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Article

Keywords: N⁶-methyladenosine (m⁶A), Epitranscriptomics, Huntington's disease, Neurodegeneration, METTL3, IGF2BP3

Posted Date: July 8th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9928562/v1>

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Additional Declarations: (Not answered)

METTL3-IGF2BP3 m⁶A axis drives mutant huntingtin stability and neurodegeneration in Huntington's disease

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Abstract:

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by CAG repeat expansion in the *HTT* gene, yet the molecular mechanisms linking mutant huntingtin (mHTT) to selective neuronal vulnerability remain incompletely understood. Emerging evidence suggests that post-transcriptional RNA regulation plays a role in HD pathogenesis; however, the contribution of epitranscriptomic modifications remains to be fully defined. Here, we identify N⁶-methyladenosine (m⁶A) RNA methylation as a conserved and modifiable regulator of HD-associated neurodegeneration. Using complementary HD mouse models (R6/2 and zQ175), human postmortem striatum, and mHTT-expressing striatal cells, we demonstrate dysregulation of the m⁶A machinery, including upregulation of the writers METTL3/METTL14 and the reader IGF2BP3, accompanied by elevated global m⁶A levels in the cortex and striatum. Pharmacological inhibition or siRNA-mediated knockdown of METTL3 reduced global m⁶A, improved cell viability, suppressed caspase-3/9 activation, and attenuated inflammatory cytokine production in HD cells. Mechanistically, knockdown of either METTL3 or IGF2BP3 significantly reduced mHTT protein levels in sdhQ^{111/111} striatal cells, identifying an m⁶A-IGF2BP3 axis that promotes mHTT accumulation and toxicity. Notably, these interventions had minimal effects in wild-type cells, indicating the selective vulnerability of mHTT-expressing cells. Together, these findings establish aberrant m⁶A RNA methylation as a driver of mHTT accumulation, neuronal apoptosis, and inflammation in HD, and highlight the METTL3-IGF2BP3 pathway as a promising epitranscriptomic therapeutic target.

Keywords: N⁶-methyladenosine (m⁶A); Epitranscriptomics; Huntington's disease; Neurodegeneration; METTL3; IGF2BP3

Introduction

Huntington's disease (HD) is a fatal, genetically inherited neurodegenerative disease that is characterized by progressive motor dysfunction, cognitive decline, and psychiatric disturbances [1]. HD is caused by a CAG trinucleotide repeat expansion in the *huntingtin* (*HTT*) gene, resulting in the production of mutant huntingtin (mHTT) protein with an elongated polyglutamine tract [2]. Although the genetic cause of HD has been well established for decades, the molecular mechanisms that translate this mutation into selective neuronal vulnerability and degeneration remain poorly understood. Emerging evidence suggests that RNA metabolism and post-transcriptional regulation play central roles in HD pathogenesis [3-5].

Among these regulatory layers, *N*⁶-methyladenosine (*m*⁶A) has recently emerged as a dynamic and reversible epitranscriptomic modification that regulates RNA fate, including RNA splicing, RNA stability, RNA export, and its translation [6, 7]. The *m*⁶A modification is written by a methyltransferase complex known as "writers," which includes METTL3, METTL14, and WTAP. These *m*⁶A modifications are removed by demethylases generally called "erasers," such as FTO and ALKBH5, and interpreted by *m*⁶A-binding proteins known as "readers," which include YTH domain proteins and insulin growth factor 2 binding proteins (IGF2BP) family members [8, 9]. These *m*⁶A regulators fine-tune gene expression programs essential for neuronal differentiation, synaptic plasticity, and stress responses [10-12]. Dysregulation of *m*⁶A signaling has been increasingly linked to neurodevelopmental and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) [13-17].

Recent studies suggest that the *m*⁶A epitranscriptome may be profoundly altered in HD [3, 18, 19]. Global transcriptomic analyses of HD models have revealed widespread RNA processing defects, altered RNA stability, and translational dysregulation [3, 18, 19]. All processes are potentially governed by *m*⁶A signaling. Moreover, several *m*⁶A regulators, such as METTL3 and IGF2BPs proteins, have been implicated in neuronal survival, mitochondrial homeostasis, and stress granule dynamics processes disrupted in HD [20, 21]. However, whether and how *m*⁶A reprogramming contributes to disease onset or progression in HD remains largely unexplored.

Here, we hypothesize that the *m*⁶A landscape is reprogrammed during HD pathogenesis and that manipulating key *m*⁶A regulators may modulate disease outcomes. In this study, we comprehensively map *m*⁶A methylation changes in HD mouse models and investigate how perturbations in the *m*⁶A machinery, particularly involving METTL3 and the *m*⁶A reader protein IGF2BP3, affect RNA stability and neuronal homeostasis in HD. Our findings reveal that *m*⁶A remodeling is not merely a consequence of mutant HTT expression but an active driver of molecular and functional alterations underlying HD progression. These

results uncover a novel epitranscriptomic layer of regulation in HD and identify the m⁶A pathway as a potential therapeutic target for mitigating neurodegeneration.

Results

HD mouse models exhibit dysregulation of m⁶A epitranscriptomic regulators: To determine whether HD pathology is associated with dysregulation of the m⁶A RNA modification pathway, we analyzed the transcriptomic expression of key m⁶A regulatory genes in HD mouse models. Using quantitative PCR (qPCR), we assessed the expression of m⁶A writers, readers, and erasers in the cortex and striatum of R6/2, a rapidly progressive transgenic HD model [22], and zQ175 mice, full length knock-in HD model [23]. In R6/2 mice, gene expression analysis revealed significant dysregulation of multiple m⁶A regulators in both brain regions (Figure 1A&B). In the cortex, several m⁶A writer genes, including METTL1, METTL2, METTL7A, METTL15, METTL16, METTL19, METTL26, METTL25B, KIAA1429, RBM15, and WTAP, were significantly upregulated (Figure 1A). Similarly, m⁶A reader genes such as YTHDC1, YTHDF2, YTHDF3, IGF2BP3, HNRNPA1, and HNRNPG were also significantly elevated when compared with their littermate WT controls (Figure 1A & Supplementary Table 1). In addition, multiple m⁶A eraser genes, including ALKBH1, ALKBH2, ALKBH4, and ALKBH5, showed increased expression in the R6/2 cortex, when compared with their littermate WT controls (Figure 1A & Supplementary Table 1). In contrast, the striatum of R6/2 mice displayed a distinct pattern of m⁶A dysregulation. Several m⁶A writer genes, including METTL1, METTL2, METTL5, METTL7A, METTL15, METTL25B, METTL26, CBLL1, KIAA1429, and ZC3H13, were significantly upregulated when compared with their littermate WT controls (Figure 1B & Supplementary Table 2). Likewise, m⁶A reader genes YTHDC1, YTHDF1, YTHDF2, YTHDF3, IGF2BP3, HNRNPA1, and FMRP were also significantly increased, whereas no significant changes were observed in m⁶A eraser gene expression in the striatum (Figure 1B & Supplementary Table 2). To further validate and extend these findings, we next analyzed the zQ175 knock-in mouse model, which more closely reflects endogenous mutant HTT expression and progressive HD pathology. In the cortex of zQ175 mice, several m⁶A regulatory genes, including METTL1, METTL16, ZC3H13, YTHDC2, IGF2BP2, and ALKBH5, were significantly upregulated (Figure 1C & Supplementary Table 3). In the striatum, robust upregulation of multiple m⁶A writers and readers was observed, including METTL1, METTL3, METTL5, METTL14, METTL17, METTL19, VIRMA, YTHDF3, IGF2BP2, IGF2BP3, and HNRNPA1, along with the eraser gene ALKBH4 (Figure 1D& Supplementary Table 4), when compared with their littermate WT controls.

To determine the regional overlap and specificity of transcriptional alterations associated with HD, we performed a comparative Venn analysis of differentially expressed genes (DEGs) in the cortex and striatum

of HD mouse models. The analysis revealed IGF2BP3 as a common dysregulated gene in cortex and striatum of both HD mouse models, R6/2 and zQ175 (Supplementary Figure 1). This suggests a role of IGF2BP3 in mHTT-induced toxicity in HD.

Elevated m⁶A regulatory proteins and global m⁶A methylation in HD brain regions: To determine whether transcriptional dysregulation of m⁶A pathway genes in HD results in functional changes at the protein and RNA modification levels, we further examined the expression of key m⁶A writer and reader proteins and assessed global m⁶A abundance in the cortex and striatum of R6/2 and zQ175 mouse models. This approach allowed us to establish whether altered m⁶A gene expression translates into coordinated activation of the m⁶A epitranscriptomic machinery in disease-relevant brain regions [24]. Western blot analysis revealed significant, region-specific upregulation of the m⁶A writer METTL3 and reader proteins, IGF2BP2, and IGF2BP3 in both the cortex (Figure 2A) and striatum (Figure 2B) of R6/2 mice compared with littermate wild-type controls, confirming that RNA-level alterations are reflected at the protein level. Similarly, the zQ175 knock-in HD model exhibited robust increases in the protein expression of METTL3, METTL14, and IGF2BP3 in cortex (Figure 2C) and METTL3, METTL14, IGF2BP2, and IGF2BP3 in striatum (Figure 2D) relative to wild-type controls, indicating conserved dysregulation of m⁶A regulatory proteins across distinct HD models. To assess the functional consequence of increased m⁶A writer and reader protein expression, we next evaluated global m⁶A RNA methylation levels using m⁶A dot blot analysis. This analysis revealed a significant elevation in global m⁶A levels in both the cortex and striatum of R6/2 and zQ175 mice compared with their respective controls (Figure 3A-D), demonstrating that protein-level changes are accompanied by enhanced m⁶A RNA methylation. Together, these findings indicate that transcriptional upregulation of m⁶A pathway components in HD leads to coordinated increases in m⁶A regulatory proteins and elevated global m⁶A modification, supporting a model in which aberrant activation of the m⁶A epitranscriptomic machinery contributes to region-specific molecular pathology in HD.

Conserved Activation of m⁶A Methylation Pathways in human HD: To determine whether the alterations observed in m⁶A-related pathways in HD mouse models are conserved in humans, we examined the protein expression of key m⁶A writers and readers in HD postmortem grade IV cortex and grade II striatal tissue and age-matched control samples. Immunoblot analyses revealed significant upregulation of METTL3, IGF2BP2, and IGF2BP3 in human HD cortex and striatal tissues (Figure 4A-D). The m⁶A methyltransferase METTL3 showed a robust increase in protein abundance in both cortex and striatum, suggesting enhanced m⁶A methylation capacity at an early (in striatum grade II) and late (cortex grade IV) neuropathological stage of disease (Figure 4A-D). Similarly, the m⁶A reader proteins IGF2BP2 and IGF2BP3

were markedly elevated, consistent with their roles in recognizing and stabilizing m⁶A-modified RNAs (Figure 4A-D). The coordinated increase in m⁶A writer and reader protein levels indicates activation of m⁶A-dependent post-transcriptional regulatory mechanisms in the HD striatum. Together, these findings demonstrate that m⁶A pathway components are significantly upregulated at the protein level in grade II human HD striatum, paralleling observations in HD mouse models. This conservation across species and disease stage suggests that dysregulated m⁶A signaling is an early and fundamental feature of HD pathology that may contribute to neuronal dysfunction in the human brain.

Upregulation of m⁶A writer and reader proteins in HD striatal cells: Given our observation of altered global m⁶A levels and dysregulated expression of key m⁶A regulatory proteins in HD mouse models and human postmortem striatal tissue, we next sought to determine whether similar epitranscriptomic alterations are present in HD-associated cellular models. To this end, we examined Q7 (sdhQ^{7/7} wild-type huntingtin) and Q111 (sdhQ^{111/111} mutant huntingtin) striatal cells to assess whether mHTT induces coordinated changes in the m⁶A RNA modification machinery at the cellular level. qPCR analysis revealed a broad transcriptional upregulation of the m⁶A machinery in Q111 cells compared with Q7 controls grown at 37 °C in serum-deprived medium (SDM) for 48 h. Specifically, transcripts encoding multiple m⁶A writers, including METTL2, METTL3, METTL4, METTL5, METTL6, METTL7A/B, METTL8, METTL14–17, METTL19, and METTL25/25B as well as m⁶A readers, YTHDF3, YTHDC1, IGF2BP1, IGF2BP3 and erasers, ALKBH1, ALKBH3–5, and ALKBH8 were significantly elevated in Q111 cells (Figure 5A & Supplementary Table 5&6). This widespread induction suggests a global remodeling of m⁶A-dependent RNA regulatory pathways in the presence of mHTT and mirrors changes observed in HD mouse models. To determine whether these transcriptional changes translate to altered protein abundance, we performed immunoblot analyses of key m⁶A regulators. Consistent with the mRNA data, METTL3 protein levels were significantly increased in Q111 cells, along with marked upregulation of the cytoplasmic m⁶A reader proteins IGF2BP2 and IGF2BP3, at both permissive (33°C) and non-permissive (37°C) temperatures (Figure 5B,C). In contrast, METTL14 protein levels were not altered, despite elevated mRNA expression, suggesting post-transcriptional or translational regulation of specific writer components. The pronounced increase in IGF2BP2 and IGF2BP3 highlights a potential shift toward enhanced m⁶A-dependent mRNA stabilization and translational control in HD cells. To assess whether altered expression of m⁶A regulators corresponds to changes in global RNA methylation, we performed m⁶A dot blot analysis. Q111 cells exhibited significantly higher overall m⁶A levels compared with Q7 controls under identical culture conditions (37 °C, SDM, 48 h), confirming that upregulation of m⁶A writers and readers is accompanied by increased RNA methylation at the cellular level (Figure 5D&E).

Collectively, these findings demonstrate that mHTT is associated with coordinated upregulation of the m⁶A epitranscriptomic machinery at both transcript and protein levels, resulting in elevated global m⁶A modification. The selective induction of METTL3 and cytoplasmic IGF2BPs readers suggests a functionally relevant axis that may contribute to altered RNA stability and translation in HD. These results provide a strong mechanistic rationale for targeting METTL3-dependent m⁶A signaling to interrogate the functional contribution of m⁶A dysregulation to HD cellular pathology.

METTL3 inhibition modulates cell viability and apoptotic signaling in HD cells: To determine whether elevated m⁶A methylation contributes functionally to HD-associated cellular pathology, we treated Q7 and Q111 cells with STM2457, a selective small-molecule inhibitor of the m⁶A writer METTL3 [25, 26]. m⁶A dot blot analysis confirmed a robust reduction in global m⁶A levels following STM2457 treatment, validating effective on-target inhibition of METTL3 activity (Figure 6A&B). Functionally, STM2457 treatment significantly improved cell viability in Q111 cells grown in SDM at 37°C (Figure 5C). Consistent with these effects on cell survival, STM2457 markedly reduced activation of the executioner caspases caspase-3 and caspase-7 in Q111 cells, indicating suppression of apoptotic signaling (Figure 6D&E). Caspase activity in Q7 cells remained largely unchanged following treatment, further supporting a disease-context-specific role for METTL3-dependent m⁶A methylation in promoting apoptosis. In addition to modulating apoptotic pathways, STM2457 treatment significantly decreased the expression and secretion of pro-inflammatory cytokines, including IL-6, INF α , IFN β TNF- α , CXCL10 and CCL5 in Q111 cells (Figure 6F). These findings suggest that METTL3 inhibition dampens inflammatory signaling associated with mHTT expression [27]. Together, these data demonstrate that pharmacological inhibition of METTL3 reduces global m⁶A methylation and mitigates both apoptotic and inflammatory responses in HD striatal cells, while sparing wild-type cells. These results identify m⁶A methylation as a modifiable epitranscriptomic regulator of neuronal survival and inflammatory signaling in HD cellular models.

METTL3–m⁶A–IGF2BP3 axis regulates mHTT stability and caspase-mediated neurodegeneration in HD:

To investigate how m⁶A RNA modification contributes to mHTT-mediated neurotoxicity, we performed siRNA-mediated knockdown of METTL3 and IGF2BP3 in Q7 and Q111 cells cultured for 48 hours in SDM. Knockdown efficiency was first validated by immunoblotting. METTL3 siRNA transfection significantly reduced METTL3 protein levels in both Q7 and Q111 cells (Figure 6A). Notably, METTL3 knockdown led to a pronounced decrease in IGF2BP3 protein, indicating that METTL3-mediated m⁶A modification is required for maintaining IGF2BP3 expression (Figure 7A&B). Functionally, METTL3 depletion in Q111 cells resulted in marked reductions of cleaved caspase-3 and cleaved caspase-9, while no significant changes were

observed in Q7 cells (Figure 7A&B). Levels of procaspase-3 and procaspase-9 remained largely unchanged, suggesting that METTL3 primarily regulates caspase activation rather than basal expression (Figure 7A&B), as caspase activation is a well-established hallmark of neuronal apoptosis and vulnerability in HD [28, 29]. These data demonstrate that METTL3 is a key upstream regulator of apoptotic signaling in mHTT-expressing cells. To directly examine the role of IGF2BP3, we further performed siRNA-mediated knockdown in Q7 and Q111 cells, which effectively reduced IGF2BP3 protein levels (Figure 7C&D). IGF2BP3 depletion recapitulated the effects of METTL3 knockdown, significantly attenuating cleaved caspase-3 and caspase-9 levels, while leaving METTL3 protein levels unaffected. These results indicate that IGF2BP3 acts downstream of METTL3 to mediate apoptotic signaling (Figure 7C&D).

Finally, we assessed mHTT protein levels following knockdown of METTL3 or IGF2BP3. METTL3 knockdown significantly decreased mHTT protein in Q111 cells (Figure 7E&F), consistent with its upstream role in mHTT regulation. Similarly, IGF2BP3 knockdown also significantly reduced mHTT levels (Figure 7G&H), supporting a model in which IGF2BP3 binds m⁶A-modified mHTT mRNA, enhancing transcript stability and translation, and thereby increasing mHTT protein accumulation and downstream caspase activation (Figure 8). Together, these findings delineate a mechanistic cascade in which METTL3 deposits m⁶A on mHTT mRNA, promoting IGF2BP3 binding, which stabilizes the transcript and enhances translation. The resulting increase in mHTT protein levels triggers caspase-3 and caspase-9 activation, leading to neuronal stress and degeneration. The METTL3-m⁶A-IGF2BP3 axis thus functions as a critical epitranscriptomic mechanism linking RNA modification to protein-level toxicity and apoptotic signaling in HD. These data support the model depicted in Figure 8, summarizing the directional regulation of mHTT expression and caspase-mediated neurodegeneration by the METTL3-m⁶A-IGF2BP3 axis.

Discussion

Our study demonstrates that dysregulation of m⁶A RNA modification is a conserved and functionally significant feature of HD across multiple model systems, including R6/2, zQ175 mice, human postmortem striatal tissue, and HD-associated Q111 striatal cells. Transcriptomic analyses revealed widespread upregulation of m⁶A writers, readers, and select erasers in both cortex and striatum of R6/2 and zQ175 mice. Notably, transcriptional changes were region-specific, with certain writers and readers selectively elevated in cortex versus striatum, suggesting that distinct neuronal populations may exhibit differential vulnerability to altered epitranscriptomic signaling. Comparative analyses identified IGF2BP3 as a consistently upregulated m⁶A reader across brain regions and HD models, implicating this factor in mHTT-induced neuronal toxicity.

Importantly, these transcriptional changes translated into elevated protein levels of METTL3, METTL14, IGF2BP2, and IGF2BP3 in HD mouse models, with a corresponding increase in global m⁶A RNA methylation. These findings establish that HD is associated not only with dysregulated m⁶A gene expression but also with coordinated activation of the m⁶A epitranscriptomic machinery, potentially enhancing RNA stability, translation, and downstream protein-level effects. The conservation of these alterations in grade IV human HD cortex and grade II striatum, with significant upregulation of METTL3 and IGF2BP2/3 proteins, indicates that m⁶A dysregulation occurs early in disease and remains till late stages of pathogenesis, and is likely to contribute to neuronal dysfunction in the human brain.

At the cellular level, Q111 striatal cells displayed broad upregulation of m⁶A writers (e.g., METTL3, METTL5, METTL14), readers (IGF2BP2/3, YTHDC1), and erasers (ALKBH1, ALKBH3-5), accompanied by elevated global m⁶A levels, recapitulating findings from mouse and human HD tissue. Pharmacological inhibition of METTL3 with STM2457 selectively reduced m⁶A levels in Q111 cells, improved cell viability, suppressed caspase-3 and caspase-9 activation, and attenuated inflammatory cytokine production (IL-6, TNF- α , IL-1 β , IFN α , IFN β , CXCL10, CCL5). These results indicate that enhanced METTL3 activity drives both apoptotic and neuroinflammatory pathways in HD neurons and highlight the selective vulnerability of mHTT-expressing cells to m⁶A dysregulation.

Mechanistically, we delineate a METTL3-m⁶A-IGF2BP3 signaling axis that directly links RNA modification to mHTT protein accumulation and neuronal apoptosis. siRNA-mediated knockdown of METTL3 in Q111 cells significantly reduced IGF2BP3 protein levels, decreased mHTT abundance, and attenuated cleaved caspase-3 and caspase-9, while procaspase levels remained largely unchanged, indicating that METTL3 primarily regulates apoptotic activation rather than basal caspase expression. Similarly, IGF2BP3 knockdown phenocopied METTL3 depletion, suppressing caspase activation and reducing mHTT protein, without altering METTL3 levels, confirming that IGF2BP3 functions downstream of METTL3. Collectively, these data support a model in which METTL3 deposits m⁶A on mHTT transcripts, promoting IGF2BP3 binding, which enhances mRNA stability and translation, resulting in elevated mHTT protein and subsequent caspase-mediated neuronal stress and degeneration (Figure 8).

The identification of the METTL3-m⁶A-IGF2BP3 axis provides several key insights. First, it positions epitranscriptomic dysregulation as an upstream driver of both apoptotic and inflammatory pathways in HD. Second, it highlights the potential for therapeutic targeting of m⁶A writers or readers to modulate mHTT levels and mitigate downstream neurotoxicity. Finally, the conservation of these changes across mouse, human, and cellular models underscores the translational relevance of m⁶A modulation in HD.

In conclusion, our findings establish m⁶A RNA modification as a central, modifiable contributor to HD pathogenesis, linking aberrant epitranscriptomic regulation to mHTT accumulation, caspase activation, and neuroinflammation. Targeting the METTL3-m⁶A-IGF2BP3 pathway represents a promising strategy to attenuate neuronal vulnerability and may inform the development of RNA-targeted therapeutics for Huntington's disease.

Materials and Methods

Cell culture and STM2457 treatment: STHdh Q7/7 (Q7) and STHdh Q111/111 (Q111) striatal cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO₂ as per our previous publication [24]. Cells were seeded at densities to achieve 60–70% confluence at the time of treatment. STM2457 (MedChemExpress), a selective METTL3 inhibitor, was dissolved in DMSO to prepare a 10 mM stock and stored at –20°C. For experiments, cells were treated with STM2457 at final concentrations of 10 μM or vehicle (0.1% DMSO) for 24–72 h, with fresh medium containing drug or vehicle replenished every 24 h.

Cell viability Assay: Cell viability was assessed using the PrestoBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's instructions. Cells were seeded in 96-well plates and allowed to adhere overnight. Following experimental treatments, culture medium was replaced with fresh medium containing 10% PrestoBlue reagent and incubated at 37 °C in a humidified 5% CO₂ incubator for 1-2 hours. After incubation, fluorescence was measured using a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Background readings from blank wells containing medium and reagent without cells were subtracted from all experimental values. Cell viability was expressed as a percentage relative to untreated control cells, which were set to 100% viability.

Gene Knockdown Using siRNA Transfection: Gene knockdown of METTL3 or IGF2BP3 was performed using small interfering RNAs (siRNAs) obtained from Thermo Fisher Scientific. Cells were transfected with gene-specific siRNAs or a non-targeting control (NTC) siRNA using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were seeded at an optimized density to achieve 50% confluency at the time of transfection. For each well, siRNA (final concentration typically 25 nM) was diluted in Opti-MEM reduced-serum medium and combined with RNAiMAX reagent diluted separately in Opti-MEM. After a 15 minute incubation at room temperature to allow complex formation, the siRNA-lipid complexes were added dropwise to the cells. Knockdown

efficiency was assessed by Western blot analysis. Non-targeting siRNA-treated cells were used as negative controls. All experiments were performed in at least three independent biological replicates.

Animal Tissues: All animal procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) and complied with all applicable ethical regulations governing the use of laboratory animals. Transgenic R6/2 and zQ175 knock-in mouse models of HD, along with age-matched wild-type littermate controls, were used in this study. Mice were housed in a specific pathogen-free (SPF) facility under controlled environmental conditions, including a 12-hour light/dark cycle, with ad libitum access to standard chow and water. Both male and female mice were included unless otherwise specified. Animals were randomly assigned to experimental groups.

Human tissues: Information regarding the human tissue samples used in this study has been described in detail in our previous publication [24].

Gene expression analysis by qRT-PCR: Total RNA was extracted from cells or tissues using Qiagen RNeasy Mini Kit according to the manufacturer's instructions and as described in our previous publications [30]. RNA concentration and purity were assessed using a NanoDrop spectrophotometer. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) following the manufacturer's protocol. Briefly, RNA was incubated with random primers and reverse transcriptase under optimized thermal cycling conditions to generate cDNA, which was subsequently diluted for quantitative PCR analysis. qRT-PCR was performed using SYBR Green chemistry on a real-time PCR detection system as per our previous publications (e.g., Applied Biosystems QuantStudio5). Each reaction was carried out in triplicate in a final volume of 5 µL containing cDNA template, gene-specific primers, and master mix. A melt curve analysis was performed at the end of the run to confirm amplification specificity in SYBR Green-based assays. Relative gene expression levels were calculated using the comparative Ct ($\Delta\Delta C_t$) method [31]. Target gene expression was normalized to the housekeeping gene β -actin. Data were expressed as fold change relative to control groups. Primer sequences were designed using standard primer design tools, and their sequences are listed in Supplementary Table 7. All experiments were performed with at least three independent biological replicates, and technical replicates were included to ensure reproducibility.

m⁶A dot-blot assay: Global m⁶A RNA methylation was assessed by dot-blot analysis. Purified total RNA (500 ng–1 µg) was denatured at 65°C for 5 min, chilled on ice, and spotted onto Hybond-N⁺ nylon membranes (GE Healthcare). Membranes were UV-crosslinked and stained with 0.02% methylene blue in

0.3 M sodium acetate (pH 5.2) to visualize total RNA as a loading control. After destaining, membranes were blocked in 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline, 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with anti-m⁶A antibody (Synaptic Systems, 1:1000) diluted in blocking buffer. After three washes in TBST, membranes were incubated with HRP-conjugated secondary antibody (1:5000) for 1 h, washed again, and developed using enhanced chemiluminescence (ECL). Signal intensities were quantified by ImageJ and normalized to methylene blue staining.

Western blotting: Western blotting was performed as described in our previous publications [32, 33]. Protein lysates from mouse tissue or Q7 and Q111 cells were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. Lysates were clarified by centrifugation (14,000 × g, 15 min, 4°C), and protein concentrations were determined by Bradford Assay. Equal amounts of protein (20µg) were resolved on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were blocked with SEA BLOCK blocking buffer (Thermo) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. After washing, membranes were incubated with IR-labelled secondary antibodies (LiCor) for 1 h at room temperature. Protein bands were visualized and images using LiCor imaging system (LiCor). Band intensities were quantified with Image Studio software and normalized to β-actin.

Acknowledgements: We sincerely thank the Department of Neurological Surgery, University of Pittsburgh, for its sustained support and for providing the funds that were instrumental in the completion of this study.

Funding: This project was supported by the Chairman's Fund of the Department of Neurological Surgery at the University of Pittsburgh.

Conflict of interest: The authors declare no competing interests. Robert M. Friedlander is the Founder and CEO of HELEXVA; however, no funding or support from this entity was used in this study.

Author contributions: **A.J.** contributed to conceptualization, methodology, investigation, validation, formal analysis, supervision, and writing, including original draft preparation, review, and editing of the manuscript. **C.S.C., O.R.A., and T.S.** performed experiments. **X.W.** maintained animal colonies. **A.M., S.A., and D.L.C.** contributed to project support and manuscript review. **R.M.F.** provided strategic direction and mentorship, motivated **A.J.** to pursue studies in epitranscriptomics, and contributed to manuscript review and editing.

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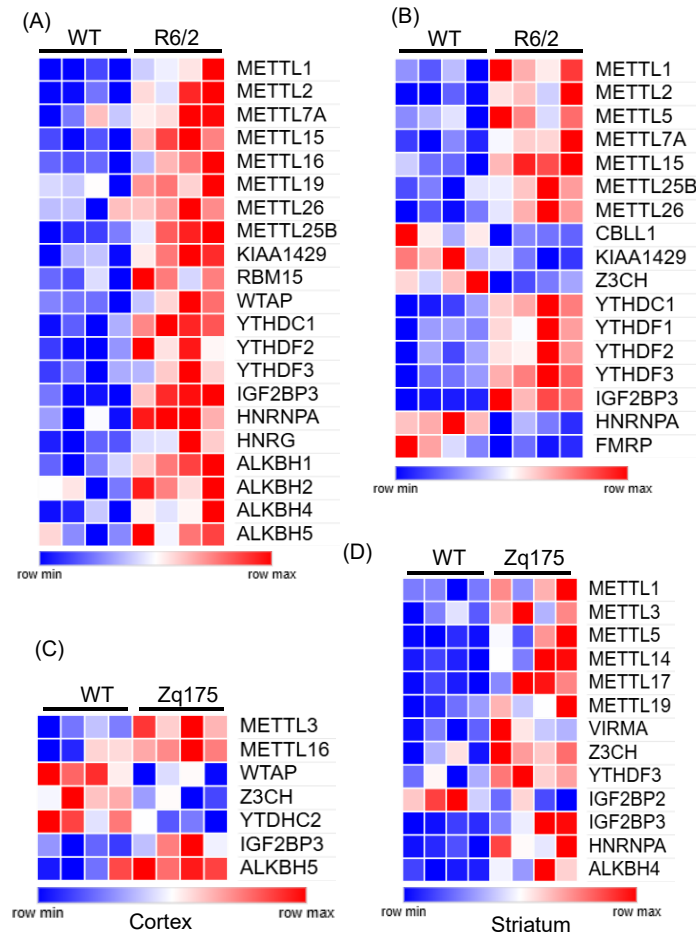


Figure 1. Epitranscriptomic Remodeling of m⁶A Regulatory Networks in the HD Mice. (A) Gene expression heatmap showing m⁶A writer, reader, and eraser genes in the cortex of WT and R6/2 mice (n = 4 per group). (B) Gene expression heatmap of m⁶A regulatory genes in the striatum of WT and R6/2 mice (n = 4 per group). (C) Gene expression heatmap showing m⁶A writer, reader, and eraser genes in the cortex of WT and Zq175 mice (n = 4 per group). (D) Gene expression heatmap of m⁶A regulatory genes in the striatum of WT and Zq175 mice (n = 4 per group). Gene expression was measured by qPCR, and relative fold changes were calculated using the $\Delta\Delta C_t$ method with β -actin as the normalization control. Statistical significance was determined using an unpaired two-tailed Student's *t* test (**P* < 0.05, ***P* < 0.01).

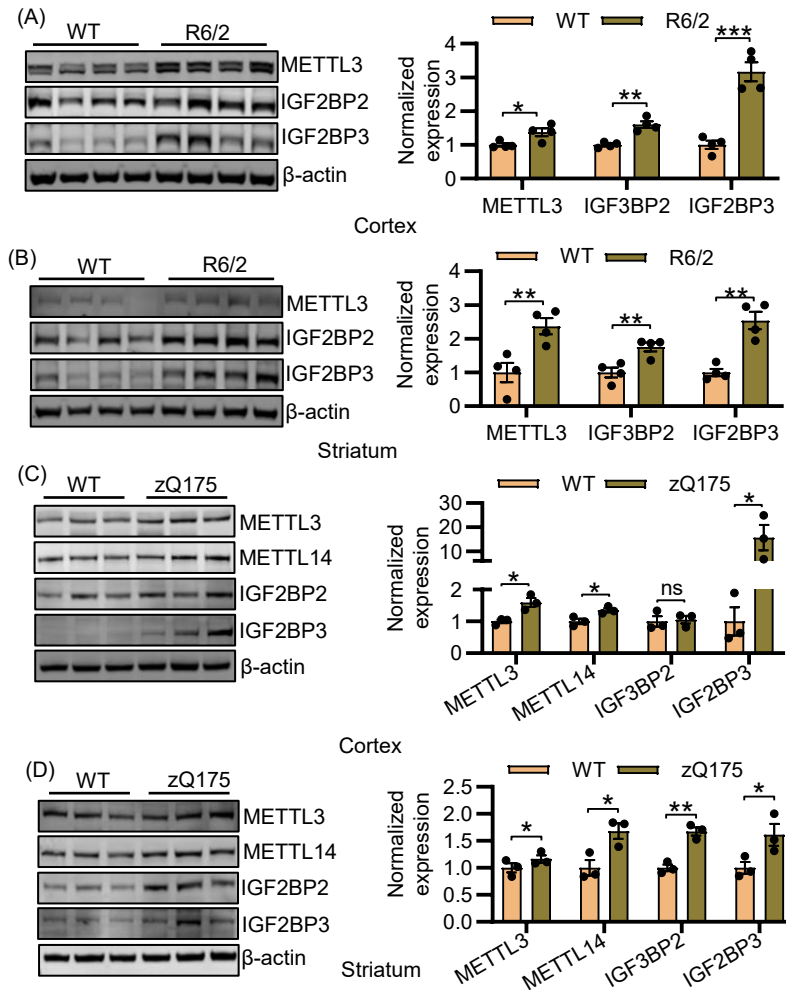


Figure 2. Deregulated m6a machinery in HD mice. (A) Representative immunoblots of METTL3, IGF2BP2, IGF2BP3, and β -actin in cortical lysates from WT and R6/2 mice. (B) Representative immunoblots of METTL3, IGF2BP2, IGF2BP3, and β -actin in striatal lysates from WT and R6/2 mice. (C) Representative immunoblots of METTL3, IGF2BP2, IGF2BP3, and β -actin in cortical lysates from WT and Zq175 mice. (D) Representative immunoblots of METTL3, IGF2BP2, IGF2BP3, and β -actin in striatal lysates from WT and Zq175 mice. Statistical significance was determined using an unpaired two-tailed Student's *t* test (**P* < 0.05, ***P* < 0.01).

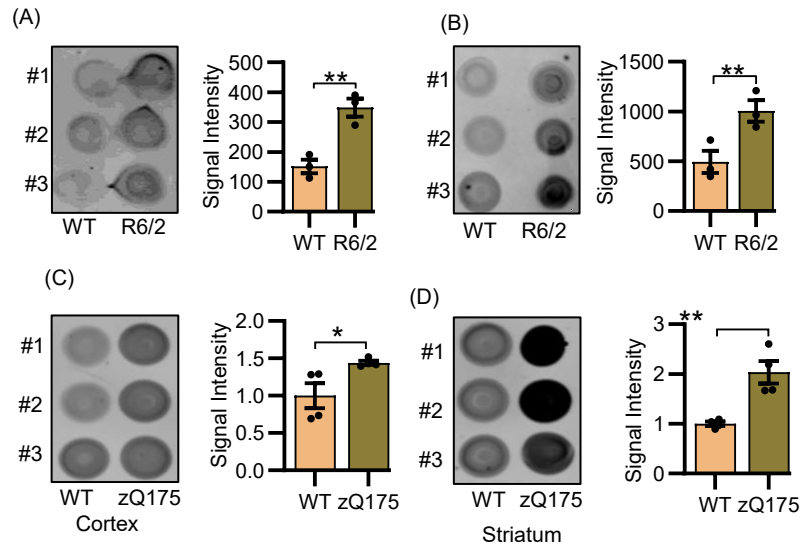


Figure 3: Global m⁶A changes in HD mice: (A) Dot blot analysis of global m⁶A levels in total cortical RNA from WT and R6/2 mice, with corresponding quantification (n = 3 per group). (B) Dot blot analysis of global m⁶A levels in total striatal RNA from WT and R6/2 mice, with corresponding quantification (n = 3 per group). (C) Dot blot analysis of global m⁶A levels in total cortical RNA from WT and Zq175mice, with corresponding quantification (n = 3 per group). (D) Dot blot analysis of global m⁶A levels in total striatal RNA from WT and Zq175mice, with corresponding quantification (n = 3 per group). Statistical significance was determined using an unpaired two-tailed Student's *t* test (*P < 0.05, **P < 0.01).

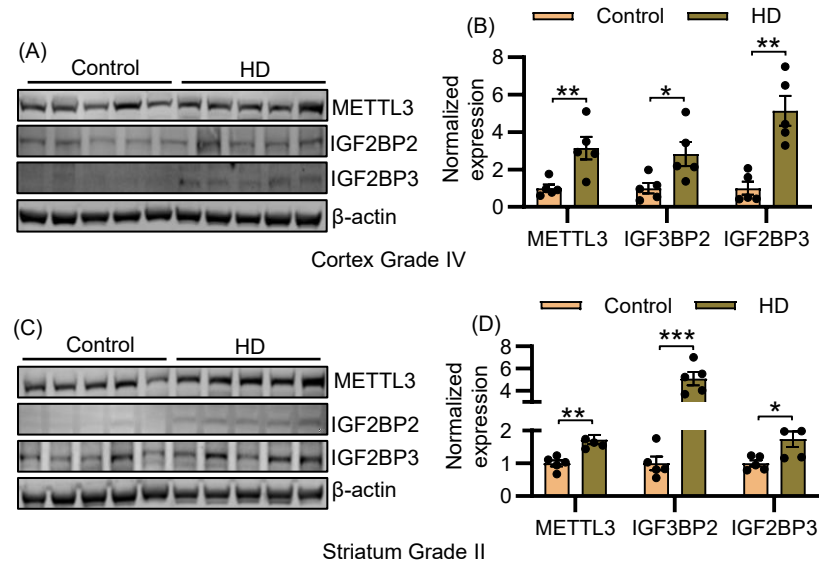


Figure 4. Altered m⁶A Regulatory Proteins in Postmortem Striatum of Huntington's Disease Patients. (A) Representative immunoblots of METTL3, IGF2BP2, IGF2BP3, and β-actin in postmortem human striatal tissue from grade II Huntington's disease (HD) cases and age-matched controls. (B) Densitometric quantification of protein expression normalized to β-actin. Data are presented as mean ± SEM (*n*=5 per group). Statistical significance was determined using an unpaired two-tailed Student's *t*-test. *P* < 0.05 was considered significant (**P* < 0.05, ***P* < 0.01).

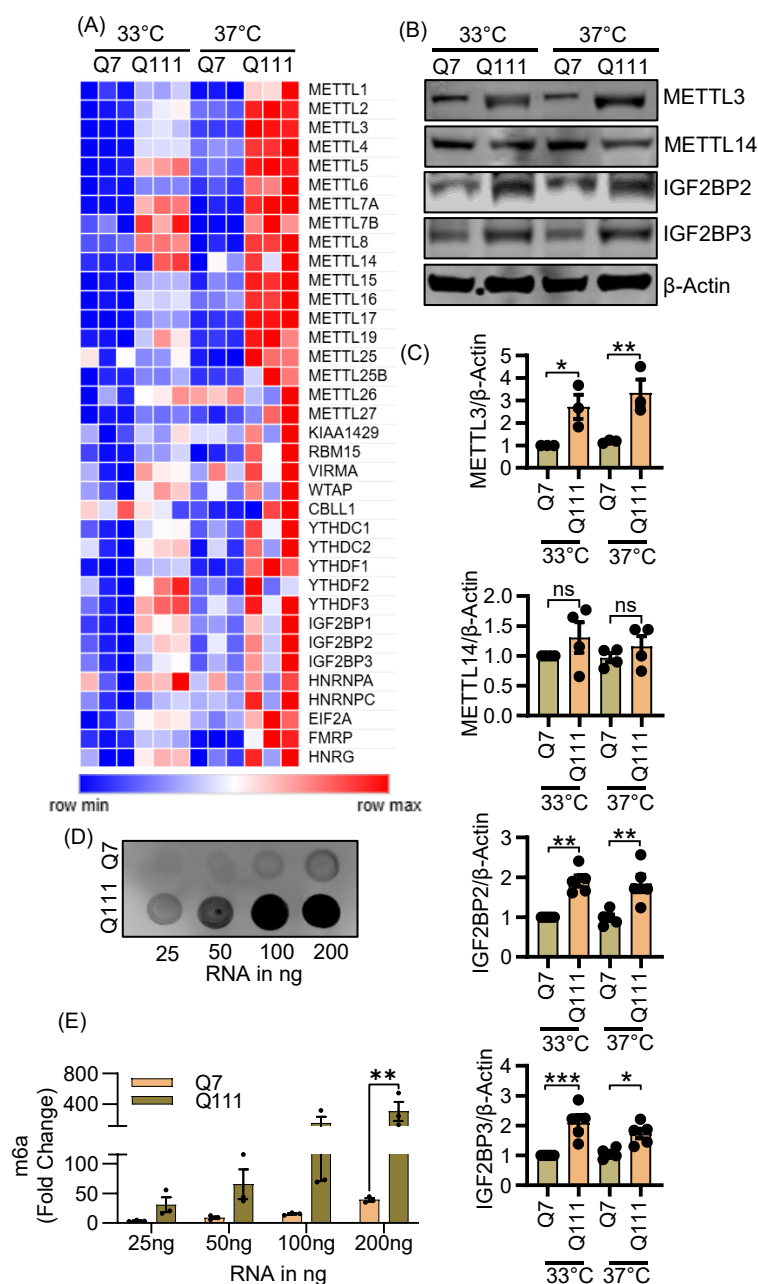


Figure 5. Deregulation of m⁶A regulatory machinery in STHdh Q7 and Q111 cells under metabolic stress.

(A) Gene expression heatmap of m⁶A writers and readers in STHdh Q7 and Q111 cells cultured in serum-deprived medium at 33°C and 37°C, assessed by qPCR (n=3 independent experiments). (B) Representative dot blot analysis of global m⁶A levels in total RNA isolated from Q7 and Q111 cells. (C) Quantification of m⁶A dot blot signals (n=3 independent experiments). (D) Representative immunoblots of METTL3, METTL14, IGF2BP2, IGF2BP3, and β-actin in Q7 and Q111 cells cultured in serum-deprived medium at 33°C or 37°C for 24 hours. (E) Densitometric quantification of protein expression normalized to β-actin (n=3

independent experiments). Data are presented as mean $\pm$ SEM. Statistical significance was determined using a student-t-test. $P < 0.05$ was considered statistically significant (* P < 0.05, ** P < 0.01).

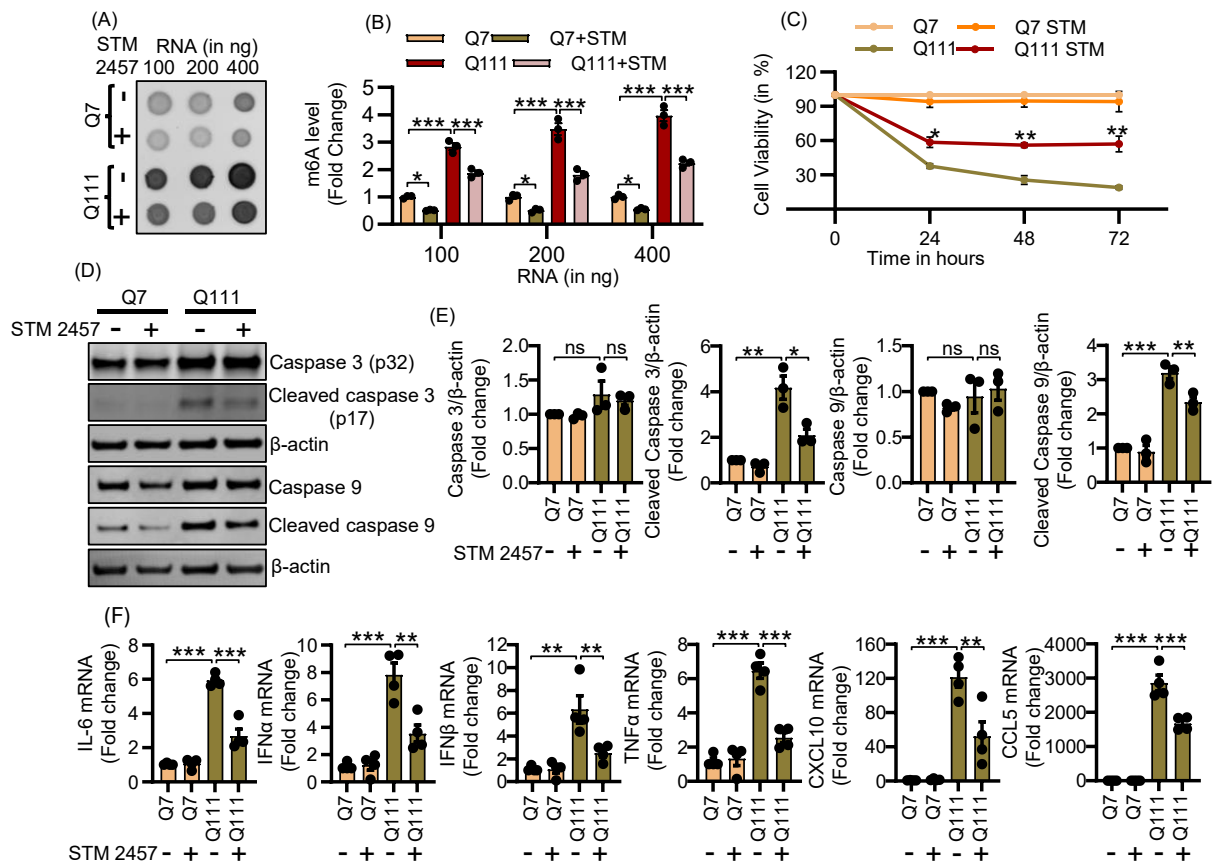


Figure 6. METTL3 inhibition attenuates cell death and caspase activation in HD cells. (A) Representative dot blot showing global m⁶A levels in Q7 and Q111 cells following treatment with the METTL3 inhibitor STM2457 ($n=3$ independent experiments). (B) Quantification of m⁶A dot blot signals. (C) Cell viability analysis of Q7 and Q111 cells treated with STM2457 for 24, 48, and 72 hours under serum-deprived conditions at 37°C. (D) Representative immunoblots of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, and β-actin in Q7 and Q111 cells following 48 hours of STM2457 treatment in serum-free medium at 37°C ($n = 3$ independent experiments). (E) Densitometric quantification of caspase protein levels normalized to β-actin. Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA as described in the Methods. $P < 0.05$ was considered statistically significant.

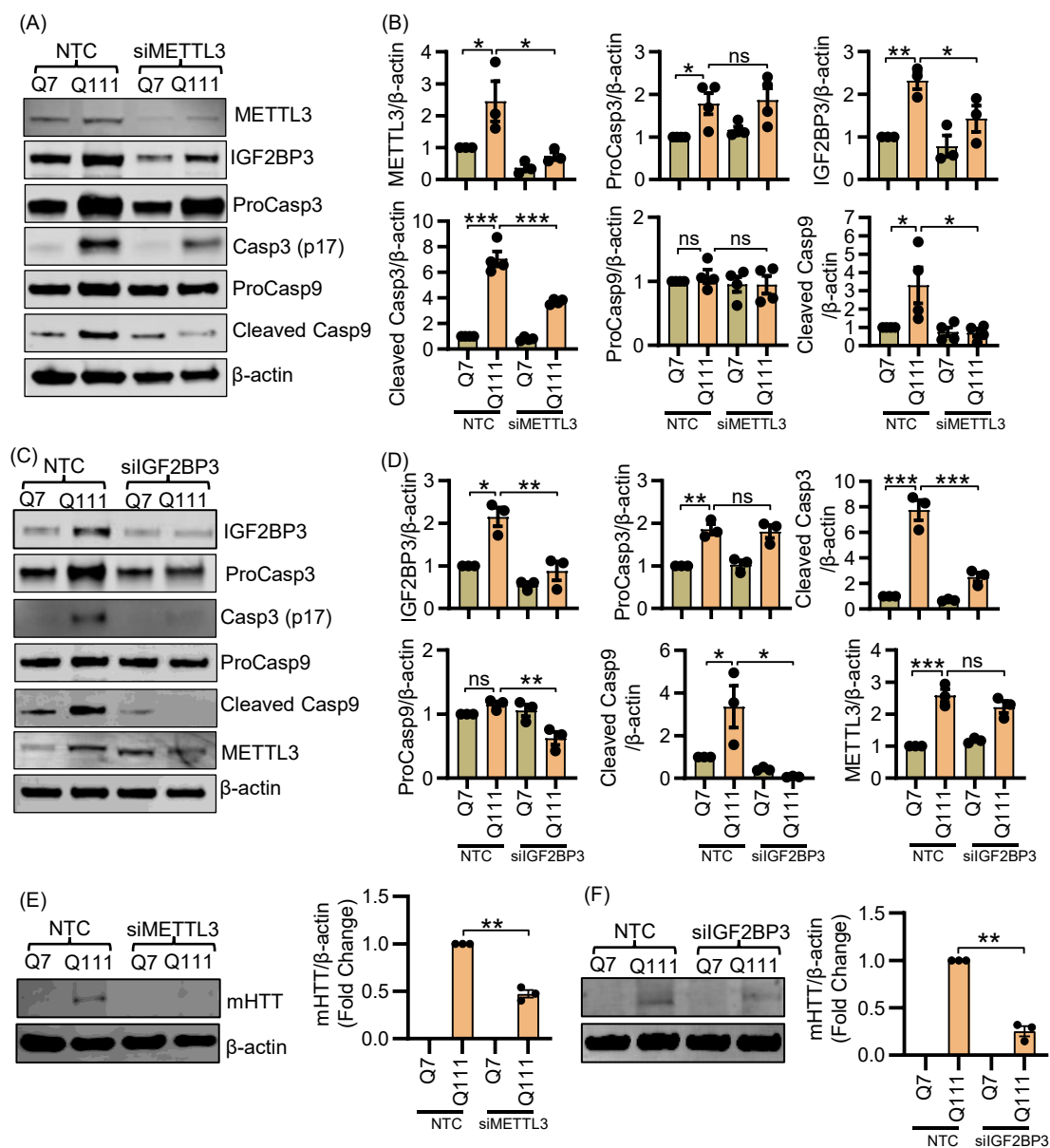


Figure 7. m⁶A modification regulates mHTT-mediated caspase activation via IGF2BP3.

(A) Representative immunoblots showing METTL3, caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, IGF2BP3, and β -actin in Q7 and Q111 cells transfected with non-targeting control (NTC) or METTL3 siRNA and cultured at 37 °C in serum-deprived media for 24 h $n=3$. (B) Densitometric quantification of caspases and IGF2BP3 protein levels normalized to β -actin following METTL3 knockdown. (C) Representative immunoblots of METTL3, caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, IGF2BP3, and β -actin in Q7 and Q111 cells transfected with NTC or IGF2BP3 siRNA cultured at 37 °C in

serum-deprived media for 24 h $n=3$. (D) Quantification of caspase activation and METTL3 expression normalized to β -actin following IGF2BP3 knockdown. (E) Effect of METTL3 or (F) IGF2BP3 knockdown on mutant huntingtin (mHTT) protein levels in Q111 cells cultured at 37 °C in serum-deprived media for 24 h $n=3$. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. $n = 3$ independent experiments for all panels.

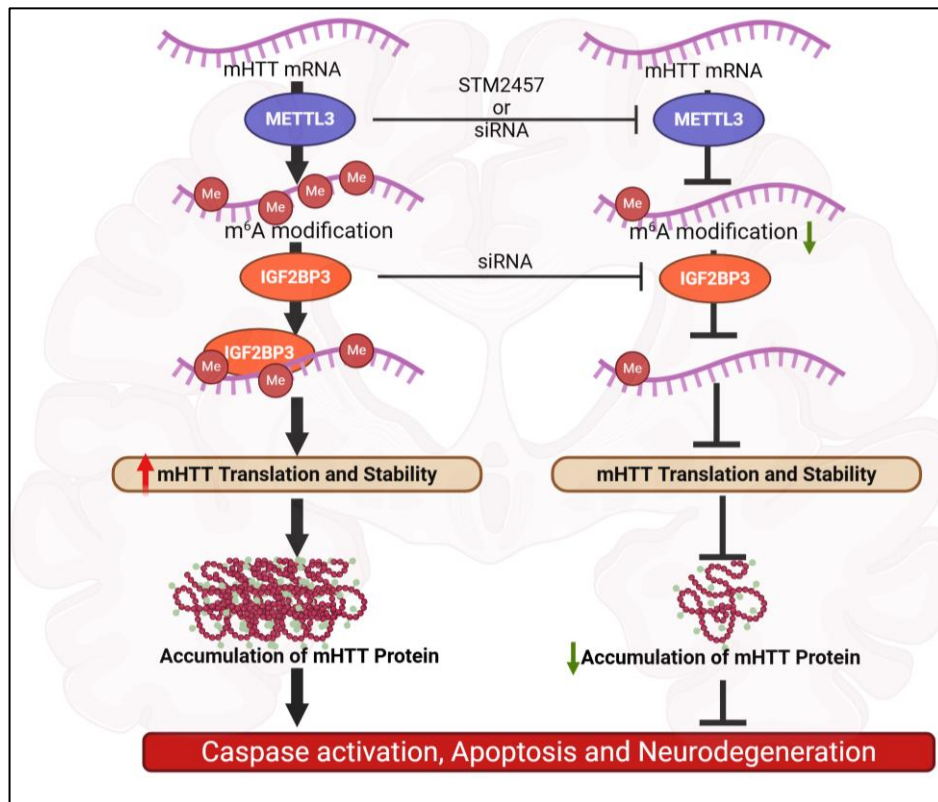


Figure 8: Schematic representation of epitranscriptomic regulation of neurodegeneration in HD via the METTL3-IGF2BP3 Axis

Supplementary Files

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