Bcl-2/adenovirus E1B 19-kDa interacting protein (BNip3) has a key role in the mitochondrial dysfunction induced by mutant huntingtin

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Abstract

Huntington’s disease (HD) is a neurodegenerative disorder caused by the expansion of a CAG repeat in the IT15 gene that encodes the protein huntingtin (htt). Evidence shows that mutant htt causes mitochondrial depolarization and fragmentation, but the underlying molecular mechanism has yet to be clarified. Bax/Bak and BNip3 are pro-apoptotic members of the Bcl-2 family protein whose activation triggers mitochondrial depolarization and fragmentation inducing cell death. Evidence suggests that Bax/Bak and BNip3 undergo activation upon mutant htt expression but whether these proteins are required for mitochondrial depolarization and fragmentation induced by mutant htt is unclear. Our results show that BNip3 knock-out cells are protected from mitochondrial damage and cell death induced by mutant htt whereas Bax/Bak knock-out cells are not. Moreover, deletion of BNip3 C-terminal transmembrane domain, required for mitochondrial targeting, suppresses mitochondrial depolarization and fragmentation in a cell culture model of HD. Hence, our results suggest that changes in mitochondrial morphology and transmembrane potential, induced by mutant htt protein, are dependent and linked to BNip3 and not to Bax/Bak activation. These results provide new compelling evidence that underlies the molecular mechanisms by which mutant htt causes mitochondrial dysfunction and cell death, suggesting BNip3 as a potential target for HD therapy.
Introduction
Huntington’s disease (HD) is a neurodegenerative disorder caused by the expansion of a CAG tract in the IT15 gene that encodes the protein huntingtin (htt). The intracellular mechanisms by which mutant htt elicits neuronal cell death are not completely understood and no neuroprotective therapy exists. Extensive evidence shows that mutant htt induces loss of mitochondrial potential (ΔΨm) and fragmentation of mitochondrial tubular network (1–3), but the underlying molecular mechanisms are still unclear. Evidence suggests that mutant htt promotes activation of certain pro-apoptotic BH3-only family proteins including Bax/Bak (4,5) and Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNip3) (6). Bax is a cytosolic protein that, once activated, translocates into the outer mitochondrial membrane (OMM); here Bax, together with Bak, forms the apoptotic pore that is the point of no return in mitochondrial apoptosis (7). Data from mouse models and human tissues converge to indicate that mutant htt expression causes Bax accumulation and translocation to the OMM (5,8-10) but whether Bax/Bak activation is a key step of the mitochondrial depolarization and fragmentation induced by mutant htt is still unclear.

BNip3 is normally expressed in tissues as an inactive conformer (11–13), and following toxic stimuli, it forms stable homodimers (14), integrates into the OMM (12,15,16) and causes loss of ΔΨm and cell death. The unique structure of BNip3 transmembrane domain (TM) suggests that dimers of the protein could trigger mitochondrial OMM changes in a Bax/Bak-independent manner (16–18) or through formation of Bax/Bak channels (19,20).

Because both BNip3 and Bax/Bak accumulation causes mitochondrial depolarization (12,16,20) and fragmentation (17,20–22), consistent with HD phenotype (2,23,24), we have tested the hypothesis that these proteins may be downstream effectors of mutant htt-induced mitochondrial dysfunction. Our data provide compelling new evidence that BNip3, but not Bax/Bak, plays a critical role in mitochondrial dysfunction and cell death induced by mutant htt, revealing a novel signaling axis.

Results
Bax/Bak double knock-out cells are susceptible to mitochondrial damage induced by mutant huntingtin
If Bax/Bak activation is a key step for mitochondrial damage induced by htt, then Bax/Bak knock-out cells should be protected from mitochondrial damages triggered by mutant htt. Thus, we tested the effects of mutant htt on mitochondrial depolarization and fragmentation in wild-type (wt) and Bax/Bak double knock-out (DKO) mouse embryonic fibroblasts (MEFs). We transfected plasmids encoding wt htt (23 CAG) or mutant htt (73 CAG and 145 CAG) in wt MEFs and measured ΔΨm by TMRM fluorescence. Wt htt did not affect TMRM fluorescence in wt and Bax/Bak DKO MEFs (mean ± SEM: empty plasmid 100 ± 7.83 versus wt htt 92.99 ± 8.24 in wt MEFs; empty plasmid 100 ± 6.00 versus wt htt 103.16 ± 5.26 in Bax/Bak DKO MEFs, P > 0.05, One-way ANOVA and Tukey test) whereas mutant htt induced ΔΨm loss both in wt MEFs (mean ± SEM: 73 CAG htt 61.50 ± 2.7 and 145 CAG htt 41.02 ± 1.41, P < 0.001 versus empty plasmid and wt htt, F = 25.94, One-way ANOVA and Tukey test) and in Bax/Bak DKO MEFs (mean ± SEM: 73 CAG htt 50.18 ± 2.72 and 145 CAG htt 42.86 ± 2.37, P < 0.001 versus empty plasmid and wt htt, F = 54.19, One-way ANOVA and Tukey test, Fig. 1A).

Because mitochondrial depolarization induces mitochondrial fission (25), we next analyzed mitochondrial fragmentation by co-transfecting plasmids encoding htt and a plasmid encoding mitochondrially targeted fluorescent protein (pDsRed2-Mito). Double transfected cells were analyzed for an automated shape-analysis of the mitochondrial structures (26): for each mitochondrial particle, we determined the aspect ratio (AR), a measure of length (27,28), and the form factor (FF), a measure of length and degree of branching (27,29).

Wt htt did not induce mitochondrial fragmentation in wt MEFs and in Bax/Bak DKO MEFs (AR medians ± 25%: empty plasmid 2.02 ± 1.54 and wt htt 2.08 ± 1.59 in wt MEFs; empty plasmid 1.94 ± 1.55 and wt htt 2.08 ± 1.56 in Bax/Bak DKO MEFs; FF medians ± 25%: empty plasmid 1.62 ± 1.24 and wt htt 1.69 ± 1.25 in wt MEFs; empty plasmid 1.55 ± 1.15 and wt htt 1.61 ± 1.17 in Bax/Bak DKO MEFs, P > 0.05, Kruskal–Wallis ANOVA on Ranks).

Mutant htt decreased both AR and FF in wt MEFs (AR medians ± 25%: 73 CAG htt 1.77 ± 1.46 and 145 CAG htt 1.82 ± 1.46, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks; FF medians ± 25%: 73 CAG htt 1.34 ± 1.07 and 145 CAG htt 1.46 ± 1.11, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks) and in Bax/Bak DKO MEFs (AR medians ± 25%: 73 CAG htt 1.82 ± 1.46 and 145 CAG htt 1.68 ± 1.39, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks; FF medians ± 25%: 73 CAG htt 1.44 ± 1.14, P = 0.038 versus empty plasmid and P = 0.022 versus wt htt; 145 CAG htt1.31 ± 1.06, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks; Fig. 1B).

Given that mutant htt induces mitochondrial depolarization, fragmentation and cell death (30), we analyzed cell viability in wt MEFs and Bax/Bak DKO MEFs transfected with wt or mutant human htt. In both cell types, overexpression of wt htt did not change cell death rate (% of death/total cells ± SEM): empty plasmid 1.20 ± 0.17 versus wt htt 0.81 ± 0.13 in wt MEFs; empty plasmid 0.99 ± 0.04 versus wt htt 0.25 ± 0.11 in Bax/Bak DKO MEFs, P > 0.05, One-way ANOVA and Tukey test). Conversely, mutant htt increased cell death rate in wt MEFs and Bax/Bak DKO MEFs (% of death/total cells ± SEM: 73 CAG htt 5.15 ± 0.52 and 145 CAG htt 3.86 ± 0.59, P < 0.001 versus empty plasmid and wt htt in wt MEFs; 73 CAG htt 1.85 ± 0.30, P < 0.001 versus empty plasmid and wt htt; 145 CAG htt 1.52 ± 0.29, P < 0.01 versus empty plasmid and wt htt in Bax/Bak DKO MEFs, F = 15.80, One-way ANOVA with Tukey test, Fig. 1C). These results show that Bax/Bak DKO MEFs, as wt MEFs, are susceptible to mitochondrial depolarization, fragmentation and cell death induced by mutant htt. This result suggests that mutant htt elicits mitochondrial damage and cell death through a molecular pathway that is independent on Bax/Bak activation.

BNip3 knock-out cells are protected from mitochondrial damage induced by mutant huntingtin
Evidence shows that mutant htt induces BNip3 activation (6). We thought that if BNip3 activation is a key step in the molecular pathway by which mutant htt induces mitochondrial damage, then BNip3 knock-out cells should be protected from mitochondrial damages induced by mutant htt.

Thus, we investigated ΔΨm and mitochondrial fragmentation in wt and BNip3 knock-out (KO) MEFs (17,31) transfected with human htt. Wt htt did not modify ΔΨm in wt and BNip3 KO MEFs (mean ± SEM: empty plasmid 100 ± 7.06 versus wt htt 118.73 ± 7.88 in wt MEFs; empty plasmid 100 ± 8.29 versus wt htt 101.52 ± 7.50 in BNip3 KO MEFs, P > 0.05, One-way ANOVA and Tukey test). Mutant htt depolarized mitochondria of wt MEFs (mean ± SEM: 73 CAG htt 55.22 ± 2.82 and 145 CAG htt 64.80 ± 3.7, P < 0.001 versus empty plasmid and wt htt, F = 28.48,
Figure 1. Bax/Bak DKO MEFs are susceptible to mitochondrial damage induced by mutant huntingtin. (A) Mitochondrial membrane potential measured by TMRM in wt and Bax/Bak DKO MEFs transfected with pcDNA3 empty plasmid (empty plasmid), wild-type htt (wt htt) or mutant htt (73CAG or 145CAG). Representative images of TMRM staining are shown. Mutant htt decreased ΔΨm both in wt and Bax/Bak DKO MEFs (One-way ANOVA test and Tukey post-test, *P<0.05, ***P<0.001 versus empty plasmid and wt htt). Data are presented as TMRM fluorescence in the ROI normalized by ROI area, where the ROI is a single cell. Data derive from two independent experiments (n=60 cells per condition). (B) Quantitative analysis of mitochondrial morphology in wt and Bax/Bak DKO MEFs co-transfected with pDsRed2-Mito and empty plasmid, wt htt or mutant htt (73CAG or 145CAG). Representative confocal images show mitochondrial shape. The dot plots show AR and FF median values (red lines) for all mitochondria (>300 mitochondria particles, 8 cells analyzed per condition). AR and FF are decreased in wt and Bax/Bak DKO MEFs expressing mutant htt (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test). (C) Viability assay with Calcein (green, living cells) and Ethidium homodimer (red, dead cells) staining performed on wt and Bax/Bak DKO MEFs transfected with empty plasmid, wt htt or mutant htt (73CAG or 145CAG). Representative images are displayed. The histogram shows the percentage of dead cells. Mutant htt increased cell death in wt and Bax/Bak DKO MEFs (One-way ANOVA test and Tukey post-test, ***P<0.001, **P<0.01). Data derive from two independent experiments.
One-way ANOVA and Tukey test). Conversely, mutant htt did not depolarize mitochondria of BNip3 KO MEFs (mean ± SEM: 73 CAG htt 97.83 ± 7.38, 145 CAG htt 106.97 ± 8.00, P > 0.05 versus empty plasmid and wt htt, F = 0.25, One-way ANOVA and Tukey test; Fig. 2A).

Because this result suggested that BNip3 KO MEFs are protected from mitochondrial depolarization induced by mutant htt, we hypothesized that BNip3 KO MEFs could also be protected from mutant htt-induced mitochondrial fragmentation. Therefore, we transfected wt and BNip3 KO MEFs with wt or mutant htt and calculated for each mitochondrial particle AR and FF values. Wt htt did not modify AR and FF in wt MEFs and BNip3 KO MEFs (AR medians ± 25%: empty plasmid 1.62 ± 1.24 and wt htt 1.69 ± 1.25 in wt MEFs; empty plasmid 1.83 ± 1.45 in BNip3 KO MEFs; FF medians ± 25%: empty plasmid 1.62 ± 1.24 and wt htt 1.69 ± 1.25 in wt MEFs; empty plasmid 1.45 ± 1.10 and wt htt 1.38 ± 1.03 in BNip3 KO MEFs, P > 0.05, Kruskal–Wallis ANOVA on Ranks). Mutant htt decreased AR and FF values in wt MEFs (AR medians ± 25%: 73 CAG htt 1.77 ± 1.46 and 145 CAG htt 1.82 ± 1.46, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks; FF medians ± 25%: 73 CAG htt 1.34 ± 1.07 and 145 CAG htt 1.46 ± 1.11, P < 0.001 versus empty plasmid and wt htt, Kruskal–Wallis ANOVA on Ranks). Conversely, mutant htt expression did not modify AR and FF in BNip3 KO MEFs (AR medians ± 25%: 73 CAG htt 1.85 ± 1.46 and 145 CAG htt 1.86 ± 1.46 versus empty plasmid and wt htt, P > 0.05, Kruskal–Wallis ANOVA on Ranks; FF medians ± 25%: 73 CAG htt 1.49 ± 1.09 and 145 CAG htt 1.39 ± 1.06 versus empty plasmid and wt htt, P > 0.05, Kruskal–Wallis ANOVA on Ranks; Fig. 2B). These results showed that BNip3 KO MEFs are resistant to mitochondrial depolarization and fragmentation induced by mutant htt and suggested that BNip3 KO MEFs can be protected by mutant htt-induced cytotoxicity.

Thus, we examined cell viability in wt and BNip3 KO MEFs transfected with plasmids encoding human htt. As expected, mutant htt protein significantly increased cell death rate in wt MEFs (% of death/total cells ± SEM 48 h after transfection: 73 CAG htt 5.15 ± 0.52 and 145 CAG htt 3.86 ± 0.59, P < 0.001 versus empty plasmid 1.20 ± 0.17 and wt htt 0.81 ± 0.13, F = 18.99, One-way ANOVA and Tukey test; Fig. 2C). Conversely, mutant htt did not increase cell death rate in BNip3 KO MEFs (% of death/total cells ± SEM 48 h after transfection: 73 CAG htt 1.52 ± 0.40 and 145 CAG htt 1.07 ± 0.28 versus empty plasmid 0.99 ± 0.23 and wt htt 0.98 ± 0.22, P > 0.05, One-way ANOVA and Tukey test). Cell death rate was significantly lower in BNip3 KO MEFs compared with wt MEFs expressing 73 CAG (48 h after transfection wt MEFs 5.15 ± 0.52, P < 0.001 versus BNip3 KO 1.52 ± 0.40, F = 18.99, One-way ANOVA and Tukey test) or 145 CAG htt (48 h after transfection wt MEFs 3.86 ± 0.59, P < 0.001 versus BNip3 KO 1.07 ± 0.28, One-way ANOVA and Tukey test; Fig. 2C). Viability assay was also performed 72 h after transfection and confirmed that mutant htt did not induce cell death in BNip3 KO MEFs (Supplementary Material, Fig. S1).

To further confirm BNip3 crucial role in mutant htt-induced cytotoxicity, we co-transfected BNip3 KO MEFs with a plasmid encoding BNip3 together with wt or mutant htt.

BNip3 KO cells expressing htt plasmid alone showed no significant variations in the rate of death among the different conditions (% of death/total cells ± SEM: 73 CAG htt 0.32 ± 0.21 and 145 CAG htt 0.27 ± 0.18 versus empty plasmid 0.32 ± 0.21 and wt htt 0.29 ± 0.20, P > 0.05, One-way ANOVA and Tukey test), whereas when BNip3 was reintroduced in BNip3 KO MEFs, the presence of mutant htt led to an increased mortality (% of death/total cells ± SEM: 73 CAG htt 4.74 ± 0.35 and 145 CAG htt 4.72 ± 0.39, P < 0.001 versus empty plasmid 0.32 ± 0.15 and wt htt 0.24 ± 0.16, One-way ANOVA and Tukey test; 73 CAG htt + BNip3 4.74 ± 0.35, P < 0.001 versus no BNip3 0.32 ± 0.21; 145 CAG htt + BNip3 4.72 ± 0.39, P < 0.001 versus no BNip3 0.27 ± 0.18, F = 56.85, One-way ANOVA and Tukey test; Fig. 2C). Overall, these experiments show that the absence of BNip3 protects MEFs from mitochondrial depolarization and fragmentation and from cell death triggered by mutant htt and suggest a central role for BNip3 activation in the mitochondrial alteration induced by mutant htt.

BNip3 blocking rescues mitochondrial potential and fragmentation in HD cell culture model

The C-terminal TM of BNip3 is critical for its homodimerization and mitochondrial integration because deletion of this domain (BNip3ΔTM) prevents BNip3 insertion into the OMM, dimers formation and cell death induction (12,18,21,33). As Figure 2 demonstrates, the absence of BNip3 protects cells from mutant htt-induced mitochondrial damage; therefore, we hypothesized that BNip3 blocking by the expression of the dominant-negative mutant BNip3ΔTM can rescue mitochondrial damage in the HD cell culture model STHdhQ111/Q111 (34). Thus, we transfected a bicistronic plasmid encoding GFP and BNip3ΔTM in STHdhQ7/Q7 and STHdhQ111/Q111 cells and analyzed ΔΨm in green fluorescent cells. As previously shown, STHdhQ111/Q111 cells displayed mitochondrial depolarization compared with STHdhQ7/Q7 (mean ± SEM: STHdhQ111/Q111 0.53 ± 0.06 versus STHdhQ7/Q7 1.00 ± 0.09, P < 0.001, Kruskal–Wallis test and Dunn’s Multiple Comparison Test) (35). BNip3ΔTM transfection did not modify ΔΨm in STHdhQ7/Q7 cells (mean ± SEM: BNip3ΔTM 1.02 ± 0.09 versus empty plasmid 1.00 ± 0.09, P > 0.05, Kruskal–Wallis test and Dunn’s Multiple Comparison Test), conversely, BNip3ΔTM transfection significantly increased ΔΨm in STHdhQ111/Q111 cells (mean ± SEM: BNip3ΔTM 0.74 ± 0.07 versus empty plasmid 0.53 ± 0.06, P = 0.030, Kruskal–Wallis test and Dunn’s Multiple Comparison Test; Fig. 3A). This result shows that BNip3ΔTM expression partially, but significantly, rescues mitochondrial depolarization in the HD cell culture model STHdhQ111/Q111. Because mitochondrial depolarization induces mitochondrial fission (25), we hypothesized that BNip3ΔTM expression may also rescue mitochondrial fragmentation in STHdhQ111/Q111 cells.

STHdhQ7/Q7 and STHdhQ111/Q111 cells were co-transfected with BNip3ΔTM (bicistronic plasmid encoding GFP and BNip3ΔTM) and pDsRed2-Mito and then were analyzed to determine AR and FF (26). The median values of FF and AR were lower in STHdhQ111/Q111 compared with STHdhQ7/Q7 (mean ± SEM: STHdhQ111/Q111 0.53 ± 0.06 versus STHdhQ7/Q7 1.00 ± 0.09, P = 0.035, Kruskal–Wallis test and Dunn’s Multiple Comparison Test; AR medians ± 25%: STHdhQ111/Q111 1.67 ± 1.00 versus STHdhQ7/Q7 2.00 ± 1.07, P = 0.001, Kruskal–Wallis ANOVA on Ranks; Fig. 3B). BNip3ΔTM expression did not modify FF and AR in STHdhQ7/Q7 cells (FF medians ± 25%: BNip3ΔTM 1.14 ± 1.00 versus empty plasmid 1.16 ± 1.00, P = 0.427, Kruskal–Wallis ANOVA on Ranks; AR medians ± 25%: BNip3ΔTM 2.00 ± 1.24 versus empty plasmid 2.00 ± 1.07, P = 0.603, Kruskal–Wallis ANOVA on Ranks) but significantly increased both the parameters in STHdhQ111/Q111 cells (FF medians ± 25%: BNip3ΔTM 1.25 ± 1.00 versus empty plasmid 1.03 ± 1.00, P < 0.001, Kruskal–Wallis ANOVA on Ranks; AR medians ± 25%: BNip3ΔTM STHdhQ111/Q111 2.00 ± 1.39 versus empty plasmid 1.67 ± 1.00, P < 0.001, Kruskal–Wallis ANOVA on Ranks). These data show that BNip3ΔTM elongates mitochondria in the HD cell culture model STHdhQ111/Q111 and suggest that BNip3ΔTM rescues mitochondrial fragmentation induced by...
Figure 2. BNip3 KO MEFs are protected against mitochondrial damage induced by mutant huntingtin. (A) Mitochondrial membrane potential measured by TMRM fluorescence in wt and BNip3 KO MEFs transfected with pcDNA3 empty plasmid (empty plasmid), wild-type htt or mutant htt (73CAG or 145CAG). Representative images of TMRM staining are shown. Mutant htt-induced mitochondrial depolarization in wt MEFs (One-way ANOVA and Tukey post-test, ***P < 0.001 versus empty plasmid and wt htt) but not in BNip3 KO MEFs (One-way ANOVA test and Tukey post-test P > 0.05). Data are presented as TMRM fluorescence in the ROI normalized by ROI area, where the ROI is a single cell. Data derive from three independent experiments (n = 70 cells per condition). (B) Quantitative analysis of mitochondrial morphology in wt and BNip3 KO MEFs co-transfected with pDsRed2-Mito and empty plasmid, wt htt or mutant htt (73CAG or 145CAG). Representative confocal images show mitochondrial shape. The dot plots show AR and FF median values (red lines) for all mitochondria (>450 mitochondria particles, 8 cells analyzed per condition). Mutant htt expression decreased both AR and FF in wt MEFs (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test) but not in BNip3 KO MEFs. (C) Viability assay with Calcein (green, living cells) and Ethidium homodimer (red, dead cells) staining performed on wt and BNip3 KO MEFs transfected with empty plasmid, wild-type htt or mutant htt (73CAG or 145CAG) and on BNip3 KO MEFs co-transfected with BNip3 and the plasmids encoding htt. Representative images are displayed. Histograms show the percentage of dead cells. Mutant htt increased cell death in wt MEFs (One-way ANOVA test and Tukey post-test, ***P < 0.001) but not in BNip3 KO MEFs (One-way ANOVA test and Tukey post-test, P > 0.05). Data derive from four independent experiments. The lower histogram show that overexpression of BNip3 in BNip3 KO MEFs causes an increase of cell death in the presence of mutant htt (One-way ANOVA test and Tukey post-test, ***P < 0.001).
Figure 3. BNip3ΔTM rescues mitochondrial potential and fragmentation in STHdh cell culture model. (A) Mitochondrial membrane potential measured by TMRM fluorescence in STHdhQ7/Q7 and STHdhQ111/Q111 cells transfected with BNip3ΔTM or the empty plasmid. The histogram represents mean TMRM fluorescence in the ROI normalized by ROI area, where the ROI is a single cell. Images are representative of three independent experiments (n = 65 cells per condition). STHdhQ111/Q111 cells show decreased ΔΨm (**P < 0.001 versus STHdhQ7/Q7), rescued by BNip3ΔTM expression (#P < 0.05 versus STHdhQ111/Q111 cells transfected with the empty plasmid).

(B) Quantitative analysis of mitochondrial morphology in STHdhQ7/Q7 and STHdhQ111/Q111 cells transfected with pDsRed2-Mito and the empty plasmid or pDsRed2-Mito and BNip3ΔTM. Eight double transfected cells for each condition were analyzed (450 mitochondrial particles). Representative confocal images are shown. The dot plots show AR and FF values for all mitochondria particles. Lines represent median values. AR and FF were lower in STHdhQ111/Q111 cells compared with STHdhQ7/Q7 (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test). Expression of BNip3ΔTM significantly increases the two parameters’ values in STHdhQ111/Q111 cells (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test).

(C) Confocal images are representative of FRAP experiments: pDsRed2-Mito signal in STHdhQ7/Q7 and STHdhQ111/Q111 cells before DsRed2 photobleaching (pre-bleach), at 0 s after photobleaching (post-bleach) and at different time points after the bleaching (15, 30, 60, 75 and 90 s). The selected photobleached ROIs are indicated and enlarged in the lower panels. The graph shows the kinetics of pDsRed2-Mito FRAP in STHdhQ7/Q7 and STHdhQ111/Q111 cells transfected with empty plasmid or BNip3ΔTM. The histogram shows the mean pDsRed2-Mito mobile fraction in STHdhQ7/Q7 and STHdhQ111/Q111 cells transfected with empty plasmid or BNip3ΔTM: STHdhQ111/Q111 show a decreased mobile fraction (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test, *P < 0.05 versus STHdhQ7/Q7 cells) rescued by BNip3ΔTM expression (Kruskal–Wallis ANOVA on Ranks and Dunn’s Multiple Comparison Test, *P < 0.05 versus STHdhQ111/Q111 empty plasmid).
mutant htt. To confirm this result, we analyzed the physical continuity between mitochondria by live cell imaging using fluorescence recovery after photobleaching (FRAP). We co-transfected STHdhQ7/Q7 and STHdhQ111/Q111 cells with pDsRed2-Mito and BNip3ΔTM (bicistrionic plasmid encoding GFP and BNip3ΔTM) and then recorded FRAP signal in red/green fluorescent cells. Because pDsRed2-Mito localizes to the mitochondrial matrix and can freely diffuse in the bleached zone by movement of molecules from surrounding unbleached zone, the time of recovery gives a measure of the mitochondrial compartment continuity (37,38).

FRAP curves were summarized by calculating the mobile fraction of mitochondrial-directed red fluorescent protein (DsRed2-Mito), which estimates mitochondrial connectivity (29,37). FRAP experiments confirmed that STHdhQ111/Q111 cells had less branched and shorter mitochondria tubules compared with STHdhQ7/Q7 cells (mobile fraction values mean ± SEM: STHdhQ7/Q7 20.85 ± 2.93 versus STHdhQ111/Q111 32.40 ± 2.67, P < 0.05, unpaired t-test). BNip3ΔTM expression in STHdhQ7/Q7 did not modify the fluorescence recovery (mean ± SEM. BNip3ΔTM transfected cells 34.44 ± 5.28 versus empty plasmid 32.40 ± 2.67, P > 0.05, unpaired t-test). BNip3ΔTM expression in STHdhQ111/Q111 caused an increase in the fluorescence recovery and significantly increased the mobile fraction value (mean ± SEM. BNip3ΔTM transfected cells 57.34 ± 12.05 versus empty plasmid 20.85 ± 2.93, P < 0.05, unpaired t-test; Fig. 3C). These results showed that BNip3ΔTM expression increases the continuity of mitochondrial network in STHdhQ111/Q111 cells and confirmed the results of Figure 3B. Taken together these data suggest that BNip3 blocking decreases mitochondrial damage induced by mutant htt.

Discussion

This paper provides new knowledge in the field of HD pathogenesis by producing the following three important novelties: (1) Bax/Bak DKO cells are not protected from mitochondrial damage induced by mutant htt, (2) BNip3 KO cells are protected from mitochondrial damage induced by mutant htt and (3) the dominant-negative BNip3ΔTM rescues mitochondrial damage in HD cells.

Because Bax/Bak levels and localization modulate the mitochondrial apoptotic pathway and influence mitochondrial fission–fusion dynamics (39,40), these proteins have long been investigated in HD models (41). In vitro studies provided controversial evidence about Bax/Bak in HD (41): mutant htt elicits accumulation of Bax protein (8) and Bax translocation from the cytosol to the OMM in some cell culture models (9) whereas, in other models, mutant htt induces Bax protein accumulation and Bax translocation from the cytosol to the OMM in some cell culture models (9) whereas, in other models, mutant htt induces Bax-independent cell death (4). In vivo studies in HD mouse models support the theory that mutant htt and fusion expression causes Bax protein accumulation and Bax translocation to the OMM (4,5,10,42), whereas Bak levels were found equal in tissues of HD mouse models and littermate controls (42). Hence, whether Bax/Bak intervene in the pathogenic pathway induced by mutant htt is still unclear. Our results show that mutant htt can induce mitochondrial depolarization, fragmentation and cell death independently of Bax/Bak expression, thus suggesting that Bax/Bak activation is not mandatory for htt-induced mitochondrial damage. Conversely, according to our results, mutant htt does not induce mitochondrial depolarization, fragmentation and cell death in BNip3 KO MEFs, thus suggesting that htt needs BNip3 protein activation to induce mitochondrial damage. How does mutant htt induce BNip3 activation? We do not have the knowledge nowadays to certainly answer this question. It has been shown that wild-type htt supports a high level of active nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in neuronal nuclei and that this function is impaired by the polyQ expansion (43). Because BNip3 is strongly transcriptionally silenced by NF-κB (44,45), we can hypothesize that mutant htt increases BNip3 mRNA levels. Nevertheless, previous findings showing that mutant htt increases BNip3 colocalization with mitochondria (6) argue in favor of a post-translational mechanism. As BNip3 undergoes several phosphorylation events at its C-terminus and phosphorylation at these residues inhibits BNip3-induced mitochondrial damage (46,47), we can speculate that mutant htt modifies BNip3 phosphorylation through impairing kinase signaling pathway (48). Future studies will clarify the molecular mechanisms by which mutant htt induce BNip3 activation; the present study provides reliable evidence that changes in mitochondrial morphology and transmembrane potential, induced by mutant htt protein, are dependent and linked to BNip3 activation.

Once activated BNip3 can induce mitochondrial damage through at least two distinct mechanisms: BNip3 can engage anti-apoptotic Bcl-2 family members to trigger Bax/Bak-dependent OMM permeability (49) or can modify the fusion–fission balance of mitochondrial networks by interacting with pro-fusion or pro-fission proteins. Showing that mutant htt toxicity is independent on Bax/Bak expression, we can speculate that BNip3 promotes mitochondria fragmentation by inhibiting pro-fusion proteins or activating pro-fission proteins. Because our data show that BNip3ΔTM rescues mitochondrial fragmentation in HD cells, our study agrees with previous results showing that TM domain of BNip3 interacts with the pro-fusion protein Optic atrophy-1 (Opal) and inhibits mitochondrial fusion (22). Equally important, BNip3 may modify the function of the pro-fusion protein Dynamin-related protein 1 (Drp1). Because evidence suggests that BNip3 induces Drp1 translocation to mitochondria in cardiac myocytes (50) and evidence shows an abnormal activation of Drp1 in HD cells (51–53), we can speculate that BNip3 induces Drp1 translocation to mitochondria in HD neurons. Yet, it is conceivable that BNip3 induces mitochondrial damage in HD cells by participating in a molecular pathway that is independent on Drp1 activation. In this context, it is important to remember that a structural study performed on bilayer lipid membranes highlighted that BNip3 alone form an ion-conducting pathway in the membrane (14). This experimental evidence, together with structural bioinformatics analyses (54), suggests that BNip3 alone can initiate the permeability of the mitochondrial outer membrane and induces ΔΨm loss. Future studies will address the mechanism by which BNip3 activation induces mitochondrial damage in HD cells. Future studies will also clarify whether BNip3 protein, being a member of Bcl-2 family proteins, already molecular targets in cancer therapy (55), may be also a therapeutic target for HD.

Materials and Methods

Cell cultures

STHdhQ7/Q7 and STHdhQ111/Q111 striatal cell lines derived from knock-in mice expressing full-length wt htt carrying 7 CAG repeats or 111 CAG repeats, respectively (34), were maintained in high glucose DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS and 400 μg/ml G418.

To induce mitochondrial stress, cells were incubated in glucose-free RPMI 1640 medium supplemented with 10% FBS, 5 mM pyruvate, 5 mM galactose and 400 μg/ml G418.

Wt, BNip3 KO and Bax/Bak DKO MEFs were maintained in DMEM supplemented with 5% FBS.
Mitochondrial morphology assay
STHdhQ7/Q7 and STHdhQ111/Q111 cells, plated on poly-d-lysine-coated coverslips at a density of 4 x 10^4, were co-transfected with pDsRed2-Mito and the bicistronic p305-GFP empty plasmid or p305-GFP-BNip3ATM using Lipofectamine 2000 (Life Technologies, 11,668), following manufacturer’s instructions.

After transfection, cells were shifted at 39°C and incubated in the stress medium for 72 h.

Then, cells were washed with PBS and fixed with 4% paraformaldehyde, coverslips were mounted with ProLong mounting with DAPI (Life Technologies, P36931) and images were acquired at confocal microscope (Nikon Eclipse C1).

MEF cells (wt, BNip3 KO and Bax/Bak DKO) were plated on collagen-coated coverslips at a density of 2.5 x 10^4. Cells were co-transfected with pDsRed2-Mito and pcDNA3 empty plasmid or pcDNA3 expressing full-length wt htt or 73 CAG or 145 CAG repeats htt, using Effectene Transfection Reagent (Qiagen, 301425).

Cells were washed with PBS and fixed with 4% paraformaldehyde 48 h after transfection; nuclei were stained with Hoechst (Thermo Scientific, 33942). Coverslips were mounted with FluoroSave Reagent (Millipore, 345789) and images were acquired by confocal microscopy (Carl Zeiss Axio Observer SD).

Using ImageJ software, raw images were binarized, and mitochondria were subjected to automated particle analyses to obtain, for each mitochondrial particle, values for aspect ratio (AR: major axis/minor axis), a measure for mitochondrial length (27,28) and form factor (FF: perimeter^2/4π·Area), a measure for mitochondrial length corresponding to a circular mitochondrion; values increase with mitochondrial elongation.

FRAP
STHdhQ7/Q7 and STHdhQ111/Q111 cells, plated on 35-mm cover glass bottom dishes, coated with poly-d-lysine, were co-transfected with pDsRed2-Mito and pcDNA3 empty plasmid or p305-GFP empty plasmid or p305-GFP-BNip3ATM, using Lipofectamine 2000, following manufacturer’s instructions.

After transfection, cells were shifted at 39°C and incubated in the stress medium for 72 h.

FRAP experiments were performed at 39°C, maintaining the cells in glucose-free, phenol red-free DMEM supplemented with 5 mM galactose. The experiments were performed using a confocal microscope (Leica Microsystems, TCS SPS A0B5) equipped with a microscope incubator (Okolab) on cells co-expressing both the transfected constructs.

pDsRed2-Mito was photobleached in a cytoplasmic ROI, and fluorescence recovery was followed for 90 s. At each time point, the mean fluorescence in the ROI (F_R00) was determined for the background fluorescence (Fbg0) and normalized for the mean fluorescence of an ROI that was not subjected to photobleaching (FPerPB0) subtracted of background fluorescence (Fbg0). to correct for the photobleaching of pDsRed2-Mito owing to the imaging procedure: F_R(t) = [F_R00 - Fbg0]/[FPerPB0 - Fbg0].

Finally, background and photobleaching-corrected fluorescence values were normalized for the fluorescence value measured just before photobleaching (F_perPB): Fnorm(t) = F_R(t)/F_perPB.

Viability assay
MEFs (wt, BNip3 KO, Bax/Bak DKO), plated on collagen-coated coverslips at a density of 3 x 10^5, were transfected with pcDNA3 empty plasmid or pcDNA3 expressing full-length wt htt, or 73 CAG or 145 CAG repeats htt or co-transfected with the htt plasmids and pcDNA3 expressing BNip3 or empty (as control condition), using Effectene Transfection Reagent.

Cells were assessed for viability 48–72 h after transfection, by staining with 3.2 μM Calcein AM (green, living cells) and 0.6 μM Ethidium homodimer-1 (red, dead cells) (Live/Dead Viability/ Cytotoxicity Kit, Life Technologies, L3224) for 30 min at 37°C in the dark. Coverslips were mounted on glass slides and images acquired using an epifluorescence microscope (Olympus Provis AX70 Research grade microscope).

Mitochondrial membrane potential
MEFs (wt, BNip3 KO, Bax/Bak DKO), plated on collagen-coated coverslips at a density of 2.5 x 10^4, were transfected with pcDNA3 empty plasmid or pcDNA3 expressing full-length wt htt, or 73 CAG or 145 CAG repeats htt, using Effectene Transfection Reagent.

Mitochondrial membrane potential was measured 48 h after transfection, by staining with 0.4 μM Tetramethylrhodamine, methyl ester (TMRM, Life Technologies, T668) (56) for 30 min at 37°C in the dark. Coverslips were then mounted on glass slides and images were acquired using an epifluorescence microscope (Olympus Provis AX70 Research grade microscope).

STHdhQ7/Q7 and STHdhQ111/Q111 cells, plated on 35-mm cover glass bottom dishes, coated with poly-d-lysine, were transfected with p305-GFP empty plasmid or p305-GFP-BNip3ATM using Lipofectamine 2000. After transfection, cells were shifted at 39°C and incubated in the stress medium for 72 h. Then cells were assessed for mitochondrial membrane potential by staining with 0.4 μM TMRM for 30 min at 37°C in the dark. Images were acquired by confocal microscopy (Nikon Eclipse C1).

Supplementary Material
Supplementary Material is available at HMG online.

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Conflict of Interest statement
None declared.

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