphorylation as a therapeutic strategy. The detailed proteomic, epigenetic, and cellular analyses offer a robust framework for future research and therapeutic development in Huntington's Disease.

9.3 Limitations of the study

1. Cell Line Model Limitations: Although the pathogenicity induced by expanded CAG repeats was validated in HD150Q and other cell line models, these models may not fully replicate the complex pathology of Huntington's Disease as observed in human patients. This limitation could impact the generalizability of the therapeutic efficacy and molecular mechanisms explored. Validation in additional models, such as mouse models and patient-derived neurons, would further strengthen the hypothesis and provide more comprehensive insights.

2. Proteomics Analysis Scope: Although label-free quantitative proteomics provided valuable insights, the method's resolution and sensitivity may not have detected all relevant proteins and subtle changes in protein expression, potentially overlooking some critical molecular alterations associated with mHTT toxicity.
3. Limited Long-Term Evaluation: The study's assessments primarily focused on short-term effects of treatments and phosphorylation status. Long-term effects, including sustained therapeutic outcomes and potential side effects, were not evaluated, which is essential for understanding the practical therapeutic potential.
4. Secretome Analysis Constraints: The impact of HD150Q cell secretome on immune cells was investigated using conditioned media, which may not encompass all relevant factors and interactions, potentially limiting the understanding of mHTT's full role in immune modulation.

9.4 Future Perspectives

Building on the comprehensive findings of this study, several future directions could further enhance our understanding of Huntington's Disease and advance therapeutic strategies:

1. Validation in *HD animal* models: While the cell line models used provided valuable insights, the validation of these findings to *in vivo* systems is crucial. Future research should involve validating the observed effects of Kinetin, BMS 345541, and Bay 11-7082 in relevant mouse models of HD. These models will help assess the efficacy and safety of these compounds in a more complex biological context, providing a clearer picture of their therapeutic potential and possible side effects.
2. Patient-Derived Cellular Models: Incorporating patient-derived neurons into the study could offer a more accurate representation of human HD pathology. These models can provide insights into how the identified therapeutic compounds affect human cells at a more relevant biological level, potentially uncovering patient-specific responses and optimizing treatment strategies.
3. Mechanistic Studies: Further elucidation of the molecular mechanisms by which HTT phosphorylation influences PRC2 activity and other cellular pathways is essential. Investigating the downstream effects of altered PRC2-mediated H3K27 trimethylation and

its impact on gene expression could reveal new therapeutic targets and refine the strategies to modulate epigenetic regulation in HD.

4. Long-Term Treatment Effects: Assessing the long-term effects of the therapeutic compounds on HD pathology will be important. Chronic administration studies in animal models or human cell cultures could provide information on the sustainability of the therapeutic benefits, potential resistance mechanisms, and any long-term adverse effects.
5. Broader Proteomic and Transcriptomic Analysis: Expanding the proteomic and transcriptomic analyses to include a wider range of cellular contexts and conditions will help in identifying additional biomarkers and therapeutic targets. Integrating these data with functional assays could elucidate how the identified proteins and pathways interact in the context of HD.
6. Investigating Alternative Compounds: Screening additional compounds with different mechanisms of action may uncover new therapeutic options for HD. Combining these with the current compounds in a synergistic manner could enhance therapeutic efficacy and address multiple aspects of HD pathology.
7. Exploring Systemic Effects: Understanding the systemic impact of mHTT expression and its influence on other organ systems beyond the central nervous system could provide a holistic view of the disease. Investigating the interplay between HD pathology and systemic inflammation or metabolic dysfunction could uncover novel therapeutic approaches.

By addressing these future research directions, we can build on the insights gained from this study and move closer to developing effective therapies for Huntington's Disease.