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Regulator of Calcineurin (RCAN1-1L) Is Deficient in Huntington Disease and Protective against Mutant Huntingtin Toxicity *in Vitro*[*S]

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Our work suggests an important new link between the *RCAN1* gene and Huntington disease. Huntington disease is caused by expansion of glutamine repeats in the huntingtin protein. How the huntingtin protein with expanded polyglutamines (mutant huntingtin) causes the disease is still unclear, but phosphorylation of huntingtin appears to be protective. Increased huntingtin phosphorylation can be produced either by inhibition of the phosphatase calcineurin or by activation of the Akt kinase. The *RCAN1* gene encodes regulators of calcineurin, and we now demonstrate, for the first time, that RCAN1-1L is depressed in Huntington disease. We also show that RCAN1-1L overexpression can protect against mutant huntingtin toxicity in an ST14A cell culture model of Huntington disease and that increased phosphorylation of huntingtin via calcineurin inhibition, rather than via Akt induction or activation, is the likely mechanism by which RCAN1-1L may be protective against mutant huntingtin. These findings suggest that RCAN1-1L "deficiency" may actually play a role in the etiology of Huntington disease. In addition, our results allow for the possibility that controlled overexpression of RCAN1-1L in the striatal region of the brain might be a viable avenue for therapeutic intervention in Huntington disease patients (and perhaps other polyglutamine expansion disorders).

Huntington disease is a genetic disorder characterized by abnormal body movements and a reduction of various cognitive functions. It is caused by an expansion of a trinucleotide (CAG) repeat encoding glutamine in the *IT15* gene, which encodes the huntingtin protein. The neuropathology of Huntington disease is characterized by neuronal death specifically in the striatal region, consisting of the caudate nucleus and the putamen. The severity of the disease is proportional to the number of glutamine repeats in the huntingtin protein. Aging (1) and disorders such as dentatorubropallidoluysian atrophy and spinobulbar muscular atrophy are also associated with polyglutamine expansion. Because short polyglutamine repeats are normally present in huntingtin and other proteins, we will call huntingtin with abnormally long expanded polyglutamines that actually cause toxic effects "mutant huntingtin." The functions of normal huntingtin and the exact mechanism by which mutant huntingtin protein actually causes Huntington disease are still unclear. It has been found, however, that phosphorylation of huntingtin is neuroprotective (2, 3). Increased phosphorylation has so far been produced either by chemical inhibition of calcineurin with FK506 or by overexpression of the dominant interfering form of calcineurin (3) or by activation of the serine/threonine kinase Akt (2). Both decreased huntingtin dephosphorylation (by calcineurin inhibition) and increased huntingtin phosphorylation (by Akt) are neuroprotective (2, 3).

It has been demonstrated in several laboratories that the *RCAN1* gene (4, 5) encodes regulators of calcineurin (6–10); thus, it may present us with a highly specific way to regulate/inhibit calcineurin. The *RCAN1* gene consists of seven exons, four of which (exons 1–4) can be alternatively transcribed or spliced to produce a number of different mRNA isoforms (4, 5). In brain, *RCAN1* is expressed predominantly in neurons rather than in astrocytes or microglia (11). We have demonstrated that at least two *RCAN1* mRNA isoforms are expressed in adult human brain and that the mRNA levels of isoform 1 are much higher than those of isoform 4 (11). We have also tested whether the various potential RCAN1 proteins (generated by alternate splicing and alternate translation start sites) are expressed in adult human brain, and we found at least three isoforms transcribed in adults: *RCAN1-1L*, *RCAN1–1S*, and *RCAN1–4* (5, 12). These isoforms have different expression patterns and different cellular distribution patterns and may also have somewhat different functions and different mechanisms of regulation. Nevertheless, all three isoforms can potentially inhibit calcineurin. Therefore, all three physiologically relevant RCAN1 proteins may have therapeutic potential for Huntington disease, and we have analyzed this possibility in our studies.

EXPERIMENTAL PROCEDURES

Human Brain Samples—We analyzed 10 Huntington disease and 10 age-matched control cases. The average age of all cases was 69 years, and there was no neuronal involvement in mortality for any of the control cases. The putamen regions were dissected from each brain and frozen until used. All tissue spec-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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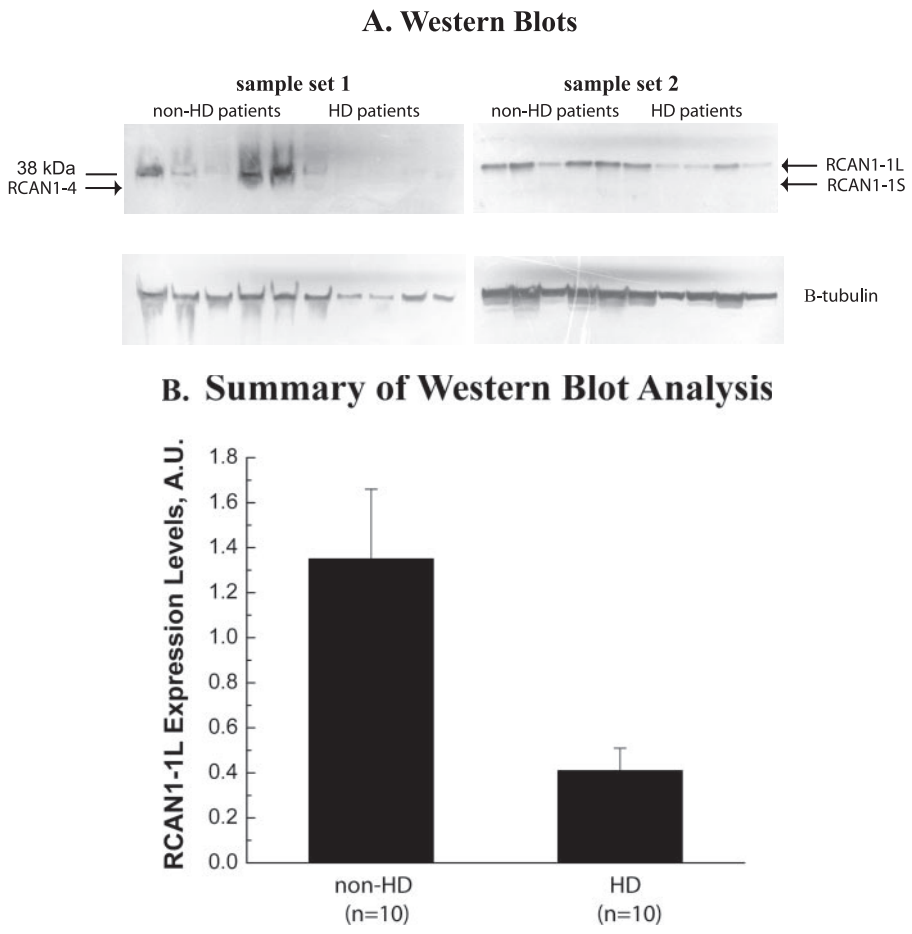


FIGURE 1. **RCAN1 protein expression in Huntington disease and control human brains.** *A*, shown are RCAN1 Western blots of post-mortem tissues isolated from the putamen region of human brains. Because of the large number, samples were analyzed on two separate gels. Each blot shows samples of non-Huntington disease (*non-HD*; *five left lanes*) and Huntington disease (*five right lanes*) patients. A total of 10 Huntington disease patients and 10 control cases were analyzed. Membranes were probed with our antibody that recognizes all RCAN1 isoforms: the "common" antibody. Signals were detected using x-ray films. The main isoform detected using this antibody has a size of ~38 kDa, which corresponds to RCAN1-1L. *B*, x-ray films were scanned, and the signals from 10 Huntington disease and 10 non-Huntington disease patients were quantified using the BioSpectrum AC imaging system. Values shown are the means ± S.E. *B* shows that expression of the RCAN1-1L protein is >70% down-regulated in Huntington disease. This down-regulation is statistically significant at $p < 0.05$ (evaluated by the *t* test). *A.U.*, arbitrary units.

imens were obtained from the Human Brain and Spinal Fluid Resource Center of the Veterans Affairs West Los Angeles Healthcare Center (Los Angeles, CA). Each sample was provided with pathological analysis, clinical diagnosis, and autolysis time.

Q15 and Q120 ST14A Cell Models—ST14A cell lines were provided to us from the Coriell Biological Material Repository by the High Q Foundation and CHDI, Inc. These cells express a human huntingtin N-terminal portion (residues 1–548). One cell line (Q15) expresses normal huntingtin with a 15-glutamine repeat region, and the second cell line (Q120) expresses mutant huntingtin with a 120-glutamine repeat region.

ST14A cells were developed from embryonic day 14 rat striatal primordia by retroviral transduction of the temperature-sensitive SV40 large T antigen (13). These cells have typical features of the medium-size spiny neurons that are affected by Huntington disease, and they are well described in the literature (13, 14). Normally, the cells were grown at +33 °C under 5% CO_2 in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS),[2] 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

ST14A Cell Differentiation Induced by Shutting down T Antigen—In our cell model, SV40 large T antigen is expressed when cells are grown at +33 °C, which allows cells to divide. When cells are placed at higher temperature (up to +39 °C), the expression of SV40 large T antigen is decreased, which stops cell division and transforms the cells into striatal-like neurons.

ST14A Cell Differentiation Induced by Hormonal Factors—Cells were first grown under normal conditions (described above) until the desired numbers of cells were produced. To induce differentiation, cells were transferred into high glucose Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1× N2 supplement (Invitrogen), 50 μM forskolin (Sigma), 250 μM isobutylmethylxanthine (Sigma), 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), 10 μM dopamine (Sigma), and 10 ng/ml recombinant human acidic fibroblast growth factor (Fisher). Cells were incubated at +33 °C under 5% CO_2.

Adenoviral RCAN1-1L Construct and Overexpression—Full-size cDNA encoding the *RCAN1-1L* isoform was cloned into an adenoviral vector using services from Vector Biolabs (Philadelphia, PA). An *RCAN1-1L* cDNA fragment carrying a hemagglutinin tag at the 5′-end (described in Ref. 15) was the kind gift of Merce Perez-Riba. To overexpress RCAN1-1L, cells were typically infected using the construct at a multiplicity of infection of 30.

Western Blot Analysis and Antibodies—The analysis was performed following standard protocols using an ECL detection system (Amersham Biosciences). The signals were typically detected using a BioChemi HR camera and quantified using a BioSpectrum AC imaging system (UVP, LLC, Upland, CA).

The "common RCAN1 antibody" was developed against exon 7 (which is common to all RCAN1 isoforms) and characterized in our laboratory as described previously (12, 16, 17). The final dilution of the RCAN1 antibody for Western blot analysis was 1:10,000. All other antibodies were commercial. Phosphorylated huntingtin protein was detected using an anti-

[2] The abbreviations used are: FBS, fetal bovine serum; GFP, green fluorescent protein.

body that specifically recognizes the huntingtin protein phosphorylated at Ser^{421}. Phosphorylated Akt1/2/3 was detected using an antibody that specifically recognizes the Akt proteins phosphorylated at Ser^{473}. Anti-phospho-huntingtin (AB9562) and anti-huntingtin (MAB5492) antibodies were from Millipore (Billerica, MA). Anti-phospho-Akt (sc-33437) and anti-tubulin (sc-9104) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Commercial antibodies were diluted as suggested by the manufacturers.

Calcineurin Assay—The assay was performed using a cellular activity kit from BIOMOL (catalog no. 207007; Plymouth Meeting, PA), which employs an RII phosphopeptide substrate. All procedures exactly followed the manufacturer's protocols. Briefly, cells were lysed, and the lysates were purified of background phosphates. Protein amounts were quantified in each sample, and equal amounts of proteins were used for each reaction. We next determined the amount of phosphates released from RII phosphopeptide substrate in each sample and ran two experimental sets: one using calmodulin buffer (containing both calcium and calmodulin) and another using EGTA buffer. Because calcineurin requires calcium and calmodulin, the activity observed in the EGTA buffer samples represents the total activity of all phosphatase enzymes minus the activity of calcineurin. Therefore, the activity of calcineurin was calculated by subtracting the activity observed in EGTA buffer from the activity observed in calmodulin buffer.

Phosphoproteomic Analysis—Cells were collected, rinsed twice with ice-cold phosphate-buffered saline, and frozen at −80 °C until used. Quantification of phosphorylated proteins was performed using services from Kinexus Bioinformatics Corp. (Vancouver, British Columbia, Canada). Kinetworks phospho-site broad coverage pathway screens (KPSS1.3) were used for this analysis. Details of the Kinetworks methodology are available and described in detail at the company's web site.

RESULTS

RCAN1-1L Is Lowered in Huntington Disease—We have examined the production of RCAN1 proteins in post-mortem human brain samples from patients affected by Huntington disease and in control cases. One of the brain regions most affected by Huntington disease is the putamen; therefore, we analyzed samples isolated from this area. The putamens of 10 Huntington disease patients and 10 control cases were compared using Western blot analysis. To detect all possible RCAN1 isoforms, we used a common antibody (directed against a portion of the peptide generated by exon 7, which is common to all RCAN1 isoforms). The analysis revealed that the main RCAN1 protein expressed in the putamen of human brains has a size of ~38 kDa, which corresponds to RCAN1-1L (Fig. 1*A*). The levels of proteins corresponding to RCAN1–1S and RCAN1–4 were very low in both control and Huntington disease samples. Even more interestingly, we found that the level of RCAN1-1L was significantly down-regulated (by an average of >70%) in brain samples affected by Huntington disease (Fig. 1*B*). This led us to hypothesize that down-regulated expression of *RCAN1-1L* might be involved in Huntington disease. Because RCAN1-1L can inhibit calcineurin, this hypothesis is in good agreement with an earlier study that reported that inhibition of calcineurin (with FK506 or by overexpression of the dominant interfering form of calcineurin) can be protective in Huntington disease (3).

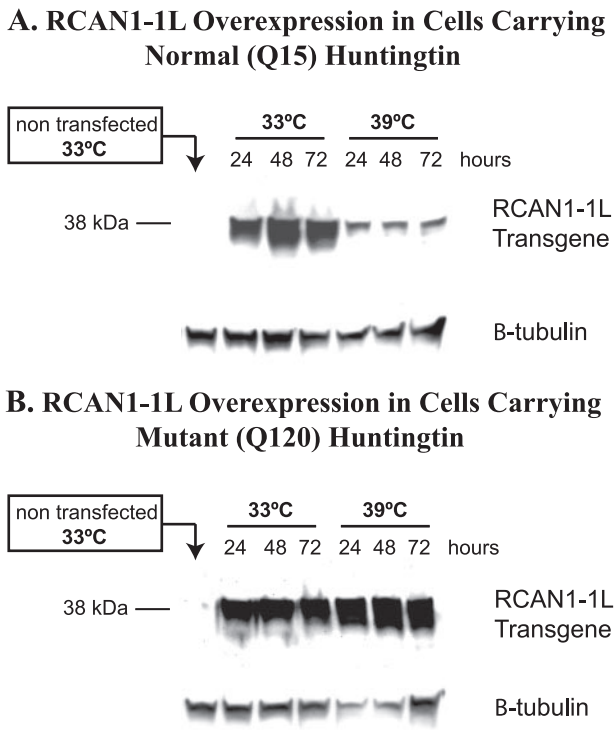
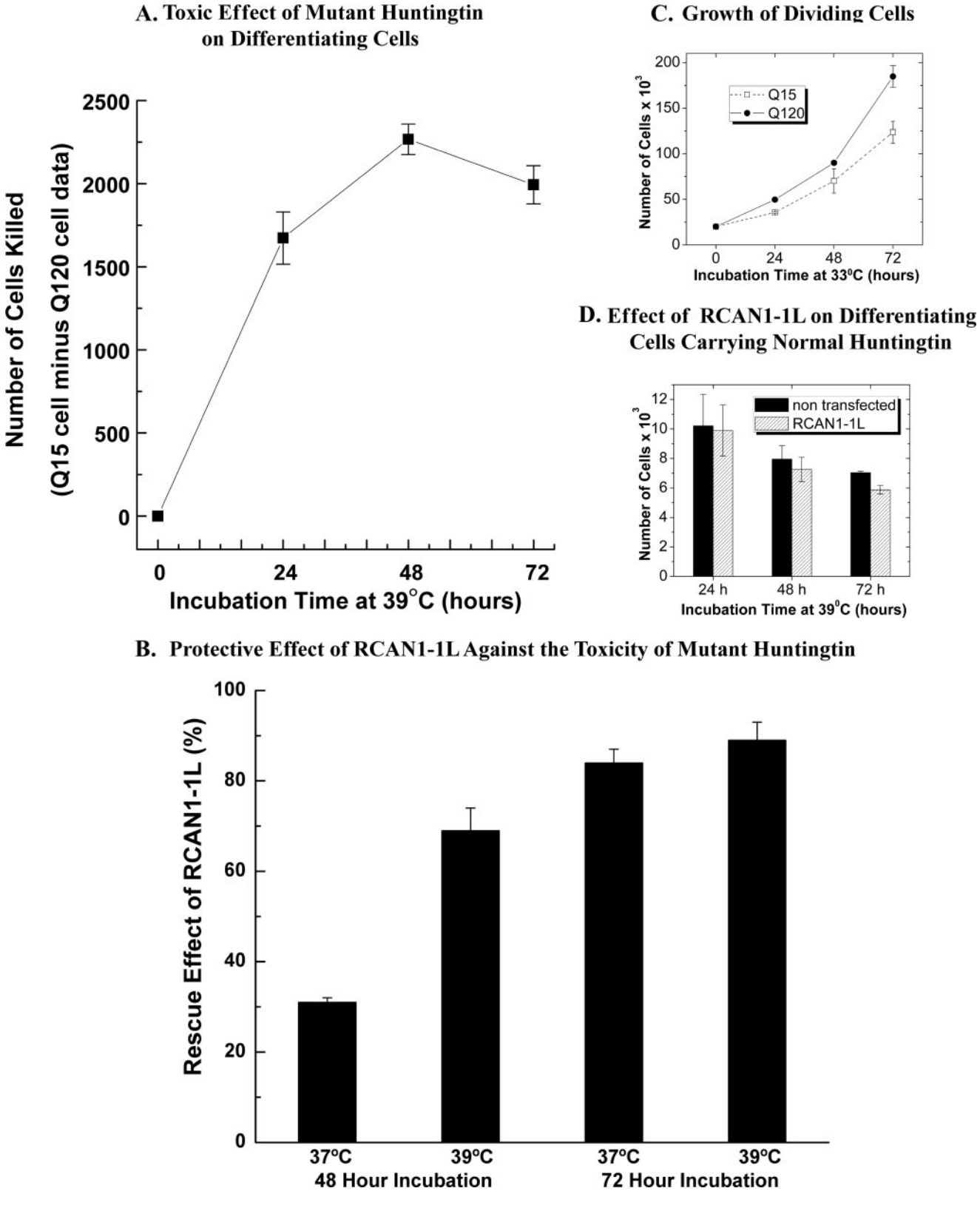


FIGURE 2. **RCAN1-1L overexpression in ST14A cells.** Cells were infected with the adenoviral construct encoding the RCAN1-1L protein at a multiplicity of infection of 30 and incubated either at 33 °C in the normal medium or at 39 °C in the medium containing no FBS for 24, 48, or 72 h. *A*, ST14A cells carrying the normal huntingtin gene (Q15); *B*, ST14A cells carrying the mutant huntingtin gene (Q120). *β*-Tubulin was included as a loading control.

Cell Culture Models for Testing the Functions of RCAN1-1L in Huntington Disease—To examine the possible effects of RCAN1-1L in Huntington disease, we used ST14A cells. ST14A cells were developed previously from embryonic day 14 rat striatal primordia by retroviral transduction of the temperature-sensitive SV40 large T antigen (13). These cells have typical features of the medium-size spiny neurons that are affected by Huntington disease, and they are well described in the literature (13, 14). In this cell model, SV40 large T antigen is expressed when cells are grown at +33 °C, which allows cells to divide. When cells are placed at higher temperature (up to +39 °C), the SV40 large T antigen is degraded, which blocks cell proliferation and reverses the phenotype back to that of a differentiating neuroblast (18), *i.e.* striatal-like neurons.

For our studies, we employed ST14A cells that expressed either normal huntingtin (which has a 15-glutamine repeat and has no negative effect on cells under any experimental condition) or mutant huntingtin (which has a 120-glutamine repeat region and is toxic when cells are induced to differentiate). To test the effect of RCAN1-1L on these cells, we created an adenoviral construct that encodes full-size *RCAN1-1L*. Cells infected with this construct produced an RCAN1-1L protein with the

expected size of ~38 kDa (Fig. 2). Both cell lines Q15 (carrying the normal huntingtin gene) and Q120 (carrying the mutant huntingtin gene) overexpressed *RCAN1-1L* under all conditions. Interestingly, the levels of RCAN1-1L overexpression in differentiating (+39 °C) Q15 cells were lower than those in dividing cells, whereas the levels of RCAN1-1L overexpression in differentiating (+39 °C) Q120 cells were higher than those in dividing cells. This finding appears consistent with the results of Figs. 3–5 and is explored further under "Discussion."



RCAN1-1L Protects Cells against Mutant Huntingtin Toxicity—Cells expressing either normal huntingtin (Q15) or mutant huntingtin (Q120) divided normally, and if anything, Q120 cells grew a little faster compared with Q15 cells (Fig. 3*A*). However, when they were induced to differentiate by raising the incubation temperature and withdrawing FBS from the medium (causing degradation of the temperature-sensitive large T antigen and cell differentiation), Q120 cells died significantly faster than did Q15 cells (Fig. 3*B*). This basic finding is in excellent agreement with the results of similar experiments reported previously (18). This accelerated cell death is attributed to the expansion of the polyglutamine stretch in the huntingtin protein. Therefore, we next used this model to test whether RCAN1-1L can rescue cells from mutant huntingtin toxicity. RCAN1-1L was overexpressed in Q15 and Q120 cells, and the cells were again differentiated by raising the growth temperature. Cell survival was estimated at two time points: 48 and 72 h. Because 39 °C might be a harsh temperature, we also studied these cells at 37 °C. We found that, under all conditions tested, RCAN1-1L had a significant rescue effect (Fig. 3*C*). The protection of Q120 cells by RCAN1-1L was greatest when cells were grown for 72 h at 39 °C; under these conditions, we observed an 89% protection by RCAN1-1L (Fig. 3*C*). It is interesting that RCAN1-1L had no beneficial effect on Q15 cells incubated at 39 °C; in fact, it actually had a slightly negative effect on the growth of Q15 cells at 39 °C (Fig. 3*D*).

We also tested the effect of RCAN1-1L on dividing cells and found that it had a slightly negative effect on the growth of both Q15 and Q120 cells (supplemental Fig. 1). To ensure that the apparent rescue properties of RCAN1-1L for differentiating Q120 cells observed in Fig. 3 (at 39 °C) are really due to RCAN1-1L and not to the viral construct by itself, we analyzed the growth of uninfected ST14A cells and cells infected with the adenoviral construct carrying green fluorescent protein (GFP). We found no measurable effect of the viral construct on these cells (supplemental Fig. 2). These data suggest that RCAN1-1L specifically abolishes the toxicity of mutant huntingtin in differentiating Q120 cells. Because the effect of RCAN1-1L on cells not expressing mutant huntingtin is negative, the real protective effect of RCAN1-1L may well be even greater than 89%.

Although the results of Fig. 3 seem to provide strong evidence that RCAN1-1L may be able to protect against mutant huntingtin cell toxicity, it might be possible that the method for inducing cell differentiation, incubation at 39 °C, induces an artifact because the growth conditions are too harsh. Arguing against this is the observation that similar results were also obtained by raising the growth temperature from 33 °C to only 37 °C (Fig. 3). Nevertheless, we felt it important to test whether the protective effect of RCAN1-1L could also be observed in a completely independent model for inducing cell differentiation. Therefore, we tried another approach in which dividing ST14A cells were induced to differentiate using hormonal factors. In these experiments, Q120 cells were grown under standard conditions at +33 °C, and hormonal growth factors were added to the medium. The cells infected with *RCAN1-1L* were compared with uninfected control cells and with cells infected with the construct carrying GFP. Cells were examined under a microscope, and only live differentiated cells that had a neuron-like morphology were counted (Fig. 4, compare *A* and *B*). Following hormonally induced differentiation, RCAN1-1L permitted better survival of ST14A cells carrying the mutant huntingtin gene (Q120). After 72 h of differentiation, there were ~46% more viable neuron-like cells in the RCAN1-1L-expressing Q120 cell population than in uninfected control cells or in cells infected with the viral vector carrying GFP (Fig. 4*C*). These results provide strong evidence that RCAN1-1L can protect against mutant huntingtin toxicity in neuronal cells.

RCAN1-1L Overexpression Causes Inhibition of Calcineurin and Phosphorylation of Huntingtin—It has been shown that inhibition of calcineurin by FK506 can protect against polyglutamine-huntingtin toxicity (3). Because RCAN1 proteins are known to regulate calcineurin, we hypothesized that RCAN1-1L might inhibit the activity of calcineurin in our system. We analyzed the effect of RCAN1-1L on calcineurin activity in dividing ST14A cells as well as in cells induced to differentiate either by hormonal factors or by raising the incubation temperature to 39 °C. Because we already observed a protective effect of RCAN1-1L against mutant huntingtin toxicity at the 48-h time point (Fig. 3), we measured calcineurin activity in cells overexpressing RCAN1-1L at this time point. We found that RCAN1-1L caused calcineurin inhibition both in dividing ST14A cells and in cells induced to differentiate by either hormonal factors or incubation at 39 °C (Fig. 5*A*). Of note, calcineurin activity significantly varied from one experiment to the next, although intra-experiment variability was small. However, calcineurin activity was always lower in cells overexpressing RCAN1-1L than in cells not expressing the *RCAN1* gene. The variability of our results could be explained by the fact

FIGURE 3. **RCAN1-1L rescues ST14A cells from the toxicity of mutant huntingtin.** *A*, toxic effect of mutant huntingtin on differentiating cells. Cells were induced to differentiate by raising the incubation temperature to either 37 or 39 °C (only 39 °C data are shown in *A*). Cells were infected using the adenoviral *RCAN1-1L* construct, and they were incubated for 24 h at 33 °C to overexpress RCAN1-1L (as described in the legend to Fig. 2). Next, the medium was replaced, and the cells were incubated at 37 or 39 °C for 48 or 72 h. Cell numbers were measured in both Q120 and Q15 cells. Q15 cells are ST14A cells that carry the normal huntingtin protein (15-glutamine repeat), whereas Q120 cells are ST14A cells that carry the mutant huntingtin protein (120-glutamine repeat). The toxic effect of mutant huntingtin on Q120 cell survival was evaluated by comparing the numbers of surviving Q15 and Q120 cells. *B*, protective effect of RCAN1-1L against the toxicity of mutant huntingtin. The protective effect of RCAN1-1L on Q120 ST14A cells was calculated as the difference between the toxic effect of mutant huntingtin on Q120 cells (mutant huntingtin-expressing) induced to differentiate at either 37 or 39 °C (as in *A*) and either overexpressing or not overexpressing the RCAN1-1L protein (encoded by the adenoviral *RCAN1-1L* construct). Values are expressed as percent rescue relative to Q15 ST14A cells carrying the normal huntingtin protein. *C*, mutant huntingtin actually allows faster growth in dividing cells. Both Q15 and Q120 ST14A cells were allowed to divide at 33 °C. Cell numbers were measured at 24, 48, and 72 h. *D*, RCAN1-1L has little effect on differentiating cells carrying normal huntingtin. Q15 cells were induced to differentiate by raising the incubation temperature to 39 °C. Cells were infected either with our adenoviral *RCAN1-1L* construct or with the viral construct alone and then incubated for 24 h at 33 °C to overexpress RCAN1-1L (as described in the legend to Fig. 2). Next, the medium was replaced, and cells were incubated at 39 °C for 24, 48, or 72 h. Cell survival was compared by measuring the number of viable cells in samples overexpressing or not overexpressing RCAN1-1L. In all cases (*A–D*), cell number was measured using a Coulter Counter (Coulter Corp.). The results shown represent the means ± S.E. of three independent experiments.

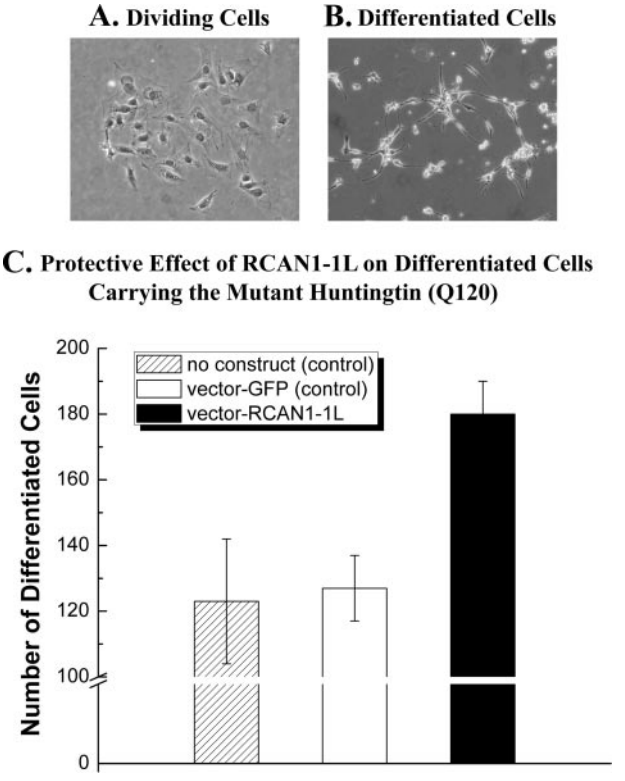


FIGURE 4. **Protective effect of RCAN1-1L against mutant huntingtin toxicity in differentiated Q120 ST14A cells.** *A*, dividing cells (magnification ×200). *B*, differentiated cells (magnification ×200). Differentiation was induced using hormonal factors as described under "Experimental Procedures." Images were taken 72 h after induction of differentiation. *C*, protective effect of RCAN1-1L on differentiated cells carrying mutant huntingtin (Q120 cells). The effect was measured in cells induced to differentiate by hormonal factors as described for *B*. Cells were infected using our adenoviral *RCAN1-1L* construct and then incubated for 24 h to overexpress the RCAN1-1L protein (as described in the legend to Fig. 2). The medium was replaced, and cells were incubated in hormonal medium for 72 h. Cell number was measured in samples infected with RCAN1-1L (*vector-RCAN1-1L*), in samples infected with the viral construct carrying GFP instead of RCAN1-1L (*vector-GFP (control)*), and in uninfected samples (*no construct (control)*). The protective effect of RCAN1-1L was evaluated by comparing the number of surviving Q120 cells. Cells were analyzed microscopically and counted manually. Only live differentiated cells (as in *B*) were counted. The number of viable neuron-like cells in the samples expressing RCAN1-1L was significantly greater than those in control cases. The results represent the means ± S.E. of three independent experiments.

that calcineurin activity in living cells does not stay constant. Calcineurin activity is regulated by several proteins, including RCAN1. To make things more complicated, it has been suggested that RCAN1 actually oscillates between stimulatory and inhibitory forms (19). These complications might also explain the fact that, so far, very few studies have reported calcineurin activities in living cells using direct measurements. Most reports judge the effect of RCAN1 proteins on calcineurin activity using indirect methods such as NFAT translocation, etc. In fact, we could identify only three articles in which the effect of RCAN1 proteins on calcineurin activity in living cells was measured directly. Interestingly, one of these articles reported the activation of calcineurin (20), whereas the other two reported the inhibition of calcineurin (21, 22). Nevertheless, considering the fact that, in our model, calcineurin activity was consistently lower in cells overexpressing RCAN1-1L than in cells not expressing RCAN1-1L, it is safe to conclude that RCAN1-1L actually did inhibit calcineurin activity in our study.

Calcineurin can dephosphorylate huntingtin, and it has been shown that huntingtin phosphorylation at Ser^{421} protects against polyglutamine-huntingtin toxicity and Huntington disease (2, 3). Therefore, we next tested whether overexpression of RCAN1-1L leads to huntingtin phosphorylation. This was done using both dividing and differentiating cells at 24, 48, and 72 h after their infection with *RCAN1-1L*. We found that huntingtin phosphorylation was significantly induced in all samples overexpressing RCAN1-1L (Fig. 5*B*). This suggests that the mechanism by which RCAN1-1L protects against polyglutamine-huntingtin toxicity involves inhibition of calcineurin and increased huntingtin phosphorylation at Ser^{421}.

Induction of Huntingtin Phosphorylation by RCAN1-1L Does Not Involve Akt (Protein Kinase B)—Besides inhibition of calcineurin, which can dephosphorylate huntingtin, increased levels of phosphorylated huntingtin can be caused by activation of Akt (protein kinase B) kinases. Indeed, it has been demonstrated that activation of Akt can phosphorylate huntingtin and is neuroprotective in Huntington disease (2). We have shown previously that RCAN1–1S can induce GSK-3 kinases (12); therefore, it seemed possible that RCAN1-1L might protect cells against mutant huntingtin toxicity by induction of Akt kinases.

Humbert *et al.* (2) did not specify exactly which Akt kinases may be involved in huntingtin phosphorylation and neuroprotection. Therefore, we tested for induction of all three known Akt kinases: Akt1, Akt2, and Akt3. This was done using two approaches: 1) phosphoproteomic analysis and 2) Western analysis. Using phosphoproteomic analysis, we first tested whether Akt1 is activated. ST14A cells were infected with *RCAN1-1L* and analyzed for the induction of Akt1 at 24 and 48 h after infection. RCAN1-1L overexpression was verified as shown in Fig. 2. The induction of Akt1 was analyzed using services from Kinexus Bioinformatics Corp. Akt1 was activated by phosphorylation at at least three different positions: Thr^{308}, Ser^{473}, and Tyr^{474}. Here, we analyzed the levels of Akt1 phosphorylated at Thr^{308} and Ser^{473} in both control and treated samples. The analysis revealed that Akt1 was not phosphorylated at Thr^{308} or Ser^{473} (Fig. 6) in any of the samples (eight samples). This suggests that Akt1 is inactive in both control and infected ST14A cells.

To address whether other Akt kinases might be induced or whether Akt1 might be phosphorylated at Tyr^{474}, we next used the Western analysis approach. We infected dividing and differentiating Q120 cells with our *RCAN1-1L* construct and analyzed the cells at 24, 48, and 72 h after infection as shown in Fig. 2. Western blots revealed no evidence of phosphorylated Akt1. In addition, neither Akt2 nor Akt3 could be detected by Western analysis, although minimal antibody dilutions, which were down to 1:200 (typical dilutions are between 1000 and 10,000), and maximal exposure times of as much as 20 min (typical times are between 1 and 5 min) were employed (Fig. 6*B*). It is unlikely that we could not detect Akt2 and Akt3 because of the low sensitivity of the technique because we could detect other phosphorylated proteins, such as GSK-3β, whose levels in ST14A cells are relatively low in comparison with other phos-

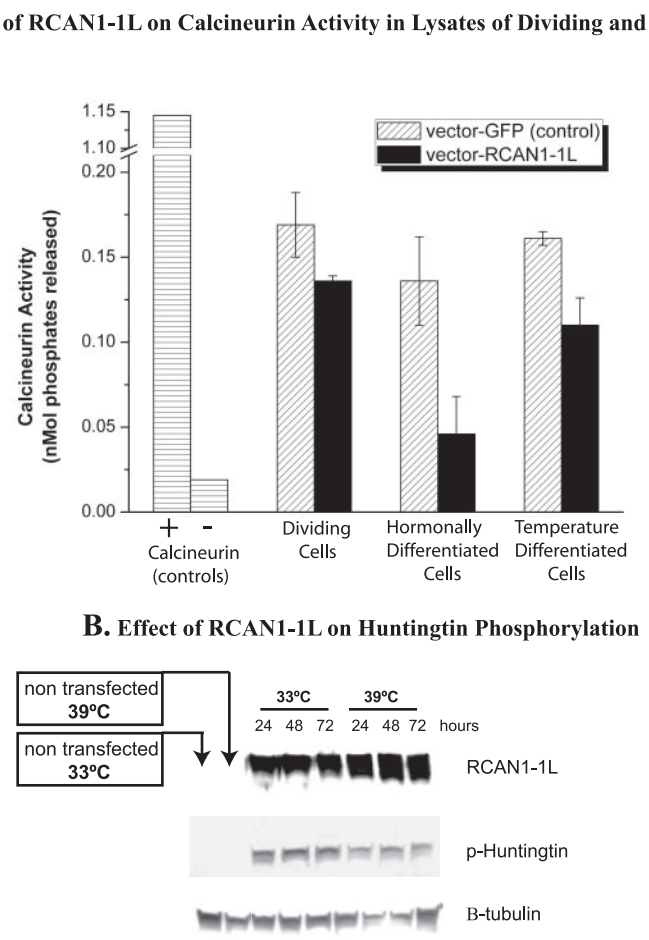


FIGURE 5. **RCAN1-1L overexpression causes inhibition of calcineurin and phosphorylation of huntingtin in ST14A cells.** *A*, effect of RCAN1-1L on calcineurin activity in both dividing and differentiated cells. The *first two bars* represent control samples for calcineurin; 5 units of calcineurin either were (+) or were not (−) added to the buffer. To analyze calcineurin activity in dividing cells, Q120 cells were infected using our adenoviral *RCAN1-1L* construct and incubated for 48 h at 33 °C in medium containing FBS. Calcineurin activity was ~29% lower in cells overexpressing RCAN1-1L (*vector-RCAN1-1L*) than in cells not overexpressing RCAN1-1L (*vector-GFP (control)*). To analyze calcineurin activity in cells differentiated using hormonal factors, Q120 cells were infected using the adenoviral *RCAN1-1L* construct and incubated for 48 h at 33 °C in hormonal medium. Calcineurin activity was ~66% lower in cells overexpressing RCAN1-1L in comparison with cells not overexpressing RCAN1-1L (control). To analyze calcineurin activity in cells differentiated by raising the incubation temperature to 39 °C, Q120 cells were infected using the adenoviral *RCAN1-1L* construct and incubated for 24 h at 33 °C. The medium was then replaced with one containing no FBS, and cells were incubated for 48 h at 39 °C. Calcineurin activity was ~32% lower in cells that overexpressed RCAN1-1L than in cells that did not overexpress RCAN1-1L (control). The results represent the mean ± S.E. of three independent experiments. *B*, effect of RCAN1-1L on huntingtin phosphorylation. Q120 cells were infected with our adenoviral construct encoding RCAN1-1L. Cells were grown either at 33 °C in normal medium or at 39 °C in medium containing no FBS for 24, 48, or 72 h, after which they were examined by Western analysis. Detection with β-tubulin was done to control the loading. The experiment was repeated with similar results. *p-huntingtin*, phosphorylated huntingtin.

phorylated proteins such as Jun and CREB1 (Fig. 6*A*). Thus, our results show no evidence of significant Akt kinase induction in our model and suggest that the phosphorylation of huntingtin is more likely caused by inhibition of calcineurin rather than by activation of Akt kinases.

DISCUSSION

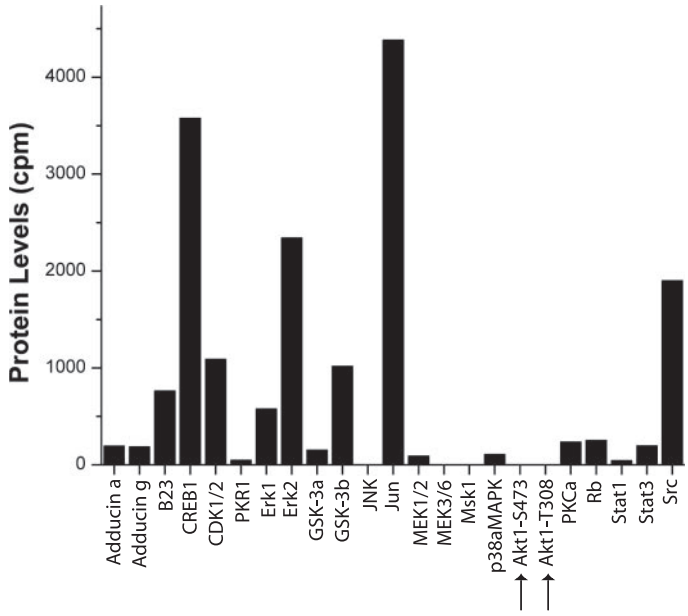
In this study, we have demonstrated for the first time that RCAN1-1L is depressed in Huntington disease, that RCAN1-1L overexpression can protect against mutant huntingtin toxicity in a cell culture model of Huntington disease toxicity, and that increased phosphorylation of huntingtin (via calcineurin inhibition) is the likely mechanism by which RCAN1-1L may be protective against mutant huntingtin. These findings suggest that RCAN1-1L "deficiency" may actually play a role in the etiology of Huntington disease. In addition, our results allow for the possibility that controlled overexpression of RCAN1-1L in the striatal region of the brain might be a viable avenue for therapeutic intervention in Huntington disease patients.

A previous study revealed that inhibition of calcineurin by the exogenous inhibitor FK506 can protect against mutant huntingtin toxicity *in vitro* (3). Because RCAN1 proteins have been demonstrated to inhibit the activity of calcineurin, we have tested if these proteins can also protect against mutant huntingtin toxicity and perhaps Huntington disease. This is especially important because the RCAN1 protein family is a major regulator of calcineurin activity *in vivo* and a potentially valuable target for Huntington disease therapy.

First, we analyzed RCAN1 expression in Huntington disease and discovered that one particular RCAN1 isoform, RCAN1-1L, is dramatically down-regulated in human brains affected by this disease. Because RCAN1-1L can potentially inhibit calcineurin and because calcineurin inhibition is protective against Huntington disease, this finding suggests that Huntington disease symptoms might actually arise as the result of down-regulated expression of RCAN1-1L. The expression of other RCAN1 isoforms, RCAN1–1S and RCAN1–4, was not significantly changed in Huntington disease, and both were observed at relatively low levels in comparison with the RCAN1-1L isoform; therefore, we focused on the RCAN1-1L isoform for our further studies.

Using ST14A cells as a model system for Huntington disease studies, we were able to demonstrate that RCAN1-1L can indeed protect against the toxicity of mutant huntingtin. It is interesting that, in our model, mutant huntingtin had a toxic effect only in differentiating but not dividing cells and that RCAN1-1L had a positive effect only on differentiating but not dividing cells. These data are in good agreement with the current view of Huntington disease as disease that affects only specific differentiated neuronal cells.

A. Levels of Phosphorylated Proteins in Q15 Cells Overexpressing RCAN1-1L



B. Levels of p-Akt kinases in Q120 Cells Overexpressing RCAN1-1L

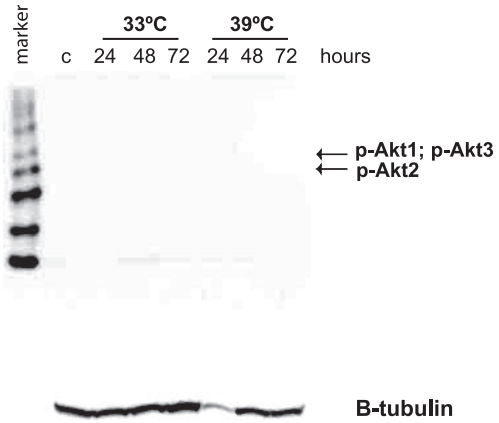


FIGURE 6. **Akt kinases are not phosphorylated in ST14A cells.** *A*, levels of phosphorylated proteins in ST14A cells. Shown are the levels of phosphorylated proteins in dividing Q15 cells that had been overexpressing *RCAN1-1L* for 48 h after infection with our adenoviral RCAN1-1L construct (as described in the legend to Fig. 2). Quantification of phosphorylated proteins was performed as described under "Experimental Procedures." *Arrows* indicate the levels of Akt1 (protein kinase Bα) phosphorylated at Ser^{473} and Thr^{308}. *JNK*, c-Jun N-terminal kinase; *MAPK*, mitogen-activated protein kinase; *PKCa*, protein kinase Cα. *B*, levels of phosphorylated Akt (*p-Akt*) kinases in Q120 cells. Cells were infected with the adenoviral construct encoding RCAN1-1L at a multiplicity of infection of 30 and incubated either at 33 °C in normal medium or at 39 °C in medium containing no FBS for the indicated time intervals. Uninfected cells are shown in the *first lane* (labeled *c* for control). Detection was performed by Western analysis. The membrane was first probed with anti-phospho-Akt antibody (1:200 dilution) and exposed for 20 min. It was then stripped, reprobed with anti-β-tubulin antibody (1:1000 dilution), and exposed for 0.5 min.

It is also remarkable that the levels of RCAN1-1L expression in differentiating (incubated at +39 °C) Q15 cells were lower than those in dividing cells, whereas the levels RCAN1-1L expression in differentiating (incubated at +39 °C) Q120 cells were higher than those in dividing cells (Fig. 2). At the same time, whereas RCAN1-1L overexpression had a negative effect on differentiating Q15 cell survival (Fig. 3*D*), it had a positive effect on differentiating Q120 cell survival (Fig. 3*C*). These data indicate that RCAN1-1L expression is more permissive in differentiated Q120 cells, which carry a mutant huntingtin gene, than in differentiated Q15 cells, which carry a normal huntingtin gene. This may be due to the fact that RCAN1-1L helps differentiated Q120 cells to overcome the toxicity of mutant huntingtin, whereas overexpression of RCAN1-1L in Q15 cells causes more harm than benefit. Thus, taken together, these observations indicate that RCAN1-1L expression might be specifically required to protect against mutant huntingtin toxicity.

Because phosphorylation of huntingtin was suggested as a protective mechanism against mutant huntingtin toxicity, we also tested whether the huntingtin protein is actually phosphorylated during cell rescue from mutant huntingtin toxicity by RCAN1-1L overexpression. We found that the huntingtin protein indeed became increasingly phosphorylated under these conditions. It is interesting that RCAN1-1L overexpression also caused increased huntingtin phosphorylation in dividing cells without increasing their survival. This suggests that huntingtin phosphorylation is an important protective mechanism in specific cell types such as differentiated striatal neurons.

As outlined in the Introduction, the phosphorylation of mutant huntingtin could theoretically be caused by inhibition of calcineurin (3), by activation of Akt (2), or by both. Here, we tested both possibilities. Overexpression of RCAN1-1L clearly caused inhibition of calcineurin in all cases tested. Moreover, the inhibition was stronger in differentiating cells than in dividing cells, which is in agreement with the fact that mutant huntingtin has a toxic effect on differentiating but not dividing cells. This indicates that RCAN1-1L protects cells by, at least in part, inhibition of calcineurin. The inhibition of calcineurin in cells overexpressing RCAN1-1L was not complete, and it varied from a high of ~66% in differentiating cells to only ~29% in dividing cells. These numbers are in a good agreement with the fact that differentiating but not dividing cells experience mutant huntingtin toxicity, and to survive, differentiating but not dividing cells might require phosphorylation of mutant huntingtin.

We also tried to address whether overexpression of RCAN1-1L might somehow stimulate Akt kinases, which would also lead to the phosphorylation of mutant huntingtin.

Because all Akt kinases require phosphorylation for their activation, this was done by detection of phosphorylated Akt kinases. We employed several approaches that all resulted in negative data, suggesting that these kinases are not induced during cell protection from mutant huntingtin toxicity by RCAN1-1L. First, we could not detect any of the known phosphorylated Akt kinases in ST14A cells by Western analysis. Second, none of the two possible phosphorylated forms of Akt1 could be detected in these cells using the more accurate and quantitative technique of phosphoproteomic analysis. Third, these results are in a good agreement with a previous study in which detection of phosphorylated forms of Akt in Huntington disease human brain samples also failed (2). Because the results were negative and unexpected, Humbert *et al.* (2) speculated that their samples may have degraded as a result of long post-mortem intervals (which is hard to avoid when working with post-mortem human tissues). We feel that, when all factors are taken into consideration, the results suggest that the phosphorylation of huntingtin is induced by inhibition of calcineurin rather than by activation of Akt kinases.

In conclusion, we have demonstrated for the first time that RCAN1-1L is depressed in Huntington disease and that RCAN1-1L overexpression can protect against mutant huntingtin toxicity in a cell culture model of Huntington disease toxicity. The mechanism of this protection involves huntingtin phosphorylation, which is caused by inhibition of calcineurin. Because we have also demonstrated that RCAN1-1L is significantly down-regulated in Huntington disease, our study suggests that Huntington disease might actually arise as the result of *RCAN1-1L* down-regulation. This suggests the possibility that up-regulated expression of RCAN1-1L in the striatal region of the brain might be used as a therapeutic approach in Huntington disease treatment. Several other diseases and aging disorders are also associated with the expansion of polyglutamine repeats. Thus, our results may have wide-ranging implications for a variety of other important diseases.

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