Early transcriptional profiles in huntingtin-inducible striatal cells by microarray analyses

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Gene expression studies conducted with mouse models of Huntington’s disease (HD) have revealed profound modifications in gene transcription. However, the complexity of in vivo tissue hampers definition of very early transcriptional modifications and does not allow discrimination between cell-autonomous changes and those resulting from intercellular activity processes. To identify early, cell-autonomous transcriptional changes, we compared gene expression profiles of clonal striata-derived cells expressing different N-terminal 548-amino-acid huntingtin fragments (with 26, 67, 105 or 118 glutamines) under the control of a doxycycline-regulated promoter. In these cells, mutant huntingtin did not form aggregates or cause cell death; therefore, the gene expression profiles report transcriptional changes reflecting early pathogenic events. We found that genes involved in cell signaling, transcription, lipid metabolism and vesicle trafficking were affected, in some cases, within 12 hours of mutant protein induction. Interestingly, this study revealed differential expression of a number of genes involved in cholesterol and fatty acid metabolism, suggesting that these metabolic pathways may play a role in HD pathogenesis.

INTRODUCTION

Huntington’s disease (HD) is an inherited neurodegenerative disorder characterized by motor, cognitive and behavioral dysfunction. It is caused by an expansion of a polymorphic CAG trinucleotide repeat in the coding region of the gene for huntingtin, a 348 kDa protein in which the CAG repeats are translated into a stretch of glutamines [poly(Q)] (1). In the normal population, up to 35 CAG repeats are present in the HD gene, while expansions of 36 or more repeats induce HD pathology. Because huntingtin is expressed ubiquitously, a most striking feature of HD is a selective neurodegeneration of the striatum and, to a lesser extent, other brain regions (2). Pathogenic events in HD appear to depend upon both the gained toxic activity conferred by the expanded poly (Q) stretch to the mutant protein and the loss of normal huntingtin function (3–8).

Caspases have been shown to cleave huntingtin in vitro in a CAG length-dependent manner, and N-terminal fragments have been detected in striatal neuron nuclei of HD patient specimens (9–11). The N-terminal portion includes the poly(Q) repeat and a proline-rich region, which are characteristic of proteins involved in gene transcription (12). With respect to the wild-type protein, mutant huntingtin differentially interacts with various transcription factors, repressors and co-activators, perhaps leading to many of the gene expression changes reported in human HD brain and laboratory models of HD (13–21).

Mouse models provide an opportunity to identify temporal and regional events in the context of intact neuronal circuitry. Interpretation of data from these complex systems may be confounded however, by tissue heterogeneity, homeostatic mechanisms, and concurrent physiologic or pathologic changes. An inducible, clonally derived, cell line expressing mutant huntingtin offers a stable and controlled genetic and transcriptional background in which to perform gene expression studies. In such a system, biological and experimental variability can be greatly reduced. Transcriptional changes can be identified that may provide insight into events that occur during embryogenesis or in presymptomatic HD patients.

To identify early, cell autonomous transcriptional changes, we engineered striatal cells to express, in a tightly regulated manner, the first 548 amino acids of wild-type or mutant huntingtin (N548-huntingtin and N548-mutant huntingtin, respectively). DNA microarray analysis revealed N548-mutant huntingtin-modulated mRNAs that encode proteins involved in lipid metabolism, signaling, vesicle trafficking and RNA processing. The novel finding that multiple genes involved in cholesterol biosynthesis are altered by N548-mutant huntingtin may provide important clues to early HD pathogenesis.

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RESULTS

Generation of TetON huntingtin-inducible striatal cells

Striatal derived ST14A cells were used to generate an inducible cell system where expression of the N-terminal fragment of mutant huntingtin is driven by a tetracycline/doxycyline (Doxy)-responsive element (TRE) (22). Both reverse tetracycline-controlled transactivator (rtTA) and TRE–huntingtin constructs were delivered by retroviral infection to maximize single integrants and consistent performance of the transgenes in the descendant cells. To reduce the risk of insertional effects or silencing of the inserted transgene, we screened over 500 clones to identify those with no background expression and tight Doxy-dependent induction of N548-mutant huntingtin.

Figure 1A shows the results of the screening for rtTA-expressing clones. Only a fraction of the clones screened are shown. Arrows indicate the product of specific amplification of the rtTA in positive clones. (B) rtTA-expressing clones were tested for their transactivation capability by transient transfection with a TRE-controlled luciferase reporter gene. Luciferase activity was measured after 48 h in the presence or absence of Doxy (1 μg/ml).

Figure 1. (A) RT–PCR screening for rtTA-expressing clones. Only a fraction of the clones screened are shown. Arrows indicate the product of specific amplification of the rtTA in positive clones. (B) rtTA-expressing clones were tested for their transactivation capability by transient transfection with a TRE-controlled luciferase reporter gene. Luciferase activity was measured after 48 h in the presence or absence of Doxy (1 μg/ml).
tested for rtTA ability to induce expression of a transiently transfected TRE–luciferase gene. Figure 1B shows that among the subclones, some had low luciferase activity in the absence of Doxy and high levels of induction (up to >500-fold increase) upon administration of the inducer. In the second step of the procedure, clone 12 was retrovirally transduced with a cDNA encoding the first 548 N-terminal amino acids of mutant huntingtin with 128 CAG repeats and under transcriptional control of a TRE sequence. Presumably as a result of retroviral processing, clones with different CAG repeat lengths were generated (Fig. 2A). Sequence analysis showed that three of the clones expressing exogenous N548-huntingtin contained 118, 105 and 104 CAG repeats [identified here as HD40(Q118), HD43(Q105) and HD27(Q104)], two clones expressed 67 CAG repeats [HD12(Q67) and HD14(Q67)], and CAG number was reduced to the human wild-type length in two clones [named HD19(Q26) and HD18(Q17)]. For each of these clones, a single sharp band was visible on agarose gel after RT–PCR, and its molecular weight was constant over high number of passages of the cells in culture.

Figure 2B and C show regulation of transgene expression in one of the clones used in this study. Typically, expression of N548-huntingtin was dependent on Doxy concentration, with a saturation effect evident at 0.5 μg/ml (Fig. 2B). Exogenous huntingtin was detectable 3–4 hours after Doxy treatment, and its expression was stable for at least 4 days following single administration of the antibiotic to the culture medium (Fig. 2C). All the other clones used in this study showed similar induction kinetics, protein levels and tight regulation of expression.

N548-mutant huntingtin in the inducible clones was detected as diffuse perinuclear staining, with no visible cytosolic or nuclear aggregates by 72 hours (Fig. 3). The mutant protein did not induce overt cellular toxicity or cell death. Nor did the presence of mutant huntingtin increased the susceptibility of the cells to proapoptotic stress driven by serum deprivation or 3-nitropropionic acid exposure (data not shown).

Analysis of gene expression upon induction of mutant huntingtin in normal growth conditions

The lines described above were used to generate gene expression profiles prior to induction of huntingtin and at 12, 24, 48 and 72 hours post induction (for a description of the procedure used, see Materials and Methods).

Thresholds applied to identify changes in gene expression were rather conservative, especially considering the small amplitude of the changes observed. To best capture temporal changes in gene expression, regression analysis was performed on the same microarray datasets (23). This allowed the identification of a few more genes that were missed by the first approach for being just below the Affymetrix thresholds used. Significant genes identified with the two independent approaches were listed together.

Results from Q60 and Q100 lines were compared with lists of genes similarly generated for parental and HD19(Q26) cells. Genes modulated in parental cells as a result of Doxy administration itself were no longer considered for analysis. Figure 4 shows the list of mRNAs modulated upon induction of mutant huntingtin at 33°C, grouped by functional families. A small number of mRNAs were differentially detected in cells expressing either expanded or non-expanded huntingtin fragment (Fig. 4A), possibly as a consequence of huntingtin functions that are not affected by the CAG expansion. Most changes, however, were unique to expanded repeat clones (Fig. 4B). Among these were changes in mRNAs associated with lipid metabolism, vesicle trafficking and RNA processing (see Discussion). Expression levels of the genes involved in lipid metabolism are shown in Figure 5.
versus HD43(Q118)). From this, we conclude that differences caused by >100 poly(Q)s cannot be distinguished from those caused by 67 poly(Q)s.

Analysis of gene expression in post-mitotic conditions

At 33°C, ST14 cells are immortalized by a temperature-sensitive variant of largeTAg, which binds and sequesters p53 and the transcriptional regulator p300/CBP [cAMP response element-binding protein (CREB)-binding protein] (for a review, see 29). At the non-permissive temperature, 39°C, the largeTAg is degraded and cells stop proliferating. Because p53 binds to poly(Q) stretches (13,18) and CBP mediates poly(Q) toxicity (14,16), we wished to see how the presence of expanded repeat huntingtin at 39°C influenced gene expression as p53 and p300/CBP are released in response to disappearance of largeTAg.

Huntingtin induction and largeTAg temperature-sensitive repression were initiated simultaneously. As expected, the disappearance of largeTAg, with consequent release of p53 and p300/CBP in the cells, resulted in extensive transcriptional changes in all cell lines (see supplemental data at www.neumetrix.info).

In addition, we identified other mRNAs that responded to the presence of expanded poly(Q). Example mRNAs that preferentially increased following largeTAg depletion in the presence of Q105-118 HD fragments were notch, steroid hormone receptor, fatty acid translocase, lecithin–cholesterol acyltransferase, phospholipase Cβ, osteopontin, glucosidase–α, cellular retinol binding protein II and receptor-linked protein tyrosine phosphatase. mRNA encoding amyloid precursor protein increased 43-fold owing to largeTAg depletion in parental cells, and increased 11-fold in Q105-118 cells. Other mRNAs that increased more in parental cells than in Q105-118 cells included α1 type I collagen, nerve cell adhesion molecule (NCAM) and insulin-like growth factor I (IGF-I). The complete data set reporting differential response to largeTAg depletion in Q105-118 cells and controls is in supplemental data (www.neumetrix.info).

Confirmation of data by RT–PCR and northern blotting

We performed semiquantitative RT–PCR and northern blotting to confirm the differential expression in mutant clones for representative genes identified in our analysis. As shown in Figures 7 and 8, the results uniformly confirmed array data, showing early and progressive downregulation (or upregulation in the case of CAIN), of mRNAs affected by N-548 mutant huntingtin.

Figure 3. Immunocytochemistry performed on HD43(Q105) clone before (A and C) and 72 hours after induction of mutant huntingtin (B and D). The antibodies used were MAb 2166 (A and B) and AP194 (C and D). A diffuse cytoplasmic staining is present; no aggregates were detectable.
Figure 4. Genes modulated at 33°C in cells expressing mutant huntingtin. Accession numbers in colored boxes indicate decreased (green) or increased (red) gene expression in the mutant clones. (A) Gene modulation in the wild-type HD19(Q26) is indicated by vertical arrows (arrow pointing up, increased expression; arrow pointing down, decreased expression). (B) Genes modulated in cells expressing mutant huntingtin only are grouped arbitrarily according to their main biological function. An asterisk indicates a potential p53 target (24–28). ‘(a)’ indicates a gene found to be modulated using the regression analysis described in (23).
We found that NF1 B1 (a silencer of gene expression), and Ssecks, were downregulated not only in the presence of N-548 mutant huntingtin, but also in parental cells exposed to 3-nitropropionic acid (3NP) (Figure 8B), a mitochondrial toxin that induces a pattern of neurodegeneration and symptomatology closely resembling HD when administered to animals in vivo (30). A more extensive analysis of gene expression upon treatment of cells with 3NP is ongoing to assess whether there is a broader overlap with events induced by mutant huntingtin.

**DISCUSSION**

The primary goal of this study was to gain insight into early cellular and molecular dysfunctions caused by expanded poly(Q) huntingtin fragments. Striata-derived cells without large intracellular aggregates likely represent an early model of HD, where influence of mutant huntingtin on gene transcription can be studied in the absence of transcriptional protein recruitment into aggregates.

N-548 mutant huntingtin modulated several mRNAs involved in cholesterol biosynthesis, monounsaturated fatty acid synthesis, and fatty acid β-oxidation, as reported in detail in Figure 5. Key enzymes of the cholesterol biosynthetic pathway, namely 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA Red) and 7-dehydroxycholesterol reductase, were found decreased at the mRNA level. Deficiency of 7-dehydroxycholesterol reductase is known to cause impaired brain development (31).

In the central nervous system, cholesterol is synthesized de novo rather than being imported from blood (32–34), and neurons depend on cholesterol provided by astroglia for normal synaptogenesis (35). Cholesterol enhances the production of presynaptic components and synaptic vesicles (36,37), and inhibitors of HMG-CoA reductase block dendrite outgrowth and axonal branching (38).

Consistent with the proposed role for cholesterol in synaptogenesis, genes involved in vesicle trafficking and synaptic vesicle formation were specifically downregulated upon induction of mutant huntingtin. Altered transcription of these genes is in agreement with a proposed role of huntingtin in vesicle trafficking (39) and with studies demonstrating endosome tubulation and autophagy (40), as well as impaired synaptic vesicle uptake (41) in cell and animal models of HD. Altered expression of genes involved in vesicle trafficking has been also shown in an animal model of HD (42).

mRNA encoding stearoyl-CoA desaturase 2, which influences membrane fluidity, was decreased in expanded poly(Q) ST14A cells and in an animal model of HD (43). These findings may further motivate the assessment of therapeutic use of essential fatty acids in HD mouse models (44). Beside downregulation of cholesterol and monounsaturated fatty acid biosynthetic enzymes, we also found reduced expression of genes involved in the rate-limiting steps of the β-oxidation pathway of fatty acids, namely medium-chain acyl coenzyme A dehydrogenase and Δ3, Δ5 enoyl-CoA isomerase. β-Oxidation is not the major metabolic pathway used by the brain to produce energy, and we speculate that the alterations observed in our striatal cell line may be more pronounced in tissues in which fatty acids are actively oxidized, such as liver and muscle.

Reduction of the β-oxidation pathway leads to esterification of long-chain fatty acids in triglycerides, which, accumulating in adipose tissue as well as in muscle and pancreatic β cells, become a risk factor for the development of type II diabetes (45). Interestingly, increased storage of fat has been observed in presymptomatic HD transgenic mice (46), and a higher incidence of diabetes has been reported in HD patients (47) and in HD animal models (46).

Another consequence of reduced utilization of fatty acids in β-oxidation is the increased utilization of glucose as a respiratory fuel to meet the demand for energy. Indeed, higher consumption of glucose in HD brains has been shown, including early stages of pathology (48). In line with these observations, enzymes involved in glycolysis and channeling of pyruvate into the Krebs cycle—GAPDH and pyruvate dehydrogenase, respectively—are upregulated in cells expressing mutant huntingtin.

Reduction β-oxidation could also explain, (at least in part), the wasting that characterizes later stages of the pathology (49,50)—a consequence of protein breakdown in HD muscle to produce energy when β-oxidation is reduced. In this perspective, administration of amino acids to HD patients might attenuate wasting.

We cannot assess here the mechanisms by which mutant huntingtin induces dysregulation of lipid metabolism. Enzymes involved in cholesterol synthesis and stearoyl-CoA desaturase are transcriptional targets of sterol regulatory element-binding proteins (SREBP-2 and SREBP-1c or -1a, respectively; for a review, see 51), which recruit p300/CBP to activate...
transcription (51). It is possible that the inhibitory action of mutant huntingtin on p300/CBP (16,14) may, in turn, lead to reduced transcriptional activity of SREBP. However, other factors may play a role as well. SREBP 1c is known to be regulated by the liver X receptors (LXRs) (52), which are members of the nuclear hormone receptor superfamily whose obligate transcriptional partners are the retinoid X receptors (RXRs) (for a review, see 53). RXRs are also obligate partners of PPARα, and PPARα/RXR heterodimers activate the transcription of the β-oxidation enzymes (for a review, see 54). Factors that affect RXR activity consequently also affect both cholesterol and fatty acid metabolism. We can speculate that the effect of mutant huntingtin on lipid metabolism pathways may be mediated by reduced RXR activity. In support of this hypothesis, in previous studies on HD transgenic animals, it was shown that >20% of the genes that were found to be modulated by mutant huntingtin were retinoid targets (20).

Finally, it has recently been reported that in HD transgenic mice and in presymptomatic and affected HD patients, the soluble form of mutant huntingtin interacts with Sp1, a transcription factor involved in the transcription of lipid metabolism genes (55,56), inhibiting its binding to DNA (57,58).

This study revealed that, in striata-derived cells, removal of largeTAg results in increased expression of many p53-regulated mRNAs and that many of these mRNAs were further modulated by mutant huntingtin (Fig. 6). Not all of the known p53 target genes were found to be modulated by N548-mutant huntingtin. This is not surprising, however, because several studies have reported on heterogeneity of expression of p53 targets in different cell models (59,60). Further investigation is required to unravel the nature of mutant huntingtin involvement in the regulation of p53-mediated pathways and its importance in HD pathogenesis.

We developed a homogeneous, tightly regulatable inducible system that lacks visible intracellular inclusions. Through increased statistical power provided by multiple clones, replicates, and timepoints, the system revealed that, within 24–48 hours, N-548 mutant huntingtin enhances expression of potential p53 target genes and interferes with the transcription of mRNAs that encode critical enzymes involved in lipid metabolism.

**MATERIALS AND METHODS**

**Cell culture**

ST14A cells, previously derived from rat embryonic striatum (59), were used to generate inducible cell lines expressing the N-terminal fragment of huntingtin. ST14A cells and all subclones produced proliferate at 33°C but become postmitotic at 39°C. Cells were grown in DMEM supplemented as described in (61), unless otherwise specified. Tet-free fetal calf serum (FCS, Clontech) was used to supplement the medium for the inducible subclones (Tet-free medium).

**Generation of inducible ST14A cell lines**

*Step 1: generation of rtTA-expressing subclones*. Transduction of rtTA into ST14A cells was performed by infection with medium conditioned by RetroPack PT67 PackagingCell Line (Clontech), stably transfected with pRevTetON vector (Clontech).

Single colonies were tested for rtTA expression by RT–PCR using primers specific for rtTA (forward 5'-tgcttaatgaggtcggaatcgaa-3'; reverse 5'-acgcggacccactttcacat-3') and the following cycling parameters: denaturation at 95°C for 7 min.; 95°C for 30 s, 52°C for 30 s, 72°C for 1 min (for 40 cycles); 72°C for 7 min. Subclones expressing the mRNA for rtTA were further analyzed for their transactivating capability and levels of background expression. Transient transfection with pRevTRE-Luc (Clontech) was performed using
Figure 7. Semiquantitative radioactive RT–PCR for some representative genes modulated by N-548 amino acid mutant huntingtin fragment. HD43(Q105) clones were induced with 1 μg/ml Doxy for the indicated time before RNA extraction. Calcineurin inhibitor (Cain), Ssecks, TBFII and NonO p54, respectively, were co-amplified with β-actin. The intensity of the gene-specific radioactive band was normalized to the β-actin product. The ratio of gene intensity to actin intensity at timepoint 0 (no Doxy) is indicated as 100% in the graphs. All the other values are expressed as percentages with respect to timepoint 0 after normalization to β-actin.
Lipofectamine 2000 (Life Technologies). Transfected cells were grown for 48 h in the presence (1 µg/ml) or absence of the inducer, doxycycline (Doxy). For assessment of luciferase activity, cells were washed with PBS and incubated for 5 min in 25 mM Tris–phosphate, pH 7.8, 2 mM EDTA, 10% glycerol, 0.5% Triton X-100 and 2 mM DTT, at room temperature. Luciferase activity was measured in 20 µl of lysis buffer by adding 100 µl of Luciferase Assay Reagent (20 mM Tricine, 0.1 mM EDTA, 1.07 mM (MgCO3)4Mg(OH)2·5H2O and 2.67 mM MgSO4, pH 7.8, containing 33.3 mM DTT, 270 µM coenzyme A, 530 µM ATP and 470 µM luciferin) in a LUMAT LB 9501 Lumimeter (Berthold). Luciferase activity was normalized to the protein content in each sample measured by a BCA-200 Protein Assay Kit (Pierce).

Step 2: generation of subclones expressing inducible N-548 huntingtin. The cDNA encoding for the first 548 amino acids of mutant huntingtin with 128 CAG (3.62) was blunt-end subcloned into the pRevTRE vector (Clontech) digested with HpaI.

The recombinant pRevTRE vector was delivered to rtTA-expressing clones by infection.

Screening for huntingtin expression and inducibility in the presence or absence of 1 µg/ml Doxy was performed by western blot. Assessment of the number of CAG expressed was done by RT–PCR and sequence analysis. Total RNA was extracted using TRIaZol Reagent (Life Technologies), and RT–PCR was carried out with primers flanking the CAG stretch (forward 5’-cgaccctggaaaagctgatgaa-3’; reverse 5’-cacaggtcttcttggtagtga-3’) using Dynazyme ExT Polymerase (Fynnzyme) in the presence of 5% DMSO. Cycling parameters were 95°C for 30 s, 56°C for 30 s (for 35 cycles), 72°C for 7 min. Amplified products were purified with the Qiaquick Gel Extraction Kit (Qiagen) and sequenced by Primm (Milano, Italy).

Characterization of inducible huntingtin expression

For the dose–response analysis, cells were grown in the presence of various concentrations of Doxy for 48 h before harvesting. For the time course of huntingtin expression, cells were grown in 1 µg/ml Doxy and harvested at the indicated time points. Cell harvesting was performed by scraping in 100 µl of ice-cold RIPA buffer (0.15 M NaCl, pH 7.2, 1% Nonidet P-40, 1% sodiumdeoxcholate, 0.1% SDS and 2 mM EDTA) containing 1 µl protease inhibitor cocktail (Sigma). Cell debris were pelleted at 10,000g for 15 min. Protein concentration was determined by the BCA-200 Protein Assay Kit (Pierce). Equal amounts of proteins (20–50 µg) of total cell lysate were used for SDS–PAGE and immunoblotting. Antibody MAb2166 (dilution 1:5000; Chemicon, Temecula, CA) was used to detect the N-terminal fragment of huntingtin. Monoclonal anti α-tubulin antibody (Sigma) was applied at 1:5000 dilution.

Immunocytochemistry

For immunocytochemistry, cells seeded onto polyornithine-coated glass coverslips were rinsed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 10 min and then incubated with MAb2166 antibody, recognizing amino acids 181–810 (Chemicon, Temecula, CA), or AP194, recognizing amino acids 1–194 (from A.H. Sharp, Baltimore, MD). Secondary fluorescinated antibody (Sigma) was used at 1:200 dilution. The cells were viewed with a Zeiss (Axioskop) microscope.

Analysis of gene expression

Time course of huntingtin induction. For analysis of gene expression at 33°C, we performed two independent time courses of induction of mutant huntingtin expression for two subclones bearing 67 CAG repeats and for two other clones bearing 118 or 105 CAG repeats. The day after plating in Tet-free medium, 1 µg/ml Doxy was added to the cells to be harvested after 72 h of induction and so on, backwards, for timepoints 48 h, 24 h and 12 h. Using this procedure, we were able to extract, on the same day and with a similar confuency, RNA from cells at different time points (12 h, 24 h, 48 h and 72 h) and from two baselines (defined as –6 and 0 h).

The parental clone expressing the transactivator rtTA but not exogenous huntingtin (Par) and one wild-type huntingtin-inducible subclone were included in each experimental set and treated with Doxy as described above. For each set of cells, two independent time courses were performed.

For analysis of gene expression at 39°C, cells were seeded at 80% confluence. The day after, cultures were shifted to 39°C for inactivation of largeTAg. At the same time, Doxy (1 µg/ml) was added to all plates except baseline controls. RNA was extracted at timepoints 48 and 72 h, when, as expected, largeTAg was no longer detectable in virtually all the cells in culture (46, 63; data not shown).

Microarray analysis. Preparation of labeled cRNA and Affymetrix Gene Chip hybridization were performed as previously described (46). Rat RGU34A oligonucleotide arrays were hybridized with 10 µg of cRNA. Data were analyzed using Affymetrix GeneChip software (version 4.0) and selected for presentation based on principles described in (23,64). Iterative comparisons of different datasets were done by spreadsheet analysis (Microsoft Excel). Datasets generated for each timepoint upon induction of mutant huntingtin were compared with the two independent baselines for the same experiment (–6 and 0 h). In addition, each timepoint was also compared to the baselines in the second experiment, giving a total of eight pairwise comparisons for each timepoint at 33°C or four comparisons for each timepoint at 39°C (in this last set of experiments, only one baseline at 0 h was produced). For example, timepoint 24 h experiment 1 was compared with each of the following: (i) baseline –6 h experiment 1, (ii) baseline 0 h experiment 1, (iii) baseline –6 h experiment 2, and (iv) baseline 0 h experiment 2. The same applied for timepoint 24 h experiment 2. For each comparison, we used the default parameters in the GeneChip4.0 software, to ‘call’ genes increased, decreased or not changed in the induced samples with respect to the baselines. Only genes that were called in at least half plus one of the comparisons (i.e. five out of eight for the experi-
Figure 8. Northern blot analysis of representative genes modulated by mutant huntingtin. (A) HD40(118) and HD43(105) clones were induced by 1 μg/ml Doxy for the indicated time. Total RNA preparations from Q105-118 clones were pooled and used for northern blotting with the following probes: sortilin cDNA (nucleotides 1378–1777) cytosolic 3HMG-CoAS cDNA (nucleotides 1253–1652) and TBF II cDNA (nucleotides 1312–1711). The graphs show relative intensity of genespecific bands normalized to the intensity of the corresponding UV-stained 18S rRNA. The ratio of gene intensity to actin intensity at timepoint 0 (no Doxy) is indicated as 100% in the graph. All the other values are expressed as percentages with respect to timepoint 0 after normalization to 18S rRNA. (B) Expression of NF1 B1 and Ssecks in the parental clone upon treatment with 3-nitroproprionic acid (3NP). Total RNA from parental cells grown for 24 h in the absence or presence of 3NP (1 mM) blotted and probed with NF1 B1 cDNA (nucleotides 241–644) or Ssecks cDNA (nucleotides 3726–4423). Normalization of expression levels was done as in (A).
ments at 33°C and three out of five for the experiments at 39°C) were considered for further analysis. To the initial list of genes ‘differently’ called, we then applied a threshold of 30% to the number of probe pairs in a given probe set that had to show changes in the same direction in order for the gene to be ‘called’. No additional fold-change thresholds were applied. Datasets were also analyzed with a randomization procedure described in (23). Significant genes identified by this method were included in the list previously described.

Semiquantitative RT–PCR. Equal amounts of total RNA were retrotranscribed. The obtained cDNAs were amplified with primers for the internal control (β-actin) and primers specifically designed for the gene of interest in the same reaction tube. PCR was performed in the presence of [α-32P]dCTP for 25 cycles. The amplification products were run on an acrylamide gel and subsequently exposed to autoradiography. Normalization was done with respect to the β-actin band. Primers and reaction conditions were as follows: NF1 B1: forward 5’-tcctcagatgacaa-3’; reverse 5’-ctctgccatgactaatct-3’; cycling parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; NonO p54: forward 5’-ctacatagaaaccagca-3’; reverse 5’-ctctccatcatgaaccact-3’; cycling parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; cytotoxic 3-hydroxy-3-methylglutaryl-CoA synthase (3HMG-CoAS): forward 5’-gtcaacagatgcacaxc-3’; reverse agtctcaggggccagac-3’; cycling parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; sortilin (Sort): forward 5’-tctccatttggtcggag-3’; reverse 5’-gtggtgcacttggtggagac-3’; cycling parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; polypyrrolidine tract-binding protein (TFBII): forward 5’-aagttgccaagctagcag-3’; reverse 5’-agtggacttgaaagggac-3’; cycling parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; calcineurin inhibitor protein (Cain): forward 5’-tcatgtagctacagcag-3’; reverse 5’-agccagtaaaccaggacc-3’; cycling parameters: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s.

Northern blots. These were performed according to standard procedures (65). Transfer efficiency was evaluated by exposing the filter to ultraviolet light (254 nm). cDNA probes were obtained by PCR with primers specific for the genes of interest and labeled with [α-32P]dCTP by random priming. Membrane hybridization was performed at 68°C in QuickHyb hybridization solution (Stratagene, La Jolla, CA). After washing, the blot was exposed to X-Omat AR film. Normalization of the signal intensity was done using the intensity of UV stained 18S ribosomal RNA band.

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