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A synergistic small-molecule combination directly eradicates diverse prion strain structures

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In the version of this supplementary file originally posted online, Edward J. Hennessy and Stephen L. Buchwald were incorrectly listed as authors. The error has been corrected in this file as of 21 December 2009.

A synergistic small molecule combination directly eradicates diverse prion strain structures

Blake E. Roberts[1*], Martin L. Duennwald[2*#], Huan Wang[1], Chan Chung[2], Nicholas P. Lopreiato[1], Elizabeth A. Sweeny[1], M. Noelle Knight[1], and James Shorter[1#].

[1]Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, 805b Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia, PA 19104.

[2]Boston Biomedical Research Institute, 64 Grove St., Watertown, MA 02472.

[*]These authors contributed equally to this work.

[#]Correspondence: jshorter@mail.med.upenn.edu or duennwald@bbri.org

Supplementary Methods, Results and Figures

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Supplementary Methods

Proteins

M was purified as described[1] and lysozyme was from Sigma.

Fiber Assembly and Disassembly

Fiber assembly or disassembly was determined by CR binding, ThT fluorescence, EM, sedimentation analysis or SDS-resistance as described [2-4]. For Congo Red (CR) binding, reactions were diluted ten-fold in AB containing CR. This yielded final concentrations of CR (10μM) and NM (0.25-0.5μM). After 30min at 25°C, absorbance was measured at 477nm and 540nm. CR binding was calculated as described [2]. Similarly for Thioflavin T (ThT), reactions were diluted ten-fold in AB containing ThT, to yield final concentrations of ThT (10μM) and NM (0.25-0.5μM). After 10min at 25°C, reactions were then excited at 450nm (bandwidth 5nm) and emission at 482nm (bandwidth 10nm) was recorded. Dilution of reactions eliminated any potential spectroscopic interference by the small molecules.

Sedimentation was at 436,000g for 10min at 25°C, except for the experiments in Fig. S6e where sedimentation was at 100,000g for 10min at 25°C. To assess SDS-resistance, AB was modified to include 150mM NaCl (instead of KCl) to circumvent solubility issues induced by potassium dodecyl sulfate. The amount of SDS-soluble (2% SDS, 25°C) NM was determined by quantitative densitometry of Coomassie stained gels. Values obtained from densitometry were converted to units of pmol by comparison to standard curves with known amounts of SDS-soluble NM. From this value, the amount of SDS-insoluble (resistant) NM was calculated.

Stability of EGCG under NM fibrillization conditions

EGCG levels were analyzed by HPLC using a Supelcosil C18 reversed-phase column (Sigma), essentially as described [5]. Briefly, at the indicated times NM (5μM) fibrillization reactions in the absence or presence of EGCG (20μM) were supplemented with 0.2% ascorbic acid and 0.05% EDTA to stabilize EGCG. This mixture was extracted twice with two volumes of ethyl acetate. Samples were vortexed for 30s and then centrifuged at 3,000g for 10min at 4°C. The upper organic phase was collected and dried using a vacuum concentrator. Residues were dissolved in 10% acetonitrile and were loaded onto the

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Supelcosil C18 reversed-phase column equilibrated in the isocratic mobile phase (14% acetonitrile, 0.086% citric acid). Eluates were monitored by absorbance at 280nm. By comparing eluates to standard solutions containing known quantities of EGCG, the EGCG concentration could be calculated.

NM-EGCG binding

NM (5μM) was incubated with EGCG (20μM) for 1h at either 25°C or 4°C. In some experiments, oligomeric NM was separated from monomeric NM by retention on a 100kDa filter (Millipore). Unbound small molecule was then removed by gel filtration using Bio-spin 6 spin columns (Bio-Rad). The amount of EGCG bound was then determined by nitroblue tetrazolium (NBT) staining as described [6,7]. EGCG catalyzes redox cycling at alkaline pH in the presence of excess glycine as a reductant, and reduces tetrazolium to formazan in the presence of oxygen [6]. After gel filtration, reactions were diluted 10-fold with 0.24mM NBT in 2M potassium glycinate, pH 10 and incubated for 1h at 25°C. The absorbance at 530nm was then measured. Control reactions lacking NM confirmed that free EGCG was completely removed by the gel filtration step. The amount of EGCG bound was estimated by comparison to standard curves of known quantities of EGCG. Reactions containing NM alone gave a negligible signal, which was subtracted from other reactions as required.

In other experiments, EGCG was coupled to Sepharose 4B (GE) as described [8]. Quenched Sepharose 4B served as a negative control. NM, NM 21-38, NM 83-110, NM 21-38, 83-110, M, or lysozyme (5μM) in assembly buffer were incubated for 1h at 4°C or 25°C with gentle rotation with either Sepharose 4B or EGCG-Sepharose 4B. Under these gentle agitation conditions, no fiber assembly occurred in this time frame. Beads were recovered by centrifugation and washed five times with assembly buffer. Proteins were eluted with SDS-PAGE sample buffer, processed for SDS-PAGE and Coomassie stained. The amount of protein bound was then determined by densitometry in comparison to known quantities of protein.

Circular dichroism spectroscopy

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CD spectra were obtained using a Jasco 715 spectropolarimeter. For CD measurements, we employed a different buffer: 5mM potassium phosphate pH 7.4 and 150mM NaCl. EGCG was dissolved directly in this buffer and not in DMSO for these experiments. NM concentration was 5μM. All spectra were measured with a 0.1cm pathlength quartz cuvette from 250 to 200nm with a step size of 1nm, bandwidth of 1nm, response time of 4s, and scan speed of 20nm/min. All spectra were buffer corrected and an average of four scans was obtained. For measurements on NM oligomers formed during NM assembly in the presence of EGCG at 25°C, NM oligomers were separated from monomers by retention on a 100kDa filter (Millipore). Oligomers were resuspended and used immediately for CD measurements. For measurements on NM oligomers that formed after disassembly of NM25 fibers with EGCG, NM oligomers were collected from the supernatant fraction after centrifugation at 100,000g for 10min.

Dot Blots

Dot blots to detect amyloidogenic NM oligomers or amyloid NM were as described [3].

Semi-denaturing detergent-agarose gel electrophoresis (SDD-AGE)

SDD-AGE was carried out as described [9] with the exception that the lysis buffer contained 1% SDS.

Hsp104 ATPase activity

Hsp104 ATPase activity was determined as described [10].

Measuring the heat shock response and unfolded protein response

Protein lysates of yeast cells treated as indicated were analyzed by immunoblot using anti-Hsp26 and anti-Hsp104 antibodies. In addition, a heat shock reporter plasmid (3XHSE-lacZ) was employed to quantify the induction of a heat shock response [11]. Alternatively, an unfolded protein response (UPR) reporter plasmid (UPRE-lacZ) was used to quantify the induction of the UPR [12]. Mid-log phase yeast cells were mixed with an equal volume of assay buffer (100mM Hepes pH 7.5, 150mM NaCl; 5mM L-Aspartate, 1% BSA, 0.05% Tween-20; 0.5% SDS, 1.2mM chlorophenolred-ß-D-galactopyranoside), incubated for 45min, and the absorbance at 578nm was measured.

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Supplementary Results

EGCG prevents the maturation molten NM oligomers that nucleate NM25 fibers

EGCG might selectively reduce the fraction of NM that forms molten NM oligomers at 25°C versus 4°C (Fig. 1a, step 1), which might inhibit fibrillization [13]. However, EGCG had no effect on molten oligomer formation at either temperature, as determined by the amount of NM retained by a 100kDa filter (Fig. S2a) or by size-exclusion chromatography (data not shown). Similar amounts of NM were retained by a 100kDa filter after 5min or 4h in the presence of EGCG at 25°C (Fig. S2b). Hence, EGCG did not increase the amount of oligomeric NM. These data suggested that EGCG might trap NM at an early stage (Fig. 1a, before step 2 or 3) in lag phase at 25°C. Indeed, NM oligomers that accumulated in the presence of EGCG at 25°C were very similar in appearance, size range (10-64nm) and mean diameter (~33nm) to the molten NM oligomers that appeared during lag phase in the absence of EGCG (Fig. S2c, d). Furthermore, early in lag phase at 25°C, but not 4°C, EGCG caused NM oligomers to become SDS-resistant (Fig. S2e), even though similar amounts of EGCG bound to NM oligomers at 25°C and 4°C (Fig. S2f). Thus, by locking oligomers in a stable structure, EGCG might prevent the maturation of molten NM oligomers at 25°C (Fig. 1a, steps 2 and 3) and preclude the nucleation of NM25 fibers.

EGCG binds directly to several regions within N

We employed EGCG-sepharose to explore EGCG-NM binding via affinity chromatography. Consistent with previous reports[6], EGCG did not interact with lysozyme (Fig. S2k). EGCG has previously been suggested to interact with natively unfolded proteins[6]. We found that EGCG binds NM directly (Fig. S2k). Surprisingly, however, EGCG did not bind to the M domain of NM, which is intrinsically unfolded[14,15]. Thus, EGCG binds to regions within N. EGCG also interacted directly with NM 21-38, which lacks the head region, but binding was reduced compared to full-length NM (Fig. S2k). EGCG binding was further reduced but not eliminated for NM 83-110 (Fig. S2k), which lacks most of the Tail sequence, and was even further reduced in an NM mutant (NM 21-38, 83-110) that lacked the Head and Tail region (Fig. S2k). These interactions were very similar at 4°C or 25°C (Fig. S2k). Thus, EGCG interacts with several regions within the unstructured N domain in a manner that precludes the formation of certain fiber strains, but

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permits the formation of others.

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Supplemental References

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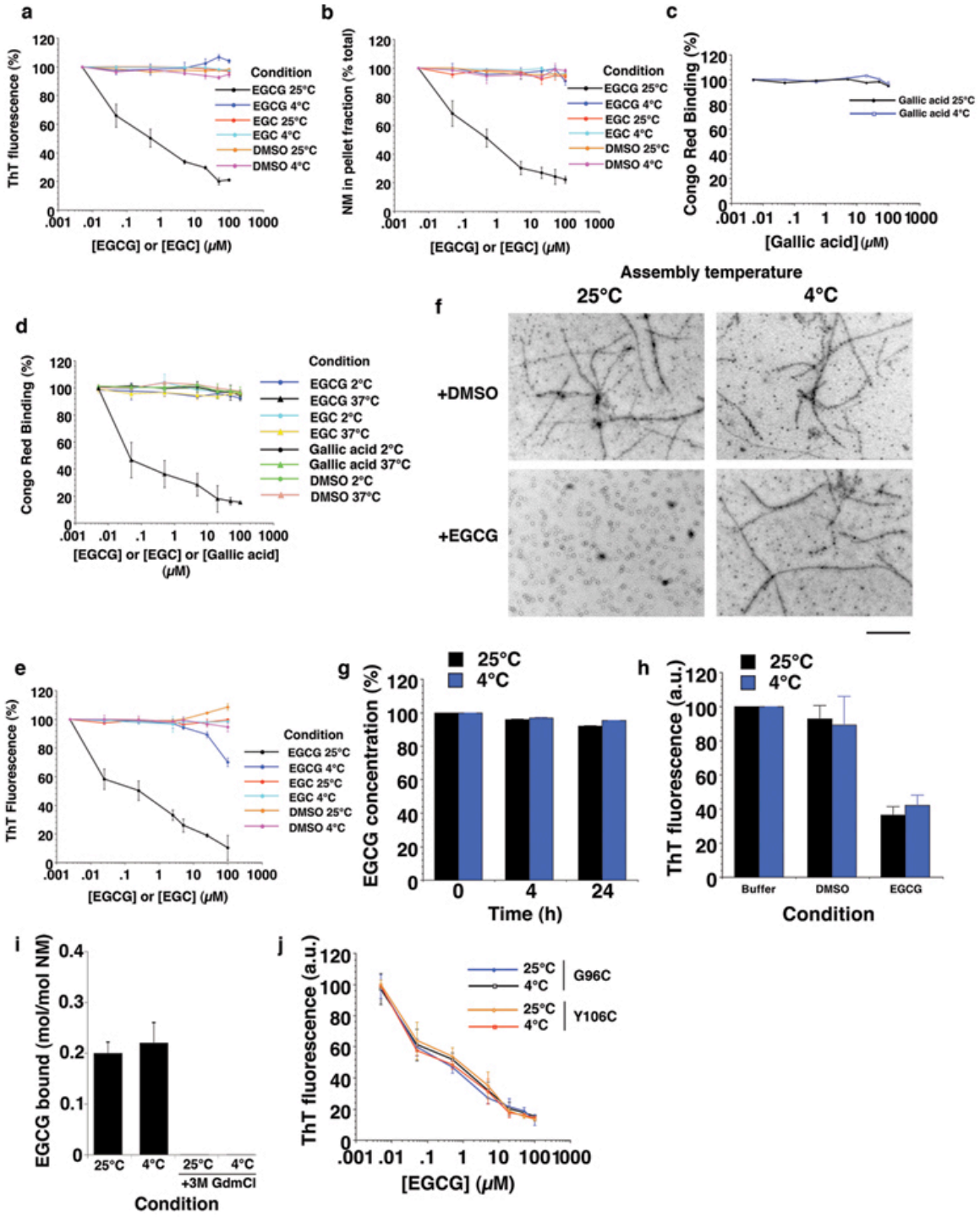

Figure S1. EGCG inhibits assembly of select Sup35 prion strains.

(a, b) Spontaneous, agitated NM (5μM) fibrillization after 4h at 25°C or 4°C in the presence of EGCG, EGC (0-100μM) or DMSO (0-1%). Fibrillization was measured by ThT fluorescence **(a)** or sedimentation analysis **(b)**. 100% reflects assembly in the absence of EGCG, EGC or DMSO. Values represent means±SD (n=3).

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(c) Spontaneous, agitated NM (5μM) fibrillization after 4h at 25°C or 4°C in the presence of gallic acid (0-100μM). Fibrillization was measured by Congo Red binding. 100% reflects assembly in the absence of gallic acid. Values represent means±SD (n=3).
(d) Spontaneous, agitated NM (5μM) fibrillization after 4h at 37°C or 2°C in the presence of EGCG, EGC, gallic acid (0-100μM) or DMSO (0-1%). Fibrillization was measured by Congo Red binding. 100% reflects assembly in the absence of EGCG, EGC, gallic acid or DMSO. Values represent means±SD (n=3).
(e) Spontaneous, agitated Sup35 (5μM) fibrillization after 6h at 25°C or 4°C in the presence of EGCG, EGC (0-100μM) or DMSO (0-1%). Fibrillization was measured by ThT fluorescence. 100% reflects assembly in the absence of EGCG, EGC or DMSO. Values represent means±SD (n=3).
(f) Sup35 was assembled as in **(e)** at 4°C or 25°C in the presence of either DMSO (1%) or EGCG (20μM) and processed for EM. Bar, 0.5μm.
(g) NM (5μM) was agitated for 0-24h at 25°C or 4°C in the presence of EGCG (20μM). At the indicated times, the amount of EGCG was determined by HPLC analysis. Values represent means±SD (n=3).
(h) Spontaneous Aβ42 (10μM) fibrillization after 24h at 25°C (black bars) or 4°C (blue bars) in the absence or presence of either DMSO (1%) or EGCG (10μM). Fibrillization was measured by ThT fluorescence. Values represent means±SEM (n=3-6).
(i) NM (5μM) was incubated with EGCG (20μM) for 1h at either 25°C or 4°C. To some reactions, 3M guanidium chloride was then added to determine if binding was reversible. Unbound small molecule was then removed by gel filtration and the amount of EGCG bound was determined by nitroblue tetrazolium staining and comparison to known quantities of EGCG. Values represent means±SD (n=3).
(j) NM cysteine variants were crosslinked under denaturing conditions with a flexible 11Å BMB crosslink at position 96 or 106. The indicated NM protein (5μM) was then assembled with agitation at 25°C or 4°C for 4h in the presence of EGCG (0.005-100μM). Fibrillization was measured by ThT fluorescence. Values represent means±SD (n=3).

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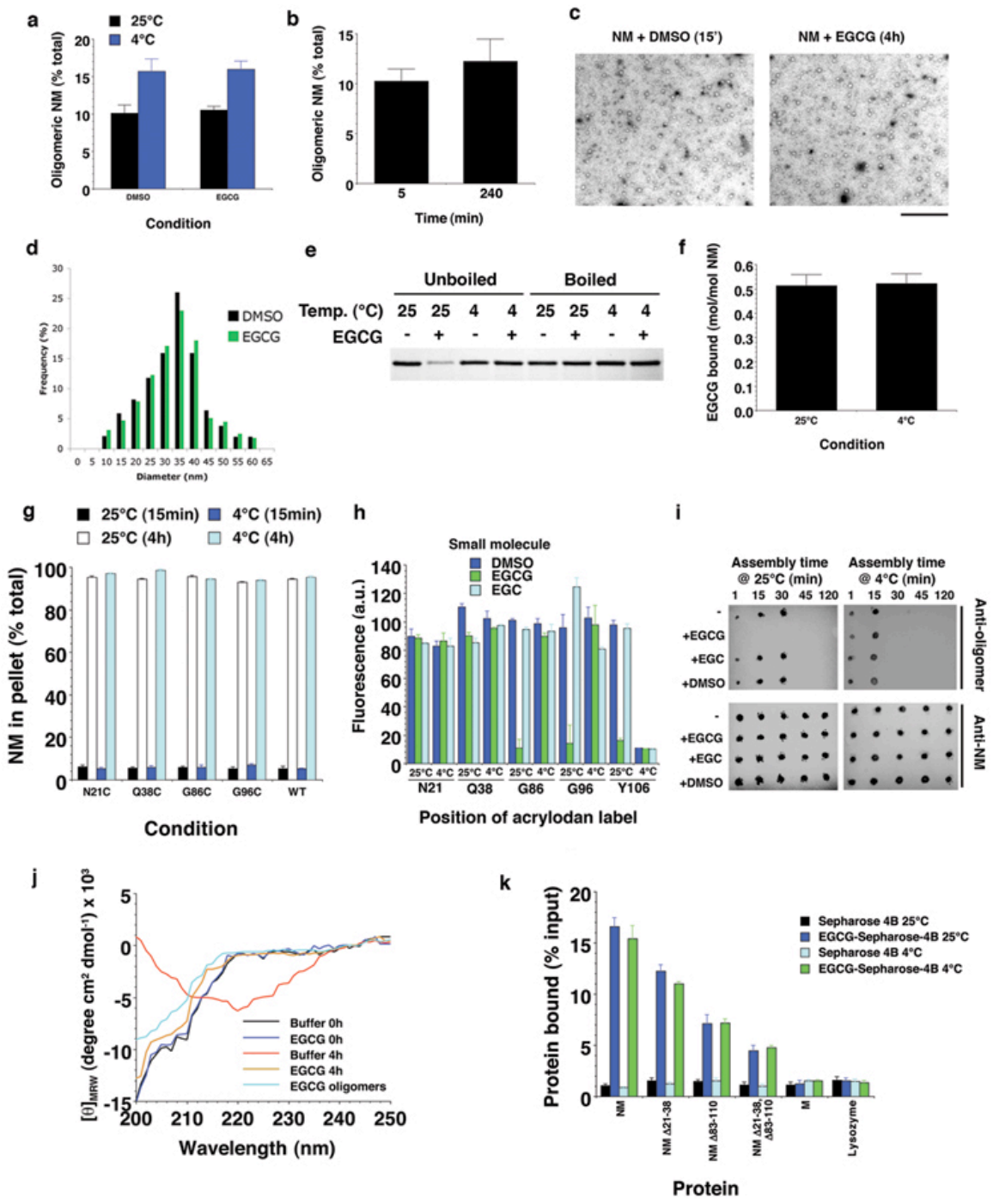

Figure S2. EGCG stabilizes NM oligomers at 25°C.

(a) NM (5μM) was incubated for 5min with agitation at 25°C (black) or 4°C (blue) in the presence of either DMSO (1%) or EGCG (20μM). Reactions were then fractionated by passage through a 100kDa molecular weight cut off Microcon filter. The retentate fraction was resuspended in SDS-PAGE sample buffer and processed for SDS-PAGE and

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Coomassie stained. The amount of oligomeric NM was then determined by densitometry in comparison to known quantities of NM. Values represent means±SD (n=3).
(b) NM (5μM) was incubated for 5min or 4h with agitation at 25°C (black) in the presence of EGCG (20μM). The amount of oligomeric NM was determined as in **(a)**. Values represent means±SD (n=3).
(c, d) NM (5μM) was incubated with agitation at 25°C in the presence of DMSO (1%) for 15min or EGCG (20μM) for 4h. Reactions were then processed for EM. Bar, 0.5μm **(c)**. The diameter of 1000 oligomers was determined for each condition and plotted as a histogram **(d)**.
(e) NM (5μM) was incubated for 15min with agitation at 25°C (black) or 4°C (blue) in the presence of either DMSO (1%) or EGCG (20μM). Reactions were then fractionated by passage through a 100kDa molecular weight cut off Microcon filter. The retentate fraction was resuspended in SDS-PAGE sample buffer, divided in two and either incubated at 25°C (unboiled) or 99°C (boiled) for 10min. Samples were then processed for SDS-PAGE and Coomassie stained. Note that after assembly at 25°C, EGCG renders NM oligomers SDS-resistant and less NM enters the gel. These SDS-resistant oligomers are disrupted by boiling in 2% SDS.
(f) NM (5μM) was incubated with EGCG (20μM) for 1h at either 25°C or 4°C. Oligomeric NM was collected as the retentate on a 100kDa filter and unbound small molecule was then removed by gel filtration. The amount of EGCG bound was determined by nitroblue tetrazolium staining in comparison to known quantities of EGCG. Values represent means±SD (n=3).
(g) Wild-type (WT) NM or the indicated acrylodan-labeled NM variant (5μM) were incubated at 25°C or 4°C for 15min or 4h with agitation. At various times the extent of fibrillization was assessed by sedimentation analysis. Values represent means±SD (n=3).
(h) Fluorescence of NM-N21C-, Q38C-, G86C-, G96C- or Y106C-acrylodan (5μM) after 15min at 25°C or 4°C in the presence of DMSO (1%), EGCG or EGC (20μM). Values represent means±SD (n=3).
(i) Spontaneous, agitated NM (5μM) fibrillization in the absence or presence of DMSO (1%), EGCG or EGC (20μM) at 25°C (left) or 4°C (right). At various times, reactions were applied to nitrocellulose and probed with anti-oligomer antibody or anti-NM antibody.
(j) NM (5μM) was assembled with agitation at 25°C in the absence (buffer) or presence of EGCG (20μM). At the indicated times (0h or 4h), CD spectra were recorded. To assess the

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secondary structure of NM oligomers (cyan trace) that accumulated in the presence of EGCG after 4h, NM oligomers were separated from monomers by retention on a 100kDa filter. The retentate was resuspended and analyzed by CD.

(k) NM, NM 21-38, NM 83-110, NM 21-38, 83-110, M, or lysozyme (5μM) were incubated for 1h at 4°C or 25°C with either Sepharose 4B or EGCG-Sepharose 4B. Beads were recovered, washed and eluted. Eluates were processed for SDS-PAGE and Coomassie stained. The amount of protein bound was then determined by densitometry in comparison to known quantities of protein. Values represent means±SD (n=3).

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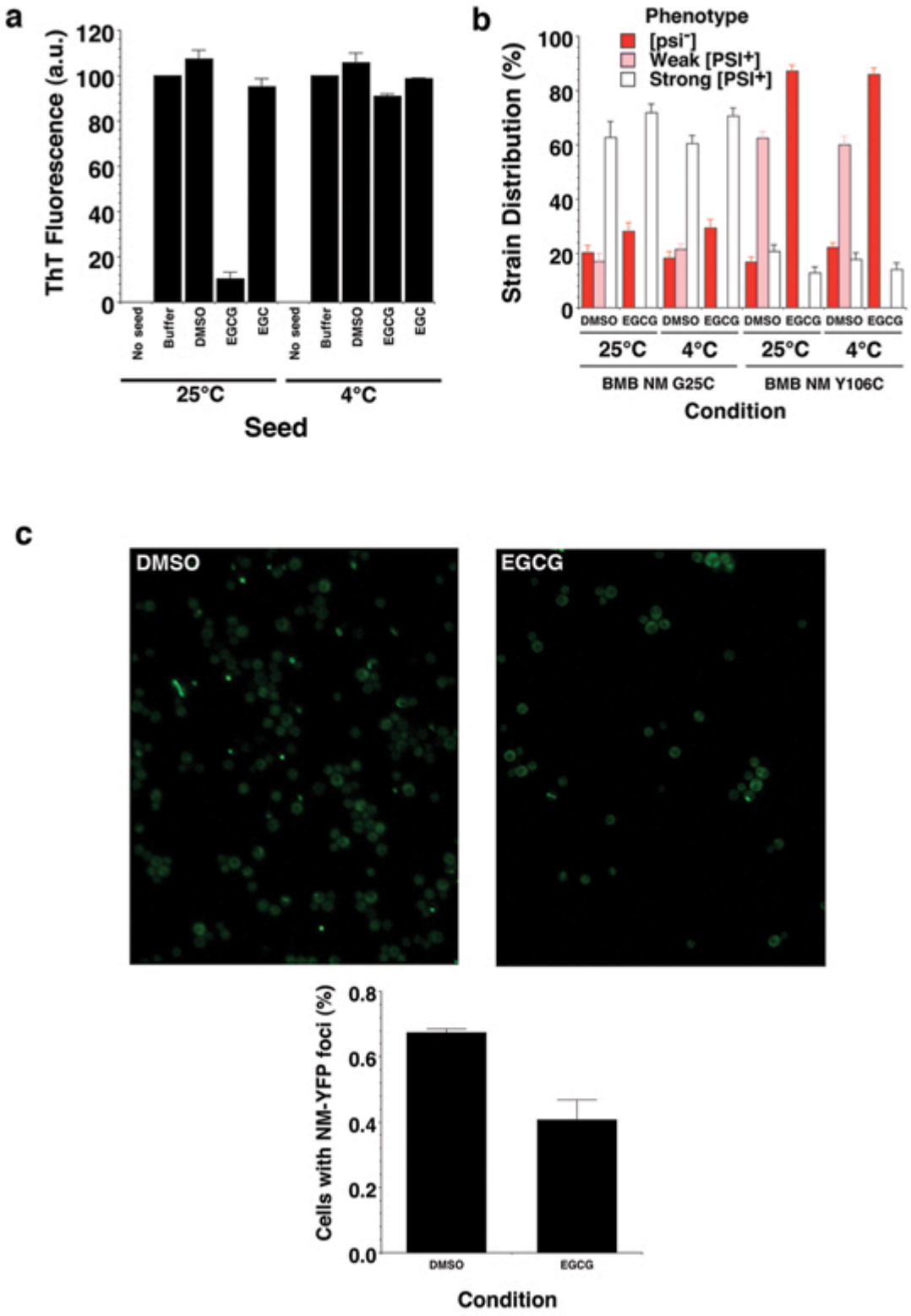

Figure S3. EGCG selectively inhibits assembly of Sup35 prion strains that encode weak [*PSI*$^{+}$].

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(a) NM (5μM) was incubated with agitation at 25°C or 4°C for 4h in the absence or presence of DMSO (1%), EGCG or EGC (20μM). Reactions were then dialyzed to remove unbound small molecule, sonicated and used to seed (2% wt/wt) fresh, undisturbed NM (5μM) fibrillization. Fibrillization was measured by ThT fluorescence. Values represent means±SD (n=3).
(b) NM cysteine variants were crosslinked under denaturing conditions with a flexible 11Å BMB crosslink at position 25 or 106. The indicated NM protein (5μM) was then assembled with agitation at 25°C or 4°C for 4h in the presence of DMSO (1%) or EGCG (20μM). Reactions were then dialyzed to remove unbound small molecule, concentrated, sonicated and transformed into [*psi*$^-$] cells. The proportion of [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] transformants was then determined. Values represent means±SD (n=3).
(c) NM-YFP was overexpressed in [*psi*$^-$] *pdr5* cells for 12h in the presence of DMSO (1%) or EGCG (125μM). Cells were then imaged and the proportion of cells containing NM-YFP foci was determined. Values represent means±SD (n=3).

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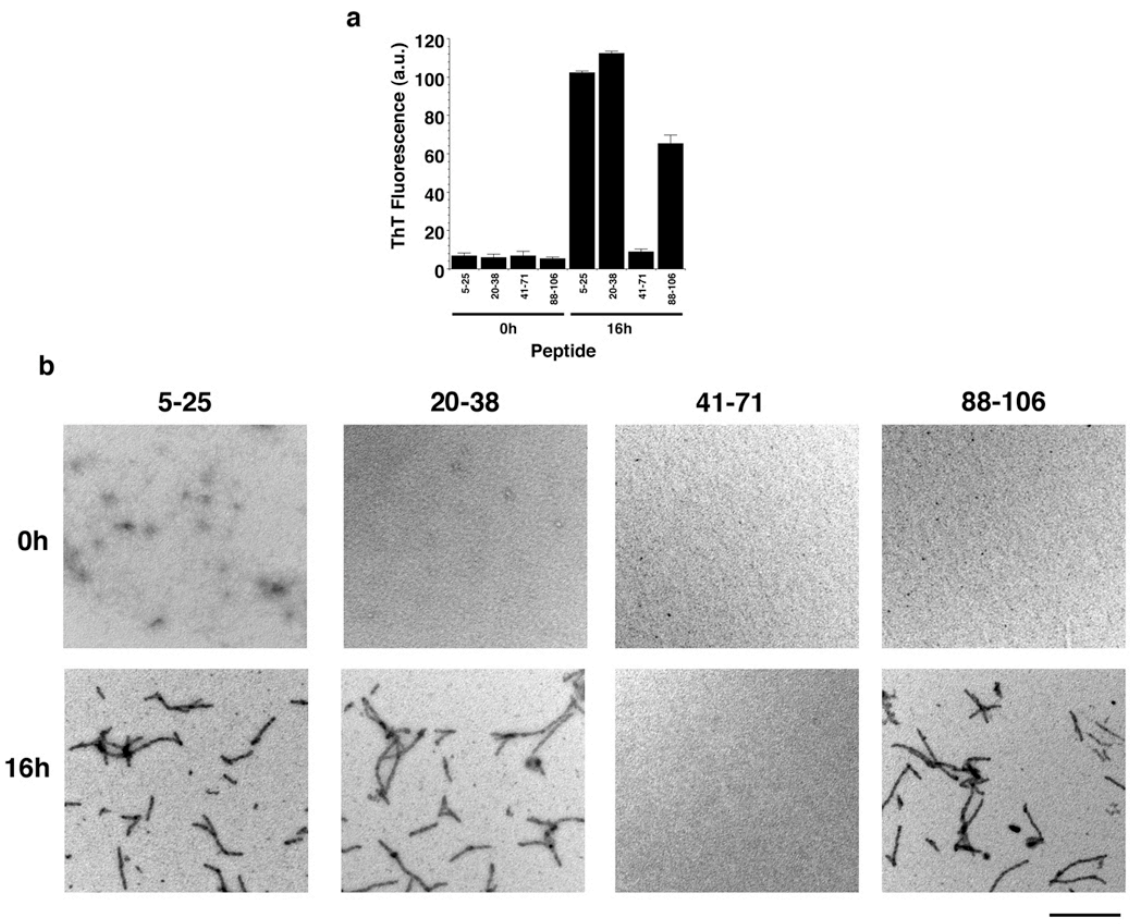

Figure S4. NM fibers assemble on immobilized N peptides comprising residues 5-25, 20-38 and 88-106, but not 41-71. (a, b) The indicated N peptides were immobilized on nitrocellulose, blocked for 24h and incubated for 0 or 16h at 25°C with NM-his (1μM) in the presence of DMSO (1%). Material was then scraped from the surface of the nitrocellulose and either processed for ThT fluorescence **(a)** or EM **(b)**. Values in **(a)** represent means±SD (n=3). Bar in **(b)** is 0.5μm.

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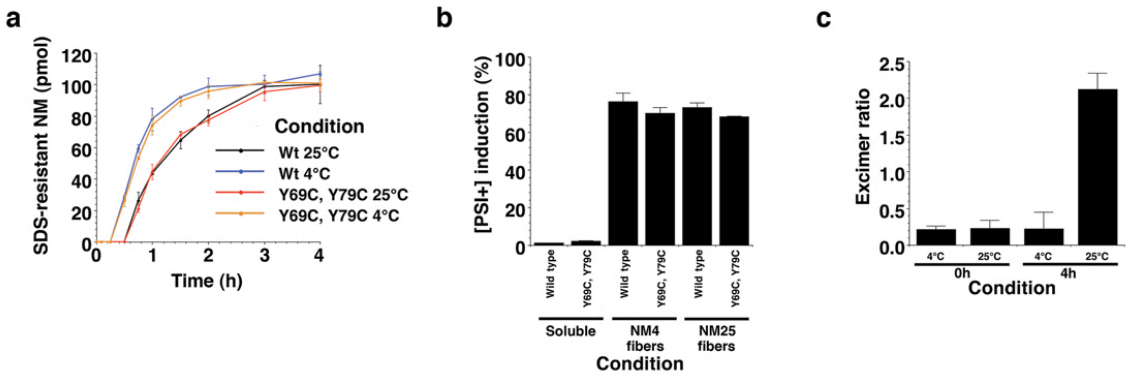

Figure S5. NM Y69C-, 79C-pyrene behaves like wild-type NM.

(a) Wild-type (Wt) NM or NM Y69C-, Y79C-pyrene (5μM) were incubated at 4°C or 25°C for 0-4h with agitation. At various times the extent of fibrillization was determined by assessing the amount of SDS-resistant NM. Values represent means±SD (n=3).

(b) Wild-type (Wt) NM or NM Y69C-, Y79C-pyrene (5μM) were incubated with agitation at 25°C or 4°C for 0h (soluble) or 4h. Reactions were concentrated, sonicated and transformed into [*psi*$^-$] cells. The proportion of transformants that were [*PSI*$^+$] was then determined. Values represent means±SD (n=3).

(c) Excimer fluorescence of NM Y69C-, Y79C-pyrene after assembly for 0h or 4h as in **(a)**. The ratio of excimer fluorescence to non-excimer fluorescence (I_{476nm}/I_{384nm}) is plotted. Values represent means±SD (n=3).

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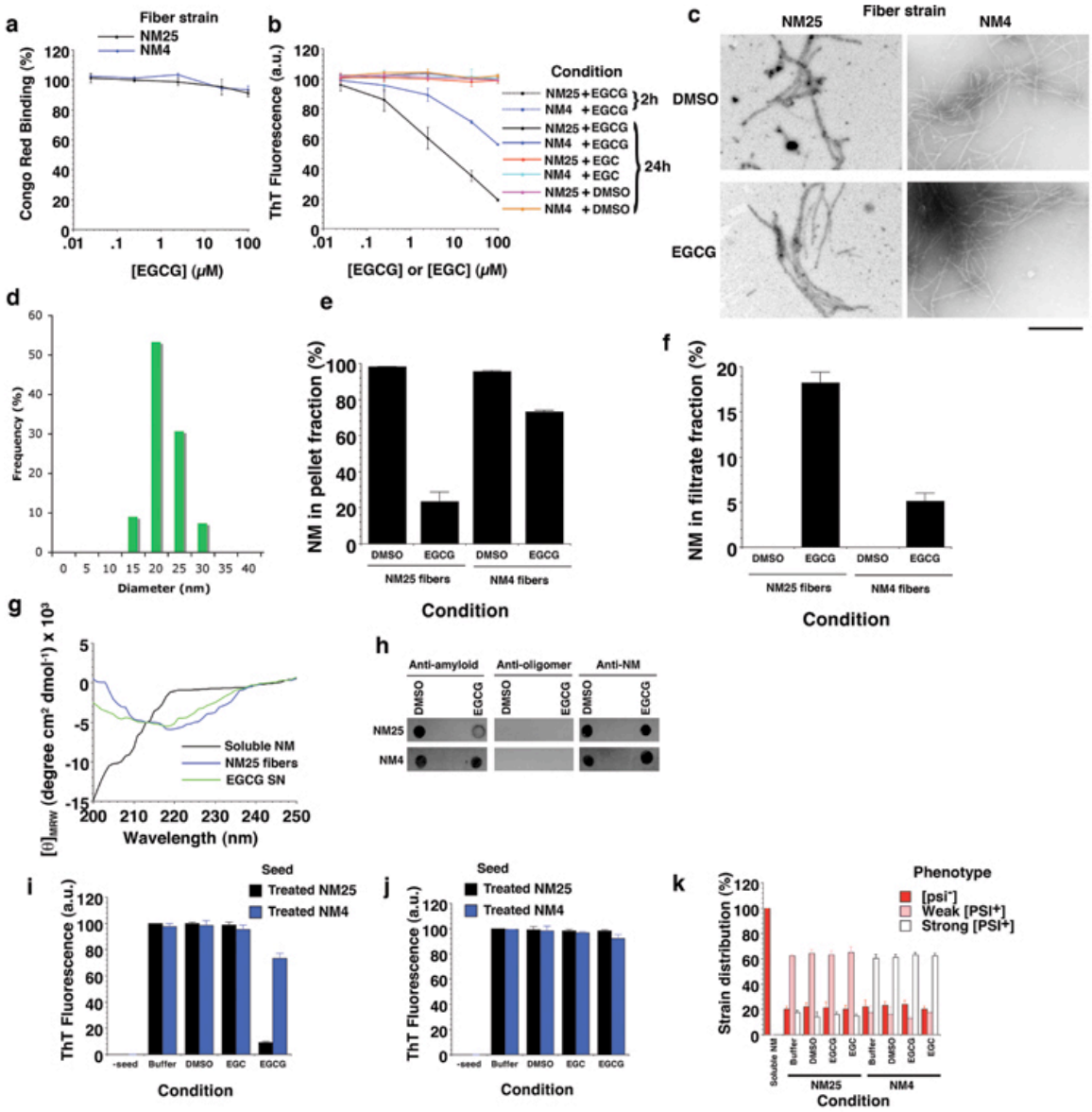

Figure S6. EGCG eliminates select NM prion strains.

(a) NM4 or NM25 fibers (2.5μM monomer) were incubated for 2h at 25°C with EGCG (0.025-100μM). Fiber integrity was then determined by CR binding. Values represent means±SD (n=3).

(b) NM4 or NM25 fibers (2.5μM monomer) were incubated for 24h at 25°C with either DMSO (1%), EGCG or EGC (0.025-100μM). Fiber integrity was then determined by ThT fluorescence. Values represent means±SD (n=3).

(c) NM4 or NM25 fibers (2.5μM monomer) were incubated with DMSO (1%) or EGCG (20μM) for 2h. Bar, 0.5μm.

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(d) NM25 fibers (2.5μM monomer) were incubated for 24h at 25°C with EGCG (20μM) and processed for EM. The diameter of 1000 oligomers was determined and plotted as a histogram.
(e) NM4 or NM25 fibers (2.5μM monomer) were incubated for 24h at 25°C with DMSO (1%) or EGCG (20μM). Reactions were then centrifuged at 100,000g for 10min and the amount of NM in the pellet fraction determined. Values represent means±SD (n=3).
(f) NM4 or NM25 fibers (2.5μM monomer) were incubated for 24h at 25°C with DMSO (1%) or EGCG (20μM). Reactions were then fractionated using a 50kDa Microcon filter. The amount of NM in the filtrate fraction was then determined. Values represent means±SD (n=3).
(g) NM25 fibers (2.5μM monomer) were incubated for 24h at 25°C with buffer or EGCG (20μM). Reactions were then centrifuged at 100,000g for 10min and the CD spectra of the supernatant fraction was determined (EGCG SN, green trace) and compared to NM25 fibers treated with buffer (blue trace) and unassembled NM (black trace).
(h) NM25 or NM4 fibers (2.5μM monomer) were incubated for 24h at 25°C with DMSO (1%) or EGCG (20μM). Reactions were then applied to nitrocellulose and probed with anti-amyloid antibody, anti-oligomer antibody or anti-NM antibody.
(i) NM25 or NM4 fibers (2.5μM monomer) were incubated with or without DMSO (1%), EGCG or EGC (20μM) for 24h. Reactions were then dialyzed to remove unbound small molecule and used to seed (2% wt/wt) fresh, undisturbed NM (2.5μM) fibrillization. Values represent means±SD (n=3).
(j, k) NM4 or NM25 fibers (2.5μM monomer) were incubated in the absence or presence of DMSO (1%), EGCG or EGC (20μM) for 2h. Reactions were then dialyzed to remove unbound small molecule and used to seed (2% wt/wt) fresh, undisturbed NM (2.5μM) fibrillization **(j)**. Values represent means±SD (n=3). Alternatively **(k)**, reaction products were dialyzed to remove unbound small molecule, concentrated, sonicated and transformed into [*psi*$^-$] cells. The proportion of transformants that were [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] was then determined. Values represent means±SD (n=3).

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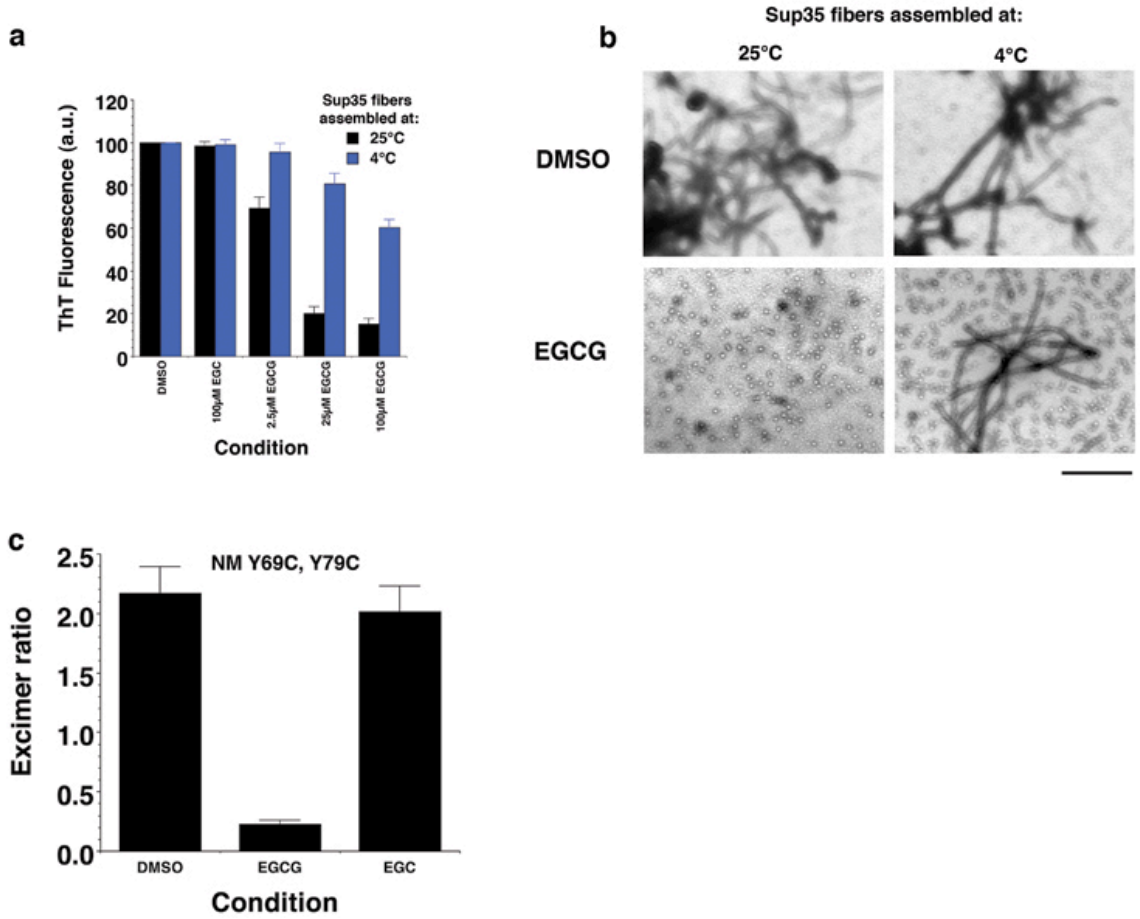

Figure S7. EGCG eliminates select Sup35 prion strains.

(a) Sup35 fibers (2.5μM monomer) assembled at 4°C or 25°C were incubated for 24h at 25°C with either DMSO (1%), EGCG (2.5-100μM) or EGC (100μM). Fiber integrity was then determined by ThT fluorescence. Values represent means±SD (n=3).

(b) Sup35 fibers (2.5μM monomer) assembled at 4°C or 25°C were incubated with either DMSO (1%) or EGCG (100μM) for 24h and processed for EM. Bar, 0.5μm.

(c) NM Y69C-, Y79C-pyrene fibers (2.5μM) were formed at 25°C and then incubated with DMSO (1%), EGCG or EGC (20μM) for 24h. The ratio of excimer fluorescence to non-excimer fluorescence (I_{476nm}/I_{384nm}) is plotted. Values represent means±SD (n=3).

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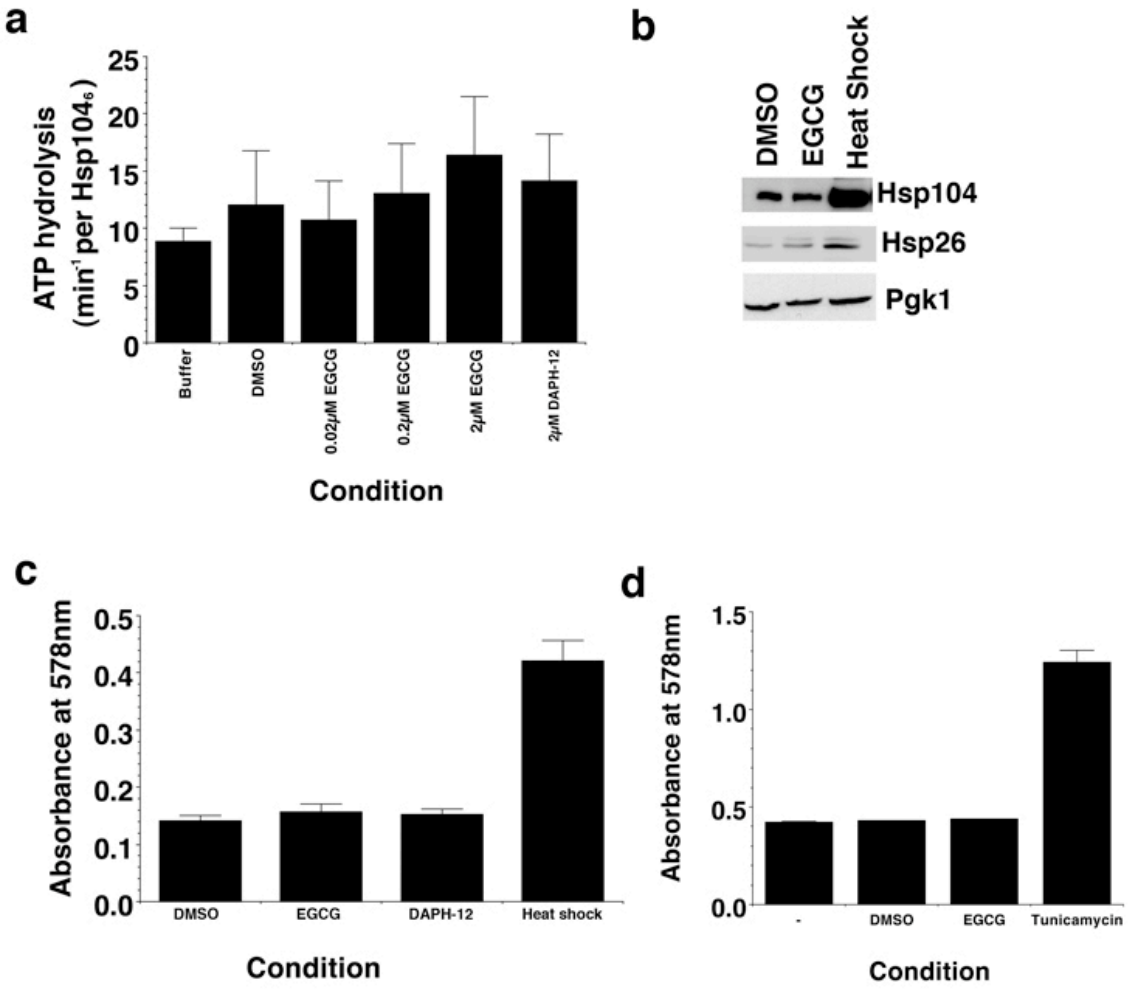

Figure S8. EGCG does not inhibit Hsp104 ATPase activity or induce a heat shock or unfolded protein response.

(a) Hsp104 (0.03μM hexamer) was incubated at 25°C for 20min in the presence of 1mM ATP and either buffer, DMSO (1%), EGCG (0.02-2μM) or DAPH-12 (2μM) and the amount of ATP hydrolysis was determined. Values represent means±SD (n=3).

(b) [*PSI*$^+$] *pdr5* yeast cells were maintained in mid-log phase for 48h in liquid culture containing either DMSO (1%) or EGCG (125μM). Alternatively, cells were heat-shocked at 42°C for 20min. Cell lysates were then analyzed by immunoblot using anti-Hsp104, anti-Hsp26 and anti-Pgk1 antibodies.

(c) Cells were treated as in **(b)** except that cells were also maintained in the presence of DAPH-12 (125μM). A heat shock reporter plasmid (3XHSE-lacZ) was employed to quantify the induction of a heat shock response. Values represent means±SD (n=3).

(d) [*PSI*$^+$] *pdr5* yeast cells were maintained in mid-log phase for 48h in liquid culture containing either DMSO (0.4%) or EGCG (200μM). Alternatively, cells were treated with

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tunicamycin (5μM) for 6h as a positive control for induction of the unfolded protein response (UPR). Tunicamycin inhibits the glycosylation of proteins in the ER and induces the UPR. An UPR reporter plasmid (UPRE-lacZ) was employed to quantify the induction of the UPR. Values represent means±SD (n=3).

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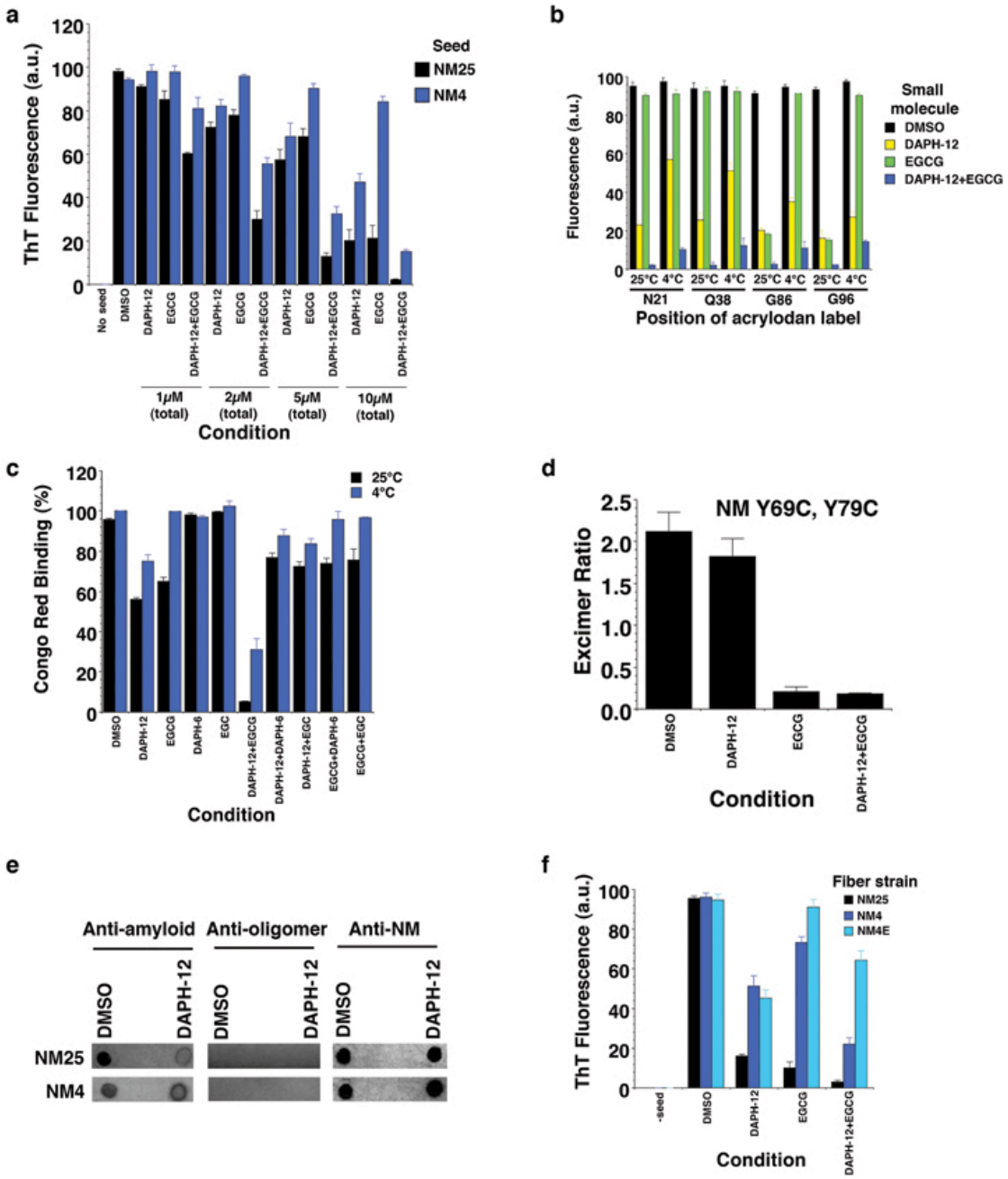

Figure S9. The effects of DAPH-12 and EGCG on NM4 and NM25.

(a) Seeded (0.5% wt/wt) NM (5μM) fibrillization after 6h in the presence of DMSO (1%), DAPH-12, EGCG or EGCG plus DAPH-12 (0.5:0.5). The total concentration of small molecule was kept constant at 1μM, 2μM, 5μM or 10μM. Fibrillization was measured by CR binding and 100% reflects assembly in the absence of DMSO, DAPH-12 or EGCG. Values represent means±SD (n=3).

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(b) Fluorescence of NM-N21C-, Q38C-, G86C- or G96C-acrylodan (5μM) after 15min at 25°C or 4°C in the presence of DMSO (1%), DAPH-12 (10μM), EGCG (10μM) or DAPH-12 plus EGCG (5μM of each). Values represent means±SD (n=3).
(c) Spontaneous, agitated NM (5μM) fibrillization after 6h at 25°C or 4°C in the presence of DMSO (1%), DAPH-12, EGCG, EGC, DAPH-6 (0.25μM) or the indicated combination EGCG (0.5:0.5). The total concentration of small molecule was kept constant at 0.25μM. Fibrillization was measured by CR binding and 100% reflects assembly in the absence of DMSO or small molecule. Values represent means±SD (n=3).
(d) NM Y69C-, Y79C-pyrene fibers (2.5μM) were formed at 25°C and then incubated with DMSO (1%), DAPH-12 (20μM), EGCG (20μM) or DAPH-12 plus EGCG (10μM of each) for 24h. The ratio of excimer fluorescence to non-excimer fluorescence (I_{476nm}/I_{384nm}) is plotted. Values represent means±SD (n=3).
(e) NM25 or NM4 fibers (2.5μM monomer) were incubated for 24h at 25°C with DMSO (1%) or DAPH-12 (20μM). Reactions were then applied to nitrocellulose and probed with anti-amyloid antibody, anti-oligomer antibody or anti-NM antibody.
(f) NM25, NM4 or NM4E fibers (2.5μM monomer) were incubated with DMSO (1%), DAPH-12 (20μM), EGCG (20μM) or DAPH-12 plus EGCG (10μM of each) for 24h. Reactions were then dialyzed to remove unbound small molecule and used to seed (2% wt/wt) fresh, undisturbed NM (2.5μM) fibrillization. Values represent means±SD (n=3).

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