Mutant ubiquitin and the proteasome in Alzheimer's disease

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Fig. 3 UBB* is a UFD substrate. A Western blot analysis with an anti-UBB* antibody of cell lysates from HeLa cells transfected with UBB*, UBB*<sup>−1</sup>, UBB*<sup>−1/K29R</sup>, UBB*<sup>−1/K48R</sup>, UBB*<sup>−1/K29,48R</sup>. Products corresponding to unmodified and ubiquitinated UBB* are indicated. B Micrographs of HeLa cells transfected with pCMS-UBB*<sup>−1</sup>/GFP that were left untreated (top) or incubated for 16 h with 30 μM lactacystin (bottom). Transfected cells were identified by GFP expression (left) and transfected cells expressing detectable levels of UBB* were visualized by immunostaining (right). Bars, 100 μm. C Western blot analysis with an anti-UBB* antibody of the steady-state levels of UBB*<sup>−1</sup> and UBB*<sup>−1/K29,48R</sup> in transiently transfected HeLa cells that were left untreated or incubated the proteasome inhibitors lactacystin (30 μM) or epoxomicin (500 nM). D The turnover of UBB*<sup>−1</sup> was determined by pulse-chase analysis in SK-N-SH neuroblastoma cells transduced with lent-UBB*<sup>−1</sup>. Intensity of the UBB* band was quantified with a phosphoimage r and the intensity at time point 0 was standardized as 100%. (A-D) One representative experiment out of three.
Fig. 4 Inhibitory activity of UBB⁺¹ requires ubiquitination at Lys29 and Lys48. (A) Micrographs of Ub⁷₆⁶-V, GFP (left) and Ub-R-GFP SH-SY5Y cells (right) transfected with UBB⁺¹, UBB⁺¹K₂⁹R, UBB⁺¹K₄⁸R, or UBB⁺¹K₂⁹,₄⁸R. The cells were stained for UBB⁺¹ (left) and analyzed for GFP fluorescence (right). Bars, 100 μm. (B) Quantification of three independent experiments as shown in A. The results are expressed as the percent of the UBB⁺¹ expressing cells with accumulated Ub⁷₆⁶-V-GFP or Ub-R-GFP levels.
Fig. 7 Overexpression of other proteasome substrates does not inhibit turnover of Ub\textsuperscript{G76V}-GFP.

Ub\textsuperscript{G76V}-GFP HeLa cells were transiently transfected with UBB\textsuperscript{1+}, FLAG\textsuperscript{Ub\textsuperscript{G76V}-nfGFP}, FLAG\textsuperscript{Ub-R-nfGFP} and FLAG\textsuperscript{p53}. UBB\textsuperscript{1+} transfected cells were stained with the anti-UBB\textsuperscript{1+} antibody while the nonfluorescent FLAG\textsuperscript{Ub\textsuperscript{G76V}-nfGFP} and FLAG\textsuperscript{Ub-R-nfGFP} constructs and FLAG\textsuperscript{p53} were stained with a FLAG-specific antibody. Representative micrographs of the immunostaining (left, red), the Ub\textsuperscript{G76V}-GFP fluorescence (middle, green), and the Hoechst 33258 counterstaining (right, blue) are shown. Note that as expected the FLAG\textsuperscript{Ub\textsuperscript{G76V}-nfGFP} and FLAG\textsuperscript{Ub-R-nfGFP} give a homogenous staining in the cytosol and nucleus, whereas FLAG\textsuperscript{p53} is localized in the nucleus. To the left are shown flow cytometric analysis of the GFP fluorescence upon transfection with the different constructs.

Chapter 4

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.
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.

Fig. 3 The proteasome reporter system in cortex cultures of Ub<sup>G76V</sup>-GFP transgenic mice. A Ub<sup>G76V</sup>-GFP transgenic cortex cultures without treatment. B Ub<sup>G76V</sup>-GFP transgenic cortex cultures treated with 1 μM epoxomicin. The reporter substrate only accumulates after proteasome inhibition.
Fig. 4 UBB$^{*+}$ is degraded by the proteasome in cortex cultures. Both the reporter protein Ub$^{G76V}$-GFP (green) and UBB$^{*+}$ (red) accumulate after treatment with 1 μM epoxomicin overnight. The lysine mutant of UBB$^{*+}$ also accumulates without inhibitor treatment.

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$^{*+}$ and Ub$^{G76V}$-GFP. Washing out the reversible inhibitor reactivates the proteasome, as shown by the degradation of Ub$^{G76V}$-GFP. UBB$^{*+}$ however, remains accumulated in a considerable amount of cells.
Fig. 6 High Ad-UBB\textsuperscript{+1} expression surpasses the threshold level and causes accumulation of Ub\textsuperscript{GFP}. Increased expression of UBB\textsuperscript{+} with adenovirus compared to lentivirus, leads to accumulation of UBB\textsuperscript{+} without inhibitor treatment. A Representative western blot of lysates of 293 cells transduced with equal MOI of LV-UBB\textsuperscript{+} (lane 1) or Ad-UBB\textsuperscript{+} (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 Ub\textsuperscript{+} and quantified with Imagepro software. Organotypic cortex slice cultures of Ub\textsuperscript{GFP}-GFP transgenic mice were transduced with B LV-UBB\textsuperscript{+}, or C Ad-UBB\textsuperscript{+}. LV-UBB\textsuperscript{+} transduction did not induce accumulation of UBB\textsuperscript{+}, while Ad-UBB\textsuperscript{+} did result in many UBB\textsuperscript{+} immunopositive cells. D UBB\textsuperscript{+} accumulation after adenoviral transduction leads to accumulation of Ub\textsuperscript{GFP} (arrows).

Fig. 4 Expression of HA-Ub-\textalpha\textbeta in cell lines. 293T were transduced with lentiviral vectors encoding HA-Ub-A\textalpha\textbeta-40, 42 or scr and stained for HA-tag (anti-HA) or A\textbeta (6E10) two days later. The amount of HA-tag positive cells indicates high transduction efficiency. In contrast, only very few cells are positive for A\textalpha\textbeta-40 of A\textalpha\textbeta-42, suggesting that A\textbeta is degraded after cleavage of the fusion proteins. A\textbeta scr is not recognized by 6E10, corresponding to the lack of 6E10 immunopositive cells in the HA-Ub-A\textbeta scr transduced cells.
**Chapter 6**

**Fig. 22** Successful transduction of human post mortem cortex culture with LV-GFP. Within a maximum of 8 h postmortem delay, 250 μm slices of frontotemporal cortex were made with a tissue chopper and put into culture free-floating in medium, which consisted of Neurobasal A medium (Gibco), complemented with 0.1 mg/ml vitamin C, B27 supplement (Gibco), 10 U/ml penicillin and 10 μg/ml streptomycin (Gibco). Lentiviral vectors were applied by dropping a 20 μl drop of medium containing 10⁶ transducing viral particles on top of a dry slice, which was replenished with medium after five minutes. At several different times after transduction, slices were fixed for 1 h in 4% formalin, after which they were stained free-floating as described previously for cortex slices of mice (Chapter 4).

**Fig. 2** Intra cellular Aβ does not inhibit the proteasome. Lentiviral transduction with LV-HA-Ub-AB1-42 of proteasome reporter cells expressing Ub<sup>G76V</sup>-GFP did not lead to accumulation of the GFP reporter after 1-4 days after transduction. As a control, 5 h epoxomicin treatment of these cells resulted in strong GFP reporter accumulation. LV-HA-Ub-AB1-40, LV-HA-Ub-ABscr and pcDNA3-SP-AB42 expression also did not result in accumulation of the reporter (not shown).

**Fig. 3** Transduction of human post mortem cortex cultures with LV-UBB<sup>11</sup> results in accumulation of UBB<sup>11</sup> in large cells. Ubi3 staining for UBB<sup>11</sup> (red), TO-PRO staining for nuclei (blue).
Fig. 4 Proteasome activity is functional in one pilot experiment of human post mortem cortex cultures. UBB\(^{+}\) accumulation seems to be highly toxic in these cells. Human post mortem cortex cultures were transduced with LV-UBB\(^{+}\) or LV-\(\text{Ub}^{\text{G76V}}\)-GFP and subsequently treated with 1 \(\mu\)M epoxomicin. \(\text{Ub}^{\text{G76V}}\)-GFP (green) accumulated in 17 cells total in 2 slices, as opposed to 3 cells in the control situation. The amount of UBB\(^{+}\) accumulating cells (red) decreased after epoxomicin treatment and they had a less healthy appearance than in the control situation.

Fig. 5 No concentration-dependent difference between cell types in vulnerability to proteasome inhibition. Transgenic \(\text{Ub}^{\text{G76V}}\)-GFP mouse cortex cultures were treated with epoxomicin in concentrations of A 100 nM, B 500 nM, C 1 \(\mu\)M. Cultures were stained for the neuronal marker NeuN (red), TO-PRO (blue) and GFP (green).

Fig. 6 Injection of A\(\beta\) fusion protein lentiviral vectors in rat hippocampus results in very few positive cells that have altered morphology. Adult Wistar rats were injected with 8x10\(^5\) TU LV-HA-Ub-A\(\beta\)1-40 or LV-HA-Ub-A\(\beta\)1-42 in 0.9 \(\mu\)l saline solution (0.2 \(\mu\)l/min) and coinjected with 0.1 \(\mu\)l with 8x10\(^5\) TU LV-GFP. After perfusion, 50 \(\mu\)m thick coronal vibratome sections were stained for anti-HA (red), GFP (green) and TO-PRO (blue).
Fig. 7 Transduction of organotypical mouse cortex slice cultures with lentiviral vectors encoding Aβ fusion proteins. A) anti-HA staining of LV-HA-Ub-Aβ1-42 transduced cortex slice. B) 6E10 positive cell in LV-HA-Ub-Aβ1-42 transduced slice. Many HA-tag positive cells were observed, in contrast to LV-injections in rat hippocampus. However, only very few Aβ positive (6E10) cells were observed, of which some seemed to form aggregates.

Fig. 8 No differences in transduction efficiency or morphology between LV: and LV:P301L cells. SH-SY5Y cells were transduced with LV: or LV:P301L and subsequently stained with tau5a6 antibody (Developmental studies Hybridoma Bank, red). Treatment of transduced cells with 100 nM epoxomicin overnight, resulted in a slight increase in immunopositive cells.

Fig. 9 LV:P301L does not lead to proteasome inhibition in Ub^Glu^GFP transgenic mouse organotypic cortex slices. Cortex cultures were transduced with LV:P301L and subsequently stained with tau5a6 (red), GFP (green) antibodies and TO-PRO (blue). τP301L expression did not induce accumulation of the GFP reporter and therefore does not seem to inhibit the proteasome.