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The DNA repair-ubiquitin-associated HR23 proteins are constituents of neuronal inclusions in specific neurodegenerative disorders without hampering DNA repair

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Intracellular inclusions play a profound role in many neurodegenerative diseases. Here, we report that HR23B and HR23A, proteins that are involved in both DNA repair and shuttling proteins to the 26S proteasome for degradation, accumulate in neuronal inclusions in brain from a mouse model for FXTAS, as well as in brain material from HD, SCA3, SCA7, FTDP-17 and PD patients. Interestingly, HR23B did not significantly accumulate in tau-positive aggregates (neurofibrillary tangles) from AD patients while ubiquitin did. The sequestration of HR23 proteins in intracellular inclusions did not cause detectable accumulation of their stable binding partner in DNA repair, XPC. Surprisingly, no reduction in repair capacity was observed in primary human fibroblasts that overexpressed GFP-polyQ, a polypeptide that induces HR23B-positive inclusions in these transfected cells. This illustrates that impairment of the ubiquitin–proteasome system (UPS) by expanded glutamine repeats, including the sequestration of HR23B, is not affecting NER.

Keywords: HR23B; Neurodegenerative disorders; Ubiquitin; Inclusions; DNA repair

Introduction

In mammals, two *H*omologues of the yeast gene *R*ad*23* exist, designated HR23A and HR23B. Both have a function in DNA repair and in the ubiquitin–proteasome system (UPS) (Schauber et al., 1998). In DNA repair, part of the HR23 proteins are in complex with XPC and function in the DNA damage recognition step of the global genome subpathway of nucleotide excision repair (GG-NER), which recognizes and removes helix-distorting lesions from the entire genome (Ng et al., 2003; Sugasawa et al., 1997, 1998). Both HR23A and HR23B stabilize the XPC protein *in vivo* and increase the affinity for damaged DNA of XPC *in vitro* (Li et al., 1997; Lommel et al., 2002; Ng et al., 2003; Sugasawa et al., 1996). It has been demonstrated that Rad23 interacts with the 26S proteasome and with ubiquitylated proteins as well (Elsasser et al., 2004; Saeki et al., 2002). In addition to a repair function, HR23B is also essential for development as indicated by the dramatic phenotype of the HR23B knock-out mouse, including impaired embryonic development and high rates of intrauterine death. In contrast, mouse models defective in XPC develop relatively normal and only exhibit a profound NER deficiency (Cheo et al., 1997; Ng et al., 2002; Sands et al., 1995). HR23B knock-out mice are smaller, display facial dysmorphology and male infertility but are GG-NER proficient due to the functional redundancy with HR23A (Ng et al., 2002). In contrast, HR23A knock-out mutants are indistinguishable from wild type because of complete functional compensation by HR23B (Ng et al., 2003).

Ubiquitylation of proteins is a general protein modification involved in many processes such as endocytosis, transcription, antigen presentation and protein degradation (Glickman and Ciechanover, 2002; Ravid and Hochstrasser, 2004). Ubiquitylation of target proteins is performed by the sequential action of the ubiquitin-activating enzyme (E1), one of a series of ubiquitin-conjugating enzymes (E2) in combination with a specific ubiquitin ligase (E3). Ubiquitin is usually covalently attached to internal lysine residues of the target protein. Since ubiquitin contains several internal lysines itself, a polyubiquitin tree can

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be formed (Glickman and Ciechanover, 2002). It is generally believed that proteins containing a lysine 48-branched ubiquitin tree are destined for degradation by the 26S proteasome. Proteins that contain a ubiquitin-interacting domain like the ubiquitin-associated domain (UBA) recognize ubiquitylated proteins creating new interactions and subsequent actions (Verma et al., 2004). Interestingly, both HR23A and HR23B contain besides an amino terminal ubiquitin-like domain (UBL), known to interact with the proteasome (Elsasser et al., 2002; Saeki et al., 2002), also two UBA domains. Recently, it has been shown that the carboxy terminal UBA domain of yeast Rad23 has a relatively high affinity for lysine 48 polyubiquitin trees (Raasi et al., 2004; Ryu et al., 2003; Varadan et al., 2005), whereas Rad23 itself is protected from degradation by an intrinsic stabilization signal (Heessen et al., 2005). This, together with the capacity to interact with the proteasome, makes HR23A and HR23B ideal as shuttles/chaperones for proteins to be degraded by the proteasome. In fact, it has been shown that Rad23, and others, are shuttles of the 26S proteasome (Varadan et al., 2005; Verma et al., 2004). Misregulation of protein degradation via the UPS is directly or indirectly involved in many human diseases including malignancies, auto-immune diseases and neurodegenerative disorders (Glickman and Ciechanover, 2002).

The formation of inclusions in specific parts of the brain is a pathological hallmark of many neurodegenerative diseases (Ciechanover and Brundin, 2003). Actually, the specific localization and the composition of these inclusions have been utilized as a diagnostic criterion of several neurodegenerative disorders, including *f*rontotemporal *d*ementia and *p*arkinsonism linked to chromosome *17* (FTDP-17), *P*arkinson *d*isease (PD), *H*untington *d*isease (HD), *S*pino*c*erebellar *a*taxia (SCAs) and *f*ragile *X* associated *t*remor/*a*taxia *s*yndrome (FXTAS). Major constituent of the inclusions is often a disease-specific mutant protein (e.g. tau, α-synuclein, huntingtin) or mutant mRNA (e.g. FMR1 mRNAs in FXTAS). For instance, an important group of neurodegenerative disorders is the polyglutamine diseases characterized by neuronal intranuclear inclusions that consist of accumulations of insoluble aggregated polyglutamine-containing proteins. Next, to mutant protein, UPS components and molecular chaperones have been detected within the inclusions, including ubiquitin and the 20S catalytic core complex of the proteasome.

Many neurodegenerative diseases share these ubiquitin-positive structures; however, their origin and function in relation to the pathology remain unclear. Although ubiquitin-positive inclusions are the pathological hallmark of many neurodegenerative disorders, it might very well be that they are secondary responses to different causes (Ciechanover and Brundin, 2003). Inclusions have been found to positively influence cell viability supporting a protective function for the inclusions (Arrasate et al., 2004; Bowman et al., 2005). Therefore, it might be that they arise as a consequence of cellular stress, thereby trying to solve a toxicity problem in the cell. Indeed, it has been shown that accumulation of mutant proteins containing long polyglutamine tracts can cause impairment of the UPS leading to cellular stress in living cells (Bence et al., 2001). In neurodegenerative diseases such as HD and SCA 1–7, expanded polyglutamine tracts in target proteins are known to be the primary source of the inclusions (Ciechanover and Brundin, 2003). Purified 26S proteasomes fail to digest these long repeats and release them instantly (Venkatraman et al., 2004). Consequently, polyglutamine-containing (mutant) proteins accumulate in time, especially in non-dividing neurons, ultimately leading to neuronal cell death. In mouse models mimicking human SCA7, no proof for UPS impairment prior to or during onset of the disease was found, suggesting that the impairment is a secondary consequence (Bowman et al., 2005).

The issue whether the formation of inclusions is a cause or an effect of toxicity is of great importance for understanding the pathogenesis of these diseases. Knowledge of the formation and of the constituents of inclusions, regardless if they have a positive or a negative influence on cell viability, can ultimately lead to the development of novel therapeutic interventions. Since HR23A and HR23B have a prominent role in shuttling proteins to the proteasome, we wondered if HR23A and/or HR23B participate in the formation of inclusions.

Materials and methods

Western blotting

Wild type and HR23B−/− mouse embryonic fibroblasts were lysed as described previously (Okuda et al., 2004). HR23B protein was detected using either fresh or pre-absorbed rabbit polyclonal antibodies against HR23B. Pre-absorption was performed by incubation of the fresh serum with nitrocellulose paper containing homogenates of HR23B−/− cells overnight at 4°C. Horseradish-peroxidase (HRP)-labeled anti-rabbit IgG serum was used as secondary antibody, allowing chemiluminescence detection with ECL (Amersham).

Immuno-neuropathology

Human brain autopsy was carried out within 24 h after death according to legal and ethical guidelines. The brain was fixed in 10% buffered-formalin solution for 6 weeks. In addition, brain tissue from the expanded-CGG mice and E13.5 embryos from WT and HR23B KO mice (Ng et al., 2002) were fixed in 3% paraformaldehyde and further processed. Paraffin-embedded sections of all brain regions underwent routine staining with hematoxylin–eosin (HE), Bodian and Congo red. From well-diagnosed cases on both clinical and neuropathological criteria (FTDP-17, PD, HD, SCA3, SCA7 and AD), specific brain regions known to contain inclusions were further analyzed for both ubiquitin and HR23B localization. In addition, brains were dissected from a mouse model for FXTAS (Willemsen et al., 2003) and immediately fixed in 3% paraformaldehyde for 24 h followed by routine paraffin embedding.

Immunohistochemistry with rabbit antibodies directed against HR23B, HR23A (Okuda et al., 2004), XP-C (van der Spek et al., 1996) or ubiquitin (1:500, Dako, Glostrup, Denmark) was performed according to standard protocols. Briefly, sections were deparaffinized, microwave-treated and blocked for endogenous peroxidase activity. Subsequently, sections were immuno-incubated with the primary antibody for 16 h followed by a secondary step with swine anti-rabbit Igs conjugated with HRP (1:100, DAKO, Glostrup, Denmark). Visualization was performed with 3,3,diaminobenzidine as substrate.

Microinjection and DNA repair synthesis

Normal human fibroblasts (C5RO) were fused using Sendai virus. Fused cells were microinjected with either GFP-polyQ or GFP as described previously (Vermeulen et al., 1994). After injection, the cells were incubated for 6 days at 37°C in standard F10 medium to recover from the injections and to form inclusions. Fluorescent (GFP) images were obtained with an Olympus IX70 microscope, PlanApo 60×/1.40 Oil immersion lens (excitation with 455–490 nm and long pass emission filter >510 nm).

After microinjection and fluorescent image recording, DNA repair capacity was determined by measuring unscheduled DNA synthesis (Vermeulen et al., 1994). Human fibroblasts were UV-irradiated (16 J/m^2, 254 nm), labeled for 2 h using [^{3}H]thymidine (20 μCi/ml) and fixed for autoradiography. Autoradiographic grains above the nuclei of injected polykaryons were counted and compared with the number of grains above nuclei of normal fibroblasts (C5RO) non-fused, assayed in parallel.

Results

Specificity of anti-HR23B serum

In order to test whether HR23B is a component of inclusion bodies, we applied an immunohistochemical approach. Our polyclonal anti-HR23B serum showed several cross-reacting bands when used on immunoblots. After multiple usages, these aspecific bands vanished while the specific HR23B band did not (Fig. 1). To increase the specificity of the serum, it was pre-absorbed prior to the actual staining. This pre-absorbed antiserum was used for our further immunohistochemical experiments. Next, the pre-absorbed HR23B antibody was applied on paraffin sections from wild type and mHR23B knock-out E13.5 embryos (Ng et al., 2002). In wild type embryos, an overall staining was detected in almost all cell types (Figs. 2A, C), whereas HR23B knock-out embryos were devoid of detectable HR23B labeling (Figs. 2B, D), illustrating the mono-specificity of our pre-absorbed antibody.

HR23B and HR23A accumulate in neuronal inclusions of FXTAS mice

Subsequently, the participation of HR23B proteins in the formation of inclusion bodies was analyzed in a mouse model of FXTAS using paraffin-embedded brain sections (Willemsen et al., 2003). Previously, it has been shown that ubiquitin-positive intranuclear inclusions accumulate in a time-dependent manner throughout the brain of FXTAS mice with very high number of ubiquitin-positive inclusions in neurons located in specific brain regions, including n. parafascicularis. In addition, these inclusions contained molecular chaperones (HSP 40) and the 20S catalytic core complex of the proteasome (Willemsen et al., 2003). To determine whether HR23B protein is co-localizing with these neuronal intranuclear inclusions in the FXTAS mouse model, an immunohistochemical analysis was performed. HR23B was abundantly present within the intranuclear inclusions throughout the brain in a similar pattern as described for the ubiquitin-labeled inclusions (Fig. 3A). In addition, cells harboring inclusions showed an overall increase in total cellular HR23B levels compared to wild type mice (Fig. 3A, compare neurons containing inclusions indicated by arrows with neurons without inclusions indicated by arrowheads). A similar labeling pattern was observed in all tested samples using HR23A-specific antibodies, although the intensity of the staining was weaker (Fig. 3a, inset for FXTAS brain; other human samples data not shown). We concluded that both HR23A and B proteins are present in relatively large quantities in FXTAS-related intranuclear inclusions.

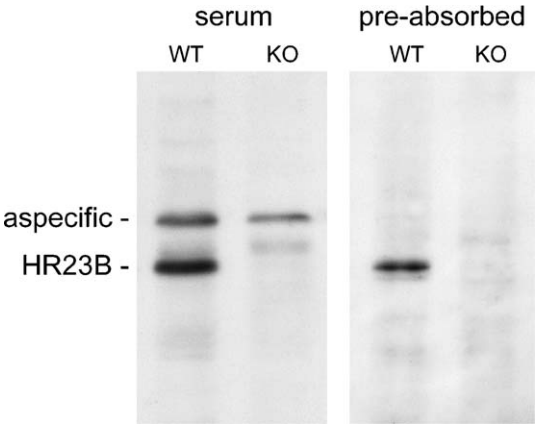

Fig. 1. Increase in the specificity of the anti-HR23B serum after pre-incubation. Immunoblots from wild type and HR23B−/− homogenates were stained with either non-used or pre-absorbed anti-HR23B serum. Besides the HR23B specific band, a higher migrating cross-reacting band can be detected if new serum is used (left panel). The cross-reacting band cannot be detected when pre-absorbed serum is applied (right panel).

HR23B accumulates in inclusions of several different neurodegenerative disorders but not in tangles from AD patients

To further gain insight whether the observed accumulation is limited to FXTAS-related inclusions and to study whether the co-localization has any pathological relevance, we stained brain sections from patients diagnosed with various neurodegenerative diseases. In brain from an FTDP-17 patient, HR23B was abundantly present in cytoplasmic Pick bodies (Fig. 3B) in a similar way as was described for ubiquitin.

Characteristic for the neuropathology of brains from AD patients are extra-cellular amyloid plaques and intracellular aggregates of hyperphosphorylated tau protein (*n*euro*f*ibrillary *t*angles; NFT). NFT are known to stain positive for ubiquitin as well (Leigh et al., 1989). However, HR23B did not accumulate to a detectable level in intracellular tangles in the cortex from an AD patient (Fig. 3C), whereas ubiquitin staining was positive in an adjacent section (Fig. 3D), illustrating that HR23B accumulation in ubiquitin-positive aggregates is not an aspecific event. Moreover, this excludes a hypothetical cross-reactivity of our anti-HR23B antibody with ubiquitin.

Expanded glutamine-coding CAG repeats in huntingtin that accumulates in intranuclear inclusion bodies in specific brain regions during lifetime, predominantly striatal neurons, cause HD. Next to ubiquitin (data not shown), HR23B was present within these intranuclear inclusions (Fig. 3e for n. caudatus).

SCAs are a clinically heterogeneous group of disorders. Several identified mutations correspond to expansions of trinucleotides repeats, including CAG repeats in SCA1, SCA2, SCA3, SCA6, SCA7, SCA17 and DRPLA. These expanded CAG repeats can be found in different target genes like ataxin-3 and ataxin-7 in SCA3 and SCA7, respectively. Previously, it has been shown that HR23B and ataxin-3 interact with each other *in vitro* using a yeast two-hybrid screen (Wang et al., 2000). Intranuclear inclusions could predominantly be detected in neurons located in the pons of SCA3 patients. Mutant ataxin-3 accumulates in these intranuclear inclusions along with ubiquitin, other components of the UPS system and molecular chaperones (Chai et al., 1999). Here, we show *in vivo* the presence of HR23B within these pontine neuronal intranuclear inclusions (Fig. 3F). In addition, we could detect small HR23B-positive cytoplasmic inclusions in pontine neurons that were devoid of intranuclear inclusions (Fig. 3G). Also in cortical brain

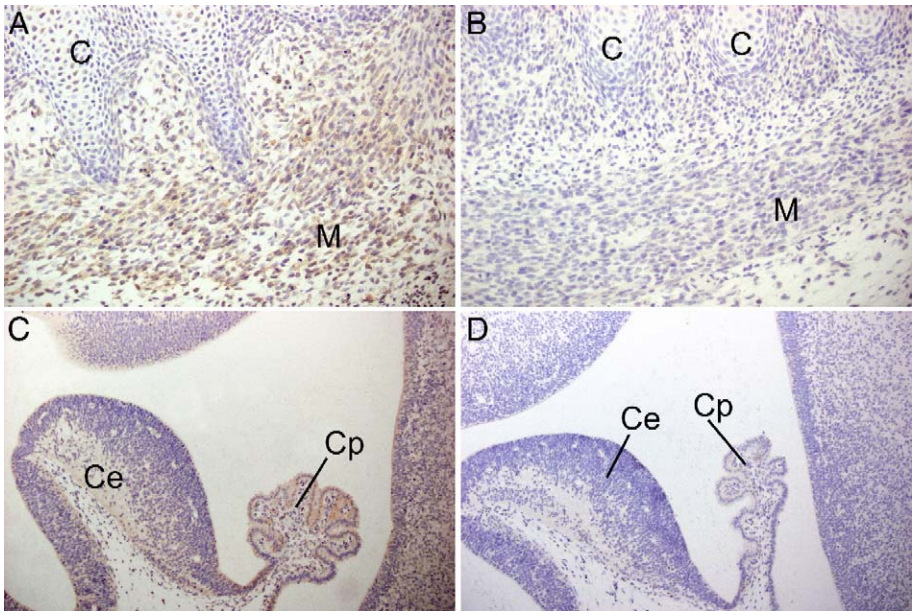

Fig. 2. Specificity of the pre-absorbed anti-HR23B serum. Paraffin sections from E13.5 embryos from WT (A and C) and HR23B (B and D) KO mice were stained with pre-absorbed anti-HR23B serum. In WT embryos, HR23B is expressed in almost all the cells at relatively low levels. A shows HR23B expression in the myotome (M). B shows HR23B expression in the choroid plexus (CP) within central part of the lumen of fourth ventricle. Similar areas in HR23B KO embryos (B and D) are devoid of HR23B expression. C = cartilage primordium, Ce = intraventricular portion of cerebellar primordium.

sections of an SCA7 patient, HR23B-positive intranuclear inclusions were present (Fig. 3H). Finally, a clear accumulation of HR23B was observed in Lewy bodies in neurons of the substantia nigra of a PD patient (Fig. 3I).

GFP-polyQ expression induces aggregate formation but does not reduce NER capacity

Since HR23B and HR23A are known to associate with the NER protein XPC (Masutani et al., 1994), we wondered whether the accumulation of HR23B caused a sequestering of the XPC protein as well. No XPC-positive inclusions could be observed in paraffin sections of the brain in the FXTAS mouse model using immunohistochemistry with monospecific antibodies against XPC (Ng et al., 2003; van der Spek et al., 1996) (Fig. 4A, left) while in a succeeding section ubiquitin did (Fig. 4A, right). Although XPC staining, predominantly nuclear, was present in normal quantities in neurons of the FXTAS mice compared to wild type mice (data not shown), we cannot exclude an impairment of the DNA repair capacity of the XPC/HR23B complex. To test the influence of inclusions on NER, both dependent as well as independent of HR23B, we measured the repair capacity in cells harboring inclusion bodies. For this purpose, we made use of a GFP-polyQ construct known to induce inclusion formation in living cells (de Pril et al., 2004). Unscheduled DNA synthesis, which is an established measurement for global genome NER capacity (Hoeijmakers et al., 1990), was used in fused normal human fibroblasts micro-injected with cDNA encoding either GFP-polyQ or GFP alone. DNA repair capacity was determined by measuring DNA-damage-induced DNA synthesis or 'unscheduled DNA synthesis (UDS)'. In this assay, cultured cells (here primary human fibroblasts) were UV-irradiated (inflicting DNA damage) and subsequently grown in the presence of tritium-labeled thymidine. The incorporated radio-actively labeled DNA synthesis marker can be visualized by autoradiography. Quantification of repair capacity is then performed by counting the autoradiographic silver grains above the nuclei. In order to avoid false positive S-phase cells (due to the incorporation of tritiated thymidine during normal replicative DNA synthesis), cells are fused using Sendai virus, 3 days after fusion fibroblasts do not replicate anymore. Six days after injection, cells were UV-irradiated with an NER-saturating dose of 16 J/m^2. This time point has been chosen because of the presence of a maximum number of inclusions. After a longer period (more than 6 days), we observed massive cell death. Prior to irradiation, pictures of microinjected cells were taken. In some, but not all, nuclear inclusions were clearly visible (Fig. 4B). Strikingly, no difference in unscheduled DNA synthesis was observed between cells injected with cDNA expressing GFP, GFP-polyQ or non-injected cells (Fig. 4B, and Table 1). Neither a difference in UDS was detected between cells expressing GFP-polyQ harboring nuclear inclusions or not (Fig. 4b and Table 1). Thus, high expression of polyglutamine repeats has no detectable effect on NER capacity. Importantly, the inclusion bodies induced by GFP-polyQ expression did stain positive for HR23B using the pre-incubated serum (Fig. 4C, middle).

Discussion

HR23B is present in neuronal inclusion of some specific neurodegenerative disorders

The exact cause and role of the UPS in the formation of inclusions in many distinct neurodegenerative disorders are not defined yet. Here, we report the accumulation of HR23B (and HR23A) in neuronal inclusion bodies in a mouse model for FXTAS, and in patient material from several but not all neurodegenerative disorders, including FTDP-17, HD, PD, SCA3 and SCA7. Interestingly, HR23B was not detected in NFT in brain tissue from AD. Antibodies recognizing ubiquitin

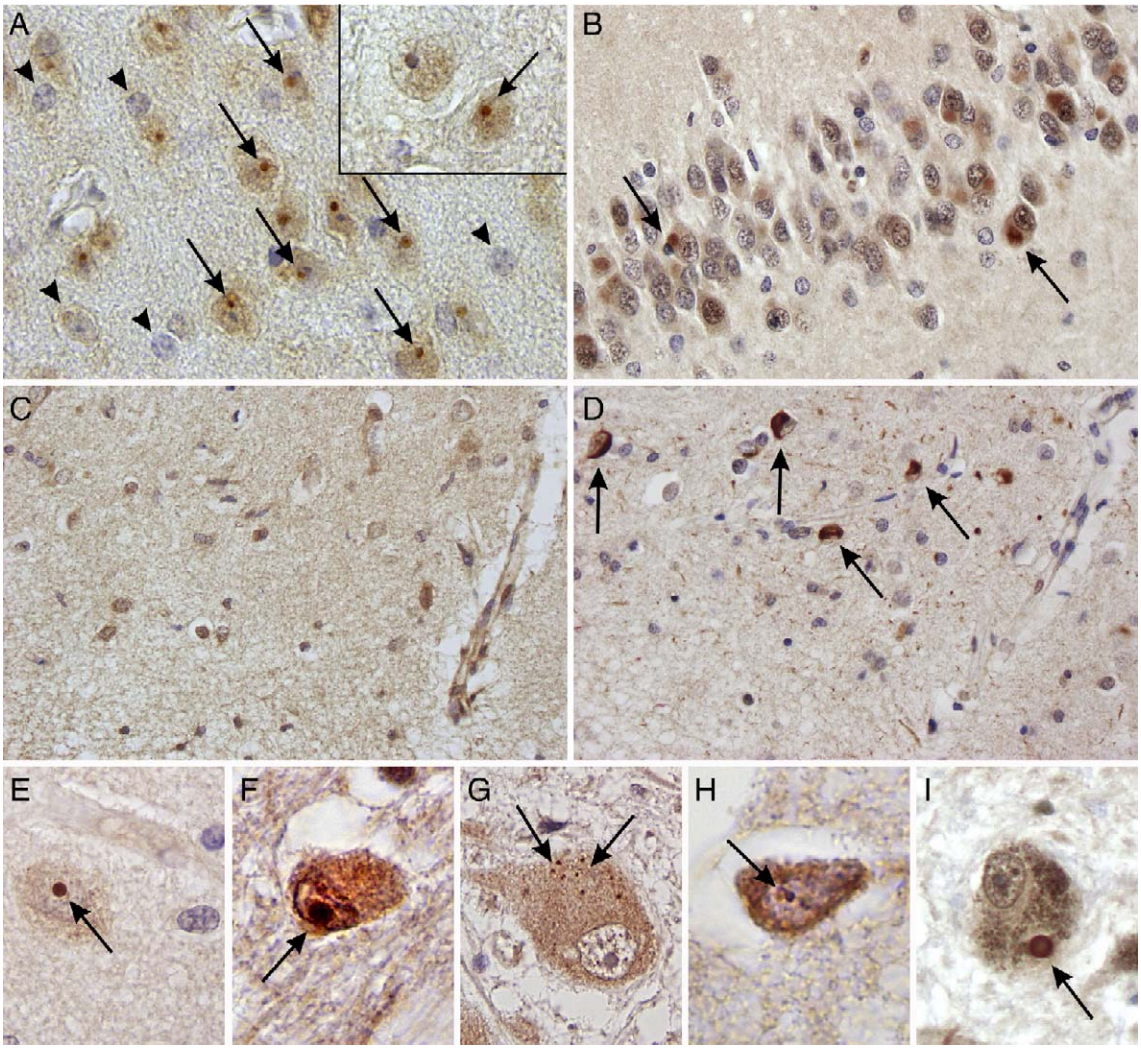

Fig. 3. HR23B accumulates in inclusions related to a variety of neurodegenerative disorders. Paraffin brain sections from an FXTAS mouse model (A), FTDP-17 (B), AD (C and D), HD (E), SCA3 (F and G), SCA7 (H) and PD (I) were analyzed for the presence of HR23B-positive inclusions. (A) HR23B-positive neuronal intranuclear inclusions (arrows) in the n. parafascicularis from a 100-week-old FXTAS mouse. Note the high expression of HR23B in these neurons compared to neurons devoid of inclusions (arrowheads). The inset shows HR23A-positive inclusions in the same area (arrows). (B) Similarly, HR23B is present in Pick bodies (arrows) in the hippocampus of an FTDP-17 patient. (C) HR23B is absent in intracellular neurofibrillary tangles of an AD patient, while ubiquitin immunolabeling of a succeeding section (D) did show the presence of ubiquitin-positive neurofibrillary tangles (arrows). (E) Neuronal intranuclear inclusions in the n. caudatus from an HD patient did stain positive for HR23B (arrow). (F) HR23B is present in neuronal intranuclear inclusion in pontine neurons of an SCA3 patient (arrow). (G) In the same sections, HR23B was also present in small cytoplasmic inclusions (arrows) in pontine neurons that were devoid of intranuclear inclusions. (H) In cortical brain sections of an SCA7 patient, HR23B did accumulate in neuronal intranuclear inclusions (arrow). (I) Finally, Lewy bodies present in neurons from the substantia nigra from a PD patient are HR23B-positive labeled (arrow).

are widely used for a post-mortem confirmation of diagnoses for one of these neurodegenerative diseases. The localization of ubiquitin-positive inclusions (brain region, cell type, nucleus or cytoplasm) is specific for different groups of neurodegenerative diseases. Here, we report a new neuropathological hallmark, the presence of HR23B within inclusions, in some specific neurodegenerative disorders. In contrast to ubiquitin, the HR23B immunolabeling could discriminate between AD and the other neurodegenerative disorders studied here. The observation that HR23B is not detected in ubiquitin-containing NFTs in AD might point to a different nature of these deposits in comparison to the inclusion bodies in other diseases tested here. However, the origin of the inclusions in the other neurodegenerative diseases examined in this study does not share a common root cause as well. The origin may vary from different mutant proteins to mutant mRNAs as the primary cause. For example, inclusions in FTDP-17 brains, a neurodegenerative disorder caused by mutations in the Tau gene, show a positive staining with the phosphorylation-dependent anti-Tau antibody AT8 (Spillantini et al., 1998). The same antibody also shows a positive labeling of the characteristic intracellular NFTs in AD patients. Although hyperphosphorylated tau is accumulating in both these deposits, only those from FTDP-17 patients stain positive for HR23B. Different cellular aspects of cells harboring inclusions may explain why HR23B is not or at low abundance accumulating in all deposits. Nevertheless, we conclude that HR23B is a significant constituent of neuronal inclusions in some specific neurodegenerative disorders, including FTDP-17,

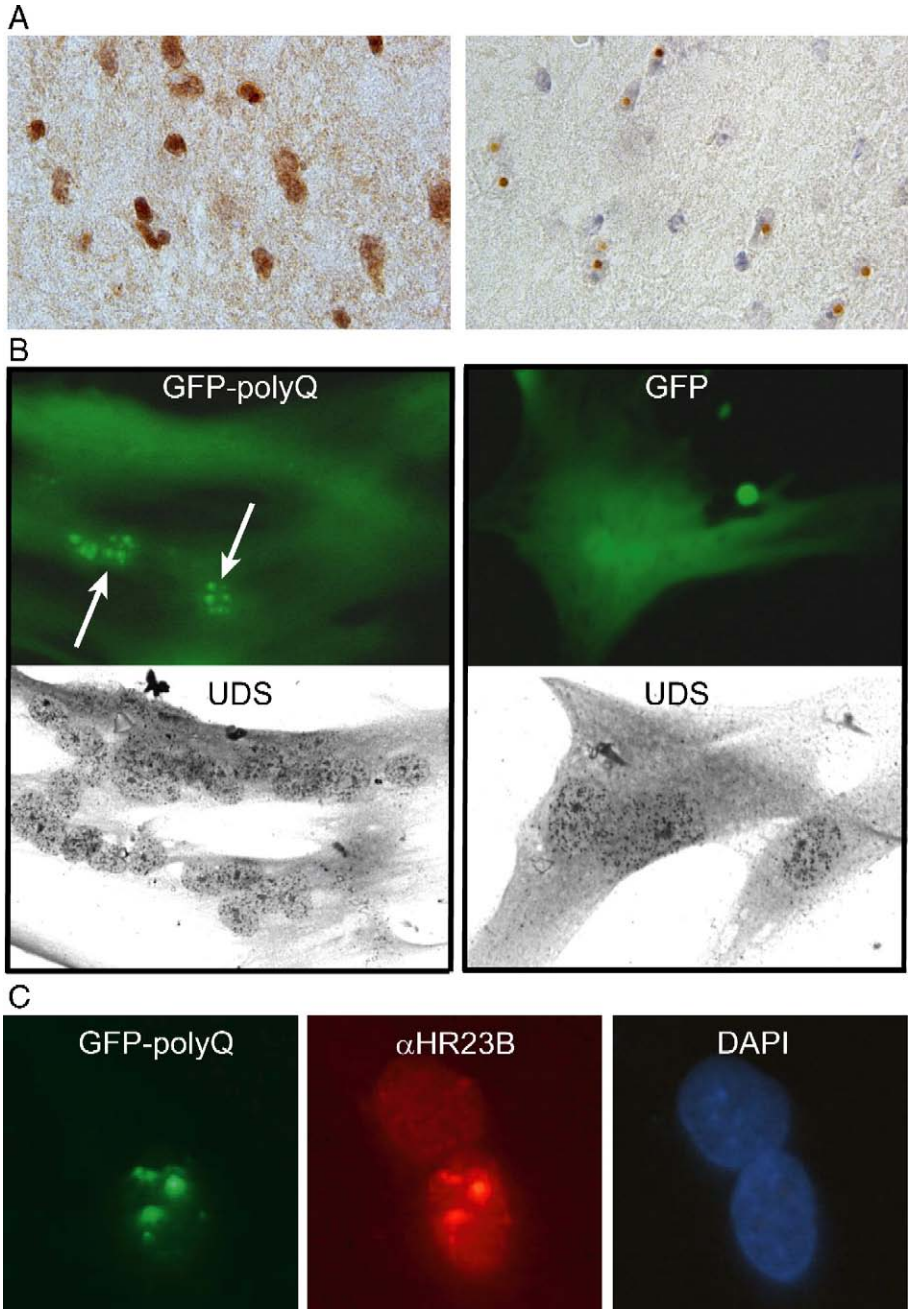

Fig. 4. Inclusion formation is not influencing NER repair capacity. (A) Paraffin section of a FXTAS mouse was analyzed for the presence of XPC using an affinity purified XPC serum. No detectable accumulation of XPC in inclusions was observed (left panel), while ubiquitin-positive inclusions (arrows) were present in a succeeding section (right panel). (B) Cells that were injected with GFP-polyQ (top left panel) did form visible inclusions (arrows) while GFP-injected cells (top right panel) did not. Repair capacity of these GFP-polyQ (below left panel)-injected cells did not change compared to GFP (below right panel) and non-injected cells. (C) HR23B (middle panel) accumulates in GFP-polyQ aggregates (left panel).

HD, FXTAS, SCA3, SCA7 and PD. Further research should focus on the presence of HR23B in inclusions/deposits of other neurodegenerative disorders that were not tested here.

A possible role for HR23B in the formation of neuronal inclusions

Inclusion formation either as a cause or a consequence of the impairment of the UPS is of importance for the understanding of many neurodegenerative diseases. Since both UBA domains of HR23B are capable of binding polyubiquitylated proteins (Raasi et al., 2004; Ryu et al., 2003), a logical explanation for the observed co-localization with ubiquitin is the sequestering of (poly)ubiquitylated proteins in general. However, this is difficult to rationalize with the absence of HR23B in ubiquitin-positive NFTs in neurons from AD patients, in spite of the notion that expression levels appeared normal in AD patient brain. Alternatively, a more fundamental role of HR23B in the formation or maintenance of these inclusions can be hypothesized. The accumulation of HR23 proteins in inclusions either as an effect

Table 1

A. Quantification of unscheduled DNA synthesis (UDS) in non-injected, GFP-injected and GFP-polyQ injected cells

	Non-injected	GFP	GFP-polyQ
UDS (grain/nucleus)	58	57	55
SEM	2	2	2
N (number of nuclei)	12	17	56

B. Quantification of UDS in GFP-polyQ injected cells with and without inclusions

	With inclusions	Without inclusions
UDS	57	58
SEM	2	2
N (number of nuclei)	16	12

of a cellular state or more functional in the cause of these structures can be of mechanistic significance. The notion that HR23B is not sequestered in all inclusions is a possible clue for future experiments to unravel these mechanisms. Next to the presence of HR23B in, ubiquitin positive, nuclear neuronal inclusions, we observed HR23B positive cytoplasmic structures in pontine neurons of an SCA3 patient. These structures did not stain positive with our ubiquitin antibodies. Although we do not know the origin or the function of these structures, it might be that these HR23B positive cytoplasmic inclusions are a prelude in the formation of the more familiar nuclear inclusions associated with SCA3. Moreover, besides the clear accumulation of HR23 proteins in the inclusions, we observed a general increase in the HR23B level in cells displaying inclusions as well. Although no correlation between HR23B protein levels and proteasomal activity *in vivo* has been made, this suggests that the UPS is indeed impaired. Extreme overexpression of either HR23A or HR23B in living cells is known to inhibit 26S proteasome-mediated breakdown (Ortolan et al., 2000; Raasi and Pickart, 2003). High levels of HR23A or HR23B might mask the polyubiquitylated substrates from the perturbed function of proteasomes, thereby relieving part of the proteasomal pressure. If so, it might be beneficial to alter HR23A or HR23B levels in target tissues. Future research monitoring the effect of either a lack or a surplus of HR23 proteins on inclusion formation is essential to understand the role of HR23A and HR23B in inclusion formation. Due to the complexity of the formation of these structures in time, it is essential to study the mechanism in an *in vivo* model. *In vitro* systems bypass the time factor and are mostly performed in an inaccurate cellular system of a pathologically irrelevant target tissue.

Polyglutamine-induced aggregates do not hamper NER

Interestingly, no difference in the repair capacity of cells microinjected with a cDNA encoding a polyglutamine repeat was observed. The construct used here is known to cause inclusion formation in time. Indeed, we could demonstrate the presence of HR23B-positive inclusions in some but not all nuclei of the microinjected cells. This is in line with the expectations, since inclusions develop in time, it is therefore hard to predict when an inclusion will be formed. Here, we used a system that forces high expression of the polyglutamine repeats known to cause impairment of the UPS and frequently resulting in inclusion formation (Bence et al., 2001; de Pril et al., 2004). Recently, it has been shown that in ubiquitylation-defective cells NER is suppressed (Wang et al., 2005). Ubiquitylation was inhibited by using temperature sensitive E1 or chemicals that block the 26S proteasome. Inhibition of the ubiquitin system has a serious effect on the homeostasis of the cell. This and the notion that several specific ubiquitylation events have a known stimulating effect on NER make the effect of ubiquitylation deficiency on NER not surprising (Hoege et al., 2002; Sugasawa et al., 2005). Here, we show that, despite the proteasomal stress and the presence of HR23B-positive inclusions, NER repair capacity as measured by unscheduled DNA synthesis was normal. This clearly indicates that NER is functionally normal in these cells regardless of the proteasomal stress and the sequestration of HR23B within inclusions. Either the impairment does not influence the homeostasis too much or NER occurs in cells despite of a high stress level. Apparently, there is no connection or influence of neurodegeneration associated inclusion formation and NER despite of the sequestering of one of the NER factors in these structures. In line with the uncoupling of DNA repair and polyglutamine induced proteasomal stress is the notion that polyglutamine diseases are in general not correlated with elevated cancer risks, which are associated with hampered GG-NER.

Complementary to ubiquitin-positive inclusions, we report here that the presence of neuronal HR23B-positive inclusions in specific brain regions is a new neuropathological hallmark of several neurodegenerative diseases, including FTDP-17, HD, PD, SCA3 and SCA7. Interestingly, HR23B did not accumulate detectably in neurofibrillary tangles from an AD patient, whereas ubiquitin did, suggesting disease specificity. Although HR23B is associated with DNA repair, we demonstrate that the NER repair capacity in living cells with impaired UPS is unaffected. In conclusion, HR23B seems to have a dual cellular function, that is, a specific role in NER and a role in the ubiquitin–proteasome degradation pathway. Furthermore, our results indicate that both cellular functions are operational independently.

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