Appendix file

Complete suppression of Htt fibrilization and disaggregation of Htt fibrils by a trimeric chaperone complex

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Plasmid cloning

pGEX-6P1-HTTExon1Q_{48}-CyPet, pGEX-6P1-HTTExon1Q_{48}-YPet as well as pGEX-6P1-HTTExon1Q_{48} plasmids were cloned by PCR-amplification of the coding sequence of HTTExon1Q_{48} from pGEX-6P1-HTTExon1Q_{48}, of CyPet cDNA from pBAD33-CyPet-His (Addgene plasmid #14030) and YPet cDNA from pBAD33-YPet-His (Addgene plasmid # 14031). The resulting PCR fragments were cloned into the plasmid pGEX-6P1. pGEX-6P1-GST-HTTExon1Q_{23}-CyPet, pGEX-6P1-GST-HTTExon1Q_{23}-YPet and pGEX-6P1-GST-HTTExon1Q_{75} plasmids were cloned by PCR amplification of the coding sequence of HTTExon1Q_{23} and HTTExon1Q_{75} from pDONR221 HTTExon1Q_{23} and pDONR221 HTTExon1Q_{75}. The resulting PCR products were digested using *EcoR*I and *Xho*I endonucleases and cloned into the plasmid pGEX-6P1-HTTExon1Q_{48} after excision of HTTExon1Q_{48} by *EcoR*I/*Xho*I endonucleases. Plasmids for expression of human HSPA8, Apg2 and DNAJB1 were obtained from the Bukau lab (Nillegoda et al., 2015). Plasmids for expression of *C. elegans dnj-12*, *dnj-13*, *hsp-110* and *hsp-1* were described previously (Nillegoda et al., 2015). *C. elegans dnj-19*, *dnj-24*, *F11F1.1*, *F44E5.4* as well as *C12C8.1* genes were amplified by PCR using complementary DNA preparations from heat-shocked three-day-old animals as a template. The above-mentioned genes were cloned into the protein expression vector pSUMO with a 6×His–Smt3 tag as previously described (Andreasson, Fiaux et al., 2008).

Site-directed mutagenesis in *hsp-1* and *hsp-110*

To create the ATPase point mutants of HSP-1 (D10S and K71E) and HSP-110 (D7S as well as N578Y E581A) we performed site directed mutagenesis using the oligonucleotides: 5'HSP-1_K71E GCCGAACGTCTTATTGGACGC, 3'HSP-1_K71E GCGTCCAATAAGACGTTCGGC, 5'HSP-1_D10S GGAATCAGTTTGGGAACTACC, 3'HSP-1_D10S GGTAGTTCCCAAACTGATTCC, 5'HSP-110_D7S GTTCTTGGATTCAGCATCGG, 3'HSP-110_D7S CCGATGCTGAATCCAAGAAC, 5'HSP-110_N578Y AGGCTGACGCCAAGTACTCGCTCGCGGAGTACGTTTACG 3'HSP-110_N578Y TCCGACTGCGGTTCATGAGCGAGCGCCTCATGCAAATGC, 5'HSP-110_E581A CCAAGGCTGACGCCAAGTACTCGCTCGCGGAGTACGTTTACGAAA, 3'HSP-110_E581A GGTTCCGACTGCGGTTCATGAGCGAGCGCCTCATGCAAATGCTTT. For amplification we used Herculase II Fusion DNA Polymerase (Agilent Technologies) and *Dpn*I (NEB) was used to digest the template plasmid. Mutagenized plasmids were transformed into DH5a *E. coli* cells and then isolated from positive clones (NucleoSpin Plasmid Kit from Macherey-Nagel) and sequenced (Source BioScience) to confirm the mutagenesis.

Protein purification

For expression of the Htt constructs the respective plasmids were transformed into *E. coli* Bl21*de3 cells containing pRARE and expression was induced by addition of 1 mM IPTG. After incubation for 4 hours at 30°C cells were

harvested and resuspended in lysis buffer (50 mM NaH_2PO_4, 5 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, pH 8.0). After cell lysis Triton-X-100 was added to a final concentration of 1% (v/v) and centrifugation was performed at 30.000g (30 min, 4° C). The resulting supernatants were applied to glutathione sepharose beads (Thermo scientific) and incubated at 4°C for 90 min. Washing of the beads was performed with washing buffer (50 mM NaH_2PO_4, 5 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton-X-100, pH 8.0). For elution the beads were incubated with elution buffer (50 mM NaH_2PO_4, 5 mM Tris, 150 mM NaCl, 1 mM EDTA, 20 mM reduced glutathione, pH 8.6) for 30 min at 20° C. Dialysis against storage buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, pH 7.4) was performed for 16 hours at 4°C. All chaperones with the exception of DNJ-19 were purified as described earlier (Nillegoda et al., 2015). For DNJ-19 cells were lysed in in 30 mM Tris-HCl, pH 7.4, 500 mM K-acetate, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail (Roche) and 10% glycerol. After centrifugation at 30.000g (30 min, 4° C), the resulting supernatants were applied to a Ni-NTA matrix (Roth) and incubated for 90 min at 4 ° C. Subsequent washing steps were performed with high-salt buffer (30 mM Tris-HCl, pH 7.4, 1 M K-acetate, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol), followed by a low-salt wash (identical to the high-salt buffer but with 50 mM instead of 1 M K-acetate). Protein elution was performed with 300 mM imidazole in the corresponding low-salt buffer. Dialysis was performed overnight at 4 °C in the presence of 4 µg His-tagged Ulp1 per mg substrate protein for proteolytic cleavage of the 6×His–Smt3 tag. The 6×His–Smt3 tag and His–Ulp1 were

removed by incubating the dialyzed proteins in Ni-NTA matrix for 10 min at 4 °C. Subsequently, the protein was applied to anion exchange chromatography using the Resource Q column (GE Healthcare).

EM sample preparation

Samples were prepared as described for the FRET assay. Concentrations of Htt proteins, chaperones as well as buffers were identical as described above for the FRET assay. At the indicated time points the samples were flash frozen in liquid nitrogen. For disaggregation experiments 3 µM of unlabeled Htt protein was diluted into aggregation buffer (30 mM HEPES-KOH pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) with 14 U PreSP (Roche) per nmol Htt for 24 h at 20°C to allow cleavage of GST domain and subsequent fibril formation. Afterwards, chaperones were added to a final concentration of 1.5 µM Htt protein, 10 µM of Hsp70, 5 µM J-protein and 5 µM HSP-110. Disaggregation was initiated by addition of 5 mM ATP as well as a regenerative system (3 mM PEP + pyruvate kinase).

Suppression of fibrilization by filter retardation analysis

GST-HttExon1Q_{48}-CyPet or GST-HttExon1Q_{75} were centrifuged (50 krpm, 40 min 4°C), the supernatant was then spit into a sample containing chaperones (30 µM HSP-1, 15 µM DNJ-13, 15 µM HSP-110) and a sample without the addition of chaperones in the aggregation buffer (see above) + an ATP regeneration system. The final concentration of GSt-HttExon1$Q_{48/75}$ constructs was 3 µM. The addition of PreSP marked the starting point (t = 0 h) of the

reaction. Samples were then taken at the indicated time points and mixed with SDS (final concentration of 2%) and DTT (final concentration of 50 mM) to stop the reaction. The samples were then spotted onto a cellulose acetate membrane (Millipore) that was pre-equilibrated in TBS containing 2% SDS. The membrane was washed twice with 0.1% SDS buffer (0.1% SDS, 150 mM NaCl, 10 mM Tris pH 8.0), blocked with 5% milk for further incubation with anti-Htt (Abcam) antibody and subsequently incubated with the secondary antibody IRDye 680 (1:10.000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor).

Disaggregation via filter retardation analysis

The GST-HttEXon1Q_{48}-Cypet or GST-HttExon1Q_{75} proteins were first fibrilized for 24 h (Q_{48}) or 48 h (Q_{75}) as described above and then centrifuged (50 krpm, 40 min 4°C) to sediment the aggregates that were subsequently re-suspended in aggregation buffer by sonication. The samples were then split into a chaperone containing sample (30 µM HSP-1, 15 µM DNJ-13, 15 µM HSP-110) and one without the addition of chaperones. Both samples contained 5 mM ATP and the ATP regeneration system. The samples were then incubated at RT for the indicated time points and then immediately mixed with SDS (final concentration of 2%) and DTT (final concentration of 50 mM) to stop the reaction. The subsequent filter retardation analysis was carried out as described above.

Nematode strains used in this study were:

Bristol strain N2 (wild type), P_{unc-54}257cAT3(Q_{45})::YFP (AT3CT(Q_{45})), P_{unc-54}::257cAT3Q63::YFP (AT3CT(Q_{63})), AM140 (rmIs132 (*unc-54*p::Q_{35}::YFP)), AM141 (rmIs133 (*unc-54*p::Q_{40}::YFP)) and P_{unc-54}::YFP. The extra-chromosomal lines (*unc-54p*::Htt513Q_{15}::YFP) and *unc-54p*::Htt513Q_{128}::YFP) were a gift from E.A. Kikis (University of the South, USA) and were integrated using γ-irradiation.

Maintenance of nematodes

Nematodes were grown on NGM plates seeded with *E. coli* OP50 strain at 20°C.

RNA interference

Synchronization of nematodes was carried out as described before (Rampelt et al., 2012). Animals were then placed as L1 larvae onto RNAi plates that were seeded with *E. coli* expressing dsRNAi against *hsp-1*, *dnj-13* or *hsp-110* or the empty vector L4440. The progeny (F1) were placed onto fresh RNAi plates for a sequential RNAi treatment. Animals were then analyzed on day of life by confocal microscopy.

Cell culture and transfection

HEK293 cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum, 1 mM glutamine (Gibco), 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). For overexpression experiments HttExon1Q_{97}-GFP (Juenemann, Schipper-Krom et al., 2013) and human DNAJB1 were used. DNAJB1 cDNA was amplified by PCR and cloned with a 5' *Hind*III site

and a 3' *Xho*I site into pcDNA3.1. Neural precursor cells (NPCs) derived from HD patient fibroblasts were cultured on Matrigel-coated plates in DMEM/F12 with 1x N2 supplement (Gibco), 1x B27 supplement (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 1 mM glutamine (Gibco), 0.05 % bovine serum albumin (Sigma Aldrich) and freshly added 1x Neurobasal (Gibco), 3 µM CHIR99021 (Stemgent), 2 µM SB431542 (Sigma Aldrich) and 10 ng/ml hLIF (Millipore) to preserve the progenitor state. For experiments, cells were transfected with jetPRIME (Polyplus-transfection) according to the manufacturer's instructions. For siRNA mediated knockdown siGENOME SMARTpool DNAJB1 siRNA (M-012735-02), HSPA4 siRNA (M-012636-02) and HSPA8 siRNA (M-017609-01) were purchased from Dharmacon and transfected in a final concentration of 50 nM. Cells were transfected twice with siRNA (24 and 48 hours before analysis). Non-targeting siRNA (Dharmacon siGENOME Non-Targeting siRNA Pool #1 D-001206-13) was used as negative control. After 48 – 72 hours, cells were harvested in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton-X100, supplemented with complete mini protease inhibitor cocktail (Roche)).

Western blot analysis and filter trap assay

Total cell lysates were boiled for 10 min in 1x Laemmli sample loading buffer (350 mM Tris/HCl pH 6.8, 10 % SDS, 30 % glycerol, 6 % β-mercaptoethanol, bromphenol blue) fractionated by SDS-PAGE gel electrophoresis and transferred to a Nitrocellulose membrane (0.45 µm pore size, Schleicher & Schuell). Western blot membranes were blocked with 5 % milk, incubated with

primary antibodies anti-DNAJB1 (1:1000, Proteintech), anti-HSPA8 (1:1000, Proteintech), anti-HSP110 (1:1000, Charles River Laboratories) and anti-β-tubulin (1:1000, Sigma-Aldrich), anti-β-actin (1:1000, Santa Cruz), and anti-GFP (1:1000, Enzo). Membranes were subsequently incubated with the secondary antibody anti-rabbit HRP (1:10.000; ThermoFisher), IRDye 680 or IRDye 800 (1:10.000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor). Filter trap assay was performed with the pellet obtained after high-speed centrifugation of the cell lysate (15 min at 14.000 rpm at 4°C). Pellet with aggregates was re-suspended in benzonase buffer (1 mM $MgCl_2$, 50 mM Tris/HCl pH 8.0) and incubated for 1 hour at 37°C with 125 U Benzonase (Merck). Reaction was stopped by addition of 2x termination buffer (40 mM EDTA, 4% SDS, 100 mM DTT fresh). Samples with 100 µg protein extract were diluted in 2% SDS buffer and filtered through a 0.2 µm pore size cellulose acetate membrane (Schleicher & Schuell), pre-equilibrated in 2% SDS wash buffer (2% SDS, 150 mM NaCl, 10 mM Tris/HCl pH 8.0) and spotted on the membrane in doublets. Membrane was washed twice with 0.1% SDS buffer (0.1% SDS, 150 mM NaCl, 10 mM Tris pH 8.0), blocked with 5% milk for further incubation with anti-ubiquitin antibody (1:100, Sigma-Aldrich), anti-Htt (Abcam), anti-HA (1:1000, Sigma) or anti-GFP antibody (1:1000, Enzo) and subsequently incubated with the secondary antibody IRDye 680 (1:10.000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor).

Primers employed for real-time PCR of NPCs:

ACTB forward 5'-TCAAGATCATTGCTCCTCCTGAG-3'; ACTB reverse 5'-ACATCTGCTGGAAGGTGGACA-3'; VIMENTIN forward 5'-GGAGCTGCAGGAGCTGAATG-3'; VIMENTIN reverse 5'-GACTTGCCTTGGCCCTTGAG-3'; BRN2 forward 5'-CGGCGGATCAAACTGGGATTT-3'; BRN2 reverse 5'-TTGCGCTGCGATCTTGTCTAT-3'.

Quantification of *in vivo* chaperone concentration by Western blotting

Synchronized N2 wild type nematodes were kept on 10 cm NGM plates seeded with OP50 at 20°C until day 4 or day 10 after hatching. Animals were separated from progeny by sedimentation in M9 medium and transferred daily onto new plates. Nematodes were harvested at day 4 or day 10 after hatching by flash freezing in liquid N_2. Nematode pellets were dissolved 1:1 in lysate buffer (20 mM HEPES, 110 mM KAc, 2 mM $MgAc_2$, 100 µM Digitionin, protease inhibitor Complete-Roche) and lysed using a Precellys® Evolution homogenizer at 6500 rpm in four cycles of 10s. Between the cycles, samples were incubated on ice for 5 to10 sec. Lysates were transferred to low-binding reaction tubes and centrifuged two times at 8000 g for 5 min at 4°C. The protein concentration of the lysate supernatants and purified proteins were determined by Bradford assay.

For quantification, 1 µg to 17.5 µg of total protein in lysates as well as dilutions between 5 ng to 120 ng of the respective purified chaperones were separated by SDS-PAGE. As a loading control, total proteins in lysates were stained with Cy5–NHS (GE healthcare) prior to SDS-PAGE as described

elsewhere (Hagner-McWhirter, Laurin et al., 2015). In brief, 0.625 pmol/µl Cy-5-NHS was used for 40 µl samples with 15 min incubation at 4°C. The reaction was stopped by adding 13.33 µl 4x loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 30% glycerol, 0.2% Bromphenol-blue, 100 mM DTT) and subsequent heating for 5 min at 100°C. Purified chaperones for dilution series were dissolved in lysis buffer as well as 2x loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 15% glycerol, 0.1% Bromphenol-blue, 100 mM DTT) and also heated for 5 min at 100°C prior to SDS-PAGE. Proteins were transfer by wet blotting (25 mM Tris pH 8, 192 mM glycine, 20% Methanol) at 100 V for 2 h onto a PVDF membrane (Roti®-Fluoro) on ice. Membranes were blocked in 5% milk/TBST for 1.5 h at room temperature and incubated with the first antibody over night at 4°C (1:5000 for HSP-1/DNJ-19, 1:2500 for DNJ-12/DNJ-13/C12C8.1/F44E5.4/HSP-110 in 3% milk/TBST). After washing with TBST, blots were incubated with goat anti-rabbit-Cy3 antibody and for F44E5.4 with goat anti-guinea pig IgG CF*555 antibody (life technologies and Sigmal Aldrich; 1:10000 in 3% milk/TBST) for 1.5 h at room temperature. Signals were detected after washing using Odyssey® Imaging System (LI-COR) at 700 nm (Cy5) and 600 nm (Cy3). Cy5 signals were quantified using LI-COR software. The Cy3 signals of the chaperones of the nematode samples and purified proteins were quantified using FIJI software. Data were analyzed from three independent experiments.