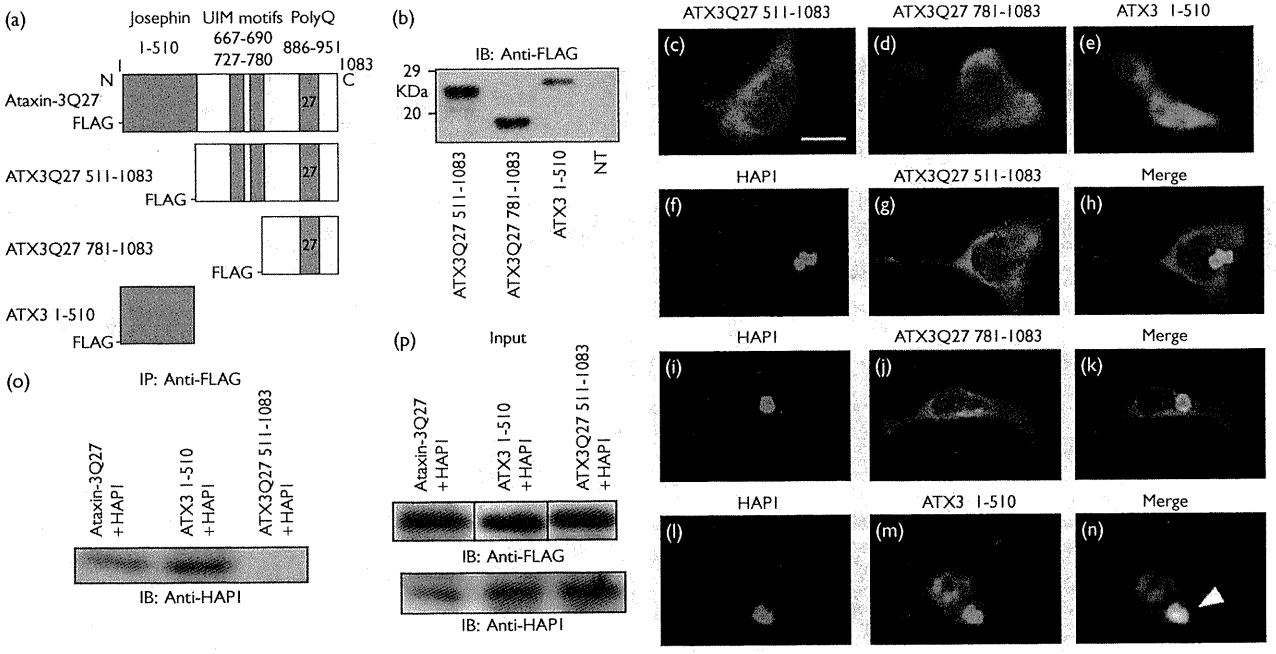
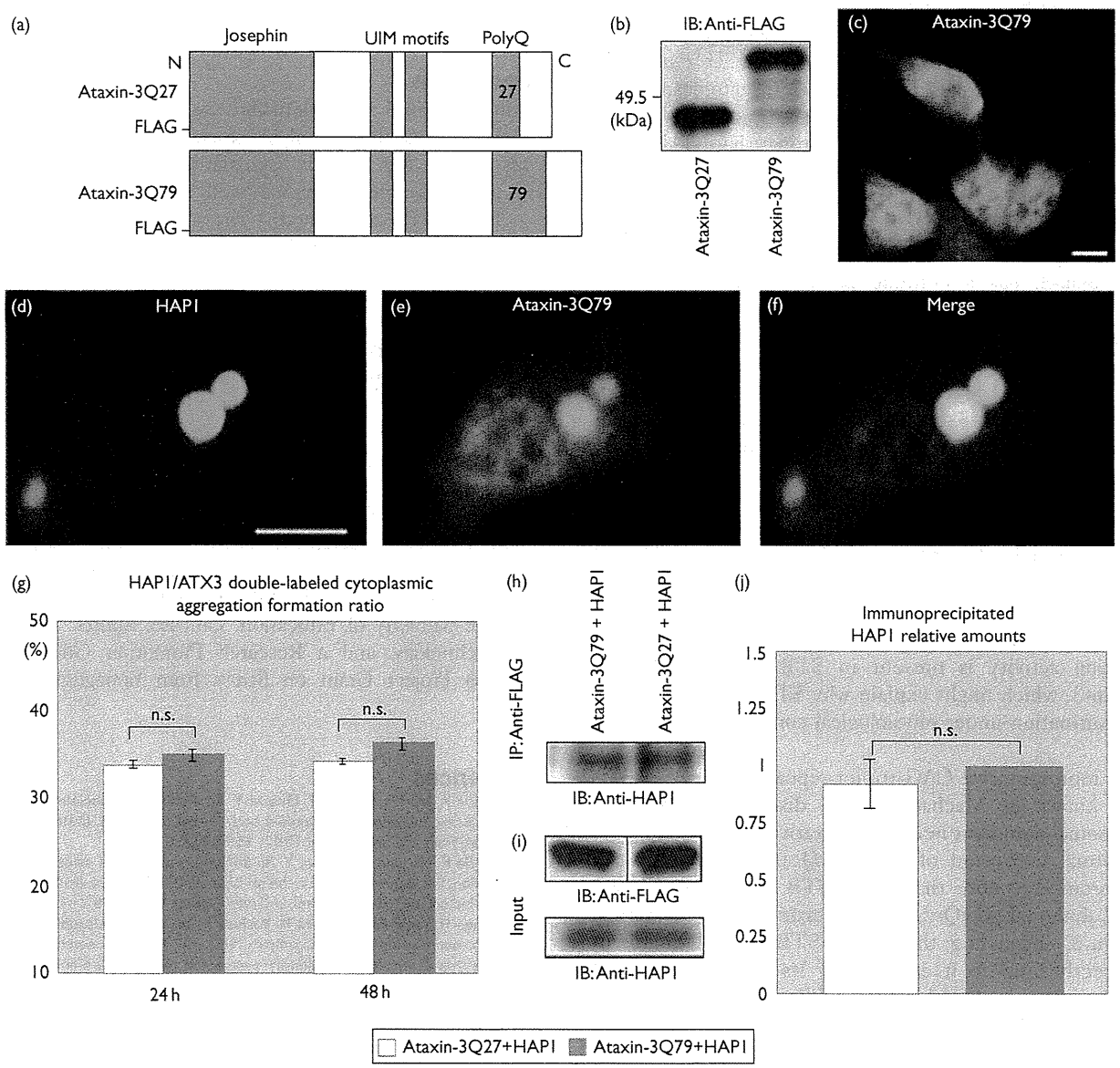
Fig. 2



Construction of ataxin-3-deletion mutants and cotransfection of huntingtin-associated protein 1 (HAP1) and these mutants. (a) Schematic representation of ataxin-3Q27 and ataxin-3-deletion mutants. Each mutant is FLAG-tagged in the *N*-terminus. ATX3Q27 511-1083, ataxin-3-lacking Josephin domain; ATX3Q27 781-1083, ataxin-3-lacking Josephin domain and ubiquitin-interacting motifs; and ATX3 1-510, Josephin domain. (b) Western blot analysis for the lysates from cells transfected with each ataxin-3-deletion mutants. (c–e) Fluorescence microscopic images of Neuro2a cells transfected with three ataxin-3-deletion mutants. (f–n) Fluorescence photomicrographs showing subcellular interactions between HAP1 and three ataxin-3-deletion mutants. Arrowhead indicates that ATX3 1-510 is associated with HAP1/stigmoid body (n). (o and p) Coimmunoprecipitation of HAP1 with ataxin-3Q27, ATX3Q27 1-510, or ATX3Q27 511-1083. Cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG M2 affinity gel. Immunoprecipitated samples are analyzed by western blotting using the anti-HAP1 antibody. Inputs are the control of proteins exogenously expressed (bar = 10 μm).

number) were surrogated as an index and chronologically compared between HAP1/ataxin-3Q27 and HAP1/ataxin-3Q79-cotransfected cells. The HA3-CAF ratios for ataxin-3Q27 and ataxin-3Q79 (HA3Q27-CAF and HA3Q79-CAF ratios) were approximately 33.7 and 34.8% in 24 h and 34.1 and 36.1% in 48 h after each cotransfection (Fig. 3g). There was no significant difference between HA3Q27-CAF and HA3Q79-CAF ratios. Coimmunoprecipitation tests were carried out to obtain biochemical evidence for the interaction of HAP1 with ataxin-3Q27 or ataxin-3Q79 (Fig. 3h–j).

Fig. 3



Interaction of huntingtin-associated protein 1 (HAP1)/stigmoid body with polyQ-expanded ataxin-3. (a) Diagrams of the primary structures of ataxin-3Q27 and ataxin-3Q79. (b) Western blot for extracts from ataxin-3Q27-transfected and ataxin-3Q79-transfected cells. (c) Fluorescence immunocytochemistry for ataxin-3Q79-transfected cells. (d–f) Fluorescence photomicrographs showing subcellular expression of HAP1 and ataxin-3Q79. Note that merged images show colocalization of HAP1 and ataxin-3Q79 (f) (bars = 10 μm). (g) Bar graph comparing HAP1/ataxin-3 double-labeled cytoplasmic aggregation formation ratios between ataxin-3Q27 and ataxin-3Q79 in time course (24 and 48 h) after the cotransfection into cells (ns, not significant). (h and i) Coimmunoprecipitation analysis of protein lysates from HAP1/ataxin-3Q27-cotransfected and HAP1/ataxin-3Q79-cotransfected cells. Inputs are the control of proteins exogenously expressed. (j) Quantification of the each immunoprecipitated HAP1 protein normalized to ataxin-3Q27 and ataxin-3Q79.

In cells coexpressing HAP1 and ataxin-3Q27, or ataxin-3Q79, HAP1 was coprecipitated. There was no significant difference in immunoprecipitated HAP1 relative amounts between ataxin-3Q27 and ataxin-3Q79.

Discussion

In this study, the most striking finding is that HAP1/STB are closely associated with normal ataxin-3Q27 and mutant ataxin-3Q79 derived from a SCA3 patient. Furthermore, even the Josephin domain alone coexpressed with HAP1 in Neuro2a cells clearly showed intimate association with HAP1/STB, whereas Josephin-deleted mutant ataxin-3 coexpressed with HAP1 turned diffuse and irrelevant to HAP1/STB in cytoplasm. Data for immunoprecipitation assay also supported the immunocytochemical results, confirming that HAP1/STB can interact with normal and mutant ataxin-3 through its Josephin domain.

The Josephin domain, which is located at the *N*-terminus of ataxin-3, has a cysteine protease sequence, which suggests potential ubiquitin protease activity [15]. Josephin domain also represses histone acetylation and transcription by binding to histone and transcriptional coactivator [16]. As HAP1/STB is intracellularly associated with ataxin-3 through its Josephin domain, it might partially modify the function of the putative ubiquitin protease or transcriptional repressor of ataxin-3. Interestingly, ataxin-3 was reported to be a typical deubiquitinating enzyme [17]. Our earlier immunohistochemical study in the rat brain showed no association between STB and ubiquitin [4], showing that HAP1/STB is a nonubiquitinated inclusion under normal conditions. Thus, it might be possible that a deubiquitinating enzyme or deubiquitination activity is present in STBs with HAP1 and ataxin-3, which might explain why STBs are spared from ubiquitination under physiological conditions.

The representative CAG-triplet-repeat disease, SCA3, is also known as Machado–Joseph disease, which is an autosomal dominant neurodegenerative disease caused by abnormal expansion of the polyQ tract [14]. It is of importance to note that HAP1/STB could also interact with abnormal polyQ-expanded ataxin3 as well as normal ataxin-3, suggesting that HAP1/STB could directly or indirectly bind to it and modify its pathophysiological involvement in SCA3. HAP1/STB also interacts with polyQ-expanded huntingtin and AR and suppresses their nuclear translocation in polyQ-dependent manner [9,10]. Thus, it could more efficiently neutralize the toxicity of the polyQ-expanded mutant forms in pathogenesis of Huntington's disease and SBMA and protect against the cell death. In SCA17 and Joubert syndrome, the affinities of HAP1/STB with pathological mutants of TATA-binding protein and Abelson helper integration site 1 are less strong than normal forms [11,12]. Nevertheless, HAP1/STB could serve as a cytoplasmic neuroprotective component interfering with 'gain-of-toxic function' of their pathological mutants [11,12]. HAP1/STB expression might raise the threshold of vulnerability for cell death and render more beneficial stability to cells with HAP1/STB than without it, as the 'HAP1/STB protection hypothesis' predicts [7]. Thus, in this study, although HAP1/STB seems to interact with ataxin-3 in polyQ-independent manner, it might be possible that HAP1/STB plays an important role in modification on physiological functions of normal ataxin-3 and on SCA3 pathogenesis attributable to ataxin-3Q79.

Conclusion

Normal ataxin-3 was identified as a new HAP1/STB interactor. In addition, polyQ-expanded ataxin-3 derived from SCA3 was closely associated with HAP1/STB through its Josephin domain as well. The findings suggest that HAP1/STB could modify the physiological function of normal ataxin-3 and pathogenesis of SCA3 attributable to the mutant ataxin-3.

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References

1 Shinoda K, Mori S, Ohtsuki T, Osawa Y. An aromatase-associated cytoplasmic inclusion, the 'stigmoid body,' in the rat brain: I. Distribution in the forebrain. *J Comp Neurol* 1992; **15**:360–376.
2 Shinoda K, Nagano M, Osawa Y. An aromatase-associated cytoplasmic inclusion, the 'stigmoid body,' in the rat brain: II. Ultrastructure (with a review of its history and nomenclature). *J Comp Neurol* 1992; **329**:1–19.
3 Fujinaga R, Yanai A, Nakatsuka H, Yoshida K, Takeshita Y, Uozumi K, *et al.* Anti-human placental antigen complex X-P2 (hPAX-P2) anti-serum recognizes C-terminus of huntingtin-associated protein 1A common to 1B as a determinant marker for the stigmoid body. *Histochem Cell Biol* 2007; **128**:335–348.
4 Fujinaga R, Takeshita Y, Uozuimi K, Yanai A, Yoshioka K, Kokubu K, *et al.* Microtubule-dependent formation of the stigmoid body as a cytoplasmic inclusion distinct from pathological aggresomes. *Histochem Cell Biol* 2009; **132**:305–318.
5 Li SH, Gutekunst CA, Hersch SM, Li XJ. Association of HAP1 isoforms with a unique cytoplasmic structure. *J Neurochem* 1998; **71**:2178–2185.
6 Gutekunst CA, Li SH, Yi H, Ferrante RJ, Li XJ, Hersch SM. The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. *J Neurosci* 1998; **18**:7674–7686.
7 Fujinaga R, Kawano J, Matsuzaki Y, Kamei K, Yanai A, Sheng Z, *et al.* Neuroanatomical distribution of huntingtin-associated protein 1-mRNA in the male mouse brain. *J Comp Neurol* 2004; **478**:88–109.

8 Li SH, Yu ZX, Li CL, Nguyen HP, Zhou XY, Deng C, *et al.* Lack of huntingtin-associated protein-1 causes neuronal death resembling hypothalamic degeneration in Huntington's disease. *J Neurosci* 2003; **23**:6956–6964.

9 Li XJ, Li SH, Sharp AH, Nucifora FC Jr, Schilling G, Lanahan A, *et al.* A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* 1995; **378**:398–402.

10 Takeshita Y, Fujinaga R, Zhao C, Yanai A, Shinoda K. Huntingtin-associated protein 1 (HAP1) interacts with androgen receptor (AR) and suppresses SBMA-mutant-AR-induced apoptosis. *Hum Mol Genet* 2006; **15**:2298–2312.

11 Prigge JR, Schmidt EE. HAP1 can sequester a subset of TBP in cytoplasmic inclusions via specific interaction with the conserved TBP(CORE). *BMC Mol Biol* 2007; **8**:76.

12 Sheng G, Xu X, Lin FY, Wang CE, Rong J, Cheng D, *et al.* Huntingtin-associated protein 1 interacts with Ahi1 to regulate cerebellar and brainstem development in mice. *J Clin Invest* 2008; **118**:2785–2795.

13 Tori F, Akelai A, Lupoli S, Siromi M, Amann-Zaicenstein D, Fumagalli MA, *et al.* Fine mapping of AHI1 as a schizophrenia susceptibility gene: from association to evolutionary evidence. *FASEB J* 2010; **24**: 3066–3082.

14 Kawaguchi Y, Okamoto T, Taniwaki M, Aizawa M, Inoue M, Katayama S, *et al.* CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nat Genet* 1994; **8**:221–228.

15 Doss-Pepe EW, Stenroos ES, Johnson WG, Madura K. Ataxin-3 interactions with Rad3 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. *Mol Cell Biol* 2003; **23**:6469–6483.

16 Li F, MacFarlan T, Pittman RN, Chakravarti D. Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor. *J Biol Chem* 2002; **277**:45004–45012.

17 Burnett B, Li F, Pittman RN. The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity. *Hum Mol Genet* 2003; **12**:3195–3205.