

**Chapter 3:  
Materials and Methods**

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### 3.1 Materials

#### 3.1.1 Cell lines

##### 3.1.1.1 *STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup> knock-in mouse striatal cells

Mouse striatal cell lines expressing full length human huntingtin protein (*STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup> knock-in mouse striatal cells obtained from Coriell Institute for Medical Research USA) were used in this study. The *STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup> cell lines are derived from the striatal neurons of transgenic mice and are extensively used in Huntington's Disease (HD) research. These cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) from Gibco, Thermo Fisher Scientific, USA. The medium was supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and a 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco). The cells were kept at 33°C in a humidified incubator with 5% CO<sub>2</sub>. *STHdh*<sup>Q7/Q7</sup> cells express the wild-type huntingtin gene with 7 CAG repeats on both alleles, serving as a control model, while *STHdh*<sup>Q111/Q111</sup> cells express the mutant huntingtin gene with 111 CAG repeats on both alleles, mimicking the pathological conditions observed in HD patients. The *STHdh*<sup>Q111/Q111</sup> cells exhibit hallmark features of HD, such as protein aggregation, mitochondrial dysfunction, and increased oxidative stress, making them invaluable for studying the molecular mechanisms of HD and for screening potential therapeutics.

##### 3.1.1.2 HD150Q cell line

Another cell line for this study was the mouse neuroblastoma Neuro 2a cell line, expressing enhanced green fluorescent protein (e-GFP)-tagged truncated N-terminal huntingtin (Tn-HTT) with 150 polyglutamine (150Q) repeats under the regulation of an ecdysone-inducible promoter, was a kind gift from Prof. Nihar Ranjan Jana (National Brain Research Centre, Gurgaon, Haryana, India) and will be referred to as HD150Q. HD150Q cells were cultured in DMEM (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco) at 37°C in a humidified 5% CO<sub>2</sub> incubator. The HD150Q cell line represents a stable dual transfectant, comprising two plasmids: pVgRXR (conferring Zeocin resistance) and pIND-HD. The selection of dual transfectant cells was accomplished by employing a concentration of 0.4 mg/ml Zeocin (InvivoGen) along with G-418 (Sigma-Aldrich). Notably, the HD150Q cells demonstrate expression of GFP-fused mutant huntingtin exclusively in the presence of Ponasterone A (Sigma-Aldrich).

### 3.1.1.3 Human lymphoblastoid cell lines (LCLs) derived from healthy controls and Huntington's Disease (HD) patients

Human lymphoblastoid cell lines (LCLs) were established from peripheral blood lymphocytes of both healthy control individuals and patients diagnosed with Huntington's Disease (HD). The LCLs were cultured under standard conditions to ensure consistent growth and viability. Specifically, they were maintained in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco). The cells were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The control LCLs serve as a baseline for normal cellular functions, while the HD patient-derived LCLs exhibit characteristic features associated with the disease, such as altered gene expression and cellular stress responses. In this study, these cell lines were utilized to examine the expression profiles of anti-inflammatory and pro-inflammatory genes using RT-qPCR.

### 3.1.1.4 THP-1 cell line

We also included THP-1 cells in this study to investigate the effects of the HD secretome on these cells. THP-1 is a human monocytic cell line derived from an acute monocytic leukemia patient. By exposing THP-1 cells to the secretome from HTT induced HD150Q cells, we aimed to assess changes in inflammatory responses and gene expression. This approach helps to elucidate the impact of HD-associated secreted factors on immune cell function. These cells were cultured in RPMI 1640 medium (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), and 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco). The cells were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

### 3.1.1.5 Sf9 cells

We also utilized Sf9 cells, derived from the fall armyworm (*Spodoptera frugiperda*), that are commonly used for the expression of recombinant proteins. Their robust growth and high protein production capabilities with desired posttranslational modifications make them ideal for expressing complex eukaryotic proteins. We used these cells for expression and purification of human full-length huntingtin (HTT) and human polycomb repressive complex 2 (PRC2). Sf9 cells were maintained in Sf-900 II SFM (serum-free media, Gibco) with 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco) at 27°C in incubator.

### 3.1.2 Chemicals and reagents

Gene specific primers were procured from Integrated DNA Technologies (IDT), USA (primer details are provided in **Table 3.1**). Kinetin, BMS 345541, Bay 11-7082, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Films, *DpnI* restriction enzyme and Cellfectin II Reagent were obtained from Thermo Fisher Scientific, USA. RNase A and buffer P1 were used from the QIAprep Spin Miniprep Kit (Qiagen, Netherlands). Ammonium Bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>), Dithiothreitol (DTT), Iodoacetamide (IAA), Acetonitrile, Formic acid, Trypsin (porcine, MS grade), Sodium Azide, H<sub>2</sub>O<sub>2</sub>, anti-FLAG M2 agarose beads, 3X FLAG peptide, HIS-Select Nickel Affinity Gel, S-adenosyl methionine (SAM), Tris, glycine, Triton-X 100, and Coomassie Brilliant Blue were sourced from Sigma-Aldrich, USA. Primary antibodies for ER stress marker proteins were purchased from Cell Signaling Technology, USA, and Anti-HTT 4-19, N-terminal, and Anti-HTT PhosphoS13-S16 were obtained from Coriell Institute for Medical Research, USA. Histone H3 and Tri-Methyl-Histone H3 (Lys27) Rabbit mAb were also purchased from Cell Signaling Technology, USA. HRP-conjugated anti-rabbit and anti-mouse antibodies were used as secondary antibodies (Cell Signaling Technology, USA). TB Green Premix Ex Taq II (Tli RNase H Plus) (SYBR Green) and PrimeScript cDNA Synthesis Kits were acquired from Takara, Japan. The ATP Determination Kit and MitoSOX Red were obtained from Invitrogen, Thermo Fisher Scientific, USA. Protease and phosphatase inhibitors were purchased from Roche. PrimeSTAR GXL DNA Polymerase was purchased from Takara Bio, Japan. Zeocin was obtained from Invivogen, and Geneticin (G418 Sulfate) was purchased from Thermo Fisher Scientific, USA. Ponasterone A was sourced from Invitrogen, Thermo Fisher Scientific, USA. Pierce C18 Desalting Spin Columns were purchased from Thermo Fisher Scientific, USA. Kanamycin, Gentamycin, Tetracycline, X-gal, and IPTG were obtained from Sisco Research Laboratories (SRL), India. Immun-Blot® PVDF Membrane, Protein Assay Dye Reagent Concentrate, and Clarity Western ECL Substrate were purchased from Bio-Rad Laboratories, USA.

### 3.1.3 Constructs

pFastBac1 vectors containing *Ezh2*, *EED*, *Suz12*, *RbAp48*, and the HTT gene with 23, 46, and 78 CAG repeats (HttQ23, HttQ43, and HttQ78) were generously provided by Prof. Marcy MacDonald from Center for Human Genetic Research, MGH, Boston, MA 02114, USA.

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**Table 3.1:** List of primers used in the study.

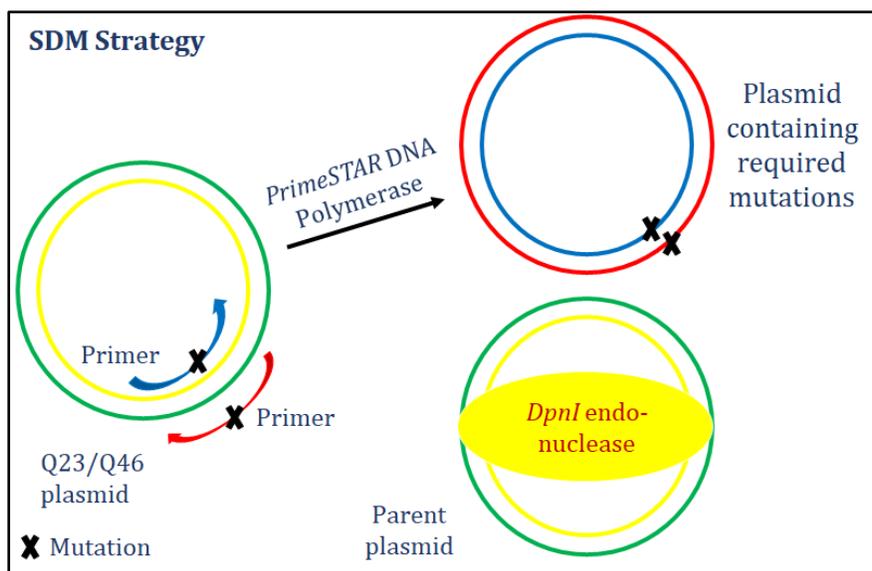
Genes	Forward Primers (5' to 3')	Reverse Primers (5' to 3')
<b>Primers for cloning</b>		
<b>HD-S13A</b>	GCCTTCGAGGCCCTCAAGTCCTTCCAGCAGC	GGACTTGAGGGCCTCGAAGGCCTTCATCAGC
<b>HD-S13D</b>	GCCTTCGAGGACCTCAAGTCCTTCCAGCAGC	GGACTTGAGGTCCTCGAAGGCCTTCATCAGC
<b>HD-S16A</b>	TCCCTCAAGGCCTTCCAGCAGCAGCAGCAGC	CTGCTGGAAGGCCTTGAGGGACTCGAAGGCC
<b>HD-S16D</b>	TCCCTCAAGGACTTCCAGCAGCAGCAGCAGC	CTGCTGGAAGTCCTTGAGGGACTCGAAGGCC
<b>Primers for RT-qPCR</b>		
<b>mBDNF</b>	TCATACTTCGGTTGCATGAAGG	AGACCTCTCGAACCTGCCC
<b>mPGC1a</b>	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
<b>mNRF1</b>	TATGGCGGAAGTAATGAAAGACG	CAACGTAAGCTCTGCCTTGTT
<b>mPERK</b>	AGTCCCTGCTCGAATCTTCT	TCCAAGGCAGAACAGATATACC
<b>mATF6</b>	GACTCACCCATCCGAGTTGTG	CTCCCAGTCTTCATCTGGTCC
<b>mXBP1s</b>	AGCAGCAAGTGGTGGATTTG	GAGTTTTCTCCCGTAAAAGCTGA
<b>mCHOP</b>	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
<b>mGAPDH</b>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<b>mB-Actin</b>	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAAACAATGCCATGT
<b>IL-6</b>	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC
<b>IL-1B</b>	CCAGGGACAGGATATGGAGCA	TTCAACACGCAGGACAGGTACAG
<b>TNF<math>\alpha</math></b>	GACAAGCCTGTAGCCCATGTTGTA	CAGCCTTGCCCTTGAAGA
<b>IL-10</b>	GAGATGCCTCAGCAGAGTGAAGA	AGGCTTGGAACCCAGGTAAC
<b>TGFB1</b>	AGCGACTCGCCAGAGTGGTTA	GCAGTGTGTTATCCCTGCTGTCA
<b>Beta Actin</b>	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<b>Primers for PCR</b>		
<b>HD CAG</b>	ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC	GCGGTGGCGGCTGTTGCTGCTGCTGCTGC
<b>CD19</b>	CCAGAACCAGTACGGGAACG	CTCGGGTTTCCATAAGACGGG
<b>CD3</b>	ACTGGCTACCCTTCTCTCG	CCGTTCCCTCTACCCATGTGA
<b>NCAM-I</b>	GGCATTTACAAGTGTGTGGTTAC	TTGGCGCATTCTTGAACATGA

### 3.2 Methodology

#### 3.2.1 Cloning, Expression and Purification of Recombinant full-length huntingtin proteins with SA or SD mutation

##### 3.2.1.1 Generation of full-length huntingtin baculovirus constructs (pFastBacQ23Htt and pFastBacQ46Htt) with Ser13 and Ser16 substituted to alanine (SA) or aspartate (SD) by site-directed mutagenesis

pFastBac vector containing *HTT* gene with 23 CAG or 46 CAG repeats were used for the mutagenesis in this study. pFastBac contains the expression cassette which is flanked by the left and right arms of Tn7, two antibiotic genes (gentamycin and ampicillin) and a multiple cloning site (MCS). The MCS has been modified to contain a polylinker containing 1X FLAG, 6X Histidine tag, TEV protease recognition site, and huntingtin gene cloned downstream of it using the restriction enzyme sites *NcoI*, *XhoI* and *SacII*. PCR based site directed mutagenesis (SDM) was carried out to modify serine 13 and 16 residues to alanine and aspartate, respectively. Partial overlapping primers were used for SDM in which base mutated was present in overlapping region of forward and reverse primers (primer details are provided in **Table 3.1**). PrimeSTAR GXL DNA Polymerase (Takara Bio, Japan) was used to carry out PCR based SDM. The PCR products obtained from SDM were subjected to *DpnI* restriction digestion at 37°C for 2 hours to digest parent template plasmid as *DpnI* cleaves only when its recognition site is methylated. After restriction digestion, a small amount of PCR products was run on Agarose gel electrophoresis to check their intactness.



Once the complete elimination of methylated parental plasmid DNA through *DpnI* digestion, the linear non-methylated plasmids were transformed into *E. coli* DH5 $\alpha$  competent cells to obtain multiple copies of circular plasmids. . The recombinant plasmids were isolated and subjected to PCR confirmation to check the cloning efficiency. These recombinant plasmids were also sent for sequencing. Further, the confirmed plasmids were transformed into *E. coli* DH10Bac competent cells harbouring bacmid and plasmid expressing transposase. The transposition event leads to generation of recombinant bacmid with huntingtin gene and are selected by antibiotics and blue-white screening. True white colonies are grown further to obtain recombinant bacmids. The Recombinant Bacmids were also confirmed by PCR using specific primers.

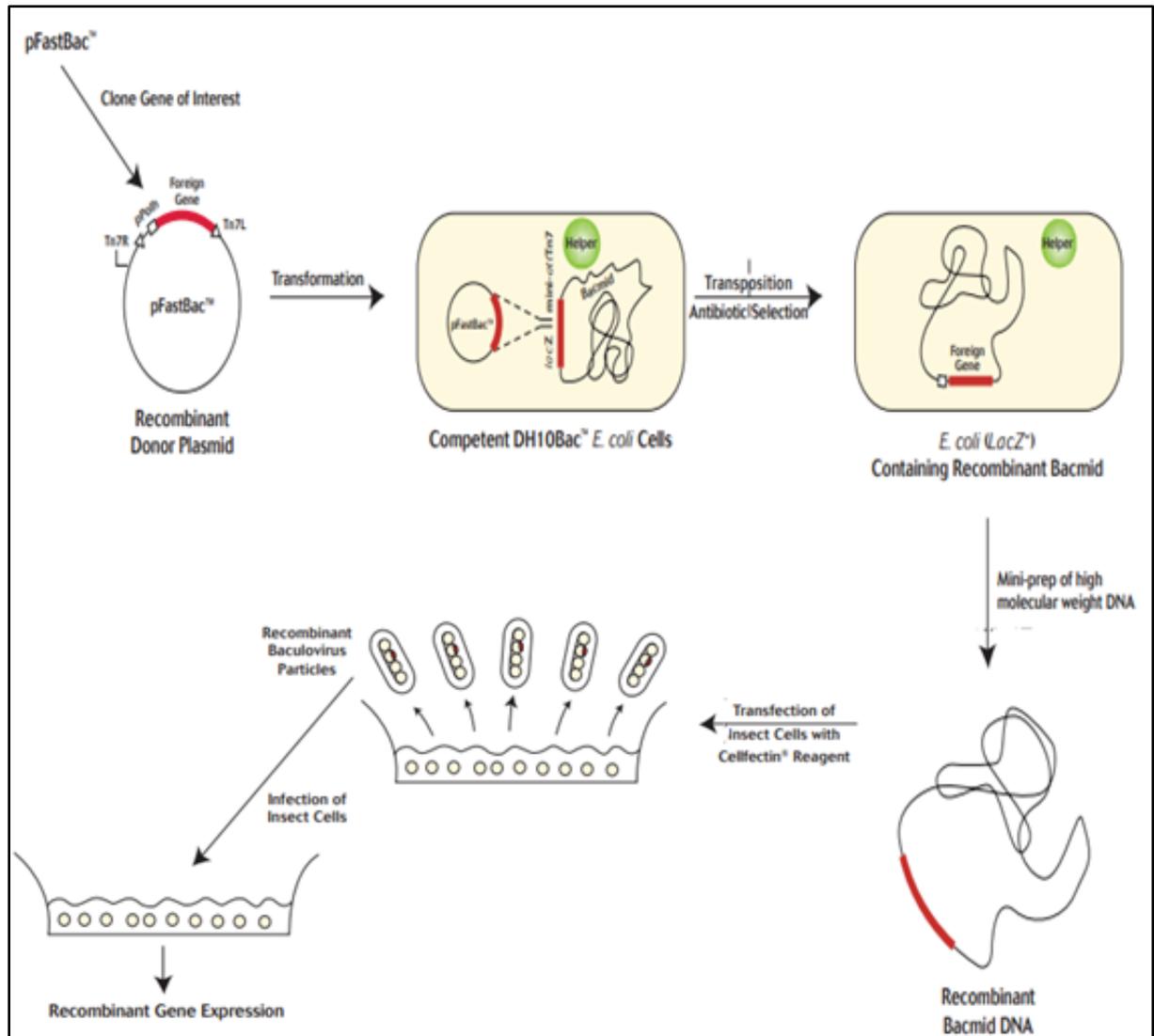
### **3.2.1.2 Expression of the huntingtin proteins with SA or SD mutation using *Sf9* insect cell Baculovirus system (from Bac-to-Bac system developed by Life Technologies-Invitrogen) [1,2]**

For expression and purification of wild type Q23, Q46 and Q78 proteins (without any modifications), pFastBac vectors containing *HTT* gene (HttQ23, HttQ43 and HttQ78) were transformed into *E. coli* DH10Bac competent cells and screened using antibiotic selection and blue white screening to obtain recombinant bacmids. The recombinant bacmids were confirmed by PCR using *HTT* gene specific primers. *Sf9* insect cells were used for transfection of recombinant bacmids and generation of baculovirus using cellfectin II (Invitrogen). After 72 hours of post-transfection, P1 baculovirus stock was collected and stored at 4°C in dark. Infected cells were lysed in 1X Laemmli buffer and loaded onto 8% acrylamide gel and subjected to SDS-PAGE. Post SDS-PAGE, the proteins were transferred onto PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) and the membrane was blocked with 5% fat-free milk in 1X TBST. Protein expression was checked using an anti-huntingtin antibody.

P1 baculovirus stock was used to generate high titer baculovirus stocks P2 and P3, respectively. Finally, *Sf9* insect cells were infected with high titer P3 virus stock to produce large amount of huntingtin protein. After 96 hours of infection, media was discarded and cells were lysed by freezing/thawing in lysis buffer (50mM Tris-Cl pH-8.0, 500mM NaCl and 5% glycerol) with protease and phosphatase inhibitors. Finally, huntingtin protein was purified using FLAG and His-tagged affinity chromatography as well as immunoprecipitation separately. Purity and integrity of the proteins was checked by Coomassie brilliant blue (CBB) staining of SDS-PAGE gel and

confirmed by western blotting. Expression and purification of recombinant huntingtin proteins with SA or SD mutation (HTTQ23 S13A/S13D/S16D and Q46 S13A/S13D/S16D) using *Sf9* insect cell-Baculovirus system and affinity chromatography was carried out same as described above.

### Strategy



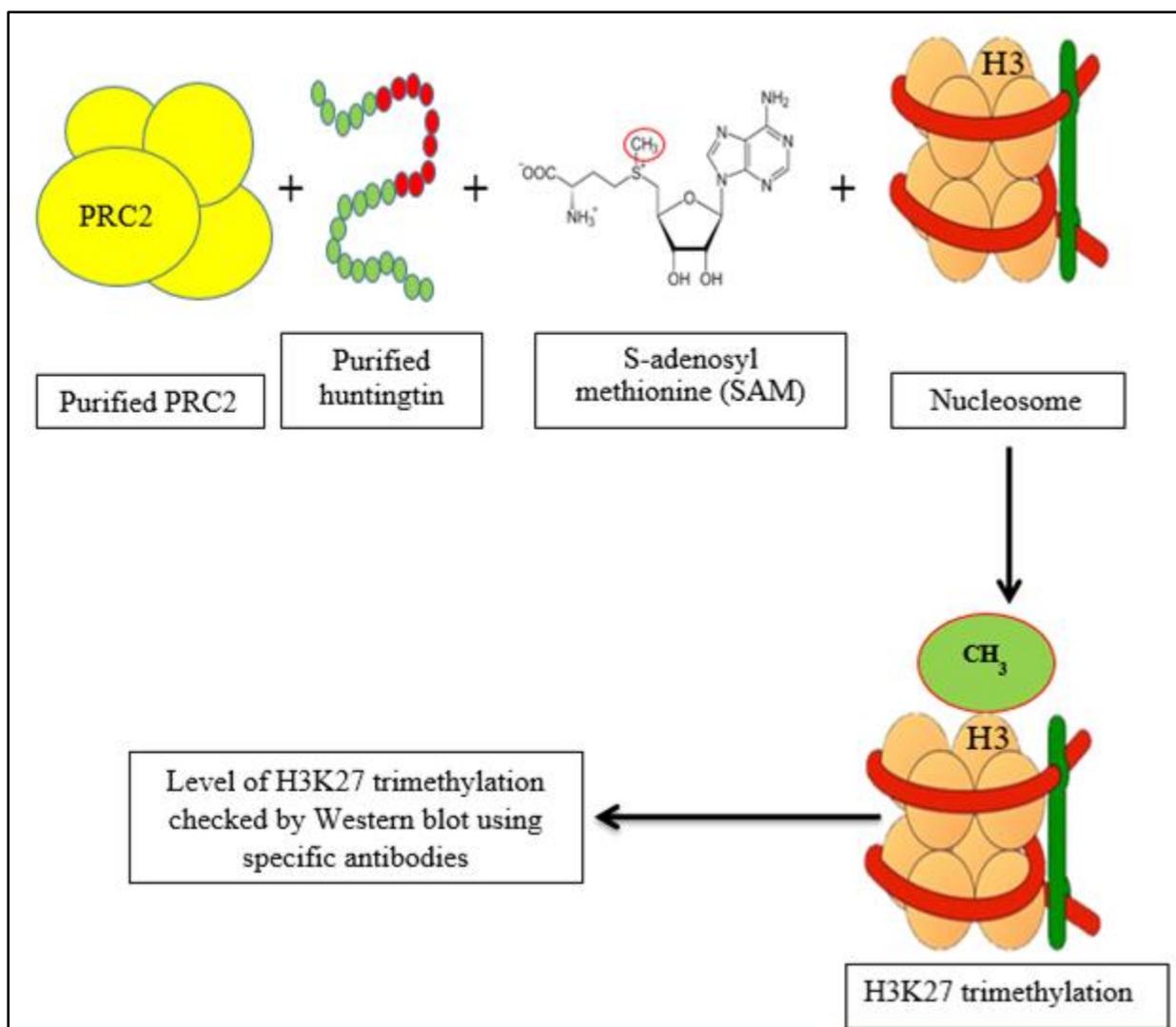
### 3.2.2 Expression and purification of the Polycomb Repressive Complex 2 (PRC2) using *Sf9* insect cell Baculovirus system and affinity chromatography

Simultaneously, pFastBac vectors containing PRC2 subunits genes (*Ezh2*, *EED*, *Suz12* and *RbAp48*) were also transformed into *E. coli* DH10Bac cells to generate recombinant bacmids. Baculovirus were generated for individual sub-units of PRC2 and then P3 stocks were mixed in

1:2 ratio for *RbAp48*, *EED*: *Ezh2*, *Suz12* to form a functional complex. Expression and purification of PRC2 protein using *Sf9* insect cell-Baculovirus system [1,2] and affinity chromatography was carried out same as described above as EZH2 has a FLAG tag.

### 3.2.3 The reconstituted *in-vitro* PRC2 activity assay

#### Strategy



The reconstituted *in-vitro* PRC2 activity assay was optimized and carried out as previously described in a 60  $\mu\text{l}$  reaction mixture [2]. The reaction components included 100 mM Tris-HCl (pH 8.3), 1 mM DTT, 2  $\mu\text{M}$  S-adenosyl methionine (SAM), 50 nM FLAG-tagged PRC2 complex, and 0.1  $\mu\text{M}$  nucleosomal array. Additionally, the reaction was performed with or without 40 nM FLAG-tagged huntingtin protein. The reactions were incubated for 4 hours at 30°C. To terminate

the reactions, sample buffer was added, and the mixtures were subjected to 12% SDS-PAGE. Following electrophoresis, the proteins were transferred onto a PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA). Specific primary antibodies, namely Histone H3 and Tri-Methyl-Histone H3 (Lys27) Rabbit mAb, were used to detect the proteins. The level of histone methylation was then compared in the presence of different serine 13 and 16 substituted huntingtin proteins. Simultaneously, FLAG-HTT levels were also checked with anti-HTT antibody for equal loading.

### 3.2.4 Analyzing effect of specific kinase and phosphatase inhibitors on *in-vivo* PRC2 activity in cell line models of HD

Mouse striatal cell lines expressing full length human huntingtin protein (*STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup> knock-in mouse striatal cells) were used to analyze effect of Kinetin (N6-furfuryladenine), BMS 345541, and Bay 11-7082 induced huntingtin S13-S16 phosphorylation on Histone Methyltransferase PRC2 activity. HTT has been shown to interact with PRC2 and enhance its H3K27 methyltransferase activity in a polyQ length dependent manner [2]. *STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup> cells were treated with Kinetin, BMS 345541, Bay 11-7082 and DMSO (as a vehicle control) in DMEM with 0.2% FBS and incubated for 24 hours at 33°C in a 5% CO<sub>2</sub> incubator. After 24 hours, the media was discarded, and cells were washed with 1X ice-cold PBS. PBS and subsequent buffers were supplemented with 5 mM sodium butyrate to retain levels of histone acetylation. These cells were processed to analyze the effect of Kinetin, BMS 345541, and Bay 11-7082 using immunoblotting.

#### 3.2.4.1 Histone extraction

Cells were resuspended in Triton Extraction Buffer (TEB: PBS with 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% (w/v) NaN<sub>3</sub>) to a density of 10<sup>7</sup> cells per mL. Lysis was performed on ice for 10 minutes with gentle agitation. The lysate was centrifuged at 6,500 × g for 10 minutes at 4°C to pellet the nuclei, after which the supernatant was discarded. The nuclei were then washed with half the volume of TEB and centrifuged again under the same conditions. The resulting pellet was resuspended in 0.2 N HCl at a density of 4 × 10<sup>7</sup> nuclei per mL, and histones were extracted overnight at 4°C. Following extraction, the samples were centrifuged at 6,500 × g for 10 minutes at 4°C to remove debris. The supernatant containing histone

proteins was collected and neutralized with 2 M NaOH at one-tenth the volume of the supernatant. Protein concentration was determined using the Bradford assay, and aliquots were stored at -20°C.

### 3.2.4.2 Western blotting

The normalized Histone-H3-Lysine-27-tri-methylation level was determined using Specific primary antibodies, namely Histone H3 and Tri-Methyl-Histone H3 (Lys27) Rabbit mAb by immunoblotting. To check if the effect is concentration dependent, *STHdh*<sup>Q7/Q7</sup> (wild type) and *STHdh*<sup>Q111/Q111</sup> (showing HD phenotype) cells were treated with varied concentrations of kinetin (0.5 μM, 1 μM and 2 μM), histones were extracted, and subjected to western blotting.

### 3.2.5 Fluorescence Microscopy

In order to visualize GFP tagged mHTT aggregates, HD150Q cells were seeded at a density of  $0.3 \times 10^6$  cells per well in 6-well plate (NEST). To induce overexpression of mutant huntingtin protein, the steroid inducer Ponasterone A (1μM) was used. Cells were treated with different concentrations of Kinetin (N6-furfuryladenine), BMS 345541, Bay 11-7082 and DMSO (as a vehicle control) for the particular time period to analyze the effect of kinetin, BMS 345541, and Bay 11-7082 on mutant huntingtin aggregation. After incubation for the particular time period, cells were stained with Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, USA) and observed using Eclipse Ti2-E inverted fluorescence microscope (Nikon, Japan) and analysed by ImageJ. For a set of experiments, the detectors gain, offset levels, and laser power were all calibrated at identical levels and remain unchanged. Numbers of mHTT puncta per field were counted manually and graph plotted for the average number of puncta per field using Graphpad prism 6 software.

### 3.2.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

HD150Q cells were seeded at a density of  $0.3 \times 10^6$  cells per well in 6-well plate and were treated as indicated. After treatment, cells were harvested, washed with ice cold 1X PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitor cocktail (Roche). After 30 minutes incubation on ice, cell lysates were centrifuged at  $13680 \times g$  for 15 minutes at 4°C and supernatant was collected for further processing. Protein concentration was determined using Bradford assay (Bio-Rad, USA), and equal amount of proteins were resolved on 10% SDS-PAGE and transferred onto PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 100 V for 120 minutes at 4°C. Following the transfer, the

membrane was blocked for 1 hour at room temperature in a 5% blocking buffer (5% non-fat dried milk or 5% BSA for phosphoproteins and 0.1% Tween-20 in TBS). The membrane was then incubated overnight at 4°C with specific primary antibody. After washing thrice with 1X TBST (TBS containing 0.1% Tween-20), the membrane was incubated with respective HRP-conjugated secondary antibody (Cell Signaling Technology, USA) for 1h at room temperature. After washing thrice with 1X TBST, the blots were incubated with chemiluminescent substrate of HRP and the bands were visualized using ChemiDoc Touch Imaging System (Bio-Rad Laboratories, USA). ImageJ image processing software (Rasband WS; NIH) was used to calculate the relative intensity of each protein's expression.

### 3.2.7 Real time PCR (RT-qPCR)

In order to analyze the effect of kinetin, BMS 345541, and Bay 11-7082 on mitochondrial dysfunction and ER stress response, HD150Q cells were treated with Ponasterone A and/or kinetin, BMS 345541, and Bay 11-7082 and incubated for 24 hours. After 24 hour incubation, cells were washed with 1X ice-cold PBS and processed to analyze the effect of kinetin, BMS 345541, and Bay 11-7082. Briefly, total RNA was extracted using TRIzol reagent (TaKaRa, Japan) followed by cDNA synthesis using PrimeScript cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions. The expressions of target genes were evaluated using gene specific primers (primer details are provided in **Table 3.1**) and TB Green Master mix (TaKaRa, Japan) in QuantStudio 3 real time PCR machine (Applied Biosystem). Reactions were run in triplicates in three independent experiments. The levels of target transcripts were determined using  $2^{-\Delta\Delta Ct}$  method. Housekeeping gene GAPDH was used as an internal control to normalize the variability of expression levels. The reaction conditions were 95°C for 2 min followed by 40 cycles of 95°C for 5sec and 60°C for 35 sec (the data was acquired at this step). The melt curves were also acquired.

### 3.2.8 Mitochondrial ROS determination

Mitochondrial Reactive Oxygen Species (ROS) were measured by MitoSOX Red (5  $\mu$ M) staining. Briefly, HD150Q cells were plated at the density of  $0.3 \times 10^6$  cells/well in 6-well plate. The cells were treated with Ponasterone A and/or kinetin, BMS 345541, and Bay 11-7082 and stained with MitoSOX Red and Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, USA). The cells were monitored under Eclipse Ti2-E inverted fluorescence microscope (Nikon, Japan). Mean

Fluorescence intensity was quantified using ImageJ and graphs were plotted using GraphPad Prism 6 software.

### **3.2.9 ATP levels**

The effect of kinetin treatment on cellular ATP production was assessed using a luminescence-based ATP determination kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. HD150Q cells were treated with 1  $\mu$ M Ponasterone A alone for various durations (24, 48, 72, and 96 hours) or in combination with kinetin, BMS 345541, and Bay 11-7082 for 48 hours as specified. Following treatment, cells were washed with ice-cold 1X PBS, lysed in 0.1% Triton X-100 on ice, and 10  $\mu$ l of cell lysate was mixed with 100  $\mu$ l of ATP determination master mix containing 25 mM Tricine buffer (pH 7.8), 5 mM  $MgSO_4$ , 0.5 mM D-luciferin, 1.25  $\mu$ g/ml firefly luciferase, 100  $\mu$ M EDTA, and 1 mM DTT. Luminescence was measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments). Protein concentrations were determined by Bradford assay, and ATP levels were normalized to protein content and expressed as a percentage of control HD150Q cells.

### **3.2.10 Cell viability assay**

The cell viability was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MTT assay is a widely used method to assess cell viability and proliferation. Briefly,  $5 \times 10^3$  cells/well were plated in 96 well plate and treated with Ponasterone A and/or different concentrations of kinetin, BMS 345541, and Bay 11-7082 for different time duration. After the designated treatment duration, the growth medium was aspirated from the cell culture plate and assay was performed by incubating cells with 0.1 mg/ml MTT for 4 h at  $NH_4HCO_3^0C$ . The viable cells convert MTT to formazan crystals. The medium-MTT mixture was carefully removed, and 200  $\mu$ l of DMSO was added to solubilize the formazan crystals. The plates were gently shaken for 10 minutes to ensure complete solubilisation followed by measurement of absorbance at 570 nm using a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments).

### **3.2.11 Identification of differentially expressed proteins associated with mHTT expression using label-free quantitative proteomics approach**

### 3.2.11.1 Sample Preparations

To get extensive quantitative data, label-free mass spectrometry analysis was performed for HD150Q control, Ponasterone A alone, Kinetin, BMS 345541 and Bay 11-7082 samples. In brief, HD150Q cells were seeded at a density of  $0.3 \times 10^6$  cells per well in 6-well plate and were treated with different concentrations of Kinetin, Bay 11-7082, and BMS 345541, as well as Ponasterone A, in DMEM with 10% FBS, and then incubated for 48 hours at 37°C with 5% CO<sub>2</sub>. After treatment, media was removed and cells were harvested, washed with ice cold 1X PBS and lysed in lysis buffer (7 M Urea, 50 mM Ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>); pH 8.0) containing a protease inhibitor cocktail (Roche). After 30 minutes incubation on ice, cell lysates were centrifuged at 13680 ×g for 15 minutes at 4°C and supernatant was collected for further processing. Protein concentration was determined using Bradford assay (Bio-Rad, USA), and equal amount of proteins (100 µg for each sample) were subjected to in-solution digestion using trypsin as per standard digestion protocol.

Briefly, Five to ten microliters of 200 mM DTT/50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, were added to the mixture and incubated for 1 hour at room temperature, achieving a final DTT concentration of 10 mM. For samples containing urea/thiourea in the lysis buffer, the reduction step was carried out at room temperature (25 to 27°C) without heating (56/60°C). Subsequently, 25 µL of 200 mM Iodoacetamide/50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, were added, the mixture was gently vortexed, and then incubated for 1 hour at room temperature in the dark, reaching a final IAA concentration of 50 mM. To consume any unreacted iodoacetamide, 10 µL of 200 mM DTT/50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, were added and the mixture was incubated for another hour at room temperature in the dark. The urea concentration was then reduced to approximately 0.5 M by adding 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 1 mM CaCl<sub>2</sub> (pH 7.6). Trypsin solution was added to achieve a final ratio of 1:50 (w/w, trypsin: protein), the mixture was gently vortexed, and incubation was performed at 37°C for 12 to 16 hours, preferably overnight. Formic acid or Trifluoroacetic acid (TFA) was added to adjust the pH to 3-4, and the pH was tested by placing < 1-µL aliquots onto pH paper. The digested protein was normally vacuum dried before storing it at -20°C. Tryptic peptides were desalted using Pierce C18 desalting spin columns with the following steps:

First, the spin column was prepared by adding 50% ACN to the C18 resin, gently tapping the column, and closing the top and bottom lids. A gentle spin was given (low rpm, 4000-5000),

allowing the resin to settle down. Next, the bottom and top lids were removed, and 50% ACN was added again, followed by gentle spinning. This process was repeated twice to activate the resin, ensuring it was never left dry during any step. Then, 100% ACN / 0.1% Formic acid (single solution) was added, gently spun, and the solution was discarded, repeating this 3-4 times. The column was equilibrated with 0.1% Formic acid by adding 200  $\mu$ L of 0.1% Formic acid, gently spinning, and discarding, repeating this 3-4 times. Before adding the sample to the spin column, a high-speed spin at 12000 rpm for 5 minutes was performed to ensure there were no undissolved or particulate-like materials; only the supernatant was carefully taken. The supernatant was added to the column, and a gentle spin (very low rpm, 800 to 1000 rpm) was applied. The flow-through was collected and added again to the column, repeating this step 2-3 times to ensure maximum binding. The column was washed with 0.1% Formic acid (100-200  $\mu$ L), and the bound peptides were eluted with 70% ACN/0.1% Formic acid (75  $\mu$ L). This elution step was repeated once more, and the collected eluants were pooled and named as Eluant 01 (E1). The eluants (E1) were vacuum dried at room temperature using a Speed vac until completely dry, resulting in very fine dots in the eluant sample. After vacuum drying, the samples were stored at  $-20^{\circ}\text{C}$  or used for Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis.

### 3.2.11.2 Information Dependent Acquisition/Data Dependent Acquisition

The nLC-MS/MS analysis was performed utilizing an Eksigent Ekspert<sup>TM</sup> Nano-LC 400 system equipped with an Ab Sciex cHiPLC System. This nano-liquid chromatography setup was directly interfaced with a TripleTOF 5600+ mass spectrometer from Ab Sciex. Initially, digested peptides were captured by a trap column with dimensions of 200  $\mu\text{m}$  x 5 cm (Eksigent). Subsequently, these peptides were separated using an analytical column measuring 75  $\mu\text{m}$  x 15 cm (Eksigent). Both columns were packed with ChromXP C18 resin, featuring a particle size of 3  $\mu\text{m}$  and a pore size of 120  $\text{\AA}$ . The analytical gradient separation for the AP-MS analysis spanned 148 minutes. Buffer A consisted of 0.1% formic acid (FA) in water, while Buffer B comprised 0.1% FA in acetonitrile (ACN). Peptides were eluted at a flow rate of 300 nL/min by progressively increasing the concentration of Buffer B. The separated peptides were ionized in positive mode and analyzed via information-dependent acquisition (IDA). The IDA protocol included an initial MS survey scan ranging from 350 to 1250 m/z, with an accumulation duration of 250 milliseconds. The top 30 precursor ions were selected for fragmentation using rolling collision energy. Subsequently, the

fragmented ions were scanned over a range of 200 to 1800 m/z, with each accumulation lasting 50 milliseconds.

### 3.2.11.3 Label Free Quantitation

Samples underwent Peptide Spectrum Match (PSM) analysis using MaxQuant software (Jürgen Cox lab). The analysis was performed with pre-defined parameters, utilizing *Mus musculus* (Mouse) UniProt-reviewed proteins as the input. Decoy proteins and contaminant FASTA files were also included to enhance the robustness of the analysis. Protein quantification was achieved through the maxLFQ algorithm, which determined the label-free intensities of the identified proteins.

### 3.2.11.4 Comparative Analysis

Identified proteins were exported and compared across samples using Venn diagrams to elucidate common and unique proteins. For fold change analysis, samples treated solely with Ponasterone A to induce mHTT expression were first compared to HD150Q control samples with no mHTT expression. To assess the impact of phosphorylated Ser13/Ser16 in mHTT, samples co-treated with Kinetin, BMS 345541 and Bay 11-7082 were compared to those treated with Ponasterone A alone.

### 3.2.11.5 Functional Enrichment Analysis

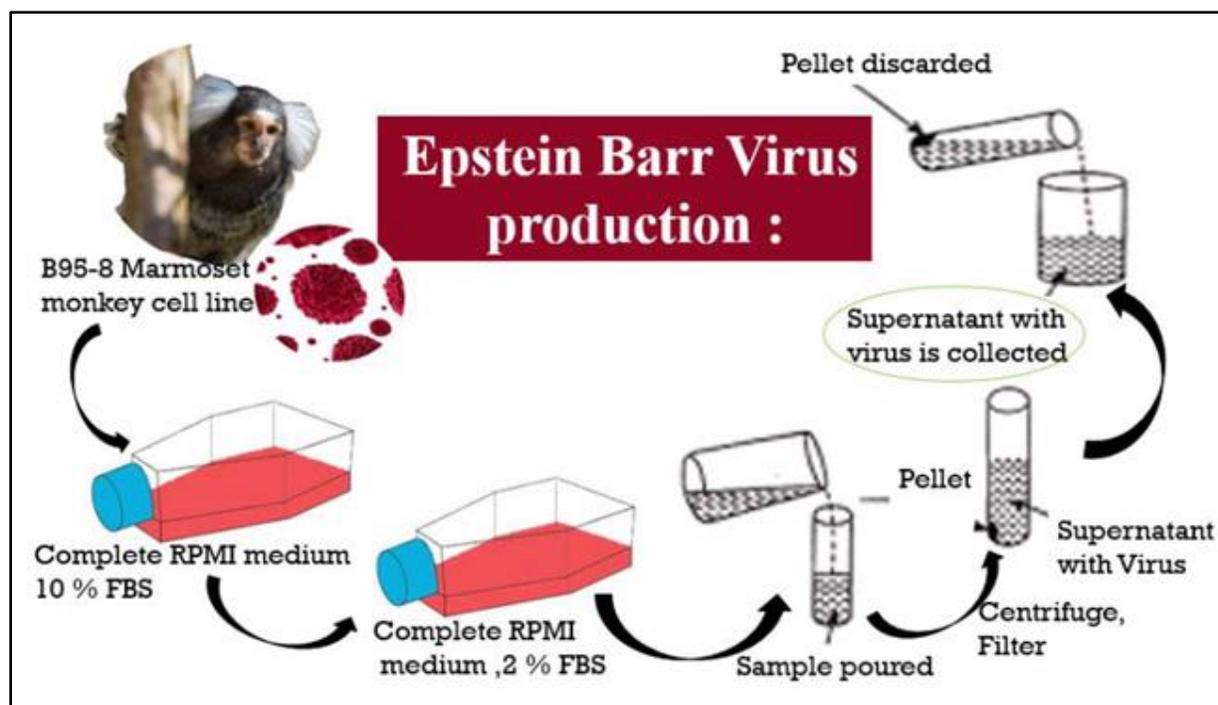
Differentially regulated proteins were subjected to overrepresentation analysis using ShinyGO 0.80. This analysis was designed to identify enriched Gene Ontology (GO) terms, including biological processes and cellular components, as well as KEGG pathways. The results are visualized as a lollipop plot, displaying the top 10 overrepresented pathways, biological processes, or cellular components. [ShinyGO 0.80 \(sdstate.edu\)](https://shinygo.ucdavis.edu/)

## 3.2.12 Generation and characterization of Lymphoblastoid Cell lines (LCLs) from Control and Huntington's Disease patients

### 3.2.12.1 Generation of Epstein Barr Virus (EBV)

The marmoset cell line B95-8 is highly permissive to Epstein-Barr virus (EBV) replication, making it an excellent source of infectious viral particles [3]. This cell line, derived from the *Callithrix* genus, was established by infecting marmoset B cells with EBV isolated from a patient with infectious mononucleosis [4]. B95-8 is utilized to produce supernatant containing

transforming, infectious virus particles and serves as the prototype laboratory strain for generating continuous B lymphoblastoid lines (LCLs) from human donors [5]. Typically, a small percentage of B95-8 cells are found in the lytic cycle under normal culture conditions. However, when cultured in medium with a low serum concentration (e.g., switching from 10% FBS to 2% FBS), the B95-8 cell line produces a substantial amount of infectious viral particles. This low-serum condition promotes the spontaneous activation of the lytic cycle, significantly increasing virus production by inducing the lytic phase in a larger proportion of cells [5].



**Figure 3.2.12.1:** A schematic representation illustrating the generation process of Epstein Barr Virus (EBV) from B95-8 cells [5].

As shown in **Fig. 3.2.12.1** B95-8 cells were cultured in complete RPMI medium containing 10% heat-inactivated fetal bovine serum (Gibco) and a 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco). When a sufficient number of cells had reached the logarithmic growth phase, the cells were centrifuged and a new culture was initiated at a density of  $2 \times 10^5$  cells/ml in complete RPMI medium containing 2% FBS. The cells were maintained under these conditions for 2 weeks at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  humidified incubator without changing the medium. At the end of the 2-week period, the culture medium was collected and centrifuged at  $300 \times g$  to sediment cells and cell debris. The supernatant was carefully collected without disturbing the pellet and filtered through

a sterile 0.45  $\mu\text{M}$  pore filter to remove all remaining cells. The filtered supernatant was stored at 4°C. This supernatant, produced under these conditions, had a virus titer high enough to be used directly, without further concentration, for the generation of LCLs.

### **3.2.12.2 Generation of Lymphoblastoid Cell Lines (LCLs)**

#### **3.2.12.2.1 Isolation of Mononuclear Cells from Blood**

Blood was diluted with an equal volume of PBS. The diluted blood was then carefully layered on Ficoll Paque, ensuring that the Ficoll Paque and the diluted blood sample did not mix during layering. The samples were centrifuged in a swing-out rotor at 20°C with  $400 \times g$  for 20 minutes, with the centrifuge brake set to zero to avoid disturbing the mononuclear cell layer that formed during centrifugation. The mononuclear cells were located in the layer ("ring") formed at the interface and were collected into a clean tube. At least three volumes of PBS were added to the cells, and the mixture was centrifuged for 10 minutes at  $200 \times g$ . The supernatant was discarded, and the washing step was repeated by filling the tube with PBS and centrifuging it again for 10 minutes at  $200 \times g$ . The cells were then resuspended in complete RPMI medium and counted, resulting in a cell suspension containing highly purified, viable lymphocytes (T and B cells) and monocytes. At this point, the purified mononuclear cells were ready to be exposed to EBV, leading to infection of B cells. Alternatively, the cells could be frozen, stored in liquid nitrogen, and infected at a later time point.

#### **3.2.12.2.2 *In-vitro* infection with EBV**

The purified mononuclear cells were exposed to virus-containing supernatant. To do this,  $3\text{-}5 \times 10^6$  cells were pelleted by centrifugation, the medium was discarded, and the cells were resuspended in 3-5 ml of B95-8 supernatant. If the virus titer was high, the number of cells could be increased. The cells were incubated with the virus-containing supernatant at 37°C for 2 hours. After incubation, the cells were pelleted by centrifugation, the supernatant was discarded, and the cells were resuspended in complete RPMI medium containing 0.4  $\mu\text{g}/\text{ml}$  cyclosporine A (CsA). 3-5 ml of the cell suspension was distributed per T25 Flask, ensuring that each flask contained  $2.8\text{-}3 \times 10^6$  cells. The cultures were maintained in a humidified incubator at 37°C with 5%  $\text{CO}_2$ . The cultures were fed weekly by replacing half of the medium with fresh medium containing CsA.

#### **3.2.12.3 Characterization of Lymphoblastoid Cell Lines (LCLs)**

Characterization of LCLs was performed using standard techniques as described below. For all assays, freshly grown cells with greater than 90% viability were harvested, washed in 1X PBS at 1000 ×g, and used for genotypic and phenotypic characterization. Marker gene analysis was conducted to verify the presence of B lymphocytes in the generated LCLs, as B lymphocytes exhibit specific surface markers like CD-19. The presence of these surface markers was used to characterize the generated cell lines. In addition to B lymphocyte marker gene analysis, marker gene analysis was also conducted for Beta-actin (housekeeping gene), T lymphocytes (CD3), and Natural Killer cells (NK cells - NCAM). Similarly, PCR and western blotting was employed to confirm the conservation of HTT polyQ repeats in the generated LCLs, which had been previously verified in fresh B lymphocyte samples.

### **3.2.13 Treatment of Lymphoblastoid Cell Lines (LCLs) with Kinetin, BMS 345541, and Bay 11-7082**

Following the establishment of Lymphoblastoid Cell Lines (LCLs) from both control and Huntington's Disease patients as described in section 3.2.12, we investigated the effects of specific treatments on inflammatory marker gene expression. LCL cells were cultured in complete RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> incubator. To assess the impact of kinetin, BMS 345541, and Bay 11-7082 on the expression levels of key pro-inflammatory (*IL-1β*, *IL-6*, and *TNF-α*) and anti-inflammatory (*TGF-β1* and *IL-10*) marker genes, cells were treated as follows.

Cells were seeded at a density of  $1 \times 10^6$  cells/ml and treated with kinetin (1 μM), BMS 345541 (300 nM), and Bay 11-7082 (1 μM) for 24 hours. Each treatment was accompanied by a vehicle control using DMSO. After the treatment period, cells were harvested by centrifugation at 300 ×g for 5 minutes. Subsequently, cell pellets were washed twice with 1X ice-cold PBS to remove any residual compounds.

Total RNA was isolated from the treated cells using the TRIzol reagent (TaKaRa, Japan), following the manufacturer's protocol. The purity and concentration of the isolated RNA were assessed using a NanoDrop spectrophotometer. For cDNA synthesis, 2 μg of total RNA from each sample was reverse transcribed using the cDNA synthesis using PrimeScript cDNA synthesis kit (TaKaRa, Japan), according to the manufacturer's instructions.

Quantitative Real-Time PCR (qPCR) was performed to analyze the expression levels of *IL-1 $\beta$* , *IL-6*, *TNF- $\alpha$* , *TGF- $\beta$ 1*, and *IL-10* (primer details are provided in **Table 3.1**). The qPCR reactions were carried out using the TB Green Master mix (TaKaRa, Japan) on a CFX Opus 96 Real-Time PCR System (Bio-Rad). The relative expression levels of the target genes were normalized to the housekeeping gene GAPDH and analyzed using the  $2^{-\Delta\Delta C_t}$  method.

This methodology facilitated the investigation into how kinetin, BMS 345541, and Bay 11-7082 treatment that enhance HTT Ser13 and Ser16 phosphorylation modulate the inflammatory response in LCLs derived from both control and HD patients, providing insights into potential therapeutic strategies for abrogation of inflammatory peripheral effects as seen in Huntington's Disease.

### **3.2.14 Investigation of the Effects of HD150Q Cell Secretome on Immune Cells Expressing Normal Huntingtin**

#### **3.2.14.1 Cell Lines and Study Design**

In this study, two HD model cell lines were utilized: HD150Q and *STHdh<sup>Q7/Q7</sup>* (control). Additionally, the THP-1 cell line and healthy control peripheral blood mononuclear cells (PBMCs) were also employed. These cell lines were selected to investigate the peripheral effects of mutant huntingtin on immune cells.

#### **3.2.14.2 Secretome Treatment of Immune Cells**

To study the effect of secretome from HD150Q cells on immune cells expressing normal huntingtin, freshly isolated human peripheral blood mononuclear cells (PBMCs), which include multiple cell types such as monocytes, macrophages, and lymphocytes, and the human monocyte cell line THP-1 were used. Freshly isolated human PBMCs and THP-1 cells were incubated with 1 ml of conditioned media from either uninduced or 48-hour Ponasterone A-induced HD150Q cells. The incubation periods were set for 24 hours to evaluate gene expression changes.

#### **3.2.14.3 RNA Isolation, cDNA Synthesis and qPCR**

Following the incubation period, RNA was isolated from the treated PBMCs and THP-1 cells using standard RNA isolation protocols. A total of 2  $\mu$ g of RNA was used for cDNA synthesis. The synthesized cDNA was then used to determine the expression levels of key pro- (*IL-1 $\beta$* , *IL-6*, and *TNF- $\alpha$* ) and anti-inflammatory (*TGF- $\beta$ 1* and *IL-10*) marker genes via real-time PCR.

### 3.2.14.4 ATP Level Measurement

To determine effect on ATP levels, *STHdh*<sup>Q7/Q7</sup> cells were incubated with 1 ml of conditioned media from either uninduced or 48-hour Ponasterone A-induced HD150Q cells for 24 and 48 hours. Post-incubation, the cells were washed with ice-cold PBS and lysed in 0.1% Triton X-100 on ice.

For ATP measurement, 10 µl of cell lysate was diluted in 100 µl of ATP determination master mix, which contained 25 mM Tricine buffer (pH 7.8), 5 mM MgSO<sub>4</sub>, 0.5 mM D-luciferin, 1.25 µg/ml firefly luciferase, 100 µM EDTA, and 1 mM DTT. Luminescence intensity was measured using the Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments).

The protein content in the samples was determined using the Bradford assay. ATP levels were then normalized to the respective protein concentrations to obtain normalized ATP levels. These normalized ATP levels were expressed as a percentage relative to control cells.

### 3.2.14.5 Effect of Ponasterone A and Kinetin-treated HD150Q secretome on ATP Levels

In additional experiments, *STHdh*<sup>Q7/Q7</sup> cells were treated with secretome derived from HD150Q cells that had been incubated with Ponasterone A and Kinetin. HD150Q cells were treated with Pon A and Kinetin for 48 hours to generate conditioned media. *STHdh*<sup>Q7/Q7</sup> cells were then incubated with 1 ml of this conditioned media for 24 and 48 hours. Post-treatment, cells were washed with 1X ice-cold PBS and prepared for ATP level analysis as previously described. This methodology was used to determine the effectiveness of Pon A and Kinetin-treated secretome in rescuing ATP levels in *STHdh*<sup>Q7/Q7</sup> cells.

### 3.2.15 Statistical Analysis

All the data in this study are shown as mean ± SEM or mean ± SD for “n” observations. Graph plotting and statistical analysis were conducted using GraphPad Prism® 6. Student's unpaired t-test was employed for comparisons between two groups for repeated measurements, while one-way ANOVA (Newman-Keuls post-test) were utilized for comparisons involving more than two groups to determine the levels of significance for each data set. Each experiment was independently repeated at least three times, with  $p \leq 0.05$  considered statistically significant. In the case of normalized graphs, data were normalized with the maximum value set as 100% and the minimum value as 0%, followed by graph plotting based on the obtained normalized values.

### 3.3 References

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