Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture

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Huntington’s disease (HD) is a progressive neurodegenerative disorder caused by an expanding CAG repeat coding for polyglutamine in the huntingtin protein. Recent data have suggested the possibility that an N-terminal fragment of huntingtin may aggregate in neurons of patients with HD, both in the cytoplasm, forming dystrophic neurites, and in the nucleus, forming intranuclear neuronal inclusion bodies. An animal model of HD using the short N-terminal fragment of huntingtin has also been found to have intranuclear inclusions and this same fragment can aggregate in vitro. We have now developed a cell culture model demonstrating that N-terminal truncated fragments of huntingtin with expanded glutamine repeats aggregate both in the cytoplasm and in the nucleus. Neuroblastoma cells transiently transfected with full-length huntingtin constructs with either a normal or expanded repeat had diffuse cytoplasmic localization of the protein. In contrast, cells transfected with truncated N-terminal fragments showed aggregation only if the glutamine repeat was expanded. The aggregates were often ubiquitinated. The shorter truncated product appeared to form more aggregates in the nucleus. Cells transfected with the expanded repeat construct but not the normal repeat construct showed enhanced toxicity to the apoptosis-inducing agent staurosporine. These data indicate that N-terminal truncated fragments of huntingtin with expanded glutamine repeats can aggregate in cells in culture and that this aggregation can be toxic to cells. This model will be useful for future experiments to test mechanisms of aggregation and toxicity and potentially for testing experimental therapeutic interventions.

INTRODUCTION

Huntington’s disease (HD) is an inherited progressive neurodegenerative disease. It is caused by an expanded CAG repeat coding for polyglutamine in a gene termed IT15 or huntingtin on chromosome 4 (1–4). The huntingtin protein has no strong homology to any other known gene product (3). The pathology in HD is restricted to the CNS, despite the widespread expression of huntingtin (5–8). There is progressive atrophy in the brains of HD patients, with the caudate and putamen (or striatum) and deep layers of the cerebral cortex especially vulnerable to neuronal cell death and gliosis.

The distribution of huntingtin in normal neurons is predominantly cytoplasmic (7–10). One group has reported localization of the normal protein to the nucleus (11,12); however, these observations have not been replicated by other groups. The protein with the expanded glutamine repeat can be detected as a band migrating more slowly on a western blot from cells or tissue derived from HD patients (7–10,13,14), consistent with the hypothesis that HD results from a genetic ‘gain of function’ mechanism. However, the pathogenesis is uncertain.

Recently a mouse model replicating some of the features of HD has been created, using the promotor and exon 1 of huntingtin with a very long expanded repeat as a transgene (15). These animals display seizures, progressive weight loss and abnormal movements and early death. Post-mortem examination of these mice revealed intranuclear neuronal inclusions in brain regions affected in HD (16). The inclusions were visible shortly before the animals developed behavioral evidence of illness, suggesting a possible pathogenic role. Experiments using protein expressed from the same...
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Figure 1. Huntingtin constructs. Schematic diagrams of constructs used. FL stands for full-length (3144 amino acids). The truncated constructs have 171 and 63 amino acids respectively using the 23Q clone (3) as a basis for numbering.

Recent studies of post-mortem brains derived from HD patients have supported the hypothesis that intranuclear neuronal inclusions involving a truncated huntingtin fragment may be important in the pathogenesis of HD (18,19). Intranuclear inclusions were observed in the cortex and striatum, the areas most affected in HD. They were denser in patients with longer glutamine repeats. These inclusions were detected with antibodies to the N-terminus of huntingtin, but not with antibodies to internal epitopes, consistent with the possibility that the inclusions were formed by an N-terminal truncation of huntingtin. Supporting this idea, a 40 kDa fragment was enriched in a nuclear fraction (18). In addition, similar aggregates were present in dystrophic neurites (18).

In order to understand the possible role of aggregation of huntingtin in different cellular compartments in HD it would be useful to have a cell culture model. In one previous study cells were transfected with full-length huntingtin with normal or expanded glutamine repeats. The localization of the protein was similar in both cases, with diffuse cytoplasmic labeling (9). The mouse model and the more recent patient studies, however, suggest the possibility that huntingtin may need to be truncated to aggregate and to localize to the nucleus. We have therefore studied the cellular localization of full-length and truncated huntingtin fragments in transient transfection experiments in cell culture. We find that N-terminal truncations of huntingtin with expanded repeats form nuclear and cytoplasmic aggregates, which can lead to increased vulnerability to cellular toxicity.

RESULTS

Cellular expression and aggregation

The constructs used in these experiments are shown in Figure 1. Full-length or truncated huntingtin was expressed transiently in N2a neuroblastoma cells or HEK 293 fibroblasts using a CMV promoter. The full-length construct coded for the unmodified human protein with either 23 or 82 glutamine repeats. In order to study the distribution in cells of the truncated protein a myc epitope tag was added to the N-terminal constructs. These had either 18 or 82 glutamine repeats. One construct expressed amino acids 1–171 and the other amino acids 1–63 (numbering based on 23 glutamine repeats as in the original published sequence; ref. 3).

Figure 2a shows western blot analysis of the expression of these constructs in cells using transient transfection techniques. Results from HEK 293 cells are shown because a greater percentage of these cells take up DNA, yielding a higher level of expression of protein. Similar results were seen with N2a cells, though the bands were less strong (data not shown). In cells transfected with the full-length constructs strong bands can be seen at the expected size for the full-length protein. Preincubation of antibody with peptide antigen blocked labeling (data not shown). Proteins containing the expanded repeats migrate slower on the gel compared with the protein with normal repeats. In addition, in many experiments bands at a lower position on the gel can be seen. These bands must contain the N-terminal region, since they can be detected with the N-terminal antibody (AP78). The band seen with the protein with the normal glutamine repeat was ∼55 or 60 kDa. The band seen with the protein with the expanded repeat was ∼70 kDa. These bands likely represent degradation of huntingtin.

When cells were transfected with the truncated huntingtin constructs bands of appropriate size were seen, with a striking dependence on the glutamine repeat length (Fig. 2b and c). The bands with the 171 amino acid constructs were ∼30 kDa for the normal repeat length and ∼50 kDa for the expanded repeat length. The bands for the 63 amino acid constructs were ∼15 kDa for the normal repeat length and 30 kDa for the expanded repeat length. These bands were all smaller then the corresponding short fragments seen in cells transfected with the full-length constructs. In addition, for both constructs with expanded repeats there was reactive material at the very top of the gel, suggesting that a portion of the protein had aggregated and could not enter the gel. The amount of material at the top of the gel was enhanced by boiling (Fig. 2c).
Figure 2. Western blot analysis of transient expression. (a) Expression of full-length huntingtin in HEK 293 cells. Western blot using AP78 or AP194 (N-terminal huntingtin antibodies) of extracts from cells transfected with full-length huntingtin expression constructs. Expression of full-length huntingtin with 23Q (lane 2) or with 82Q (lane 3) is compared with untransfected cells (lane 1). (b) Expression of N-terminal Myc fusions of huntingtin. Western blot probed with c-Myc monoclonal antibody (9E10; Zymed) of extracts from transfected HEK 293 cells (lanes 2–5) compared with untransfected cells (lane 1). (c) A blot of protein from cells transfected with N63 and boiled before loading more clearly showing aggregation at the top of the gel (arrow).

Figure 3. Cytoplasmic localization of full-length huntingtin (23Q and 82Q). N2a cells were transfected with 0.5 μg HD-FL-23Q (left) or HD-FL-82Q (right). Cells expressing huntingtin were stained with mAb2166 (amino acids 181–823 of huntingtin; Chemicon) and appear green (a and d). Nuclei were visualized using TOTO-3 and appear red (b and e). (c and f) Merged images (huntingtin green, DNA red).

Table 1. Cellular localization of Huntingtin

<table>
<thead>
<tr>
<th>Construct (n)</th>
<th>Diffuse only (%)</th>
<th>Aggregates (%)</th>
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<tr>
<td>FL-23Q (137)</td>
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<td>1</td>
</tr>
<tr>
<td>FL-82Q (124)</td>
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<td>1</td>
</tr>
<tr>
<td>N171-18Q (123)</td>
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<td>6</td>
</tr>
<tr>
<td>N63-18Q (104)</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
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<td>82</td>
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<tr>
<td>N63-82Q (120)</td>
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<td>100</td>
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<td>N171-82Q</td>
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<td>42%</td>
</tr>
<tr>
<td>N63-82Q</td>
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<td>66%</td>
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Number of aggregates

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<th>Fl-82Q</th>
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<tbody>
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<td>2.2</td>
</tr>
<tr>
<td>N63-82Q</td>
<td>3.6</td>
<td>7.7</td>
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**Figure 4.** Aggregation of the N171 truncation with an expanded repeat. (Left) Normal repeat protein showing a cytoplasmic localization. (Middle and right) Nuclear and cytoplasmic aggregation of repeat expanded protein. N2a cells were transfected with 0.25 µg pcDNA3.1-N171-18/82Q-Myc-His. Cells expressing N171 were visualized with a c-Myc monoclonal antibody (9E10; Zymed), shown in green. Nuclei are shown in red and in the merged image N171 is in green and nuclei red.

**Cellular localization**

Cells transfected with the full-length construct generally showed diffuse cytoplasmic labeling (Fig. 3). This was seen whether the glutamine repeat length was in the normal range or was expanded. Label was not detected in the nucleus as defined by TOTO-3 labeling of DNA. Quantitation of labeled cells was consistent with these observations (Table 1).

Cells transfected with the N171 construct also showed diffuse cytoplasmic labeling when the construct had a normal glutamine repeat length (Fig. 4). However, when the glutamine repeat length was expanded the distribution of label was dramatically different (Fig. 4 and Table 1). Most cells had punctate discrete localization of label. This was present both in the cytoplasm and the nucleus. In general the label in the nucleus tended to have a rounder more compact appearance and the label in the cytoplasm was more heterogeneous in size and often had a more irregular appearance.

Cells transfected with the N63 construct again had diffuse cytoplasmic labeling if the glutamine repeat length was within the normal range (Fig. 5 and Table 1). However, essentially all cells transfected with the N63 construct with the expanded repeat had punctate labeling and almost no cells had diffuse cytoplasmic labeling. In addition, these cells tended to have more labeling in the nucleus and a greater number of distinct punctate foci (Fig. 5 and Table 1).

Double label experiments for huntingtin and ubiquitin were done using cells expressing N171-82Q or N63-82Q (Fig. 6). Aggregates of huntingtin protein (especially large aggregates) were often positive for ubiquitin as well.

**Cellular toxicity**

Cells transfected with the expanded truncated huntingtin constructs had increased vulnerability to treatments with staurosporine 48 h post-transfection compared with cells transfected with the normal polyglutamine construct or a control lacZ construct (Fig. 7). This difference was significant at 4 h post-staurosporine (Fig. 7), but no longer significant at 8 h, when most of the cells were no longer viable (data not shown). Assays performed at 24 h post-transfection showed no differences in vulnerability between expanded and normal huntingtin transfections. Thus aggregation precedes detectable toxicity, consistent with a causal role.

**DISCUSSION**

In this study we have examined the cellular distribution of full-length and truncated huntingtin when expressed transiently...
in fibroblasts and neuroblastoma cells. We find that when the truncated protein has an expanded glutamine repeat it forms aggregates in both the cytoplasm and the nucleus. The nuclear localization is most striking for the shorter truncated fragment.

These results are similar to those from mouse models and patients with HD. In the mouse model and in HD patients nuclear inclusions are seen. In the mouse model the protein is derived only from the N-terminal truncation injected as a transgene and does not contain substantial amounts of the full-length normal protein. Similarly, in HD patients the inclusions appear to be formed predominantly from an N-terminal truncation of the protein. Our results are similar to these observations in that the full-length protein with an expanded repeat had an almost purely cytoplasmic diffuse localization while the N-terminal truncations with expanded repeats formed nuclear aggregates. As in the mice and patients, the aggregates were often ubiquitinated.

In addition, there are some differences between our results in cell culture and the observations in patients and mice. In our experiments we see cytoplasmic aggregation as well as nuclear aggregation. In HD patients dystrophic neurites containing the N-terminal fragment of huntingtin are observed, but their location within the cell appears to differ somewhat from the aggregations we see in cell culture, which are predominantly perinuclear. In addition, the inclusion bodies seen in mice and in patients are most often single, discrete and relatively round, whereas the aggregates seen in this study are often multiple and of variable size. Some of these differences may be ascribed to the different time course of development of the inclusions. In mice they develop over a period of weeks; in patients they presumably can develop over a period of years. However, in our experiments they develop over only 24 h.

Nuclear inclusions have now been observed in several other glutamine repeat diseases besides HD, including SCA-1, SCA-3 and DRPLA (19–21). In addition, nuclear aggregates have been observed in a mouse model of SCA-1 (20). Furthermore, our results are similar to recent cell transfection experiments involving both ataxin-1 (20) and ataxin-3 (21). Thus nuclear inclusions are emerging as a distinguishing hallmark of the known glutamine repeat diseases (22) and quite possibly additional disorders (23).

Several lines of evidence are consistent with the hypothesis that the truncated protein with the expanded repeat forms aggregates. The N63 construct (and to a lesser extent the N171 construct) with expanded repeats yielded reactivity at the very top of the gels in
western blot experiments. These aggregates appeared to have a β-sheet conformation, as indicated by their birefringence when stained with Congo red and examined with polarized light microscopy. Similar western blot data have been generated for SCA-3 and also with congo red and examined with polarized light microscopy. One possibility is that small truncated products may be able to diffuse into the cytoplasmic and thus its presence in the nucleus is abnormal. One possibility is that small truncated products may be able to diffuse into the nucleus even in non-striatal cells. Thus it is possible that proteolytic cleavage is the event with cell specificity.

**MATERIALS AND METHODS**

**Huntingtin expression plasmids**

**FL-23Q.** The full-length huntingtin construct, pRc/CMV-FL-23Q, was generated by subcloning a 10.3 kb NotI fragment containing the huntingtin cDNA from pEBV-HIS-FL-23Q (originally assembled by triple ligation of a C-terminal cDNA clone of 9543 bp and an N-terminal cDNA clone of 823 bp) into pRc/CMV (Invitrogen).

**FL-82Q.** N-Terminal fragments with expanded CAGs were generated by PCR using genomic DNA isolated from patient lymphoblastoid cell lines using the sense primer 5′-GGCCCGAG-GGCTCCCGGAGACTGC-3′ and the antisense primer 5′-GGCT-GAGGCAAGCCGGCTGTGC-3′. PCR products were gel purified and subcloned into pCRII (Invitrogen). The PCR fragment with the expanded CAG repeat substituted for the normal cDNA was removed from pCRII using an AlwNI site (bp 501) and an NcoI site (bp 314) and ligated into a Bluescript plasmid (Stratagene) containing the N-terminal 1011 bp of the huntingtin cDNA.

**Differential susceptibility of transfected N2A cells to staurosporine-induced apoptosis**

Figure 7. Cellular toxicity of the expanded polyglutamine construct. N2a cells transfected with an N171–82Q construct show accelerated death (48 h post-transfection) relative to cells transfected with either control lacZ or HD N171–19Q constructs after a 4 h exposure to 500 nM staurosporine (P < 0.05). Results represent quantitation from 10 randomly selected fields for each construct in three independent experiments.

**Results**

**In vitro** experiments of Scherzinger et al. (17) showed formation of aggregates of the N-terminal fragment of huntingtin using both filter assays and western blots. These aggregates appeared to have a β-sheet conformation, as indicated by their birefringence when stained with Congo red and examined with polarized light microscopy. In our experiments nuclear aggregation and toxicity are seen even in these transient transfection experiments with fibroblasts and neuroblastoma cells. In contrast, in patients with HD nuclear inclusions predominate in particular neurons in the brain, which correspond well to the neurons which are affected in the disease (18,19). The reason for this cell specificity is unclear. Our experiments show that once huntingtin is truncated it can aggregate in the nucleus even in non-striatal cells. Thus it is possible that proteolytic cleavage is the event with cell specificity.

**Aggregation of huntingtin** appears to render cells more vulnerable to toxic stimuli. Similarly, a fragment of ataxin-3 was toxic to cells (24). We cannot determine the relative contribution of nuclear versus cytoplasmic aggregation to toxicity in our experiments, since both truncated constructs yielded aggregation in the two compartments.

**One issue relates to the mechanism of initiation of aggregation** (29). One possibility raised by recent experiments with SCA-1 is that there is a nidus formed by binding to another protein. Ataxin-1 binds a nuclear protein called leucine-rich acidic nuclear protein (LANP) (30) more tightly than ataxin-1 has an expanded repeat. Both proteins are then located in an insoluble fraction in the nuclear matrix (20,30). Huntingtin can associate with several other proteins, but none of them have a prominent normal nuclear localization (31–38).

Aggregation of huntingtin appears to render cells more vulnerable to toxic stimuli. Similarly, a fragment of ataxin-3 was toxic to cells (24). We cannot determine the relative contribution of nuclear versus cytoplasmic aggregation to toxicity in our experiments, since both truncated constructs yielded aggregation in the two compartments.

A final question relates to the cell specificity of the inclusions. In our experiments nuclear aggregation and toxicity are seen even in these transient transfection experiments with fibroblasts and neuroblastoma cells. In contrast, in patients with HD nuclear inclusions predominate in particular neurons in the brain, which correspond well to the neurons which are affected in the disease (18,19). The reason for this cell specificity is unclear. Our experiments show that once huntingtin is truncated it can aggregate in the nucleus even in non-striatal cells. Thus it is possible that proteolytic cleavage is the event with cell specificity.
cDNA from bp 314 to 823) into pcDNA3.1-Myc-HisA (Invitrogen) to generate a fusion to the Myc epitope and a His tag.

The truncated huntingtin expression vectors with 63 amino acids, pcDNA3.1-N63-18/82Q-Myc-His, were created by subcloning a fragment of the huntingtin cDNA (from bp 314 to 503) generated by PCR using high fidelity Taq polymerase (Boehringer Mannheim). The sense primer 5′-GATCGGATCCATGGCGAAGCTTGATGAAAG-3′ introduces a BamHI site (shown in bold) upstream of the initiation codon of the huntingtin cDNA and the antisense primer 5′-GATCCCTCGAGCGGTGGCGGCTGTTGCTG-3′ creates an XhoI site at bp 503 in the huntingtin cDNA. The 189 bp product was digested with BamHI and XhoI and subcloned into pcDNA3.1-Myc-HisA.

Cell culture and transfection

Human embryonal kidney cells (HEK 293) cells, obtained from ATCC, were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) supplemented with penicillin and streptomycin and 5% fetal calf serum. Neuro-2a cells (N2a), from ATCC, were maintained in 50% OptiMEM (Gibco BRL), 50% high glucose DMEM (Gibco BRL) supplemented with penicillin and streptomycin and 5% fetal calf serum. In preparation for western blot analysis cells were seeded 24 h before transfection in 6-well plates at a density of 2.5 × 10^5 cells/well. For immunofluorescence experiments chamber slides (4-well; Labtek) were seeded at a density of 0.5 × 10^5 cells/well. One hour before transfection the medium was changed.

Cells were transfected using a modification of the calcium phosphate transfection method (39). Briefly, 2× BBS [50 mM N,N-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid (Sigma), 0.28 M NaCl, 1.5 mM Na_2HPO_4, pH 6.95] was substituted for 2× HEPES. Precipitates were added to cells and incubated at 35°C in a 2–4% CO_2 incubator overnight. Cells were washed once in serum-free medium and fresh medium was added to the monolayer. Cells were incubated for a further 8–24 h before harvesting. For toxicity experiments cells were co-transfected with 2.5 µg of a green fluorescent protein (GFP) construct (40) as a maker for transfection.

Western blot analysis

Cells were transfected with 5 (full-length constructs) or 2.5 (N-terminal truncations) µg DNA. Transfected cells were washed once in phosphate-buffered saline (PBS) and harvested in 10 mM Tris, 1 mM EDTA buffer, pH 7.5, supplemented with complete protease inhibitors (Boehringer Mannheim). Extracts were sonicated and the protein concentrations were determined (Coomassie protein assay reagent; Pierce). Total extracts (50 µg) were resolved by SDS–PAGE (N-terminal truncations using 4–15% gradient ready gels from BioRad and full-length huntingtin using 6% SDS–PAGE). Proteins were transferred onto nitrocellulose using Tris–glycine (Ameresco), 20% methanol buffer. Blots were blocked for 1 h at room temperature with PBS (5% milk). Primary antibodies (1 µg/ml) were incubated for 1 h at room temperature in PBS, 5% milk. Blots were washed for 4 × 5 min with PBS/milk before incubation with peroxidase-conjugated secondary antibodies (Amersham). Bands were detected using enhanced chemiluminescence (Amersham).

Indirect immunofluorescence

N2a cells were transfected with 0.5 µg DNA for the full-length constructs (pRe/CMV-Fl-18/82Q) or 0.25 µg truncated constructs [pcDNA3.1-N171(63)-18/82Q-Myc-His]. At 48 h post-transfection cells were washed with PBS and fixed for 10 min at –20°C in methanol/acetic acid (3:1). Cells were air dried prior to re-hydration with PBS (2 × 5 min). Slides were blocked with PBS, 5% normal goat serum (Vector) for 30 min at room temperature. Primary antibodies were diluted (2 µg/ml for c-Myc monoclonal, 1:500 for mAb 2166 and 1:100 for Dako polyclonal anti-ubiquitin) in PBS, 5% normal goat serum, slides were incubated with the primary antibody for 1–2 h at room temperature and then washed with PBS, 1% normal goat serum for 4 × 5 min. Secondary antibodies (FITC-conjugated goat anti-mouse) were diluted to a final concentration of 1 µg/ml and incubated for 30 min at room temperature. Slides were washed for 3 × 5 min in PBS and then incubated with 1 µM TOTO-3 (a double-stranded DNA stain; Molecular Probes) diluted in PBS for 30 min at room temperature. Excess stain was removed by a brief wash in distilled water. Slides were mounted with Vectashield (Vector) and visualized by confocal laser microscopy. To define the nuclear or cytoplasmic localization of aggregates scans were done at several points on the z-axis and the scan which best defined aggregate localization based on quantitation was used for the figures.

Quantitation of label

The cellular distribution of huntingtin constructs transiently expressed in N2a cells was assessed by an observer using coded slides. A number of cells (n in Table 1) were selected by random sweeps at 60× for anti-myc or anti-huntingtin staining. Labeling was scored as either diffuse or containing aggregates. The cellular distribution was further examined by confocal microscopy using single 1.0 µm thick optical sections. Aggregates were scored as nuclear only if completely surrounded by DNA labeling. Similar results were obtained in transfected HEK 293 cells (not shown).

Cellular toxicity

N2a cells were grown, plated and transfected with constructs as described above. At 36 h after transfection staurosporine (Sigma) was added to the cell medium at a concentration of 500 nM. Staurosporine was removed after a 4 h incubation and the cells then incubated with 1 µM propidium iodide. Ten percent of the cells were imaged by confocal microscopy using a Zeiss Axiovert inverted microscope and GFP filter set (EX 485, DM 510, BA 515–565). Cell death was monitored with propidium iodide immunofluorescence and phase contrast microscopy. Cell counts per condition were totalled and compared between the various constructs. Reported results are compiled from three independent experiments.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Subsequent to the submission of our paper a complementary study was published by Martindale et al. (Nature Genet., 18, 150–154).

REFERENCES


