Chapter 2:
Aims and Objectives

2.1 Rationale and Hypothesis

Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disorder caused by an expanded CAG repeat in the *HTT* gene, leading to the production of mutant huntingtin (mHTT) protein with an abnormally long polyglutamine tract. Despite extensive research since the identification of the mutation over 30 years ago, there is no cure available for HD. Current therapeutic strategies primarily focus on silencing the mutant allele or modifying huntingtin post-translationally, with some success in animal models but limited translational impact in clinical settings.

Phosphorylation of huntingtin at specific residues has emerged as a potential modulator of its toxicity. Notably, phosphorylation at serine residues Serine13 and Serine16 has been shown to confer protective effects, reducing mHTT aggregation and ameliorating HD phenotypes in transgenic mouse models. The precise mechanisms underlying these protective effects, particularly their relationship with the Polycomb repressive complex 2 (PRC2) and mitochondrial function as well as ER stress, remain unclear and warrant further investigation. This study aims to elucidate the role of Ser13 and Ser16 phosphorylation in modulating mHTT toxicity, with a focus on PRC2 activity and normalization of dysregulated pathways.

We hypothesize that Ser13/Ser16 phosphorylation alleviates mutant huntingtin (mHTT) toxicity by influencing PRC2 activity and normalization of dysregulated pathways. Specifically, we propose that phosphomimetic substitution of these residues rescue the toxic phenotype of mHTT, leading to reduced PRC2 overactivation. This hypothesis is grounded in prior findings that demonstrate the protective nature of Ser13/Ser16 phosphorylation in HD models and seeks to extend these observations by exploring their mechanistic underpinnings.

To test this hypothesis, we have employed a combination of *in-vitro* and cell-based assays, utilizing recombinant huntingtin proteins with phosphomimetic Serine to Aspartic acid (SD) or phospho-resistant Serine to Alanine (SA) substitutions at Ser13 and Ser16. We have assessed the impact of these mutations on PRC2-mediated histone modifications and studied mHTT aggregation, mitochondrial bioenergetics, ER stress and mHTT toxicity in cell line models, including $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ mouse striatal cells and HD150Q cells as well as patient-derived lymphoblastoid cell lines. The outcomes of this study provide critical insights into the therapeutic potential of targeting huntingtin phosphorylation in HD.

2.2 Proposed Objectives

- **Objective 1: Cloning, Expression and Purification of Recombinant full-length huntingtin proteins with SA or SD mutations.**

- Generation of full-length huntingtin baculovirus constructs (pFastBacQ23*HTT* and pFastBacQ78*HTT*) with Ser13 and Ser16 substituted to alanine (SA) or aspartate (SD) by site-directed mutagenesis.
    - Design of mutagenesis primers to generate Serine to Alanine and Aspartate mutants at position 13 and 16 as reported previously (**Gu *et al.*, 2009**).
    - Carry out mutagenesis as per the instructions (QuikChange II XL SDM kit from Agilent technologies).
    - Confirm the clones by sequencing.

- Expression and purification of the huntingtin proteins with SA or SD substitutions using *Sf9* insect cell Baculovirus system and affinity chromatography.
    - Generation of recombinant bacmid DNA carrying huntingtin gene with SA or SD substitutions by transformation of pFastBac clones into *E. coli* DH10Bac cells.
    - Transfection of recombinant bacmid DNA into *Sf9* insect cells using Cellfectin reagent (Invitrogen).
    - Isolation of recombinant baculovirus and their amplification.
    - Infection of large culture of *Sf9* insect cells with high titer recombinant baculovirus to express recombinant huntingtin.
    - Purification of the recombinant huntingtin using FLAG affinity chromatography.

- **Objective 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.**

- Analyzing the impact of these substitutions on huntingtin's facilitation of reconstituted PRC2 histone H3K27-tri-methylase activity *in vitro*.
    - Set-up the *in vitro* assay as described previously (**Seong *et al.*, 2010**).

- Determine the levels of Histone-H3-Lysine-27-tri-methylation using specific antibody (Cell Signaling Technology) by immunoblotting and quantify with ImageJ (NIH). Normalize with total Histone H3 levels.

- Analyzing effect of specific kinase and phosphatase inhibitors that impact phosphorylation status of Ser13 and Ser16 of normal and mutant huntingtin on PRC2 activity in cell line models of HD.

  - Treat cultured *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ cells with different kinase and phosphatase inhibitors along with vehicle.

  - Determine the effect of PRC2 mediated Histone-H3-K27-tri-methylation using specific antibody (Cell Signaling Technology) by western blotting and quantify with ImageJ (NIH). Normalize with total Histone H3 levels.

- **Objective 3: Deciphering the global impact of phosphorylation status of Ser13 and Ser16 on mutant huntingtin (mHTT) toxicity.**

- Study neuroprotective effects of specific kinase and phosphatase inhibitors that modulate HTT phosphorylation by-
- Determining mHTT aggregation in HD 150Q cells.
- Investigating mutant huntingtin (mHTT) toxicity in Mitochondrial Dysfunction.
- Analyzing ER stress markers playing role in mHTT toxicity by Real Time PCR and Western Blotting.
- Assessing cell survival by MTT assay.

- **Objective 4: Identification of differentially expressed proteins associated with mHTT expression using quantitative proteomics approach.**

- **Objective 5: Studying the peripheral effects of mutant huntingtin (mHTT) expression in cell line models of HD.**

- Establishment and Characterization of EBV-Infected Lymphoblastoid Cell Lines (LCLs) from healthy control and HD patients.

  - Epstein Barr Virus (EBV) Production.

- Generation of Lymphoblastoid Cell Lines (LCLs).
- Characterization of Lymphoblastoid Cell Lines (LCLs).

- Effects of specific kinase and phosphatase inhibitors on Inflammatory Marker Gene Expression in HD LCL Cells.
    - Checking the expression of different key pro-inflammatory (*IL-1β*, *IL-6*, *TNF-α*) and anti-inflammatory (*TGF-β1*, *IL-10*) marker genes by Real Time PCR (RT-qPCR).
- Impact of HD150Q Cell Secretome on Immune Cells Expressing Normal Huntingtin.