Chapter 5:

Results and Discussion 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

This part of the study aims to investigate the role of mutant huntingtin phosphorylation at Ser13 and Ser16 residues on Polycomb Repressive Complex 2 (PRC2)-mediated epigenetic modifications implicated in HD. mHTT phosphorylation at these sites was induced by treatment of three compounds- Kinetin, BMS 345541, and Bay 11-7082, as reported earlier. Understanding the molecular mechanisms by which these compounds influence HD pathology is crucial for developing targeted therapies. Each compound has shown potential in modulating key pathways involved in HD, and this study provides comprehensive insights into the role of mHTT phosphorylation in PRC2 complex mediated gene regulation.

Kinetin, a plant-derived cytokinin, modulates Casein Kinase 2 (CK2) activity, enhancing phosphorylation of huntingtin's N-terminal domain at Ser13 and Ser16 [1]. CK2, a key kinase in HTT phosphorylation, facilitates mutant huntingtin clearance via proteasomal and lysosomal pathways [2]. Functioning as an ATP analog, kinetin is particularly effective under reduced intracellular ATP levels, a characteristic of Huntington's disease. By augmenting CK2-mediated phosphorylation, kinetin mitigates mHTT aggregation and cytotoxicity, altering its subcellular localization and promoting degradation [1].

BMS 345541, a selective IKKβ inhibitor within the NF-κB pathway, modulates mHTT phosphorylation at Ser13 and Ser16 [3]. Paradoxically, IKKβ inhibition enhances N17 domain phosphorylation via compensatory kinase activation, altering mHTT's nuclear-cytoplasmic distribution and promoting cytoplasmic degradation while reducing nuclear aggregation, a hallmark of HD [3]. Furthermore, BMS 345541 attenuates neuroinflammatory responses, which contribute to HD progression [4,5]. By modulating NF-κB activity, which interacts with chromatin remodeling, BMS 345541 may indirectly reduce PRC2-mediated H3K27me3 levels, upregulated in HD.

Unlike BMS 345541, Bay 11-7082 inhibits IKK via irreversible alkylation of cysteine residues in its kinase domain, blocking NF-κB signaling and attenuating inflammatory and stress responses elevated in HD models [3,6,7]. While it similarly enhances N17 phosphorylation, Bay 11-7082 exerts broader proteostatic effects [3]. Its impact on PRC2 activity likely stems from indirect NF-κB suppression, reducing PRC2 chromatin recruitment and H3K27me3 levels.

Huntington's disease is characterized by the presence of mutant huntingtin (mHTT) protein aggregates, which contribute to neuronal dysfunction and death [8,9]. Phosphorylation of mHTT,

especially at serine residues 13 and 16 (Ser13/Ser16), has been identified as a crucial modification that can alleviate its toxic effects. This phosphorylation alters the subcellular localization and aggregation properties of mHTT, thus reducing its harmful impact on cells [10,3,11]. In this study, the effects of three specific compounds- Kinetin, BMS 345541, and Bay 11-7082 on the phosphorylation status of mHTT at Ser13/Ser16 were investigated. This was carried out using two cell models: *STHdh*$^{Q7/Q7}$ cells, homozygous for the wild-type HTT gene, and *STHdh*$^{Q111/Q111}$ cells, homozygous for the mutant HTT gene with an expanded polyglutamine tract. The goal was to assess if these compounds could increase mHTT phosphorylation at these specific serine residues, thereby mitigating their toxicity and providing therapeutic insights for HD by targeting the molecular mechanisms of mHTT toxicity.

Additionally, HD is also associated with epigenetic alterations that contribute to disease progression [12]. PRC2 plays a crucial role in chromatin remodeling and gene expression regulation through its catalytic subunit, EZH2, which tri-methylates Histone H3 at lysine 27 (H3K27me3) [13]. In HD, dysregulation of PRC2 activity and subsequent epigenetic alterations contribute to neurodegeneration and disease progression [14]. Previous research has shown that huntingtin associates with the PRC2 and increases its activity in a polyQ length dependent manner and leads to abnormal gene silencing [15].

This study examined the effects of Kinetin, BMS 345541, and Bay 11-7082 on PRC2-mediated H3K27me3, a marker of epigenetic silencing, in *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ cells. Additionally, we assessed PRC2-mediated H3K27me3 activity using site-directed mutagenesis-generated phosphomimetic (Ser→Asp) and phospho-resistant (Ser→Ala) Ser13/16 HTT proteins to further understand the impact of these modifications on the regulation of gene expression in HD. Phosphomimetic mutants may mimic the charge-dependent effects of phosphorylation, disrupting mHTT-PRC2 interactions, whereas phospho-resistant mutants could stabilize these interactions, providing a direct link between phosphorylation status and PRC2 dysregulation. By combining pharmacological and genetic approaches, this work elucidates how Ser13/16 phosphorylation modulates PRC2 activity, offering mechanistic insights into epigenetic dysfunction in HD and the therapeutic potential of targeting mHTT post-translational modifications.

5.1 Expression and Purification of Polycomb Repressive Complex 2 (PRC2) using the *Sf9* Insect Cell Baculovirus System and Affinity Chromatography

The Polycomb Repressive Complex 2 protein was expressed and purified simultaneously, using the donor plasmid pFastBac, which contained the gene for each subunit of the PRC2 complex. Four sets of pFastBac plasmids were constructed, each carrying the genes for one of the four core subunits: *Ezh2*, *EED*, *Suz12*, and *RbAp48* (**Fig. 5.1.1**). Each donor plasmid set was transformed into *E. coli* DH10Bac host cells and subjected to antibiotic selection and blue-white screening. (**Fig. 5.1.2**). The bacmids containing the PRC2 subunit genes were then isolated from the culture of true white colony of each sub-unit using the alkaline lysis method (**Fig. 5.1.3**). The presence of the genes of interest was confirmed through PCR using gene specific primers (**Fig. 5.1.4**).

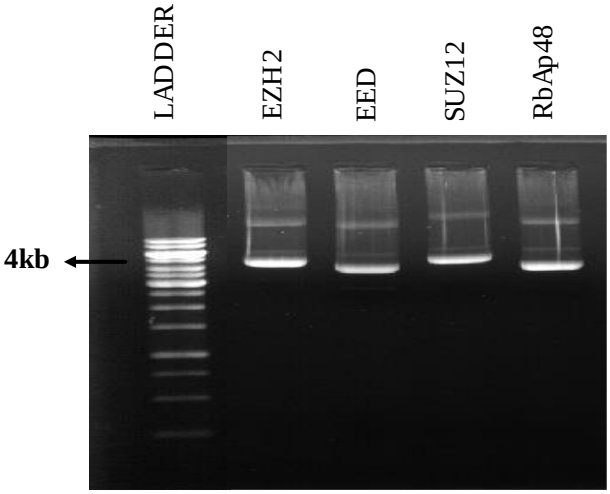


Figure: 5.1.1

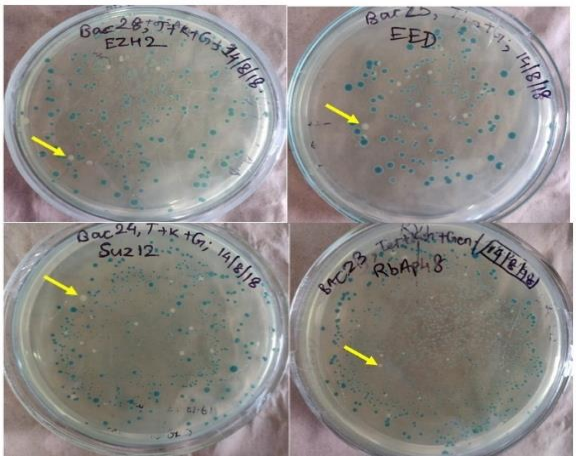


Figure: 5.1.2

Figure 5.1.1: 0.8% Agarose gel showing pFastBac vectors of PRC2 subunits (*Ezh2*, *EED*, *Suz12* and *RbAp48*).

Figure 5.1.2: Blue-white Colonies observed upon transformation of plasmids of PRC2 subunits into *E. coli* DH10Bac cells.

These bacmids were subsequently transfected into *Sf9* cells, an insect cell line derived from the pupal ovarian tissue of *Spodoptera frugiperda*, resulting in the generation of the P1 viral stock in the culture supernatant. To amplify the viral stock, *Sf9* cells were infected with the P1 stock and incubated for 8-10 days to produce the P2 stock, which was further used to infect the cells until there is ~90% cell death to generate a high-titer P3 viral stock. Protein expression was monitored at each stage by western blotting using an anti-EZH2 antibody (**Fig. 5.1.5 and Fig. 5.1.6**).

However, protein expressions for the remaining subunits could not be checked by immunoblotting due to the unavailability of specific antibodies.

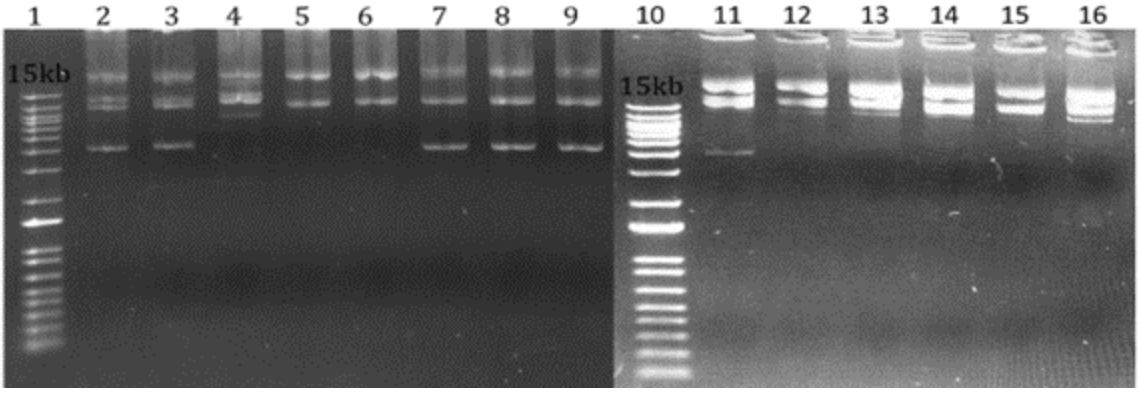


Figure 5.1.3: 0.8% Agarose gel showing bacmids of PRC2 subunits. (1- DNA ladder, 2-5 *Ezh2*, 6-9 *EED*, 10- DNA ladder, 11- *RbAp48* and 12-16 *Suz12*).

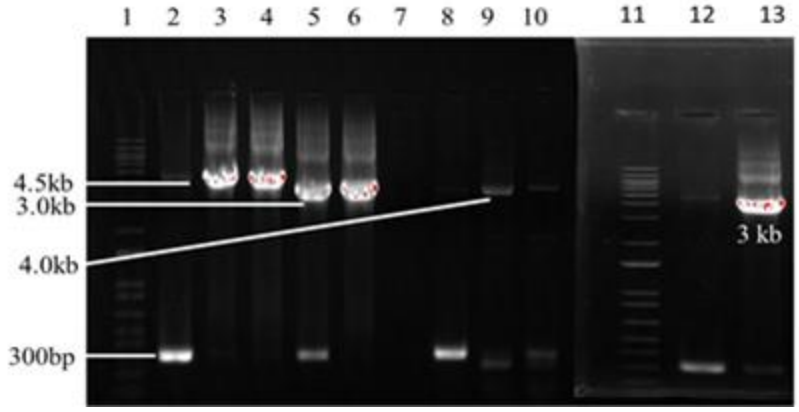


Figure 5.1.4: 0.8% Agarose gel showing PCR confirmation of bacmids of PRC2 subunits. (1 &11 DNA ladder, 2 &12- vector controls, 3-4 *Ezh2*, 5-6 *EED*, 7-8 & 13- *RbAp48*, 9-10 *Suz12*).

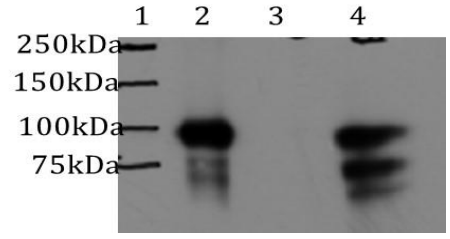


Figure 5.1.5: Western Blot image showing EZH2 expression at P2 stage. (1- Protein marker, 2- purified EZH2 as a control, 3- Transfection control and 4- EZH2 expressed at P2 stage).

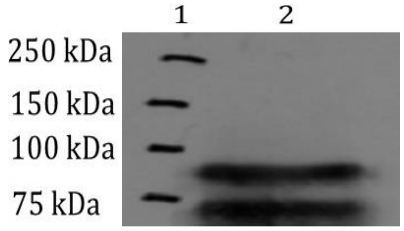


Figure 5.1.6: Western blot image showing EZH2 expression at P3 stage. (1- Protein marker and 2- EZH2 expressed at P3 stage).

PRC2 is a multi-subunit complex comprising two larger subunits, Ezh2 and Suz12, and two smaller subunits, EED and RbAp48. To express the PRC2 complex in *Sf9* cells, the cells were co-infected with high-titer P3 viral stocks containing baculovirus particles for each individual subunit (**Fig. 5.1.7**). This co-infection facilitated the expression of the PRC2 complex in the *Sf9* cells. Subsequently, the complex was purified using FLAG affinity chromatography. The eluted fractions were analyzed by SDS-PAGE and western blotting to assess protein purity. As shown in **Fig. 5.1.8**, a protein band was observed at approximately 85 kDa for EZH2 and SUZ12, and two bands were observed at approximately 50-55 kDa for EED and RbAp48, respectively. The protein expression of EZH2 was further confirmed by western blotting (**Fig. 5.1.9**).

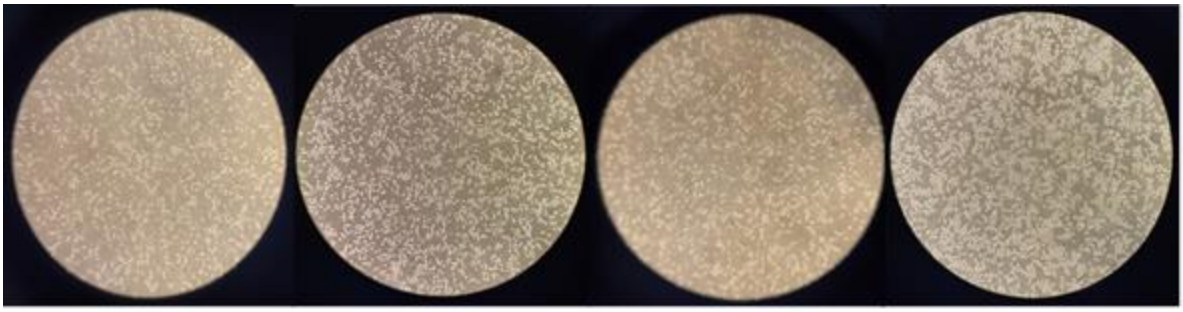


Figure 5.1.7: Representative microscopy images showing *Sf9* cells after 48 hours post-incubation with EZH2, EED, SUZ12 and RbAp48 P3 virus. Images were captured at 10X magnification.

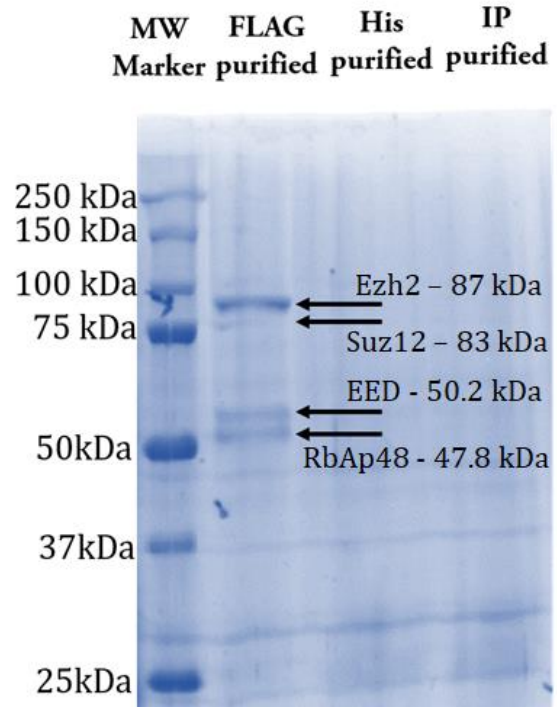


Figure: 5.1.8

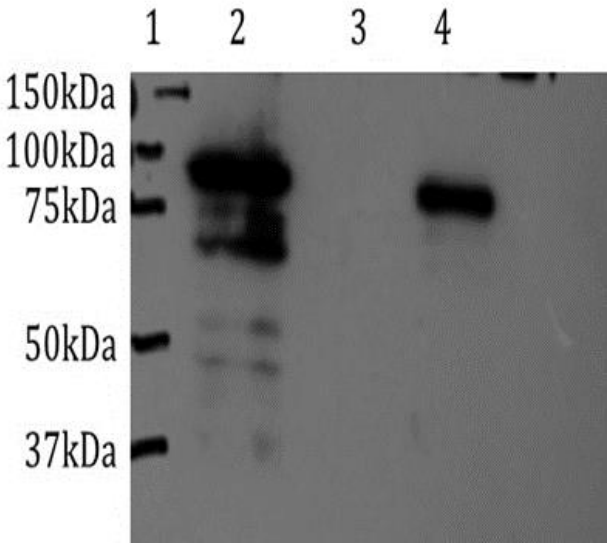


Figure: 5.1.9

Figure 5.1.8: SDS-PAGE gel showing purified PRC2 by different purification methods. (1-protein markers, 2- FLAG affinity, 3- His-tagged affinity purification and 4- immunoprecipitation).

Figure 5.1.9: Western Blot image showing purified PRC2 expression by different purification methods. (1-protein markers, 2- FLAG affinity purification, 3- His-tagged affinity purification and 4- immunoprecipitation).

5.2 Impact of Huntingtin Phosphomimetic Mutations on PRC2-Mediated H3K27 Trimethylation Activity in a PolyQ-Dependent Manner

In the cell-free assay designed to evaluate the H3K27 trimethylation activity of PRC2, purified PRC2 was added to the microcentrifuge tubes containing nucleosomal arrays. In this context, the presence of the methyl group donor S-adenosyl methionine (SAM) facilitates the trimethylation of Histone 3 at the lysine 27 residue by catalytic activity of EH2subunit. Our assay was performed both in the absence and presence of purified wild-type huntingtin (HTT), as depicted in **Fig. 5.2.1**. It was expected that PRC2 would interact with HTT, leading to an increase in H3K27 trimethylation levels in a polyQ-dependent manner. The degree of H3K27 trimethylation in each condition was determined using western blot analysis with anti-H3K27me3 specific antibodies, which are selective for Histone 3 trimethylated at the K27 residue. As shown in **Fig. 5.2.2**, the presence of mutant huntingtin (HTT Q78) with expanded poly-Q repeats resulted in a significantly higher PRC2-mediated H3K27me3 signal compared to HTT Q23 and HTT Q46. This observation corroborates the previous reports that HTT interacts with PRC2 in a polyQ-dependent manner, thereby enhancing PRC2 H3K27 trimethylation activity.

In addition, we conducted a reconstituted *in- vitro* PRC2 Histone H3K27-trimethylase activity assay using purified huntingtin proteins with serine-to-alanine (SA) or serine-to-aspartate (SD) mutations. As illustrated in **Fig. 5.2.3A** and **B**, mutant huntingtin (HTT Q46) with expanded poly-Q repeats generated a more pronounced PRC2-mediated H3K27me3 signal than HTT Q23. Interestingly, the Histone H3K27-trimethylase activity of huntingtin proteins with SD mutations (Q23 S13D, S16D, and Q46 S13D, S16D) was significantly reduced, as shown in **Fig. 5.2.3A** and **B**. This suggests that the substitution of Ser13 and Ser16 residues with phosphomimetic aspartate, which is known to rescue the mutant huntingtin phenotype, exerts its effect in a PRC2-dependent manner.

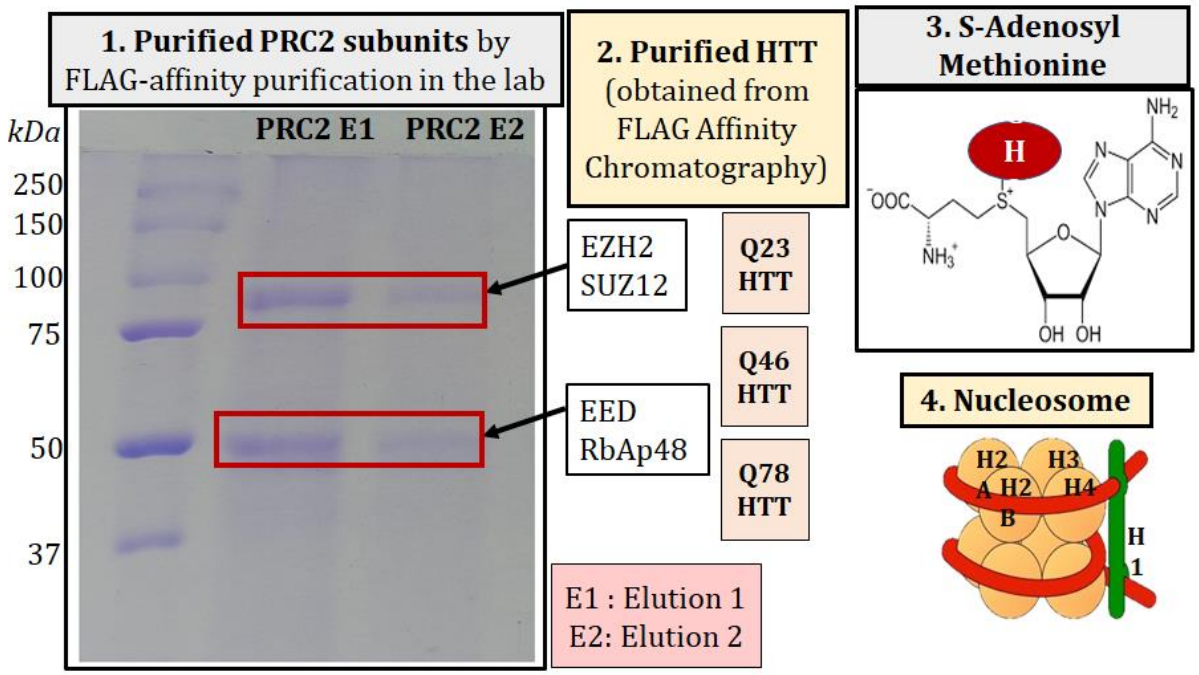


Figure 5.2.1: Pictorial representation showing *in- vitro* Assay for PRC2 mediated H3K27me3.

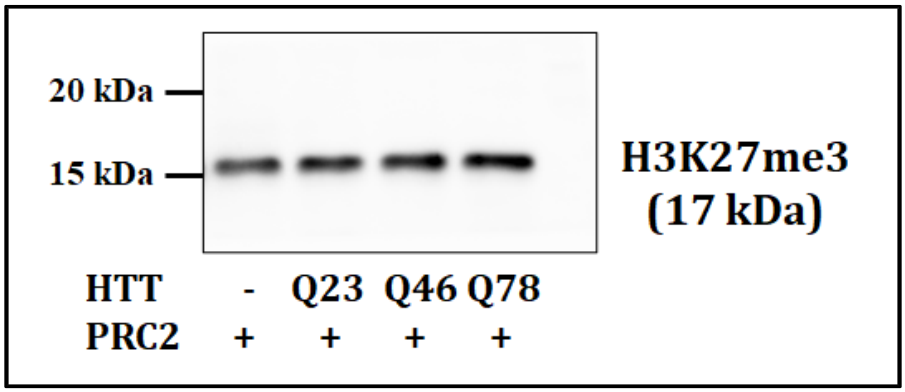
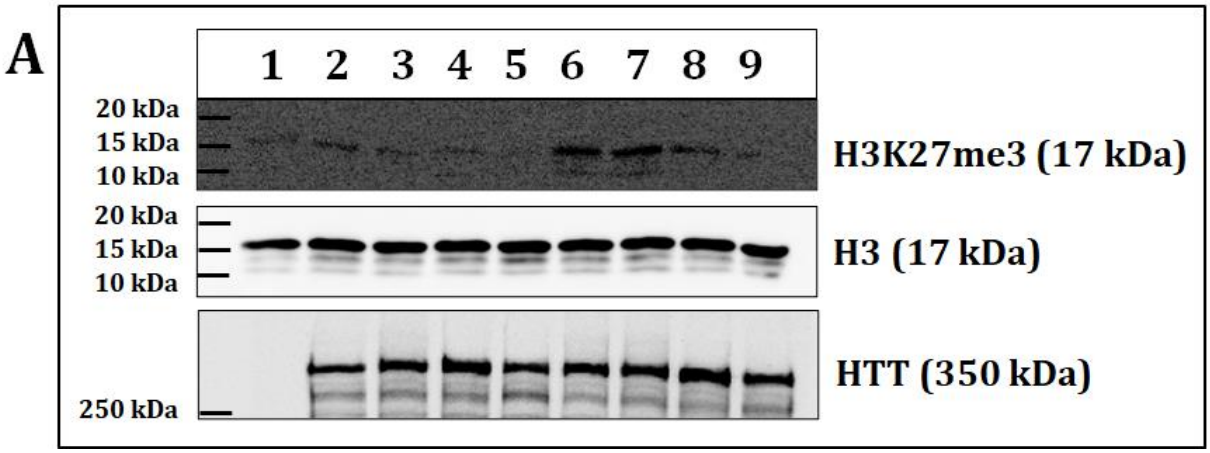


Figure 5.2.2: Western Blot image showing *in- vitro* Assay for PRC2 mediated H3K27me3.



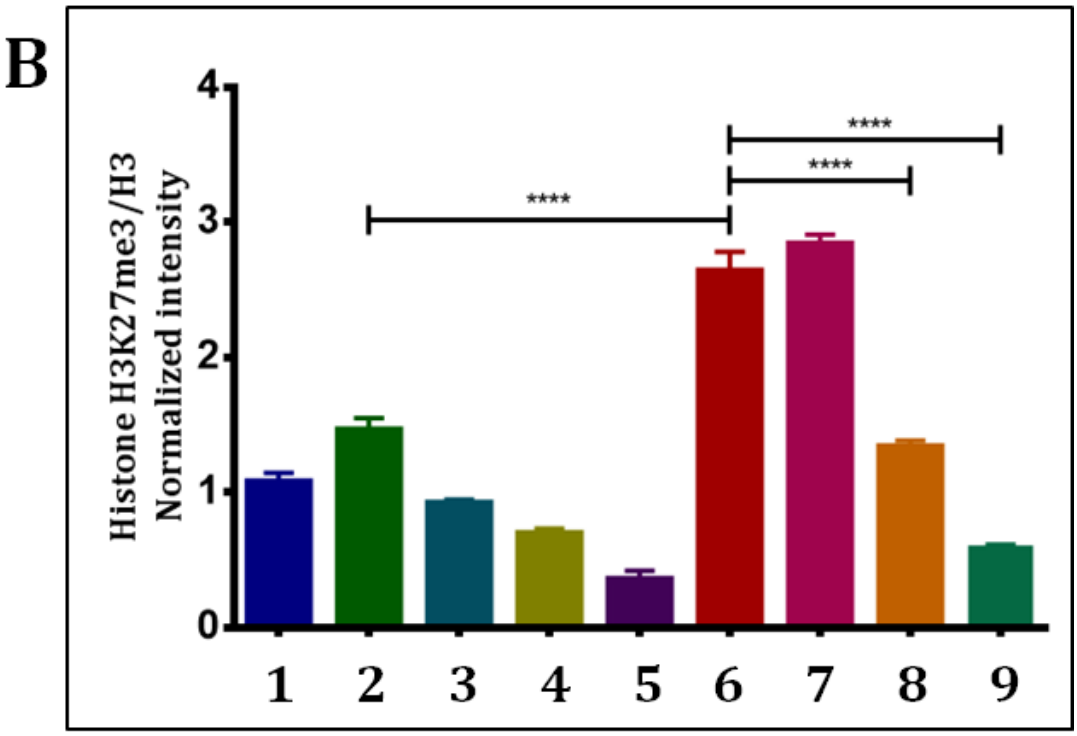
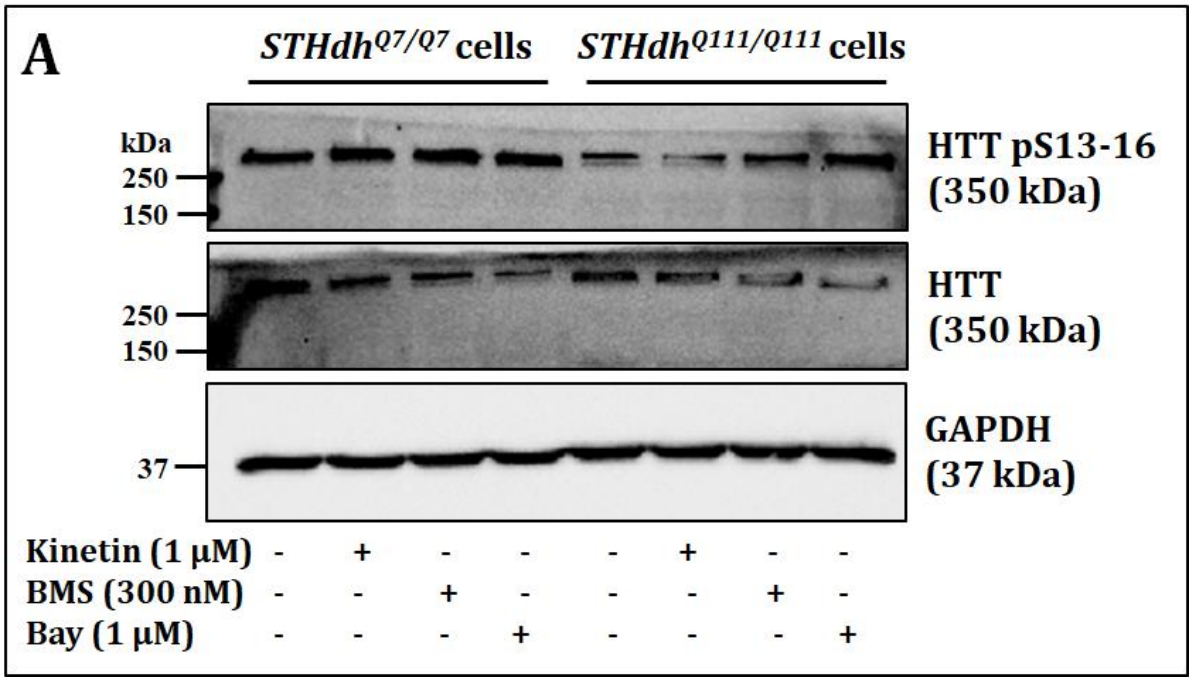


Figure 5.2.3: (A) Western Blot image and (B) Graphical representation for densitometric analysis showing *in- vitro* Assay for PRC2 mediated H3K27me3 with purified huntingtin proteins (HTT Q23 and Q46) with SA or SD mutation. (1- PRC2 alone 2- PRC2 + HTT Q23 wild type 3- PRC2 + HTT Q23 S13A 4- PRC2 + HTT Q23 S13D 5- PRC2 + HTT Q23 S16D 6- PRC2 + HTT Q46 wild type 7- PRC2 + HTT Q46 S13A 8- PRC2 + HTT Q46 S13D 9- PRC2 + HTT Q46 S16D).

5.3 Kinetin, BMS 345541, and Bay 11-7082 Increases Phosphorylation of Ser13/Ser16 in Mouse Striatal Cells Expressing Mutant Huntingtin

To explore the impact of Kinetin, BMS 345541, and Bay 11-7082 on mHTT phosphorylation at Ser13/Ser16, individual treatments were administered to *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ cells in DMEM supplemented with 0.2% FBS. DMSO alone served as a vehicle control. Following a 24-hour incubation period, cells were harvested, rinsed with 1X ice-cold phosphate-buffered saline (PBS), and subsequently subjected to western blot analysis. The anti-N17-S13pS16p antibody from the Coriell Institute for Medical Research, USA, was employed for this analysis. As anticipated, treatment with Kinetin, BMS 345541, and Bay 11-7082 significantly augmented HTT phosphorylation at Ser13/Ser16 in *STHdh*$^{Q111/Q111}$ cells compared to untreated cells (**Fig. 5.3.1A** and **B**).



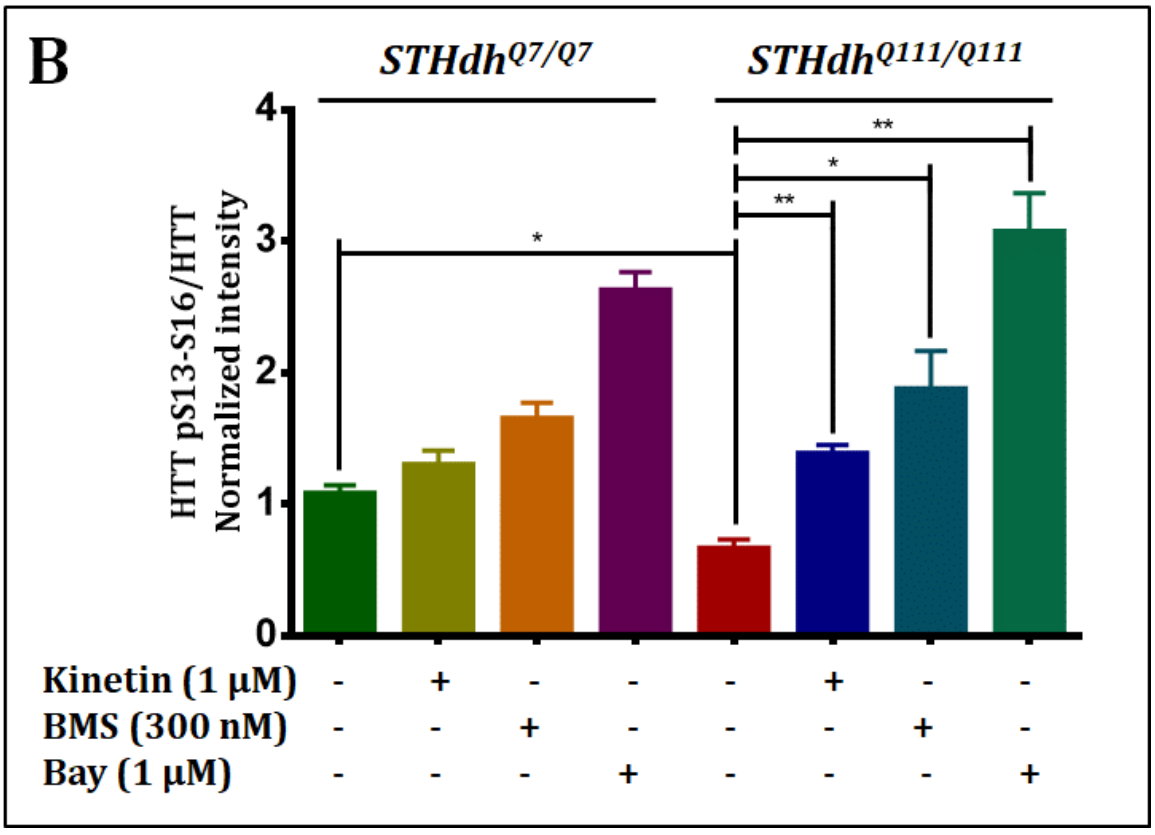
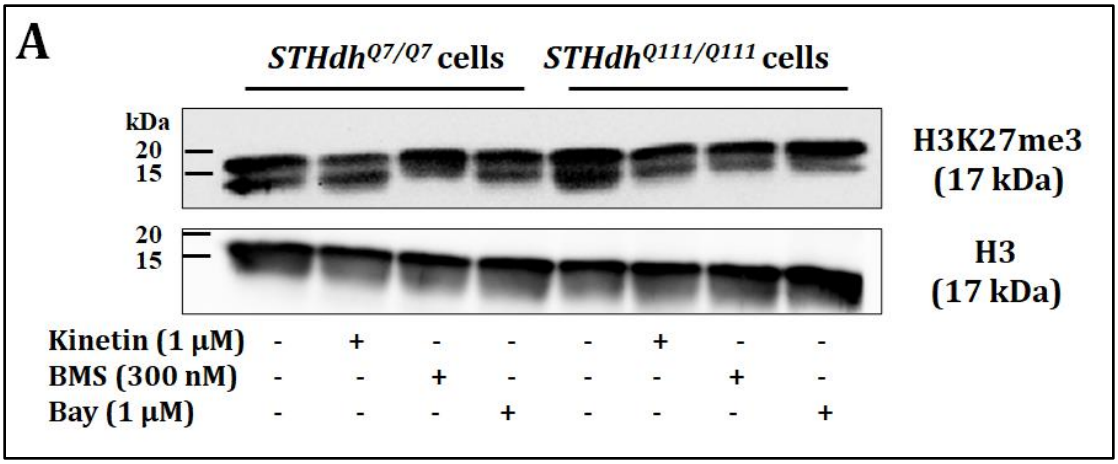


Figure 5.3.1: (A) Western Blot image and (B) Graphical representation for densitometric analysis showing pSer13-pSer16 level in Kinetin, BMS 345541, and Bay 11-7082 treated *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ cells (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**).

5.4 Kinetin, BMS 345541, and Bay 11-7082 reduces PRC2-Mediated Histone H3 Lysine 27 Trimethylation in Mouse Striatal Cell Lines

Mouse striatal cell lines, specifically *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$, were maintained under controlled conditions in DMEM supplemented with 10% FBS and 1X penicillin-streptomycin at 33°C in a 5% CO_2 incubator. To investigate the effects of Kinetin, BMS 345541, and Bay 11-7082 on PRC2-mediated Histone H3 Lysine 27 trimethylation, these cells were treated with each compound individually in DMEM containing 0.2% FBS, using DMSO as a vehicle control, for a duration of 24 hours. Following the incubation period, the cells were washed with 1X ice-cold PBS and then processed according to the protocol detailed in the methodology section to assess the effects of Kinetin, BMS 345541, and Bay 11-7082.

To determine the level of Histone H3 Lysine 27 trimethylation (H3K27me3), immunoblotting was performed. This assay aimed to investigate the modulation of PRC2 activity in response to the treatments with Kinetin, BMS 345541, and Bay 11-7082. Previous studies have demonstrated that huntingtin interacts with the polycomb repressive complex 2 (PRC2) and enhances its activity. PRC2 is a critical transcriptional regulator that mediates gene silencing through H3K27 trimethylation. It has been established that mutant huntingtin significantly enhances this activity compared to normal huntingtin [15]. As depicted in **Fig. 5.4.1A** and **B**, the level of H3K27 trimethylation was significantly reduced in *STHdh*$^{Q111/Q111}$ cells treated with Kinetin, BMS 345541, and Bay 11-7082 compared to the untreated control *STHdh*$^{Q7/Q7}$ cells. This suggests that mHTT Ser13 and Ser16 phosphorylation mediated by these compounds may influence the interaction between huntingtin and PRC2, thereby modulating its activity and reducing the associated trimethylation of Histone H3 at lysine 27.



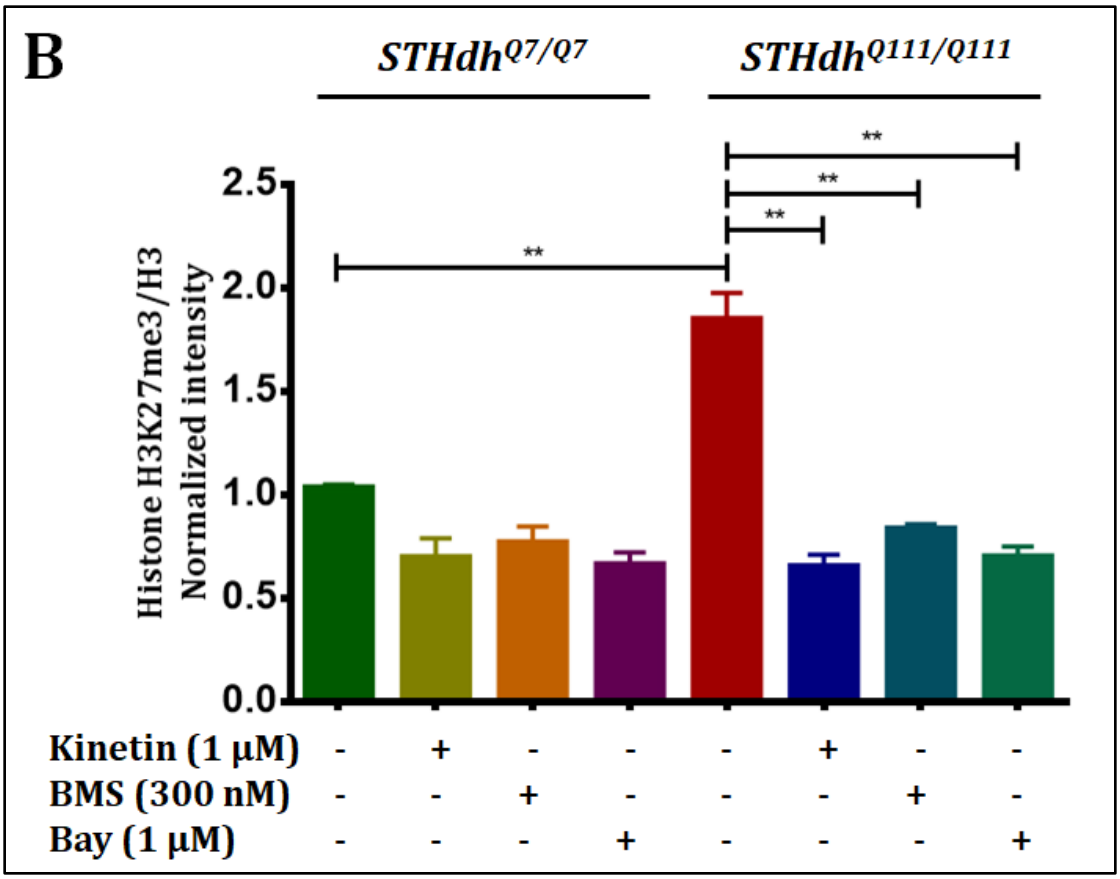


Figure 5.4.1: (A) Western Blot image and (B) Graphical representation for densitometric analysis showing Histone H3K27me3 activity in Kinetin, BMS 345541, and Bay 11-7082 treated *STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q111/Q111}$ cells (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**).

5.5 Discussion

In this part of the study, we explored a unique facet of Huntington's Disease pathology, focusing on the impact of phosphorylation status of mHTT at Ser13/Ser16 on Polycomb Repressive Complex 2 activity using 3 small molecules, reported earlier to increase phosphorylation at these sites. Our findings provide significant insights into the molecular mechanisms underlying gene expression dysregulation in HD and potential therapeutic targets.

We successfully expressed PRC2 sub-units using the *Sf9* insect cell baculovirus system and purified the complex by affinity chromatography. This methodological success allowed for subsequent functional studies on PRC2's role in HD. The use of this expression system ensured high yield and purity, facilitating accurate enzymatic assays [16]. Further, this study revealed that phosphomimetic mutations in huntingtin affect PRC2-mediated H3K27 trimethylation in a polyQ-

dependent manner. Specifically, these mutations modulated the enzymatic activity of PRC2, leading to altered Histone methylation patterns. This finding is significant as it links post-translational modifications of huntingtin with epigenetic regulation in HD, consistent with earlier reports that epigenetic dysregulation contributes to HD pathology [17-19,14,20,21].

We observed that Kinetin, BMS 345541, and Bay 11-7082 treatment increased the phosphorylation of Ser13/Ser16 in mouse striatal cells expressing mutant huntingtin. This phosphorylation event is crucial for modulating huntingtin's function and interactions [22,10,23-27]. The increase in phosphorylation suggests these compounds can alter the post-translational landscape of huntingtin, potentially reducing its toxic effects. In mouse striatal cell lines, treatment with Kinetin, BMS 345541, and Bay 11-7082 led to a significant reduction in PRC2-mediated H3K27 trimethylation. This effect indicates that these compounds can modulate epigenetic marks associated with gene repression. Given the role of PRC2 in regulating gene expression, this reduction could have widespread effects on neuronal gene expression profiles, potentially alleviating some aspects of HD pathology [28].

This part of the study highlights the complex interplay between post-translational modifications and epigenetic regulation in HD. By elucidating these mechanisms and demonstrating the potential of Kinetin, BMS 345541, and Bay 11-7082 to modulate these pathways, we provide a foundation for the development of targeted therapies for HD. Future research should focus on translating these findings into clinical applications, offering hope for more effective treatments for HD patients. Additionally, studies should aim to elucidate the precise mechanisms by which these compounds exert their effects, explore their efficacy in *in vivo* models of HD, and investigate their impact on other aspects of HD pathology, such as neuronal function and behavior, to provide comprehensive insights into their therapeutic potential.

5.6 References

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