SUPPLEMENTARY METHODS

Cell cultures

Human SH-SY5Y neuroblastoma cells (A.T.C.C., Manassas, VA) were cultured in DMEM, F-12 Ham with 25 mM HEPES and $NaHCO_3$ (1:1) and supplemented with 10% (v/v) FBS, 1.0 mM glutamine and antibiotics. Cell cultures were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C and grown until they reached 80% confluence for a maximum of 20 passages.

Wild type human embryonic kidney 293 cells (wt HEK293 cells) stably expressing the tetracycline (tet) repressor (Flp-In T-REx HEK-293) were grown in DMEM supplemented with 10% FBS and 1.0% antibiotics, 5μg/ml Blasticidine and 100μg/ml of Zeocin were also added to the cell culture medium to retain the tetracycline repressor and the Flp-In recombination site.

Stable tetracycline-inducible V5-HSP-expressing cell lines - HEK293 cells expressing Hsp70 (HSPA1A) and HEK293 cells expressing Hsp22 (HSPB8) - were generated by co-transfecting them with the respective V5-tagged HSP-encoding pcDNA5/FRT/TO and the Flp recombinase-expressing plasmid pOG44 and selected with 100μg/ml Hygromycin as described before (16,17). The two clones were maintained in culture by adding 5μg/ml Blasticidine and 100μg/ml Hygromycin B to the cell culture medium. To induce HSP expression, tetracycline was added to the cell culture medium at a final concentration of 1μg/ml, 24-48h prior to each experiment. Transgene expression in the stable cell lines was verified by Western blot and immunofluorescence using mouse monoclonal anti-V5 antibodies against the V5-tag (Invitrogen, Eugene, OR). All cell cultures were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C.

MTT reduction assay

Aggregate cytotoxicity was assessed in SH-SY5Y cells seeded in 96-well plates using the MTT assay. Preformed HypF-N oligomers were resuspended in the cell culture medium, incubated for 1 h

at a corresponding monomer concentration of 12 μM in the absence or presence of 2.4 μM Hsp70 and 2.4 μM ATP and then added to SH-SY5Y cells for 24h.

In another set of experiments, 150 μM H_2O_2 was incubated for 1 h in the cell culture medium in the absence or presence of each chaperone (chaperone concentration as described in the *Methods* section) or 100 μM vitamin E and then added to SH-SY5Y cells.

The MTT assay was also carried out in wt HEK293 and in the two chaperone overexpressing clones. Preformed HypF-N oligomers were resuspended in the cell culture medium, incubated for 1 h at a corresponding monomer concentration of 12 μM in the absence or presence of extracellular Hsp70 or αBcr and then added to the three clones for 24h. In all these experiments, the molar ratios of HypF-N:chaperone used, and the analyses performed, were as described in the *Methods* section.

Finally, in another experimental set, preformed oligomers of HypF-N, $A\beta_{1-42}$ and IAPP (12 μM monomer concentration) were incubated in the cell culture medium for 1 h in the absence or presence of different concentration of rabbit polyclonal anti-HypF-N antibodies (Primm, Milan, Italy), monoclonal mouse 6E10 anti-Aβ antibodies (Signet, Dedham, MA) and rabbit polyclonal anti-IAPP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, and then added to SH-SY5Y cells for 24h.

Measurement of intracellular Ca^{2+} and ROS, calcein release and caspase-3 activity and oligomer internalisation

In order to evaluate the time scale of intracellular Ca^{2+} dyshomeostasis, ROS production**,** membrane permeabilisation, caspase-3 activation and oligomer internalisation, preformed HypF-N oligomers (12 μM monomer concentration) were incubated for 5, 15, 30 and 60 min in the absence or presence of αBcr, Hsp70, Clu, Hp or α_2M, and then added to SH-SY5Y cells seeded on glass coverslips for 60 min at 37 °C. To detect intracellular Ca^{2+} and ROS production, cells were then loaded with 10 μM fluo3-AM (Molecular Probes, Eugene, OR) or with 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes), respectively, as described previously (14,15). To assess

membrane integrity disruption, SH-SY5Y cells were preloaded with 2.0 μM calcein-AM (Molecular Probes) prior to oligomer exposure, as previously described (15). Caspase-3 activity was evaluated by loading cells with FAMFLICA ™ Caspases 3&7 solution [Caspase 3&7 FLICA kit (FAM-DEVDFMK), Immunochemistry Technologies, LLC, Bloomington, MN, USA], after treatment with oligomers.

To analyze oligomer internalisation the cells were counterstained for 10 min with 50μg ml^{-1} Alexa Fluor 633–conjugated wheat germ agglutinin and fixed in 2% (w/v) buffered paraformaldehyde for 10 min at room temperature (20 °C). After plasma membrane permeabilization with a 3% (v/v) glycerol solution for 5 min, the coverslips were incubated for 60 min with 1:1,000 diluted rabbit polyclonal anti-HypF-N antibodies (Primm) and then for 90 min with 1:1,000 diluted Alexa Fluor 488–conjugated anti-rabbit secondary antibodies. Cells were also treated with nontoxic HypF-N oligomers or the native protein (12 μM monomer).

Cell fluorescence was analysed by confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with laser sources for fluorescence measurements at 488 nm and 633 nm and a Leica Plan Apo 63X oil immersion objective. A series of optical sections (1024X1024 pixels), 1.0 μM in thickness, were taken through the cell depth for each examined sample. The confocal microscope was set at optimal acquisition conditions, e.g. pinhole diameters, detector gain and laser powers. Settings were maintained constant for each analysis. To quantify the green fluorescence intensity arising from HypF-N oligomers inside the cells, the images were analysed at median planes parallel to the coverslip for 10-22 cells using ImageJ software (NIH, Bethesda, MD, USA). The intracellular fluorescence intensity was expressed as F/F_0, where F_0 (taken as 100%) represents the intracellular fluorescence of cells treated with HypF-N oligomers and F represents the observed intracellular fluorescence after subtraction of the baseline fluorescence.

SUPPLEMENTARY FIGURES

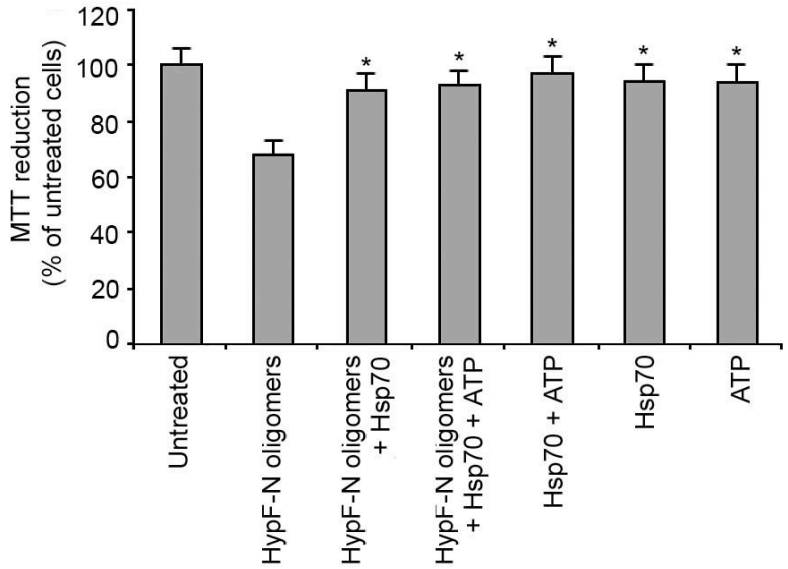

Fig. S1. Reduction of HypF-N oligomer toxicity by extracellularly added Hsp70 with or without ATP. Preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 μM for 1 h without or with Hsp70 (protein:chaperone molar ratio as described in the *Methods* section) and 2.4 μM ATP and then incubated with SH-SY5Y cells. The values shown are means ± SD. The asterisks indicate a significant difference ($p \leq 0.01$) relative to the experiment with oligomers without chaperones. Unlike for canonical, intracellular chaperone activity by Hsp70, which requires ATP, here Hsp70 was found to be similarly effective in reducing the toxicity of preformed HypF-N oligomers with or without ATP.

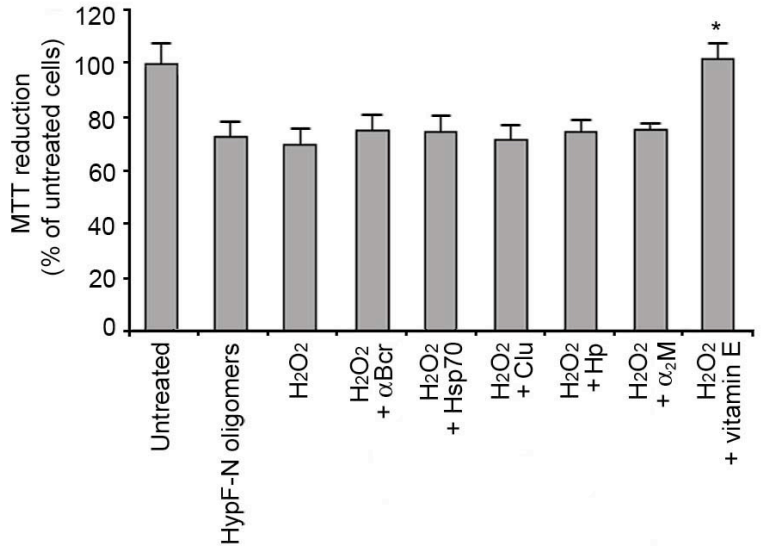

Fig. S2. Effect of extracellularly added chaperones on H_2O_2 toxicity. 150 μM H_2O_2 was incubated for 1 h in the cell culture medium in the absence or presence of chaperones (H_2O_2:chaperone molar ratios as described in the *Methods* section) or 100 μM vitamin E and then added to SH-SY5Y cells. The concentration of the various chaperones were identical to those indicated in Fig. 1. The values shown are means ± SD. The asterisk indicates a significant difference ($p \leq 0.01$) relative to the experiment with 150 μM H_2O_2 without chaperones. We found that 150 μM H_2O_2 decreased the MTT reduction of the cells to a level similar to preformed protein oligomers under our conditions. None of the five chaperones could significantly reduce H_2O_2 toxicity, whereas incubation with the antioxidant vitamin E during the H_2O_2 treatment increased MTT reduction to the levels observed for untreated cells. The protective effect mediated by chaperones cannot therefore be reproduced with other cellular insults, appearing to be specific for oligomer-mediated toxicity.

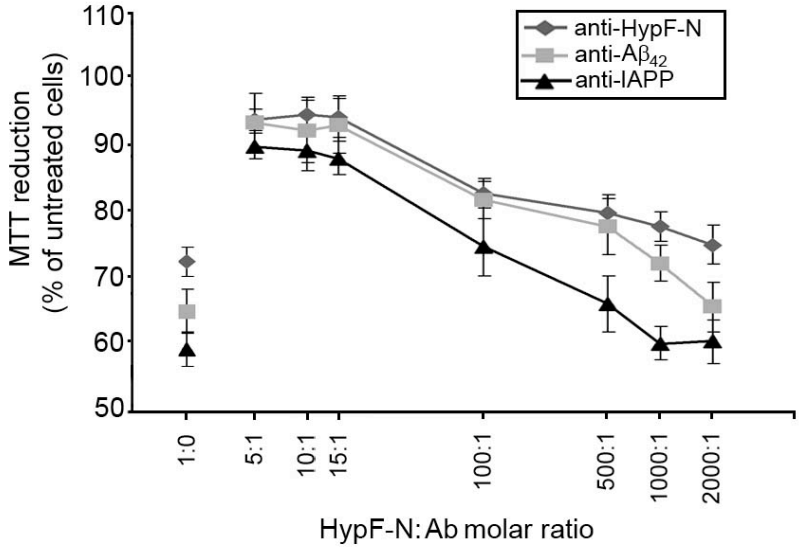

Fig. S3. Reduction of protein oligomer toxicity by antibodies. Preformed oligomers of HypF-N, $A\beta_{42}$ and IAPP, as indicated, were resuspended in the cell culture medium, incubated for 1 h at a corresponding monomer concentration of 12 μM in the absence or presence of their respective sequence-specific antibodies and at the indicated protein:antibodies molar ratio and then added to SH-SY5Y cells. The scale on the *x* axis is logarithmic. Cell viability was expressed as percent of MTT reduction in treated cells with respect to untreated cells (taken as 100%). The values shown are means ± SD.

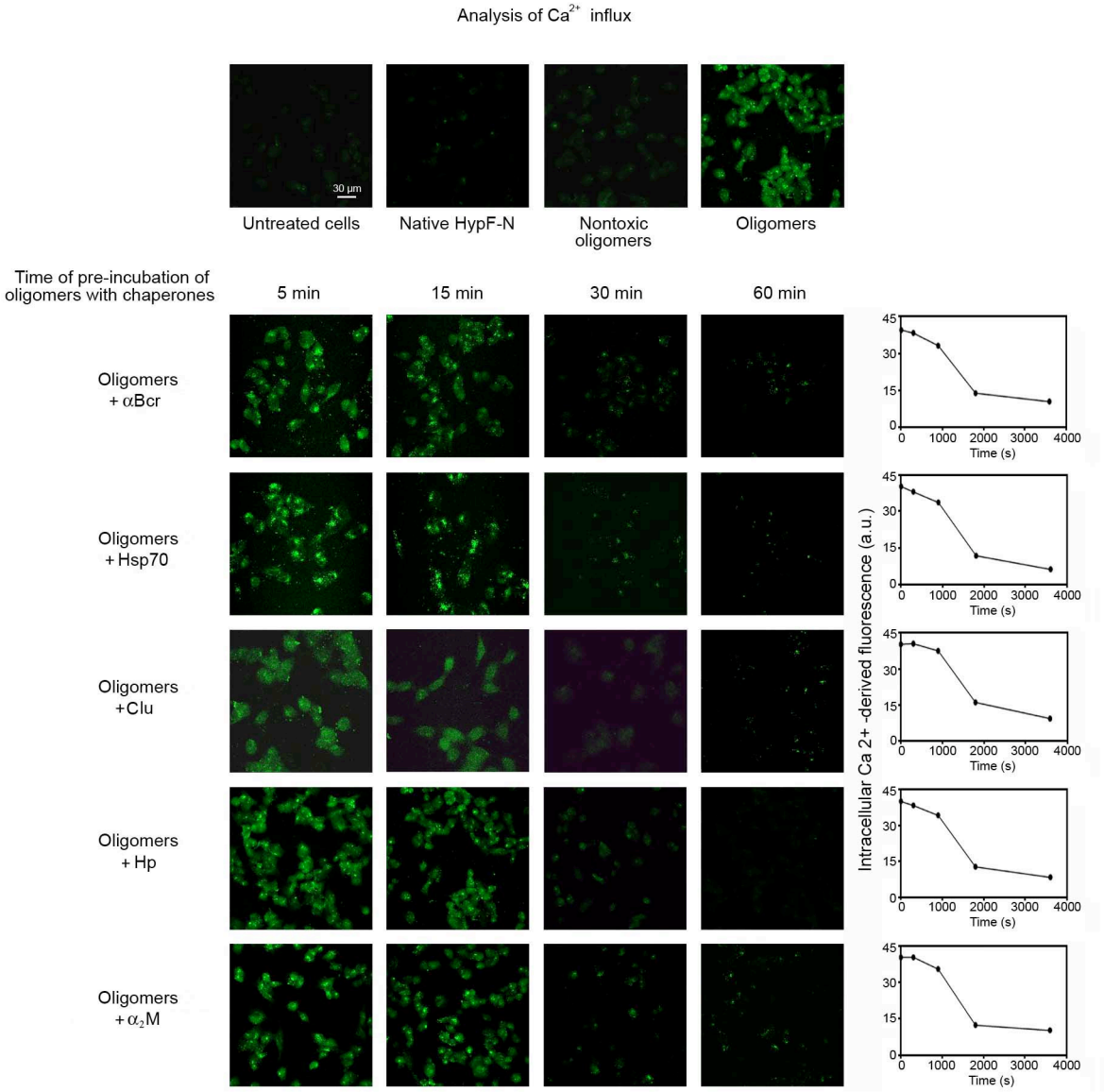

Fig. S4. Representative confocal scanning microscope images showing intracellular Ca^{2+} levels in SH-SY5Y cells. Top panel: images obtained with untreated cells, cells exposed for 1 h to the native protein, nontoxic HypF-N oligomers and toxic oligomers (12 µM monomer). Lower panel: preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 µM in the absence or presence of the indicated chaperones (protein:chaperone molar ratios as described in the *Methods* section) for the indicated time lengths and then added to SH-SY5Y cells for 1 h. The kinetic plots show the mean fluorescence per cell associated with intracellular Ca^{2+} versus time elapsed after pre-incubation of the oligomers with each chaperone. In all images the green fluorescence arises from the intracellular Fluo3 probe bound to Ca^{2+}. Ca^{2+} influx from the cell culture medium to the cytosol mediated by the oligomers was inhibited by the five chaperones, with an effect dependent on the time of pre-incubation of the oligomers with the chaperones.

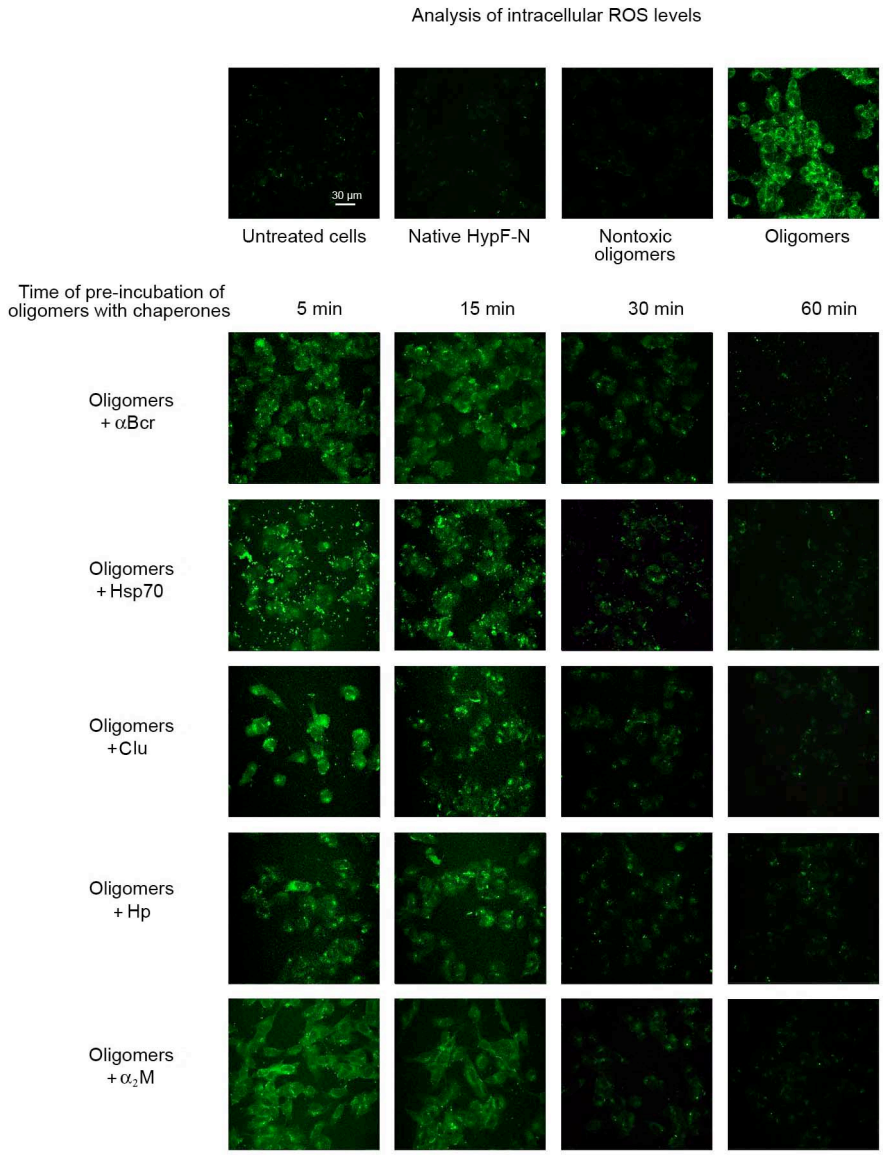

Fig. S5. Representative confocal scanning microscope images of SH-SY5Y showing intracellular ROS levels. Top panel: images obtained with untreated cells, cells exposed for 1 h to the native protein, nontoxic HypF-N oligomers and toxic oligomers (12 µM monomer). Lower panel: preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 µM in the absence or presence of the indicated chaperones (protein:chaperone molar ratio as described in the *Methods* section) for the indicated time lengths and then added to SH-SY5Y cells for 1 h. The green fluorescence arises from the CM-H_2DCFDA probe that has reacted with ROS. Intracellular ROS production mediated by the oligomers was inhibited by the five chaperones with an effect dependent on the time of pre-incubation of the oligomers with the chaperones.

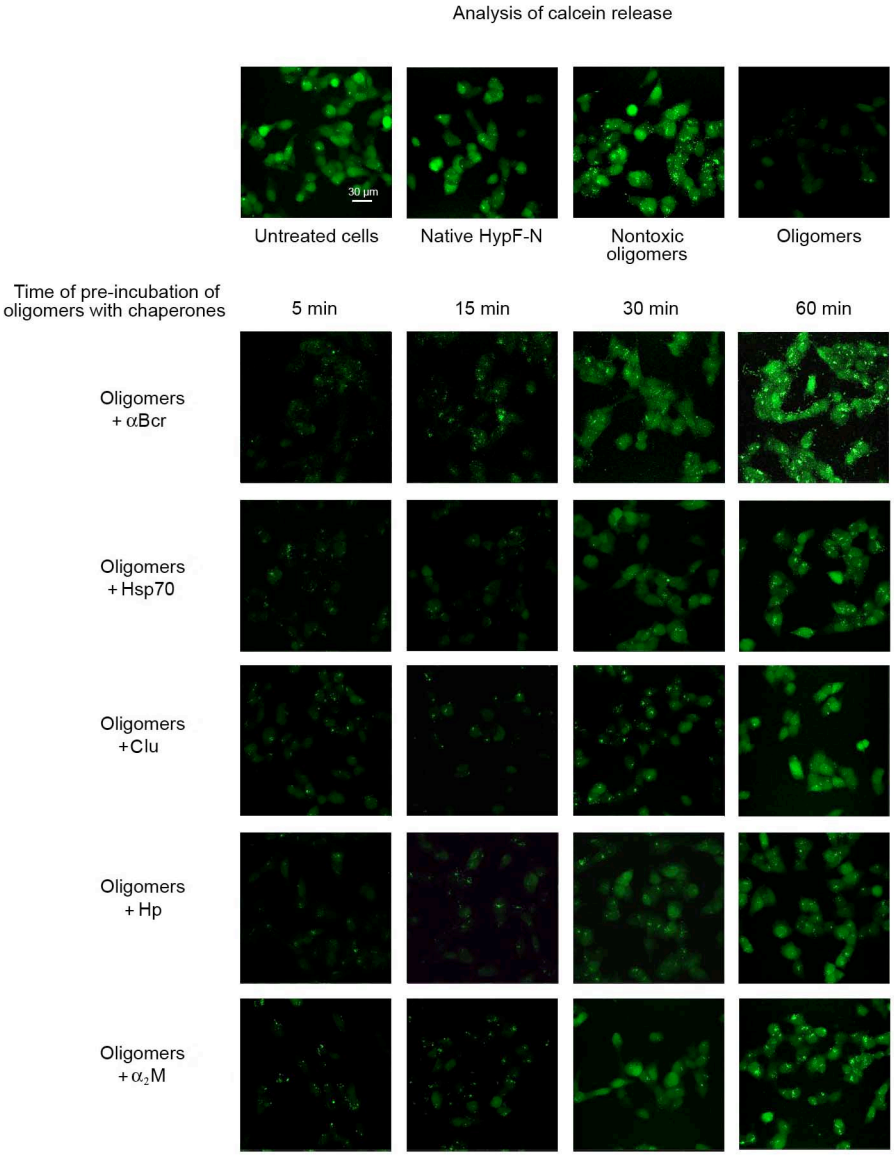

Fig. S6. Representative confocal microscope images showing the release of intracellular calcein in SH-SY5Y cells. Top panel: images obtained with untreated cells, cells exposed for 1 h to the native protein, nontoxic HypF-N oligomers and toxic oligomers (12 μM monomer). Lower panel: preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 μM in the absence or presence of the indicated chaperones (protein:chaperone molar ratio as described in the *Methods* section) for the indicated time lengths and then added to SH-SY5Y cells for 1 h. The green fluorescence arises from the calcein entrapped inside cells. Calcein leakage mediated by the oligomers was inhibited by the five chaperones with an effect dependent on the time of pre-incubation of the oligomers with the chaperones.

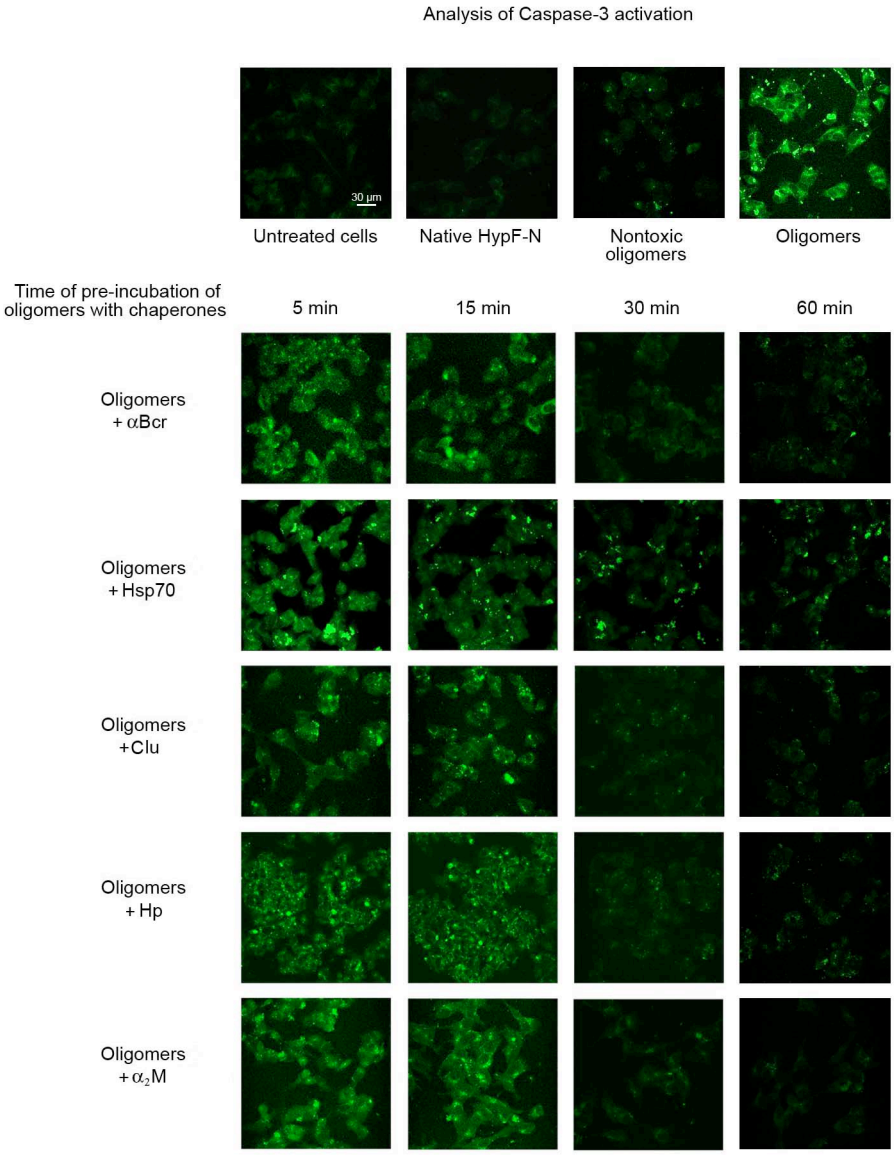

Fig. S7. Representative confocal microscope images showing caspase-3 activation in SH-SY5Y cells. Top panel: images obtained with untreated cells, cells exposed for 1 h to the native protein, nontoxic HypF-N oligomers and toxic oligomers (12 µM monomer). Lower panel: preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 µM in the absence or presence of the indicated chaperones (protein:chaperone molar ratio as described in the *Methods* section) for the indicated time lengths and then added to SH-SY5Y cells for 1 h. Caspase-3 activity was assessed using the fluorescent probe FAM-FLICA™ Caspase 3&7 (green). Caspase-3 activation mediated by the oligomers was inhibited by the five chaperones with an effect dependent on the time of pre-incubation of the oligomers with the chaperones.

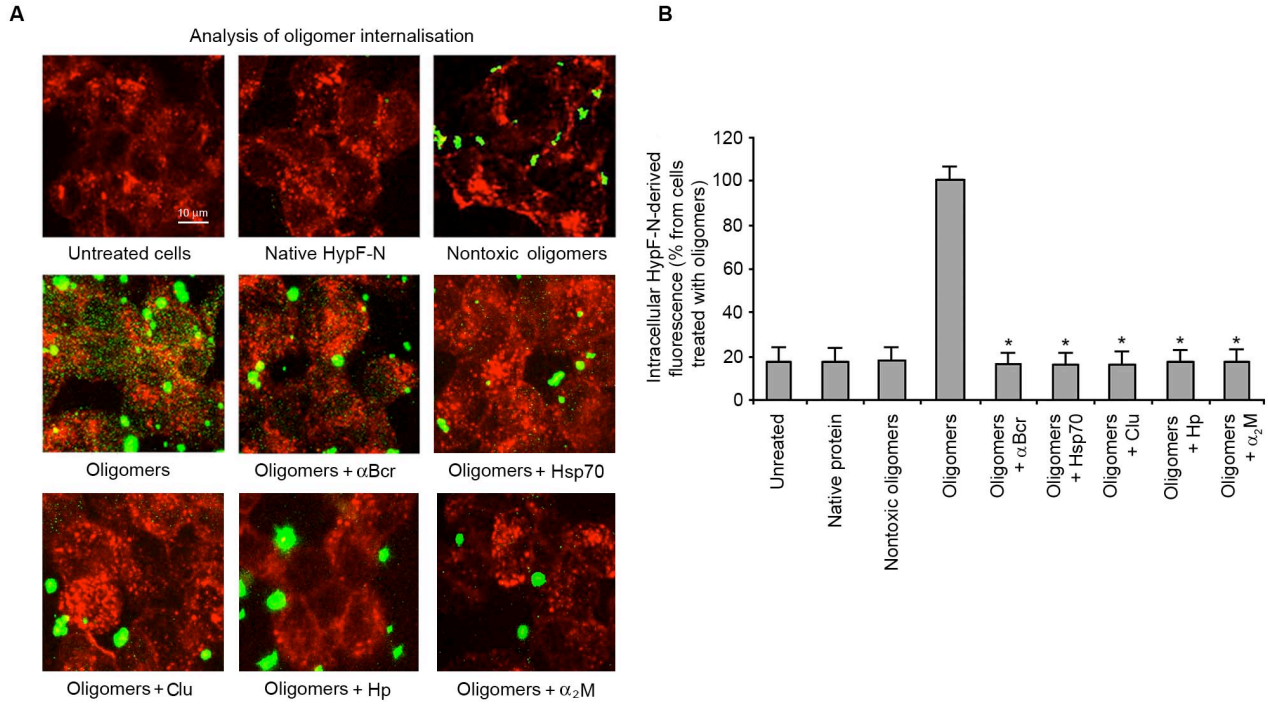

Fig. S8. (*A*) Representative confocal microscope images showing the internalisation of HypF-N oligomers into SH-SY5Y cells. Preformed oligomers of HypF-N were resuspended in the cell culture medium in the absence of cells, incubated at a corresponding monomer concentration of 12 µM for 1 h without or with the indicated chaperones (protein:chaperone molar ratio as described in *Methods*) and then incubated with SH-SY5Y cells for 1 h at 37°C. After plasma membrane permeabilisation with a 3% glycerol solution, counterstaining was performed with Alexa Fluor 633-conjugated wheat germ agglutinin to detect the plasma membranes (red) and with 1:1000 diluted rabbit polyclonal anti-HypF-N antibodies and 1:1000 diluted Alexa Fluor 488-conjugated anti-rabbit secondary antibodies (green) to detect the oligomers. (*B*) Quantification of the green fluorescence arising from HypF-N oligomers inside the cells (median planes). The values reported are means ± SD of three independent experiments. The asterisks indicate $p \leq 0.01$, relative to the experiment with oligomers. The analysis indicates that HypF-N oligomers, unlike the native protein and nontoxic oligomers, are internalised following pre-incubation for 1 h in the absence of chaperones. The green fluorescence arising from anti-HypF-N antibodies appears inside the cells, rather than outside or attached to the membrane, only upon treatment with the toxic oligomers. This was shown by analysing the confocal images at median planes parallel to the coverslip (*B*). Pre-incubation of the oligomers with each chaperone led to little or no HypF-N entry. The oligomers are predominantly detected outside or attached to the cells, but not within them, as confirmed by analysing the confocal images at median planes (*B*). These observations show that the chaperones inhibit oligomer internalisation, at least under the conditions used here, rather than stimulating their intracellular degradation following endocytosis.

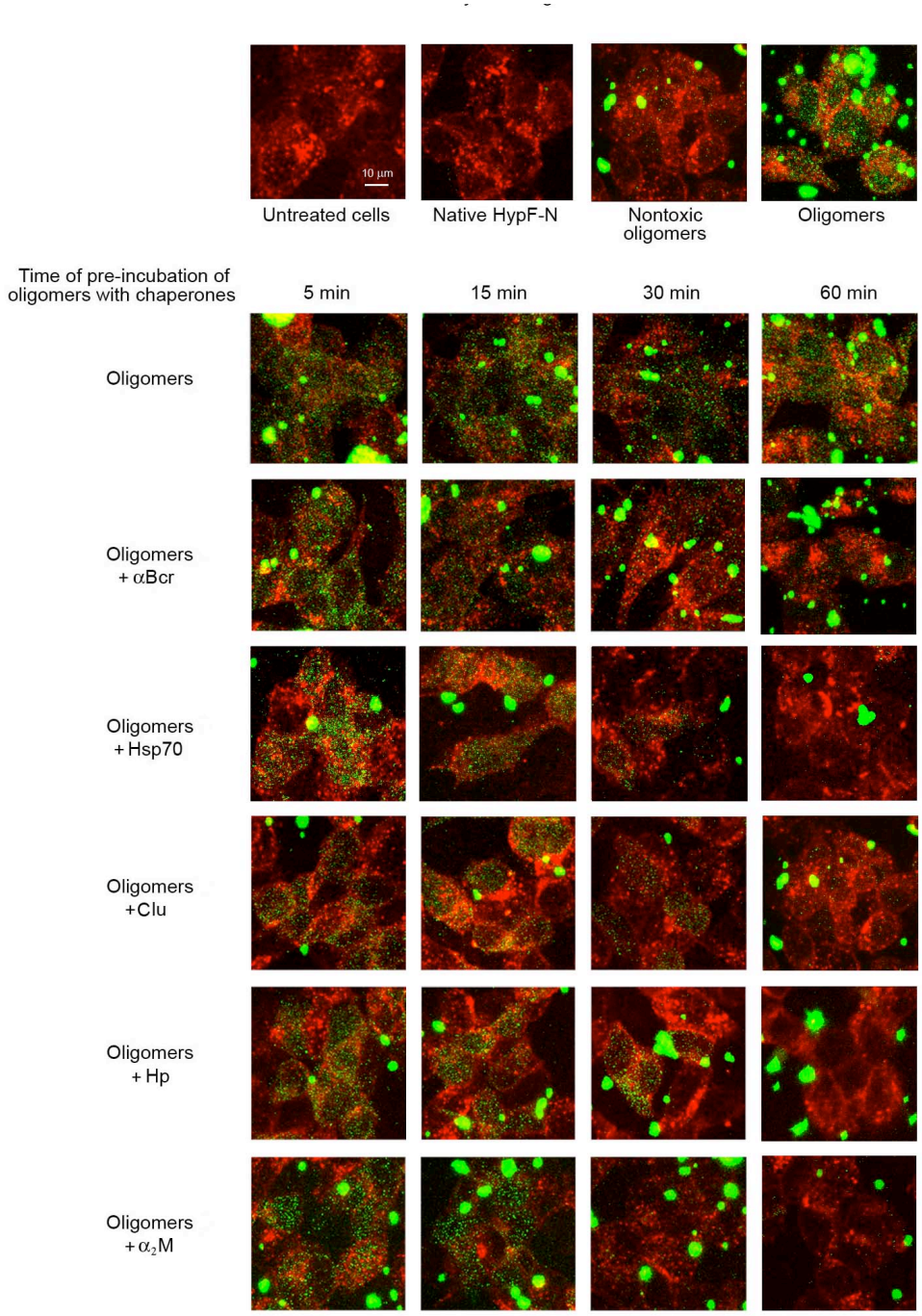

Fig. S9. Confocal microscope images showing HypF-N oligomers within SH-SY5Y cells. Top panel: images obtained with untreated cells, cells exposed for 1 h to the native protein, nontoxic HypF-N oligomers and toxic oligomers (12 μM monomer). Lower panel: preformed oligomers of HypF-N (12 μM monomer) were pre-incubated with or without the indicated chaperones (protein:chaperone molar ratio as described in *Methods*) for the indicated times and then added to SH-SY5Y cells for 1 h. After membrane permeabilisation, counterstaining was performed as described in Figure S8. The images show that HypF-N oligomer internalisation was inhibited by the five chaperones with the extent of inhibition dependent on the time of pre-incubation. The oligomer-associated fluorescence was detected outside the cells in all cases.

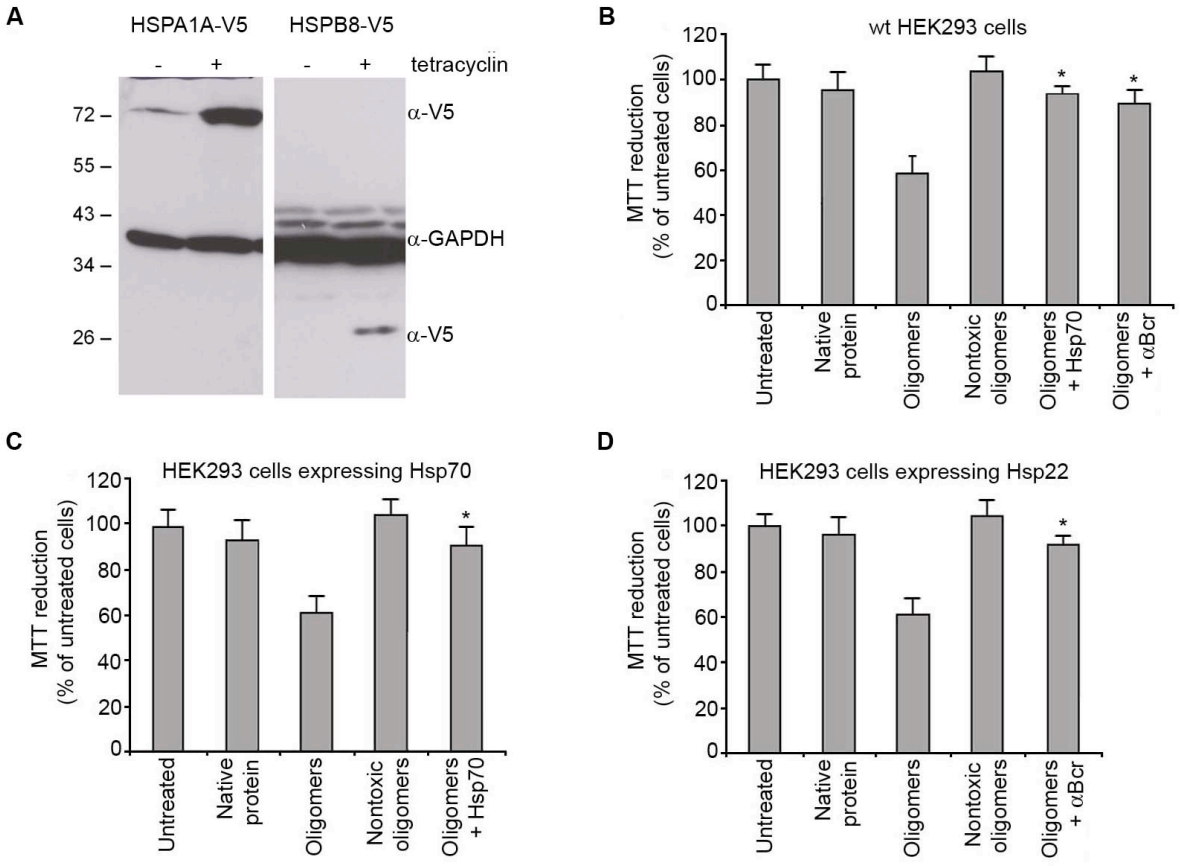

Fig. S10. Effect of HypF-N oligomers on HEK293 cells expressing intracellular chaperones. (*A*) Typical western blot of the tetracyclin-regulated expression of HEK293 cells overexpressing Hsp70 (HSPA1A) or Hsp22 (HSPB8). Prior to the experiment, cells were cultured for 24h with (+) or without (-) tetracyclin added to the cell culture medium after which they were processed for SDS-PAGE and western blotting. Blots were stained with anti-V5 antibodies to monitor the expression of the transgenic chaperones tagged with V5 and with anti-α-GAPDH as a control. The results indicate that the V5-tagged transgenic chaperones are correctly expressed in the cells over-expressing the chaperones. (*B-D*) Preformed oligomers of HypF-N were resuspended in the cell culture medium, incubated at a corresponding monomer concentration of 12 μM for 1 h in the absence or presence of Hsp70 or αBcr (protein:chaperone molar ratio as described in the *Methods* section) and then added to wild-type HEK293 cells (*B*), HEK293 cells overexpressing Hsp70 (*C*) and HEK293 cells overexpressing Hsp22 (*D*). Cell viability was expressed as percent of MTT reduction in treated cells with respect to untreated cells (taken as 100%). The values shown are means ± SD of three independent experiments. The asterisk indicates a significant difference ($p \leq 0.01$) relative to the experiment with oligomers without chaperones. The results show that the overexpression of these intracellular chaperones did not protect the cells against HypF-N oligomer cytotoxicity.

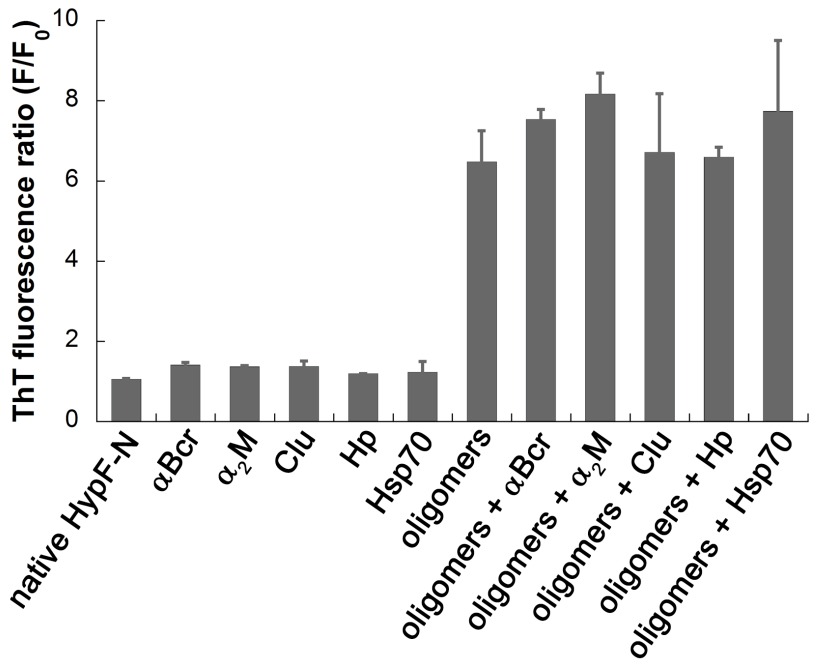

Fig. S11. Chaperones do not disaggregate HypF-N oligomers. ThT fluorescence at 485 nm (excitation 440 nm) in the presence of native HypF-N, αBcr, α_2M, Clu, Hp, Hsp70, and HypF-N oligomers after 1 h incubation in phosphate buffer in the absence or presence of αBcr, α_2M, Clu, Hp and Hsp70. After the incubation, the samples were added to a solution of 25 μM ThT dissolved in 25 mM phosphate buffer at pH 6.0, in order to obtain a 3.7-fold molar excess of dye. The final HypF-N monomer concentration was 6 μM in each case. The ratio between the ThT fluorescence in the presence (F) and absence (F_0) of proteins is reported; data are means ± SD of three independent experiments. The HypF-N concentration was 48 μM (in monomer units) in the 1 h incubation solution prior to addition of ThT.

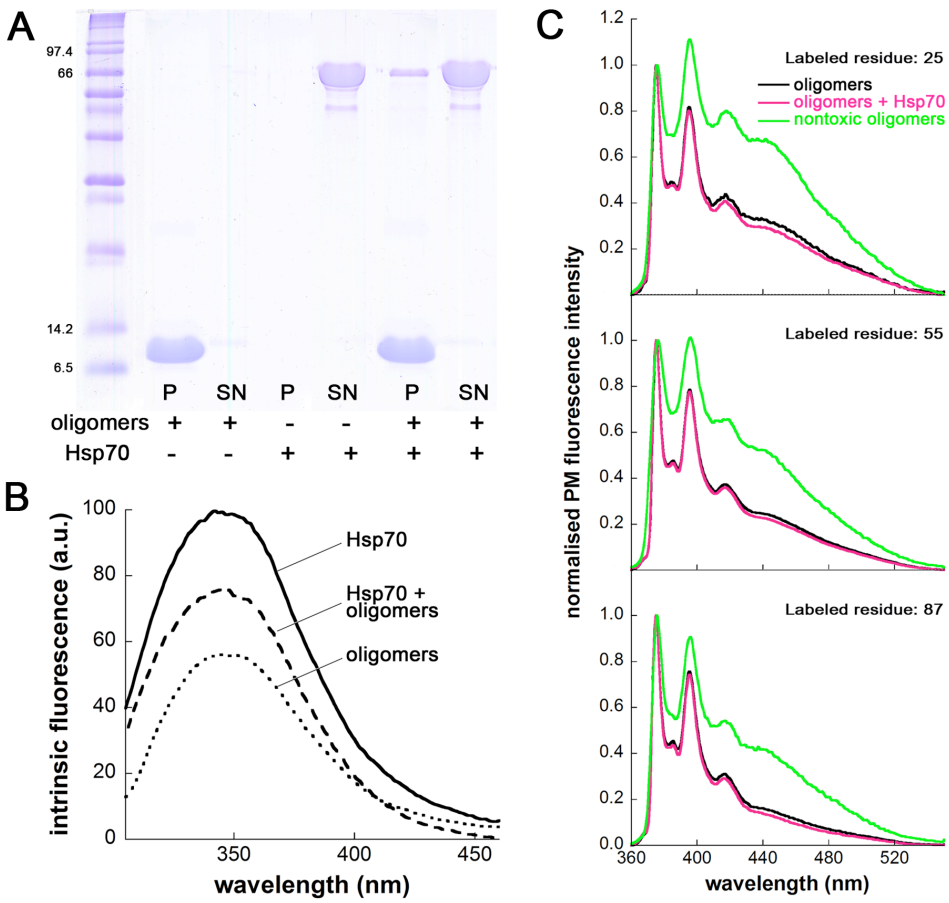

Fig. S12. Effect of Hsp70 on HypF-N oligomers. (*A*) SDS-PAGE of the insoluble (P) and soluble (SN) fractions obtained from samples containing preformed HypF-N type A oligomers (lanes 2, 3), Hsp70 (lanes 4, 5) and preformed type A oligomers treated for 1 h with Hsp70 (lanes 6, 7). HypF-N concentration was 48 μM (monomer concentration). The bands at ~ 10 and 70 kDa indicate HypF-N and Hsp70 respectively. (*B*) Intrinsic fluorescence spectra of the SN fractions obtained after centrifugation of samples containing preformed HypF-N type A oligomers (dotted line), Hsp70 (solid line) and HypF-N type A oligomers + Hsp70 (dashed line). The spectrum of HypF-N oligomers has been subtracted from that of Hsp70 + HypF-N oligomers to eliminate its contribution. All spectra are the means of three independent experiments. (*C*) Fluorescence emission spectra of samples containing HypF-N oligomers labeled with PM at positions 25 (top), 55 (middle) and 87 (bottom). Mutants of HypF-N containing a single cysteine at either position 25, 55 or 87 were labeled with PM, incubated to form the toxic oligomers and then 4-fold diluted, to a corresponding monomer concentration of 12 μM, into 20 mM phosphate buffer, pH 7.0, 25 °C. The spectra refer to a 1 h incubation under these latter condition in the absence (black) and in the presence of Hsp70 (pink). The spectra have been normalized to the intensity of the peak centered at 375 nm. Spectra of nontoxic oligomers (green) labelled at the same positions are also reported (14).

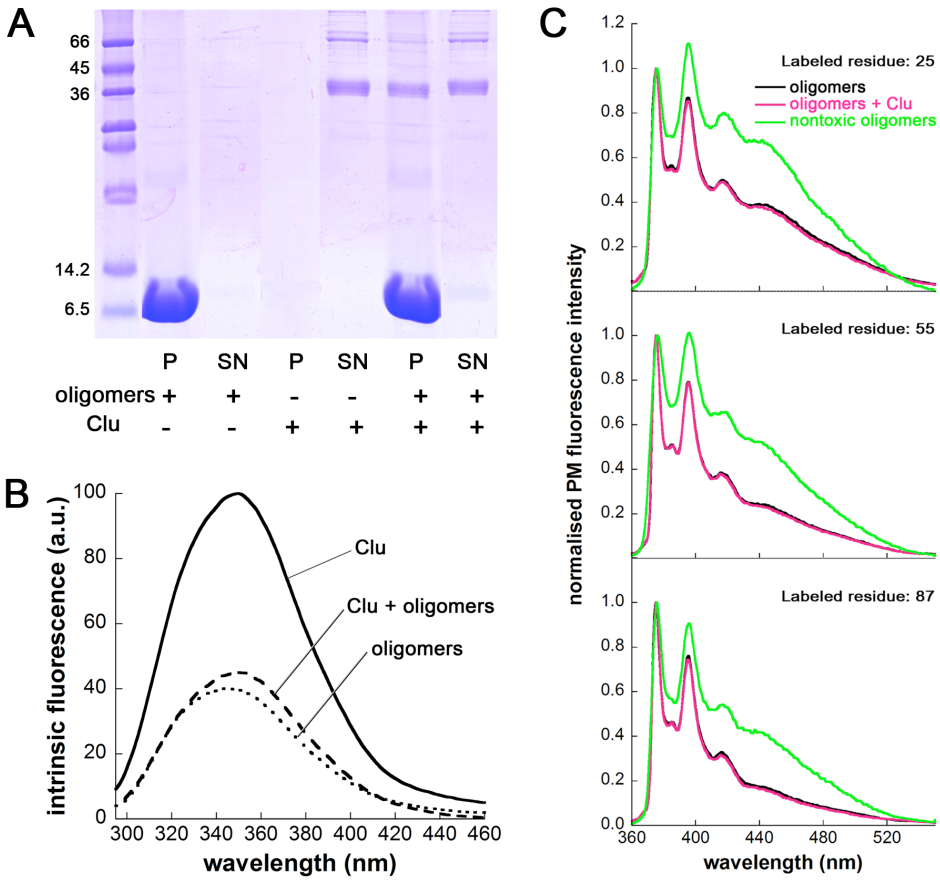

Fig. S13. Effect of Clu on HypF-N oligomers. (*A*) SDS-PAGE of the insoluble (P) and soluble (SN) fractions obtained from samples containing HypF-N oligomers (lanes 2, 3), Clu (lanes 4, 5) and oligomers treated for 1 h with Clu (lanes 6, 7). The bands at ~ 10 kDa and bands ranging from 36 to 66 kDa indicate HypF-N and Clu, respectively. Under reducing conditions, the α and β subunits of Clu co-migrate to about 36 kDa and lesser amounts of unprocessed (single chain) and variably glycosylated Clu are also visible at higher molecular weights. HypF-N concentration was 48 μM (monomer concentration). (*B*) Intrinsic fluorescence spectra of the SN fractions obtained after centrifugation of samples containing HypF-N oligomers (dotted line), Clu (solid line) and HypF-N oligomers + Clu (dashed line). The spectrum of HypF-N oligomers has been subtracted from that of Clu + HypF-N oligomers to eliminate its contribution. All spectra are the means of three experiments. (*C*) Fluorescence emission spectra of samples containing HypF-N oligomers labeled with PM at positions 25 (top), 55 (middle) and 87 (bottom). Mutants of HypF-N containing a single cysteine at either position 25, 55 or 87 were labeled with PM, incubated to form the toxic oligomers and then 4-fold diluted, to a corresponding monomer concentration of 12 μM, into 20 mM phosphate buffer, pH 7.0, 25 °C. The spectra refer to incubation under these latter condition without (black) and with Clu (pink). The spectra were acquired at 12 μM HypF-N. The spectra have been normalized to the intensity of the peak centered at 375 nm. Spectra of nontoxic oligomers (green) labelled at the same positions are also reported (14).

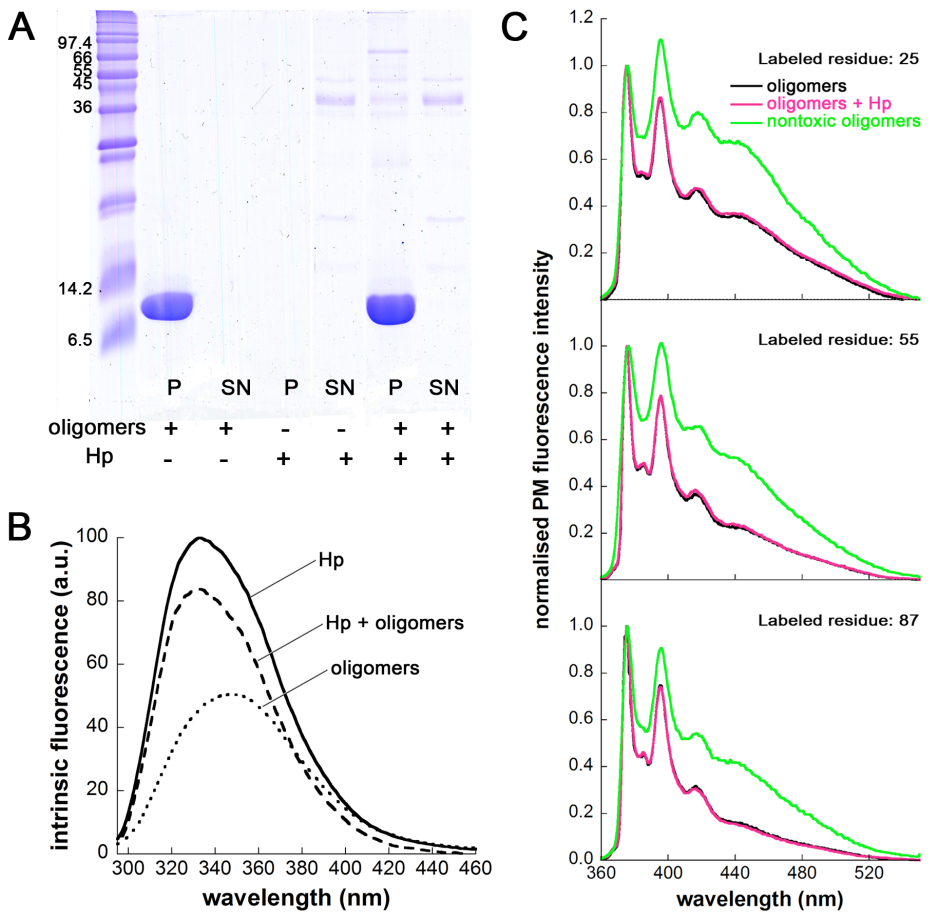

Fig. S14. Effect of Hp on HypF-N oligomers. (*A*) SDS-PAGE of the insoluble (P) and soluble (SN) fractions obtained from samples containing HypF-N oligomers (lanes 2, 3), Hp (lanes 4, 5) and HypF-N oligomers treated for 1 h with Hp (lanes 6, 7). The bands at ~ 10 kDa and bands ranging from 14 to 70 kDa indicate HypF-N and Hp, respectively. Hp generates a wide range of bands due to its varied polymeric structure (supplementary reference 1). HypF-N concentration was 48 µM (monomer concentration). (*B*) Intrinsic fluorescence spectra of the SN fractions obtained after centrifugation of samples containing HypF-N oligomers (dotted line), Hp (solid line) and HypF-N oligomers + Hp (dashed line). The fluorescence emission spectra (excitation at 280 nm) were acquired at 37 °C. The spectrum of HypF-N oligomers has been subtracted from that of Hp + HypF-N oligomers to eliminate its contribution. All spectra are the means of three experiments. (*C*) Fluorescence emission spectra of samples containing HypF-N oligomers labeled with PM at positions 25 (top), 55 (middle) and 87 (bottom). Mutants of HypF-N containing a single cysteine at either position 25, 55 or 87 were labeled with PM, incubated to form toxic oligomers and then 4-fold diluted, to a corresponding monomer concentration of 12 µM, into 20 mM phosphate buffer, pH 7.0, 25 °C. The spectra refer to incubation under these latter condition without (black) and with Hp (pink). The spectra were acquired at 12 µM HypF-N. The spectra have been normalized to the intensity of the peak centered at 375 nm. Spectra of nontoxic oligomers (green) labelled at the same positions are also reported (14).

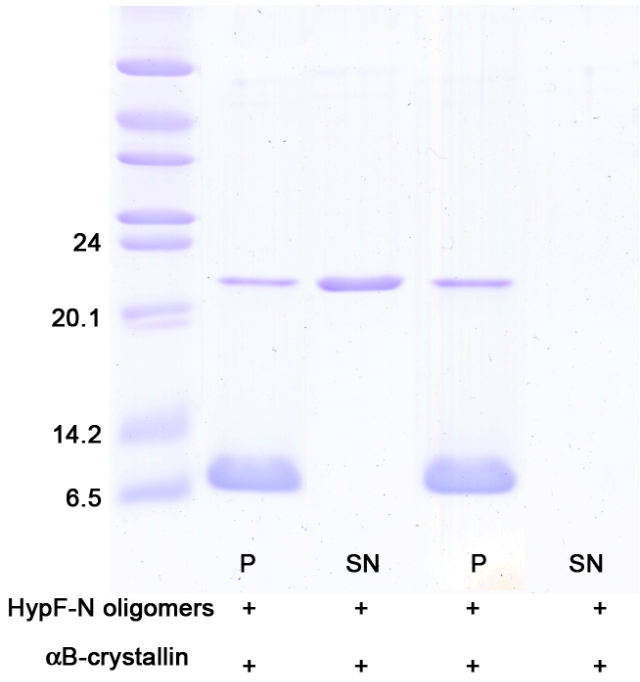

Fig. S15. The binding between αBcr and HypF-N oligomers is stable. Preformed HypF-N oligomers (48 μM monomer) were incubated for 1 h with αBcr. The resulting mixture was then centrifuged and the SN and P fractions analysed with SDS-PAGE (lanes 2,3). A fraction of αBcr remains in the SN, whereas a fraction is in P (lanes 2,3), due to the binding to the HypF-N oligomers. A P fraction of a similarly prepared sample was resuspended in phosphate buffer (pH 7.0) as before, incubated for 1 h, centrifuged and then analysed with SDS-PAGE (lanes 4,5). All the chaperone is found in the P fraction, while the SN fraction is empty. This result indicated that the binding between αBcr and HypF-N oligomers was stable because of the same amount of chaperone in the P fraction after washing it. The bands at ~ 10 and 22 kDa indicate HypF-N and αBcr monomers, respectively.

Supplementary figures references

1. Hooper DC and Peacock AC (1976) Determination of the Subunit Composition of Haptoglobin 2-l Polymers Using Quantitative Densitometry of Polyacrylamide Gels. *J Biol Chem.* 251:5845-5851 .