Mutant ubiquitin and the proteasome in Alzheimer's disease

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Critical levels of Alzheimer associated mutant ubiquitin cause a shift from substrate to inhibitor of the proteasome

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Submitted
ABSTRACT

UBB*+1 is a mutant ubiquitin that accumulates in the pathological hallmarks of Alzheimer’s disease. Besides being an efficient proteasome substrate, UBB*+1 can also act as a potent inhibitor. To explain this paradox, we hypothesized that UBB*+1 contributes to proteasome inhibition only after exceeding a threshold of accumulation. This hypothesis was studied in organotypic cortex cultures of mice. A GFP-based proteasome substrate, UbG7V-GFP, was used to monitor proteasome activity. Like UbG7V-GFP, UBB*+1 was efficiently degraded in cortex cultures after lentiviral transduction, and only accumulated after additional proteasome inhibition. After washing out the reversible inhibitor MG132, proteasome activity was restored, as demonstrated by regained capacity to degrade UbG7V-GFP. UBB*+1 however, remained accumulated in many cells, corroborating our hypothesis. In cortex cultures of UbG7V-GFP transgenic mice it was demonstrated that accumulated UBB*+1 inhibits the proteasome in this system. These results indicate that accumulated UBB*+1 in Alzheimer brain may have reached a critical threshold from which UBB*+1 can contribute to proteasome inhibition and neurodegeneration.
INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting an increasingly large group of elderly (Cummings et al., 1999; Selkoe, 2001). In the last few years, evidence has accumulated that supports the premise that the ubiquitin proteasome system (UPS) plays a role in many neurodegenerative diseases, including AD (Ciechanover and Brundin, 2003).

The UPS normally is responsible for the majority of protein degradation in the cell. Ubiquitin (Ub) tags proteins for degradation through a complex enzymatic machinery, consisting of Ub activating (E1), Ub conjugating (E2) and Ub ligating (E3) enzymes. Through this pathway, isopeptide bonds are formed between the C-terminal Gly residue of Ub and the ε-amino groups of Lys residues in proteins. By the sequential addition of mono-Ub to a Lys residue of substrate-bound Ub, a polyubiquitin tree is formed, which targets the protein for degradation by the 26S proteasome - for a review see (Glickman and Ciechanover, 2002). The proteasome is a large enzymatic complex, consisting of a 20S core, which can be flanked by several regulatory particles, such as 19S (Ferrell et al., 2000). The 20S and 19S particles together form the 26S proteasome, which is mainly responsible for the degradation of polyubiquitinated proteins.

UBB$^{+1}$ is a mutant form of Ub which is the result of a dinucleotide deletion in the mRNA of the ubiquitin-B gene (Van Leeuwen et al., 1998b). UBB$^{+1}$ accumulates in the neuropathological hallmarks of AD and other neurodegenerative diseases, with the exception of synucleinopathies (De Pril et al., 2004; Fischer et al., 2003). UBB$^{+1}$ has paradoxical properties; on the one hand it acts as a ubiquitin-fusion-degradation (UFD) substrate for the proteasome, on the other hand however, it is a specific inhibitor of the proteasome when expressed at high levels (Lindsten et al., 2002) and eventually causes apoptotic-like cell death (De Vrij et al., 2001). We hypothesized that high levels of UBB$^{+1}$ accumulation will cause a shift in UBB$^{+1}$ properties from proteasome substrate to proteasome inhibitor after exceeding a threshold.

The balance between substrate and inhibitor properties of UBB$^{+1}$ was found to be highly variable between different systems (De Vrij et al., 2001; Fischer et al., 2003; Lindsten et al., 2002). In rat brain it was shown that injected lentivirus encoding UBB$^{+1}$ intriguingly did not lead to accumulation of the protein (Fischer et al., 2003), in contrast
to cell line studies. Therefore, we chose to study our threshold hypothesis in a system relevant to the situation in the human brain, where UBB⁺¹ is most likely degraded in young control individuals and only accumulates in certain specific neurodegenerative diseases (De Pril et al., 2004; Fischer et al., 2003). We made use of organotypic cortex slice cultures of mice, which represent an elegant system to study brain cells in their original context.

Transgenic mice expressing a previously characterized GFP-based proteasome reporter, Ub


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-GFP (Dantuma et al., 2000b; Lindsten et al., 2003; Neefjes and Dantuma, 2004), were used to validate the ability to manipulate proteasome activity in our organotypic cortex culture system. The proteasome reporter carries a UFD-signal consisting of an uncleavable Ub moiety that is target for polyubiquitination and subsequent degradation by the proteasome, comparable to UBB⁺¹ (Lindsten et al., 2002). Therefore, cells that express this proteasome reporter will only accumulate GFP when proteasomal degradation is inhibited. For a direct comparison of this reporter substrate with lentivirally induced UBB⁺¹, organotypical cortex slice cultures of wild type C57BL/6 mice were transduced with lentiviral vectors encoding either Ub


-GFP or UBB⁺¹. The results of using a reversible proteasome inhibitor that allows recovery of proteasome activity after strong inhibition supported our threshold hypothesis for UBB⁺¹. Quantification of this effect was performed in human neuroblastoma cells.

RESULTS

Viral transduction in organotypical mouse cortex cultures

In AD, UBB⁺¹ accumulates mostly in neurons, while in other neurodegenerative diseases, such as progressive supranuclear palsy, UBB⁺¹ also clearly accumulates in glial cells of white matter (Fischer et al., 2003). Organotypical cortex slice cultures form an elegant system to study different types of brain cells in their original context. In this study, C57BL/6 mouse cortex slice cultures were efficiently transduced with viral vectors encoding
several proteins of interest. Transduction of wildtype cortex slice cultures with lentivirus (LV) encoding Ub-M-GFP - a control fusion protein resulting in a stable form of GFP (Dantuma et al., 2000b) - resulted in many immunopositive cells (Fig.1) two days after transduction. The population of cells that was transduced consisted mainly of astrocytes, although also neuronal cells were observed, as demonstrated by NeuN/GFAP double staining in LV-Ub-M-GFP transduced cultures (Fig.2).

Fig. 1 Successful lentiviral transduction of organotypic mouse cortex slice cultures. Wild type organotypic mouse cortex slice cultures were transduced with LV encoding the stable control GFP fusion protein Ub-M-GFP. GFP stained with αGFP/Cy2. See Color figures.

Fig. 2 Lentivirus targets a heterogeneous cell population in cortex slice cultures. NeuN (blue) and GFAP (red) double staining on LV-Ub-M-GFP transduced cultures revealed mostly GFAP labeled GFP positive cells, but also NeuN positive neurons. Arrows indicate transduced NeuN positive neurons. See Color figures.
The proteasome reporter system in mouse cortex slice cultures

To verify if the proteasome reporter system works in our culture system, organotypic cortex slices of Ub676V-GFP/2 transgenic mice (Lindsten et al., 2003) were cultured and subsequently treated with the proteasome inhibitor epoxomicin. GFP staining confirmed that the reporter only accumulated in the cortex slice cultures after inhibitor treatment (Fig.3).

In order to compare lentivirally induced UBB+1 to Ub676V-GFP, also lentiviral vectors of Ub676V-GFP were produced and applied to cortex cultures of wild type mice. Lentiviral transduction of the proteasome reporter only resulted in accumulation of Ub676V-GFP after treatment of the cultures with epoxomicin (Fig.4). Lentiviral transduction of wildtype cortex slice cultures with UBB+1 intriguingly also did not lead to accumulation of the protein (Fig.4), in contrast to lentiviral transduction of cell lines (Lindsten et al., 2002). Apparently, UBB+1 was very efficiently degraded in cortex slice cultures, as demonstrated by the accumulation of UBB+1 after proteasome inhibition by epoxomicin (Fig.4). The lysine-mutant of UBB+1, UBB+1K29,R33R, is not a target for ubiquitination (Lindsten et al., 2002) and therefore accumulated in transduced cortex cultures regardless of proteasome inhibition, as expected (Fig.4).

Fig.3 The proteasome reporter system in cortex cultures of Ub676V-GFP transgenic mice. A Ub676V-GFP transgenic cortex cultures without treatment. B Ub676V-GFP transgenic cortex cultures treated with 1 µM epoxomicin. The reporter substrate only accumulates after proteasome inhibition. See Color figures.

UBB+1 threshold in mouse cortex cultures

To test our threshold hypothesis for UBB+1 we made use of the reversible proteasome inhibitor MG132 that allows reactivation of the proteasome after strong inhibition. Applying MG132 to
Fig. 4 UBB+1 is degraded by the proteasome in cortex cultures. Both the reporter protein UbG76V-GFP (green) and UBB+1 (red) accumulate after treatment with 1 μM epoxomicin overnight. The lysine mutant of UBB+1 also accumulates without inhibitor treatment. See Color figures.

lentivirally transduced cultures for 16 hours resulted in strong accumulation of both UbG76V-GFP and UBB+1 (Fig. 5). When MG132 treated cultures were rinsed and allowed to recover, proteasome activity was restored, as demonstrated by the regained capacity to completely degrade the GFP reporter protein (Fig. 5). Nevertheless, this restored proteasome activity was not capable of degrading the accumulated UBB+1 in all cells (Fig. 5). The number of cells containing accumulated UBB+1 after washout of the inhibitor was increased compared to the number of UBB+1 positive cells after initial transduction. These experiments indicate that although UBB+1 is processed more efficiently in mouse cortex cultures than it is in human neuroblastoma cells, UBB+1 is less efficiently degraded than UbG76V-GFP, which can be due to the fact that it inhibits the proteasome system. Intrinsically, the ability of UBB+1 to inhibit its own degradation in this system, does not prove that it also inhibits the proteasome in general, like we found in cell lines (Lindsten et al., 2002). Therefore, we also transduced cortex cultures of UbG76V-
GFP transgenic mice to study if accumulated UBB+1 also results in accumulation of the reporter in this system. However, similar to wild type cortex slice cultures, lentiviral UBB+1 transduction did not lead to UBB+1 accumulation unless additional proteasome inhibitors were applied. This treatment would certainly lead to GFP accumulation in the transgenic cultures regardless of UBB+1 expression, making it impossible to distinguish proteasome inhibition by UBB+1. Therefore, we found a way to induce higher expression levels of UBB+1, which, according to our threshold hypothesis, might trigger UBB+1 accumulation by itself. Such high expression levels of UBB+1 were achieved by making use of adenoviral in stead of lentiviral transduction of UBB+1. We confirmed the increased expression of adenoviral compared to lentiviral UBB+1 by transducing 293 cells with equal multiplicities of infection of either LV-UBB+1 or Ad-UBB+1 and comparing protein levels on western blot - performed as described in (De Vrij et al., 2001). Adenoviral transduction resulted in 4- to 5-fold expression compared to lentiviral transduction (Fig.6A). Transduction of UbG76V-GFP transgenic cortex cultures with Ad-UBB+1 indeed resulted in accumulation of UBB+1 in many cells.

Fig.5 The threshold effect of UBB+ accumulation in cortex cultures. 16 hour incubation of transduced cultures with the reversible inhibitor MG132 results in accumulation of both UBB+1 and UbG76V-GFP. Washing out the reversible inhibitor reactivates the proteasome, as shown by the degradation of UbG76V-GFP. UBB+1 however, remains accumulated in a considerable amount of cells. See Color figures.
(Fig. 6C), in contrast to lentiviral transduction (Fig. 6B). Most cells that were positive for UBB*+1 clearly accumulated the GFP reporter (Fig. 6D), indicating that UBB*+1 is capable of inducing proteasome inhibition in this system.

The threshold effect that was observed with lentiviral transduction followed by reversible proteasome inhibition was difficult to quantify in this culture system. Therefore, experiments in human neuroblastoma cells were designed to quantify the threshold hypothesis for UBB*+1 accumulation.

**Fig. 6** High Ad-UBB*+1 expression surpasses the threshold level and causes accumulation of UBB*+1-GFP. Increased expression of UBB*+1 with adenovirus compared to lentivirus, leads to accumulation of UBB*+1 without inhibitor treatment. A. Representative western blot of lysates of 293 cells transduced with equal MOI of LV-UBB*+1 (lane 1) or Ad-UBB*+1 (lane 2). Equal amounts of protein were loaded per lane, as confirmed by Coomassie staining of total protein load of the same lanes shown on the right. The blot was stained with Ubi3 and quantified with Imagepro software.

Organotypic cortex slice cultures of UbG76V-GFP transgenic mice were transduced with B LV-UBB*+1, or C Ad-UBB*+1. LV-UBB*+1 transduction did not induce accumulation of UBB*+1, while Ad-UBB*+1 did result in many UBB*+1 immunopositive cells. D UBB*+1 accumulation after adenoviral transduction leads to accumulation of UbG76V-GFP (arrows). See Color figures.

**UBB*+1 threshold in human neuroblastoma cells**

Human neuroblastoma cells were transduced with lentiviral vectors encoding UBB*+1, UBB*+1K29R,K358R or UbG76V-GFP. Three days after transduction cells were treated with the irreversible proteasome inhibitor epoxomicin or the reversible inhibitor MG132. Inhibitors were left on for 16 hours or washed out after five hours of treatment and grown for 16 hours in normal medium. All conditions were fixed at the same time (Fig. 7).
The lysine mutant of UBB⁺¹, UBB⁺¹K29,R38 is not degraded by the proteasome and therefore accumulated in all transduced cells (36.7% of total cell number) (Fig.7). Without inhibitor treatment UBB⁺¹ was degraded in the majority of transduced cells, but accumulated in a considerable amount of them (8.6% of total, ~25% of transduced cells), as expected (De Vrije et al., 2001; Lindsten et al., 2002). The GFP reporter UbG76V-GFP was completely degraded by the proteasome in virtually all cells (0.9% of total). After treatment with either proteasome inhibitor overnight all three proteins accumulated to about the same levels (~40% of total), demonstrating that transduction levels between the different lentiviral constructs were comparable. Washing out the reversible inhibitor MG132 led to almost complete remission of the accumulation of UbG76V-GFP (1.7% of total). UBB⁺¹ however, remained significantly more accumulated (17.2% of total, p=0.007) in this condition than in the control situation (8.6% of total) (Fig.7). These results show that proteasome inhibition by UBB⁺¹ indeed seems subject to a threshold level of UBB⁺¹ accumulation. Accumulated UBB⁺¹ was capable of sustaining proteasome inhibition by itself in cells that were able to degrade UBB⁺¹ before treatment with the reversible inhibitor.

![Fig. 7 Quantification of the threshold effect of UBB⁺¹ in human neuroblastoma cells. Reversible proteasome inhibition in lentivirally transduced SH-SY5Y cells. No virus (contr), LV-UBB⁺¹ (UBB⁺¹), LV-UBB⁺¹K29,R38 (Lys) and LV-UbG76V-GFP conditions were treated with no inhibitor (c), MG132 overnight (o/n) or MG132 for 6 hours, which was allowed to wash out overnight (MG132 w) and the same treatments with the irreversible inhibitor epoxomicin. The reporter protein UbG76V-GFP was almost completely degraded in cells after washout of the inhibitor (1.7% accumulation), while UBB⁺¹ remained significantly more accumulated (17.2%, p=0.007, asterisk) than in the control situation. Results of two representative experiments, each performed in duplo.](image-url)
UBB⁺¹ has paradoxical properties as both a substrate and an inhibitor of the proteasome. The differences in UBB⁺¹ processing between cell lines, cortex slice cultures and in vivo experiments, further complicate the understanding of the mechanisms of action of UBB⁺¹. These differences can be partly explained by the threshold hypothesis as shown in this study. In mouse cortex slice cultures, UBB⁺¹ was found to accumulate in more cells after reversed proteasome inhibition than in the control situation and compared to the UFD reporter protein. This effect was quantified in neuroblastoma cells and found to be significant. An interesting issue that remains to be resolved is why UBB⁺¹ accumulates relatively easy in human neuroblastoma, HeLa or 293 cells after lentiviral transduction, but is degraded in virtually all cells in mouse cortex slice cultures transduced with the same virus. This effect was also observed earlier in lentiviral UBB⁺¹ injection in rat hippocampus, which also showed degradation of UBB⁺¹ (Fischer et al., 2003). Human to murine species differences between the cortex cultures and neuroblastoma cell lines were ruled out as a cause for this discrepancy as human SH-SY5Y and rat N2a neuroblastoma cells did not show different numbers of UBB⁺¹ positive cells after lentiviral transduction (results not shown). A more probable explanation could be a difference in transduction efficiency of lentivirus in cell lines versus cortex cultures. In earlier reports, it has also been described that higher expression levels are obtained in transfection of tumorigenic cells versus normal cells (Wadia et al., 2004). In contrast to lentiviral UBB⁺¹, adenoviral induced UBB⁺¹ expression leads to accumulation of the protein not only in cell lines, but also in cortex slices. Adenoviral expression is stronger than lentiviral expression, as we demonstrate in this study in cell lines. In cortex slice cultures, this increased expression of UBB⁺¹ apparently surpasses the threshold that lentiviral expression does not reach in this system. Alternatively, the difference in accumulation of UBB⁺¹ in cortex slice cultures transduced with lentivirus compared to adenovirus could also reflect an influence of certain viral proteins expressed by the adenoviral vector or a difference in tropism of the viruses. In the latter case, adenoviral transduction would target a subpopulation of cells that is more vulnerable to proteasome inhibition than lentivirally transduced cells are. It is known from earlier reports that lentivirus is more capable of transducing neurons than adenovirus is (Ehrengruber et
al., 2001). Besides this difference, in which lentivirus only seems to have a broader tropism than adenovirus, it is not known if adenovirus could transduce an entirely different cell population than lentivirus. In our cultures, no obvious difference was observed between cells transduced with lentivirus or with adenovirus. Therefore, we believe that the higher expression levels of adenovirally transduced UBB⁺¹ compared to lentiviral transduction are responsible for the difference in UBB⁻¹ accumulation.

In AD brain, proteasomal inhibition is found in affected brain areas (Keck et al., 2003; Keller et al., 2000a). The threshold for UBB⁺¹ accumulation is clearly reached in AD brain, as UBB⁺¹ immunoreactivity is highly associated with the pathological hallmarks of AD. However, since UBB⁺¹ normally is an efficient substrate for the proteasome, it is not likely that UBB⁺¹ accumulation forms the initial trigger for proteasome inhibition in AD. Other AD-related mechanisms probably precede UBB⁺¹ accumulation and are more prone to elicit inhibition of the proteasome. UBB⁺¹ will then serve as an endogenous reporter of proteasome inhibition, as suggested earlier (Fischer et al., 2003). However, besides this reporter function, we now show that UBB⁺¹ will most likely contribute to proteasome inhibition in the cells in which it accumulates. PHF-tau, Aβ and oxidative stress have all been implicated to play a role in proteasome inhibition (Gregori et al., 1995; Keck et al., 2003; Keller et al., 2000b; Zhu et al., 2004a) and therefore might form the initial trigger for proteasome inhibition and UBB⁺¹ accumulation in AD. Recently, an interesting link was demonstrated between Aβ mediated toxicity and proteasome activity, through the E2-25K enzyme and UBB⁺¹ (Song et al., 2003).

In summary, the current study indicates that UBB⁺¹ properties shift from proteasome substrate to inhibitor after a critical level of accumulation is reached. It is notable that the experiments are performed in a physiologically relevant setting of organotypic cortex slice cultures, in which sub threshold levels UBB⁺¹ are efficiently degraded. In our view, this situation is comparable to AD neuropathology, in which UBB⁺¹ probably is efficiently degraded until the threshold is finally reached after a gradual increase in UBB⁺¹ levels due to proteasome inhibition by other AD-related mechanisms. The present study indicates that once this threshold is reached, UBB⁺¹ will act as a potent inhibitor of the proteasome. Therefore, we hypothesize that UBB⁺¹ is involved in neurodegeneration in AD.
by contributing to proteasome inhibition once a threshold of UBB⁺¹ accumulation is reached.

**MATERIALS AND METHODS**

Organotypic cortex slice cultures

Organotypic cortex slice cultures were made from five day old C57BL/6 wild type or C57BL/6 transgenic Ubᶜ⁷⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻{-
were used in dilutions of 1:4000, 1:500 and 1:400 respectively, followed by Cy2 and Cy3 staining (1:800). Nuclei were visualised with TO-PRO (1:1000, Molecular Probes). Subsequently, slices were mounted in mowiol (0.1M Tris-HCl pH8.5, 25% glycerol, 10% w/v mowiol 4-88) and analyzed with confocal laser scanning microscopy (Zeiss 510).

**Viral constructs**

First generation recombinant adenoviral vectors AdUBB\(^{+1}\) and AdwtUb were generated, purified and titereed as described elsewhere (De Vrij et al., 2001; Hermens et al., 1997). Adenoviral vectors were based on the Ad5 mutant dl309 (Jones and Shenk, 1979) and employed the cytomegalovirus immediate early (CMV) promoter to drive transgene expression. Titration of double CsCl gradient-purified Ad-CMV-UBB\(^{+1}\) and Ad-CMV-wtUb on the permissive cell line 911 (Fallaux et al., 1996) revealed titres of 1×10\(^{9}\) plaque forming units/ml.

DNA encoding GFP, Ub-M-GFP, Ub\(^{G76^{Wh}}\)-GFP, UBB\(^{+1}\) or the control lysine mutant of UBB\(^{+1}\), UBB\(^{1K294^{Wh}}\), was cloned into the lentiviral transfer plasmid pRRLsin-ppThCMV. Lentivirus was produced according to Naldini et al (Naldini et al., 1996a; Naldini et al., 1996b) by co-transfecting the transfer lentiviral plasmid, the VSV-G envelope plasmid (pMD.G.2) and the packaging plasmid (pCMVΔR8.74) into 293 T human kidney cells using a calcium phosphate method. After two days the lentivirus (LV) was harvested, filtered and further concentrated by ultra-centrifugation at 20000 rpm at 16°C for 2.5 h. The virus was then resuspended in phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH\(_2\)PO\(_4\), 1.44 g/l Na\(_2\)HPO\(_4\); pH 7.4) + 0.5% BSA (Sigma). Virus titres were determined with an HIV-1 p24 core profile ELISA kit (Perkin Elmer Life Sciences, USA) and correlated to titres determined by counting GFP fluorescent cells of an LV-Ub-M-GFP stock. In this way titres of adenoviral and lentiviral stocks could be correlated.

**Cell lines**

SH-SY5Y human neuroblastoma cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco) containing 10% foetal
calf serum (FCS) (Gibco) and supplemented with 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco) (DMEM-10% FCS). Cells were cultured on 0.2% gelatin coated glass coverslips in 24-wells plates (Nunc, Denmark) one day prior to lentiviral transduction. The next day the cells were transduced with lentiviral vectors with a multiplicity of infection (MOI) of 10. Medium containing the lentiviral vectors was left on overnight, after which medium was replenished with culture medium containing 4 µM all-trans retinoic acid (Sigma).

After transduction cells were fixed in 4% formalin in phosphate-buffered saline (PBS), pH 7.4 for one hour or longer. In between staining steps cells were rinsed with PBS, pH 7.4. Rabbit polyclonal ubi3 (anti-UBB+I) was used in a dilution of 1:1000 overnight in Supermix. The secondary anti-rabbit Cy3 antibody was diluted 1:200 and nuclei of cells were stained with TO-PRO-3 (Molecular Probes, 1:1000). Coverslips were mounted in mowiol. Images were acquired by confocal laser scanning microscopy (Zeiss 510) with three different lasers emitting at 488, 543 and 633 nm to excite GFP, Cy3 and TO-PRO-3, respectively. Cells were quantified by hand with ImagePro software (Media Cybernetics, Silver Springs, MD). For each coverslip, a Cy3 image and the corresponding TO-PRO image were acquired in five fields. The experiments were performed in duplicate. The total number of cells was quantified by counting the nuclei. The number of transduced cells was quantified by counting the immunopositive cells in the Cy3 or GFP images. Statistics was performed by applying single-factor ANOVA between groups, based on α=0.05.

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