Mutant Huntingtin: Nuclear translocation and cytotoxicity mediated by GAPDH

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The pathophysiology of Huntington's disease reflects actions of mutant Huntingtin (Htt) (mHtt) protein with polyglutamine repeats, whose N-terminal fragment translocates to the nucleus to elicit neurotoxicity. We establish that the nuclear translocation and associated cytotoxicity of mHtt reflect a ternary complex of mHtt with GAPDH and Siah1, a ubiquitin-E3-ligase. Overexpression of GAPDH or Siah1 enhances nuclear translocation of mHtt and cytotoxicity, whereas GAPDH mutants that cannot bind Siah1 prevent translocation. Depletion of GAPDH or Siah1 by RNA interference diminishes nuclear translocation of mHtt.

Huntington's disease | Siah | polyglutamine

Huntington's disease (HD) is a genetically dominant disorder caused by expansion of a polyglutamine (polyQ) sequence in the N-terminal region of Htt (1). In cell culture models, overexpression of mutant Htt (mHtt) with the expanded polyQ, especially the N-terminal mHtt fragments, elicits cytotoxicity that requires nuclear translocation of the N-terminal fragment (2, 3). In a variety of HD animal models, nuclear accumulation of mHtt is also critical for HD pathophysiology (4–7). Accordingly, understanding the mechanism of the nuclear translocation may shed light on the neurotoxicity and potential therapy for HD.

Recently, we described a cell-death cascade involving GAPDH and the ubiquitin-E3-ligase Siah1 (8). Apoptotic insults stimulate inducible or neuronal forms of nitric oxide (NO) synthase with NO *S*-nitrosylating GAPDH, conferring upon it the ability to bind to Siah1, a ubiquitin-E3-ligase. Siah1, which possesses a nuclear localization signal (NLS), elicits the translocation of GAPDH to the nucleus. In the nucleus, GAPDH stabilizes the rapidly turning over Siah, enabling it to degrade its nuclear protein targets, leading to cell death. We wondered whether the GAPDH/Siah1 cascade participates in the nuclear translocation of mHtt. In the present study, we demonstrate that GAPDH, together with Siah, facilitates nuclear translocation of mHtt and resultant neurotoxicity.

Results

Htt and other proteins with polyQ repeats have been reported to bind to GAPDH (9, 10). To determine whether such binding is relevant to the nuclear targeting of mHtt and its cytotoxicity, we have explored this interaction in detail. First, we directly demonstrate *in vitro* protein binding of GAPDH to an N-terminal fragment of Htt (His-Htt-N171-23Q) (Fig. 1*A*), which was confirmed in a yeast two-hybrid assay (Htt-N427, data not shown). We then examined potential interactions of wild-type Htt (wtHtt) and mHtt with GAPDH and Siah1 in HEK293T cells by monitoring coimmunoprecipitation (Fig. 1*B*). Siah1, but not GAPDH, levels are elevated in whole-cell lysates with mHtt compared with wtHtt, presumably reflecting stabilization of Siah after cytotoxic insult (8). GAPDH precipitates to the same extent with wtHtt and mHtt, whereas Siah1 coprecipitates much more with mHtt than with wtHtt. Under basal conditions, Siah1 self-degrades through its ubiquitin-E3-ligase activity mediated by its RING finger domain (11). To prevent confounding effects of Siah self degradation, we transfected Siah1 lacking the RING finger domain (Siah1ΔRING), which provides total lysate levels of Siah1ΔRING in wtHtt cells comparable to those of mHtt cells. Nonetheless, substantially greater Siah1ΔRING coprecipitates with mHtt than with wtHtt. Accordingly, Siah1 does bind more avidly to GAPDH/Htt complexes containing expanded polyQ.

To determine whether GAPDH and Siah1 are responsible for the nuclear translocation of mHtt, we conducted subcellular fractionation of N2a cells, a mouse neuroblastoma cell line, after transfection with GAPDH or with a mutant GAPDH in which lysine-225 is replaced with alanine (GAPDH-K225A) (Fig. 2*A*). We previously showed that K225 is critical for the binding of GAPDH to Siah1, so that GAPDH-K225A fails to translocate to the nucleus (8). In the presence of mHtt, we observe substantial levels of GAPDH, but not GAPDH-K225A, in the nucleus (data not shown). Nuclear mHtt levels are also augmented after transfection of GAPDH. Transfection of GAPDH-K225A leads to substantially less nuclear mHtt. Thus, GAPDH and its interaction with Siah1 appear critical for nuclear translocation of mHtt, and both mHtt and GAPDH accumulate within the nuclear fraction.

To assess the domains of Siah1 that are critical for nuclear translocation of mHtt, we cotransfected N2a cells with wild-type Siah1, Siah1 lacking its NLS (Siah1ΔNLS), or SiahΔRING (Fig. 2*B*). Transfection with Siah1 or Siah1ΔNLS reduces nuclear as well as total cell levels of mHtt, presumably because the ubiquitin-E3-ligase activity of Siah1 degrades mHtt. The ratio of nuclear to total mHtt in cells is tripled after Siah1 overexpression. Siah's NLS mediates the nuclear translocation of mHtt, because Siah1ΔNLS fails to augment the ratio of nuclear to total mHtt. In contrast, overexpression of Siah1ΔRING leads to increases of both indicators. Thus, both GAPDH and Siah mediate nuclear translocation of mHtt.

Many studies have established an important role for nuclear translocation of mHtt in eliciting cytotoxicity (2, 3). Accordingly, we monitored cell death in N2a cells using various GAPDH and Siah1 constructs to determine whether GAPDH and Siah1 modulate mHtt-induced cytotoxicity (Fig. 3*A*). Overexpression of GAPDH or Htt-Associated Protein-1 (HAP-1) does not cause notable cell death. By contrast, overexpression of mHtt is cytotoxic, and combined transfec-

Conflict of interest statement: No conflicts declared.

Abbreviations: HD, Huntington's disease; polyQ, polyglutamine; Htt, Huntingtin; mHtt, mutant Htt; wtHtt, wild-type Htt; RNAi, RNA interference; Siah1ΔRING, Siah1 lacking the RING finger domain; GAPDH-K225A, mutant GAPDH in which lysine-225 is replaced with alanine; NLS, nuclear localization signal; Siah1ΔNLS, Siah1 lacking NLS; HA, hemagglutinin.

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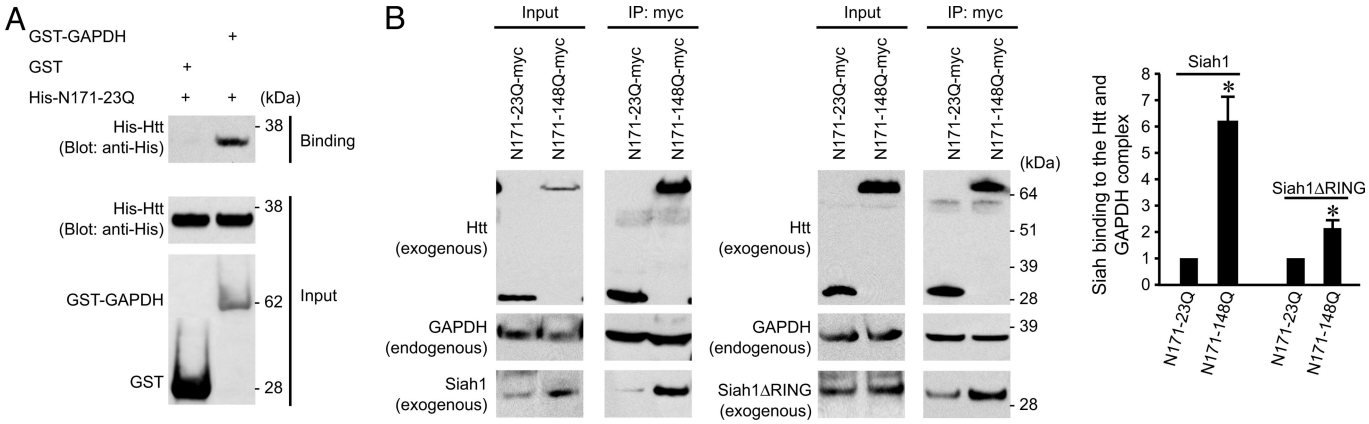

Fig. 1. Htt, GAPDH, and Siah1 form a ternary complex. (*A*) GAPDH binds to Htt *in vitro*. GST-tagged GAPDH (GST-GAPDH) and His-tagged Htt containing N-terminal 171 amino acids and 23 polyQ (His-N171-23Q) were purified from *Escherichia coli*. *In vitro* binding assay was performed on glutathione beads. (*B*) Htt, GAPDH, and Siah1 form a ternary complex in HEK293T cells transfected with myc-tagged Htt N171-23Q or N171-148Q, and HA-tagged Siah1 or Siah1ΔRING, as shown by coimmunoprecipitation (co-IP). The Htt protein complex was immunoprecipitated by myc antibody and immunoblotted with Htt (EM48), GAPDH, and HA antibodies. (*Left*) Siah1, a rapidly turning-over protein, is stabilized (input) and recruited to the Htt-GAPDH complex (IP) to a greater extent with N171-148Q than with N171-23Q. (*Center*) Siah1ΔRING is resistant to self degradation, leading to comparable protein levels in N171-23Q and N171-148Q-transfected cells (input). More Siah1ΔRING is recruited to the Htt-GAPDH complex by N171-148Q than by N171-23Q (IP). PolyQ expansion does not affect the interaction of GAPDH and Htt. (*Right*) Densitometric analysis of coIP shows that mHtt is better than wtHtt in recruiting Siah1 and Siah1ΔRING. Data represent the mean and SEM of two independent experiments (*t* test, *, $P < 0.01$).

tion with GAPDH augments this toxicity, whereas combined transfection with HAP-1 does not. Enhanced toxicity with GAPDH is observed in mHtt with two different lengths of the N-terminal fragment. Overexpression of expanded polyQ itself also elicits cytotoxicity that is substantially increased by GAPDH transfection, whereas overexpression of a nonpathogenic glutamine tract (23 glutamines) is not cytotoxic, even in the presence of overexpressed GAPDH. We also monitored the influence of Siah1 on cytotoxicity. In the absence of mHtt, neither Siah1ΔNLS nor Siah1ΔRING is cytotoxic, consistent with our previous studies (8). In cells overexpressing mHtt, transfection with Siah1ΔRING markedly increases cell death, whereas overexpressing Siah1ΔNLS does not. We did not examine Siah1 by itself, because the intrinsic cytotoxicity of Siah1 would confound evaluations of mHtt toxicity.

Because GAPDH is a major glycolytic enzyme, it is conceivable that the protein interaction of GAPDH and mHtt may influence cytotoxicity via changes in cellular energy status. Accordingly, we examined the influence of mHtt on GAPDH catalytic activity in N2a cell extracts and on intracellular levels of ATP (Fig. 3*B*). Overexpression of mHtt does not affect GAPDH catalytic activity. In confirmation of our earlier study (8), mutation of cysteine-150 to serine abolishes catalytic activity of the transfected mutant GAPDH. GAPDH transfection doubles cellular ATP levels with a similar augmentation observed in cells overexpressing mHtt. GAPDH-C150S does not influence cellular ATP.

Most of the experiments described above involved overexpression of proteins. To assess the importance of endogenous GAPDH and Siah1 for nuclear translocation of mHtt, we depleted proteins by RNA interference (RNAi). In N2a cells, as we reported in other cells, treatment with RNAi almost totally depletes mRNA levels for both GAPDH and Siah1 (data not shown). As described (8, 12), we added pyruvate to maintain cellular energy status. In control cells, about half of the inclusions detected by immunofluorescent staining are intranuclear. Depletion of GAPDH or Siah1 reduces nuclear inclusion levels by 50–60% (Fig. 4 *A* and *B*). By contrast, there is a substantial increase of perinuclear inclusions in the GAPDH and Siah1-depleted cells with no major change in cytoplasmic inclusions. We recently reported that nuclear translocation of Htt in human lymphoblasts involves specific perinuclear sites (13). These findings suggest that GAPDH and Siah1 are important for regulating trafficking between the perinuclear and nuclear compartments rather than between the cytoplasm and the perinuclear region.

Discussion

In the present study, we provide a mechanism for nuclear translocation of mHtt that involves a ternary complex of mHtt, GAPDH, and Siah1.

A role of GAPDH/Siah1 in mediating the nuclear translocation of mHtt reflects a function for GAPDH/Siah1. In our previous study, we showed that nuclear translocation of GAPDH and Siah1 mediates cell death induced by a variety of stressors (8, 14). GAPDH stabilizes Siah1 in the nucleus and augments Siah1-associated toxicity. By contrast, in the present study, Siah1ΔRING, which by itself is nontoxic, augments mHtt-induced cytotoxicity. Thus, GAPDH and Siah1 influence the sorting of mHtt to the nucleus, independent of the GAPDH/Siah1 death cascade (8). There exist two distinct forms of human Siah, Siah1 and Siah2 (15). Most of the experiments in the present study have used Siah1. In preliminary studies, deletion of Siah2 by RNAi also reduces the nuclear translocation of mHtt (B.-I.B. and S.H.S., unpublished observations).

Our findings implicate the GAPDH/mHtt interaction in HD pathology. In early studies describing the binding of Htt to GAPDH, it was speculated that altered glycolytic activity of GAPDH might play a role in the pathophysiology (9, 16). We observed that augmentation of mHtt cytotoxicity by GAPDH is unrelated to decreases in GAPDH glycolytic activity or ATP content of cells. Similarly, Beal and coworkers (17) as well as Shapira and coworkers (18) have failed to find altered GAPDH activity in brains of patients with HD, although there is a report of a slight change of GAPDH in the caudate of HD brain (19). Presumably, in neurons with mHtt, oxidized GAPDH translocates to the nucleus together with Siah, facilitating nuclear translocation of mHtt. Chuang and coworkers (20) recently detected nuclear accumulation of GAPDH in a transgenic mouse model of HD, fitting with our findings. Nuclear GAPDH in HD fibroblasts migrates aberrantly in glycerol gradient sedimentations, suggesting that GAPDH in patient tissues is incorporated into a protein complex of a large molecular weight,

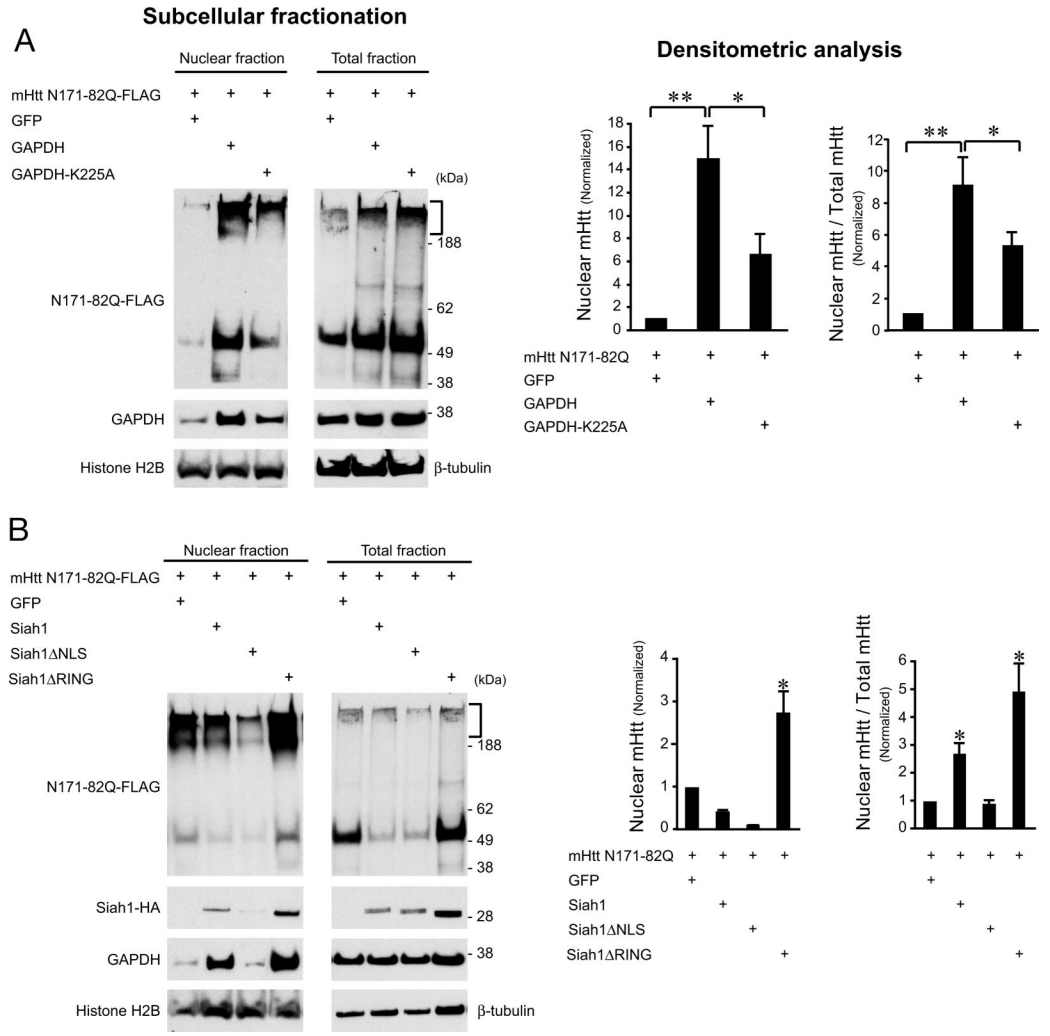

Fig. 2. Overexpressed GAPDH and Siah1 enhance nuclear targeting of mHtt. (*A*) Overexpressed GAPDH augments nuclear targeting of mHtt (N171-82Q) 10- to 15-fold in N2a cells transfected with FLAG-tagged mHtt and GAPDH. The binding of GAPDH to Siah1 is critical for nuclear targeting of mHtt, because GAPDH-K225A, which fails in binding to Siah1 and translocating to the nucleus, leads to significantly less nuclear mHtt than GAPDH (*t* test, *, $P < 0.01$; **, $P < 0.001$). (*B*) Overexpressed Siah1 augments nuclear targeting of mHtt in N2a cells transfected with mHtt and Siah1. Overexpressed Siah1 and Siah1ΔNLS degrade both total and nuclear mHtt through the ubiquitin-E3-ligase activity of Siah1, as Siah1 lacking ubiquitin-E3-ligase activity (Siah1ΔRING) only increases nuclear targeting of mHtt 2- to 3-fold without degrading mHtt. It is noticeable that the ratio of nuclear to total mHtt is tripled by overexpression of Siah1, but not Siah1ΔNLS (ANOVA, *, $P < 0.01$). The bracket indicates SDS-insoluble mHtt aggregates. Data represent the mean and SEM of three independent experiments.

probably with mHtt (21). In the present study, such a complex is implied by the smearing of GAPDH immunoreactivity together of that of mHtt near the gel top in Western blots (data not shown).

Li and coworkers (22) have described an alternate means whereby mHtt might enter the nucleus. They showed that N-terminal fragments of Htt bind to the nuclear pore protein translocated promoter region (Tpr) that participates in nuclear export. A lesser binding of mHtt to this protein is associated with greater nuclear accumulation. Reducing the expression of Tpr increases nuclear accumulation of mHtt. Conceivably, diminished interactions of mHtt with Tpr function in concert with the GAPDH-Siah1 system in mediating nuclear translocation.

Exact mechanisms whereby nuclear mHtt elicits cytotoxicity are still unclear. There are several candidate proteins that interact with mHtt and mediate cytotoxicity, including cAMP response element-binding protein, Sp1, and p53 (23). In both cellular and animal models of HD, p53 is up-regulated, and blockade of p53 diminishes mitochondria-associated cellular dysfunctions and cytotoxicity as well as behavioral abnormalities of HD mice (24). Abnormalities of p53 in HD are not evident in spinocerebellar ataxia type-1 (SCA1) (25). Interestingly, ataxin-1, atrophin-1, and the androgen receptor, whose polyQ expansions are responsible for SCA1, dentatorubral pallidoluysian atrophy, and spinobulbar muscular atrophy, bind to GAPDH in a manner similar to Htt (9, 10). In preliminary studies, we showed that cytotoxicity induced by mutant atrophin-1 is also increased by overexpression of GAPDH (A.S. and S.H.S., unpublished data). These results suggest that GAPDH may have a common role in modulating the pathophysiology of polyQ diseases.

Materials and Methods

Reagents and Constructs. Unless otherwise noted, reagents were obtained from Sigma. All Htt, GAPDH, and Siah plasmids were previously described (8, 24). Short oligomers of RNAi to GAPDH, Siah1, Siah2, and control were synthesized (Dharmacon Research, Lafayette, CO), following the sequences previously described. Antibodies against GAPDH (Biogenesis, Bournemouth, U.K.), Htt EM48 (Chemicon), hemagglutinin

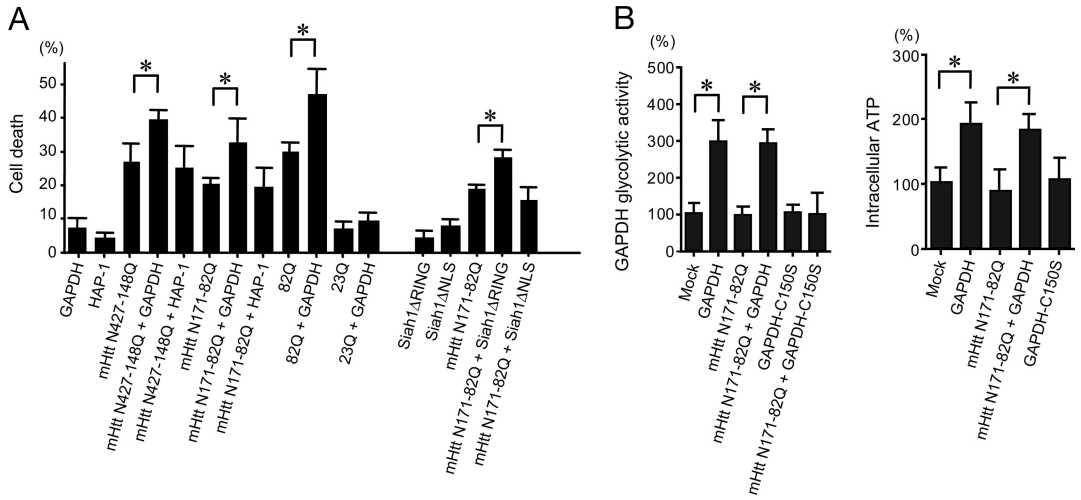

Fig. 3. Overexpressed GAPDH and Siah1ΔRING increase mHtt cytotoxicity. (*A*) Overexpressed GAPDH and Siah1ΔRING augment mHtt cytotoxicity in N2a cells transfected with Htt and GAPDH, Htt-Associated Protein-1 (HAP-1), Siah1ΔRING, or Siah1ΔNLS for 72 h. GAPDH, but not HAP-1, augments cytotoxicity of mHtt N171-82Q, mHtt N427-148Q (mHtt containing N-terminal 427 amino acids and 148 polyQ), and 82 polyQ (82Q), suggesting the specificity of GAPDH. GAPDH is not toxic in itself or with 23 polyQ (23Q). Siah1ΔRING, but not Siah1ΔNLS, significantly augments mHtt toxicity. (*B*) Enhanced mHtt cytotoxicity by GAPDH is independent of GAPDH glycolytic activity or intracellular ATP levels. Overexpressed GAPDH augments glycolytic activity and the ATP level, which is not affected by mHtt N171-82Q expression. GAPDH-C150S harbors a mutation at the catalytic center, debilitating enzymatic activity of GAPDH (*t* test, *, $P < 0.01$).

(HA) (Sigma), FLAG (Sigma), Myc (Roche Applied Science, Indianapolis), Histone H2B (Upstate Biotechnology, Lake Placid, NY), and β-tubulin (Upstate Biotechnology) were purchased.

***In Vitro* Binding.** His-tagged Htt N171-23Q and GST-tagged GAPDH proteins were prepared and used for *in vitro* binding by following the detailed protocol previously described (8, 24, 26).

Coimmunoprecipitation. Homogenates of HEK293T cells transfected with 2 μg of myc-tagged Htt N171-23Q/148Q and 6 μg of HA-tagged Siah1 or Siah1ΔRING (60-mm dishes) for 48–60 h were prepared in ice-cold 500 μl of RIPA lysis buffer (150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/50 mM Tris·Cl, pH 8.0) containing protease inhibitors and 1 mM EDTA. Htt was immunoprecipitated with 2 μg of myc antibody, and the presence of GAPDH and Siah1 or Siah1ΔRING in the Htt protein complex was examined through immunoblotting with anti-GAPDH and HA antibodies. Efficiency of immunoprecipitation of Htt was confirmed with the EM48 antibody. Siah binding to the Htt and GAPDH complex was analyzed by densitometry.

Nuclear Fractionation. By using N2a cells transfected with 2 μg of FLAG-tagged mHtt N171-82Q and 6 μg of GFP, GAPDH, GAPDH-K225A, Siah1-HA, Siah1ΔNLS-HA, or Siah1ΔRING-HA (60-mm dishes) for 48–60 h, the nuclei were obtained from the cell lysates in buffer B (10 mM Tris·Cl, pH 7.4/0.4% Nonidet P-40/0.25 M sucrose/10 mM $MgCl_2$/10 mM KCl/1 mM DTT, and protease inhibitors without EDTA) by centrifugation over a 2-M sucrose cushion (10 mM Tris·Cl, pH 7.4/1.7 M sucrose/10 mM $MgCl_2$/10 mM KCl/1 mM DTT) at 100,000 × *g* for 1 h (14). Immunoblotting of nuclear and total fractions was performed with FLAG, GAPDH, and HA antibodies. Histone H2B and β-tubulin levels were used as loading controls. Levels of nuclear mHtt and the ratio of nuclear to total mHtt were analyzed by densitometry.

GAPDH Enzymatic Activity and Intracellular ATP Levels. GAPDH glycolytic activity and intracellular ATP in N2a cells were measured as described (8, 27).

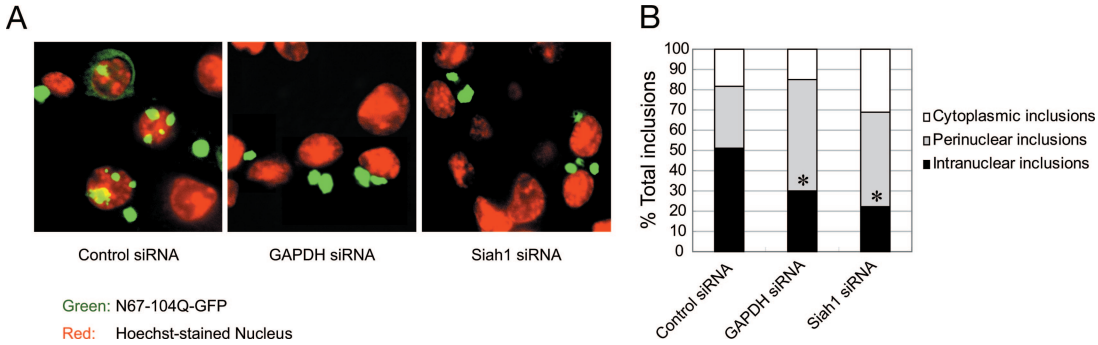

Fig. 4. Depletion of GAPDH and Siah1 blocks nuclear targeting of mHtt. mHtt containing N-terminal 67 amino acids and 104Q fused to GFP (N67-104Q-GFP) was transfected into N2a cells pretreated with siRNA to GAPDH or Siah1. Medium was supplemented with 1 mM pyruvate to avoid toxicity of GAPDH siRNA. Intranuclear inclusions are frequently observed with control RNAi but not with GAPDH siRNA or Siah1 siRNA. When nuclear targeting of mHtt is significantly less efficient without GAPDH or Siah1, and mHtt is accumulated in the perinuclear region. Each graph bar in *B* corresponds to the scoring of ≈200 inclusions from randomly chosen fields 36 h after transfection performed in triplicate (*t* test, *, $P < 0.01$).

Cell Culture and Transfection. N2a and HEK293T cells were cultured in DMEM containing 10% FBS and nonessential amino acids. Pyruvate-supplemented culture media were used to compensate potential energy deficits by RNAi to GAPDH, according to the established protocol (8). Lipofectamine 2000 (Invitrogen) was used for transfection, following the manufacturer's protocol. The ratio of plasmids to lipofectamine 2000 was 1:4 (wt/wt), and that of RNAi to lipofectamine 2000 was 1:50,000 (wt/wt) to get the highest transfection efficiency with minimal toxicity. For cotransfection of Htt and GAPDH or Siah1 into a 60-mm dish culture, 2 μg of Htt and 6 μg of GAPDH or Siah1 plasmids was used to ensure coexpression. For cotransfection of Htt and RNAi into a 60-mm-dish culture, 0.4 ng of RNAi, 2 μg of plasmid, and 20 μg of lipofectamine 2000 were used.

Cell Death Assay. Cytotoxicity was monitored by using a lactate dehydrogenase (LDH) assay kit (Sigma). LDH in the media and within N2a cells was measured. In parallel, we monitored the transfection efficiency by GFP signal. The LDH ratio of media supernatant to total cell extract was standardized by the transfection efficiency to obtain the percentage of dead cells.

Confocal Microscopic Quantification of Nuclear Targeting of mHtt. Thirty-six hours after the first transfection of RNAi to GAPDH, Siah1, or Siah2 into N2a cells, RNAi and N67-104Q-GFP were introduced together, and the cells were analyzed 24~36 h later. In this way, residual GAPDH and Siah1 were depleted by the time mHtt was expressed. Visible mHtt inclusions in the cytoplasmic, perinuclear, or nuclear domains scored under confocal microscopy (PerkinElmer UltraVIEW) were used to assess nuclear targeting of mHtt. Inclusions bound to the nuclear membranes without nuclear entry were considered "perinuclear." The nuclei were stained with Hoechst 33258 (Invitrogen). Subcellular localization was determined by overlaying GFP, the Hoechst signal, and bright-field images. In the same condition, immunoblotting was also performed to confirm microscopic quantifications (data not shown).

Densitometry and Statistical Analysis. Quantitative densitometric analysis of immunoblotting was performed by using the EAGLESIGHT program (Stratagene). Two-tailed P values were calculated by Student's t test and ANOVA by using MINITAB 13 (Minitab, State College, PA).

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