

A computational combinatorial approach identifies a protein inhibitor of superoxide dismutase 1 misfolding, aggregation, and cytotoxicity

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Molecular agents that specifically bind and neutralize misfolded and toxic superoxide dismutase 1 (SOD1) mutant proteins may find application in attenuating the disease progression of familial amyotrophic lateral sclerosis. However, high structural similarities between the wild-type and mutant SOD1 proteins limit the utility of this approach. Here we addressed this challenge by converting a promiscuous natural human IgG-binding domain, the hyperthermophilic variant of protein G (HTB1), into a highly specific aggregation inhibitor (designated HTB1_M) of two familial amyotrophic lateral sclerosis-linked SOD1 mutants, SOD1^{G93A} and SOD1^{G85R}. We utilized a computational algorithm for mapping protein surfaces predisposed to HTB1 intermolecular interactions to construct a focused HTB1 library, complemented with an experimental platform based on yeast surface display for affinity and specificity screening. HTB1_M displayed high binding specificity toward SOD1 mutants, inhibited their amyloid aggregation *in vitro*, prevented the accumulation of misfolded proteins in living cells, and reduced the cytotoxicity of SOD1^{G93A} expressed in motor neuron-like cells. Competition assays and molecular docking simulations suggested that HTB1_M binds to SOD1 via both its α -helical and β -sheet domains at the native dimer interface that becomes exposed upon mutated SOD1 misfolding and monomerization. Our results demonstrate the utility of computational mapping of the protein-protein interaction potential for designing focused protein libraries to be used in directed evolution. They also provide new insight into the mechanism of conversion of broad-spectrum immunoglobulin-binding proteins, such as HTB1, into target-specific proteins, thereby paving the way for the development of new selective drugs targeting the amyloidogenic proteins implicated in a variety of human diseases.

Amyotrophic lateral sclerosis (ALS)² is a fatal neurodegenerative disease characterized by the loss of upper and lower motor neurons (1). About 10% of ALS cases are familial (fALS), having a genetic origin, whereas the rest are sporadic (2). About 20% of fALS cases are caused by mutations in the enzyme copper/zinc superoxide dismutase 1 (SOD1), an enzyme that reduces oxidative stress by converting reactive oxygen species into oxygen and hydrogen peroxide (2, 3). More than 150 mutations in SOD1 (either single point or deletion) have been linked to fALS (4–6). These are distributed throughout the protein sequence with no specific pattern (4, 7), and, importantly, most are associated with misfolding and aggregation of SOD1 (8–10). Even though the mechanism by which SOD1 is associated with neuron degeneration is not clear, it has been hypothesized that gain of function(s) acquired by misfolded SOD1 proteins allows them to interact with homologous (other SOD1 molecules) or heterologous (unrelated) proteins, thereby leading to misfolding and aggregation of the former and hampering the normal functions of the latter proteins (6, 8, 11–13). In the “homologous mechanism,” misfolded SOD1 induces misfolding of folded SOD1 conformers in a prion-like manner, and those misfolded conformers eventually form amyloid-like aggregate structures (7, 14, 15). Recently, we identified the aggregation-triggering segment of SOD1, “LSGDHC,” which is located in the Greek key loop. This segment is presumably exposed upon misfolding and interacts with folded SOD1 proteins to trigger aggregation (7). Interaction of misfolded SOD1 with heterologous proteins may cause toxicity by a variety of mechanisms; for example, by binding to Derlin-1, a component of the endoplasmic reticulum associated with the protein degradation pathway, and hampering its function (3, 16, 17).

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This article contains supplemental Figs. S1–S10, Tables S1–S4, and Experimental Procedures.

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² The abbreviations used are: ALS, amyotrophic lateral sclerosis; fALS, familial amyotrophic lateral sclerosis; SOD, superoxide dismutase; YSD, yeast surface display; SMD, steered molecular dynamics; SPR, surface plasmon resonance; EGFP, enhanced GFP; EBFP, enhanced blue fluorescent protein; IRES, internal ribosome entry site; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide; IP, immunoprecipitation; ThT, thioflavin T; TEM, transmission electron microscopy; AAV, adeno-associated virus; RC, resistance coefficient; PE, phycoerythrin; BGU, Ben-Gurion University of the Negev; DMSU, DNA Microarray and Sequencing Unit; NIBN, National Institute for Biotechnology in the Negev.

Similarly, in the mitochondrial compartment, misfolded SOD1 may interact with voltage-dependent anion channel 1 (VDAC1), Bcl-2, and other protein import machinery of the mitochondria, thereby altering their functions (13). Thus, inhibition of misfolding and subsequent aggregation of SOD1 appears to be of paramount importance for preventing the toxicities associated with both homologous and heterologous interaction pathways (3, 18, 19).

SOD1^{G93A} is the most extensively researched fALS-linked mutant of SOD1, being studied in both cellular and animal ALS models (3, 7, 20, 21). Nevertheless, there are only a few reports of development of molecular agents targeting SOD1^{G93A} for the treatment of ALS, and none has yet been approved by the Food and Drug Administration. A few compounds have been reported to extend the life of transgenic SOD1^{G93A} mice by 10–15%, but, likewise, none of these have been successfully translated into a treatment for fALS (22, 23). To date, there are only two Food and Drug Administration-approved drugs for ALS, Rilutek (riluzole) and Radicava (edaravone), and these drugs are not curative; they merely marginally slow disease progression (24). Among the few compounds that are in phase III trials, dextramipexole and ceftriaxone failed to slow disease progression, and CK2017357 is still under examination (25–28). Therefore, there is a pressing need for new therapeutics that will halt or significantly slow the progression of the disease.

With the aim to target SOD1 misfolding and aggregation, some studies have used *in silico* approaches to develop therapeutic agents (29–31). For example, the computational approach of Ray *et al.* (30) yielded several small molecules that stabilized the SOD1 dimer interface, but their role in inhibiting SOD1 aggregation was not determined. We and others have applied different methods with the aim to develop molecules that inhibit the aggregation of SOD1 (7, 31, 32). Benmohamed *et al.* (31), for example, used a cell-based screening approach to identify several small molecules that inhibit the aggregation of SOD1^{G93A}. However, their most potent compound increased the life span of SOD1^{G93A} transgenic mice by no more than 13% compared with untreated mice, again exemplifying the need for better therapeutics for fALS (33). Using phage display, Ghadge *et al.* (32) evolved a single-chain variable fragment to bind mutant SOD1 proteins and showed that the evolved variants prevented the aggregation and cytotoxicity of the SOD1^{A4V} mutant form in NSC-34 cells. However, the evolved variants did not display specificity for misfolded SOD1, as it also bound to SOD1^{WT}, thereby limiting their application as therapeutics. Moreover, antibodies generated against the SOD1 dimeric interface did not increase the life span of ALS mice by more than 13%, probably because their large size limited their penetration into motor neuronal cells (34). Recently, using a novel computational analysis of the dynamics of protein surfaces, we identified a 20-amino acid SOD1-derived peptide (designated SE-12) that prevented amyloid aggregation of SOD1 mutants (7). Nevertheless, the need for a high inhibitor to SOD1 ratio and the self-aggregation propensity of this peptide limit its application as a drug candidate.

In this study, we sought to improve the efficiency of the individual experimental and computational approaches by employ-

ing a combined strategy in which a quantitative screening approach for selecting desirable features was complemented with computational analysis to map the interaction-prone regions of a starting protein scaffold (35, 36). The scaffold chosen was HTB1, a 58-residue hyperthermophilic variant of protein G (3, 37–39). Being a small, compact protein that is stabilized by a hydrophobic core, HTB1 possesses high thermal and chemical stabilities. An additional advantage of this protein is that the residues within both its α -helix and β -sheet regions in HTB1 are highly tolerant to substitution or incorporation of additional amino acids (37, 40, 41). A backbone dynamics analysis of the HTB1 surface revealed 12 positions within the α -helix and β -sheet regions in which intermolecular contacts would be expected to have the strongest impact on binding affinity. Based on these positions, a focused HTB1 library was constructed and screened using a yeast surface display (YSD) platform. The screening yielded HTB1_M, a selective binder of two fALS-related mutants, SOD1^{G93A} and SOD1^{G85R}. Here we show that HTB1_M prevented amyloid aggregation and inhibited misfolding and cytotoxicity of SOD1^{G93A} in cellular ALS models.

Results

Design of the focused HTB1 mutant library

To develop molecular agents that would specifically target misfolded SOD1 mutant proteins, we applied a combinatorial affinity maturation technique using YSD (38, 39). With this technique, a combinatorial library of a protein of interest, randomized at specified positions, is expressed on the surface of yeast as a C-terminal fusion to the yeast Aga2p protein (42), and the clones are then screened for improved molecular features, such as target affinity, by using FACS (43).

In this study, HTB1 (37) served as the YSD scaffold to produce a focused HTB1 mutant library that allowed us to cover most of the sequence space for the randomized HTB1 positions. Specifically, we used a recently developed computational algorithm based on a steered molecular dynamics (SMD) simulation to map the dynamic landscape of the HTB1 surface (35, 44). The algorithm identifies surface areas that contain clusters of static residues, the so-called “stability patches.” Intermolecular interactions that involve contact residues positioned within the stability patches are expected to make a greater contribution to the binding free energy of the complex than the residues positioned within mobile surface region (35). We hypothesized that, by focusing our library on the residues within the existing stability patches, the probability of identifying efficient HTB1 binders would increase. The clusters of static residues on the HTB1 surface comprised Lys-6, Ile-8, Ala-26, Glu-27, Glu-29, Lys-30, Ile-31, Lys-33, Tyr-35, Glu-44, Lys-52, and Thr-55; of these, Lys-6, Ile-8, Glu-44, Lys-52, and Thr-55 belong to the β -sheet region and the rest to the α -helix region (Fig. 1). These residues were subjected to randomization using an assembly PCR technique, yielding a stability patch-focused library with a diversity of about 3×10^5 clones. The library was used as the starting point for affinity maturation by YSD.

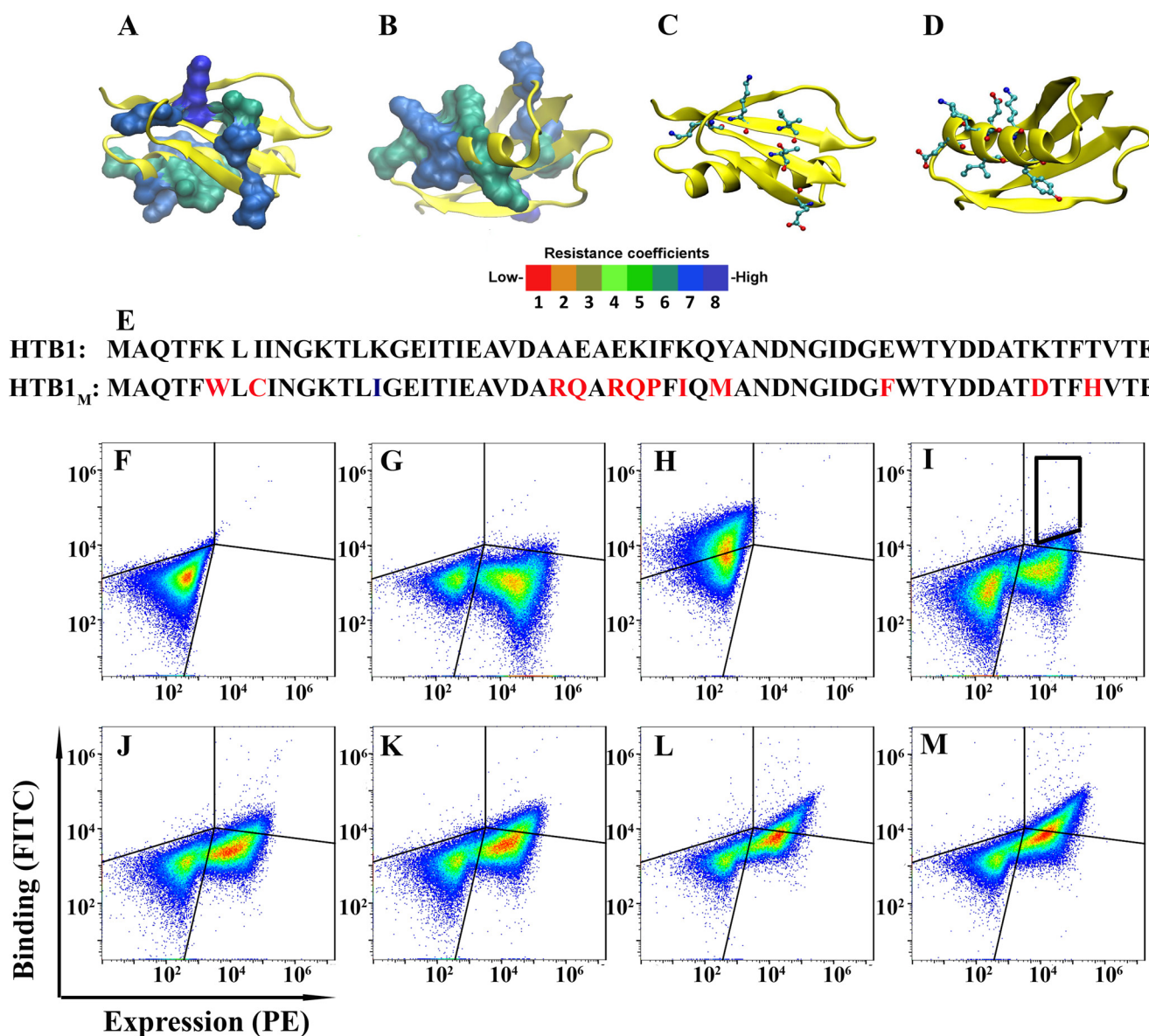


Figure 1. Construction of the stability patch-focused HTB1 library and affinity maturation experiment. *A–D*, 12 indicated surface positions harboring static residues (with RC ≥ 6 , see “Experimental Procedures”) located within the β -sheet (*A* and *C*) and α -helix (*B* and *D*) regions of the HTB1 structure (yellow) were selected for randomization. *A* and *B*, the static residues are depicted in the surface representation and colored according to their RCs. *E*, amino acid sequence of HTB1 and its engineered variant, HTB1_M. Mutations are colored in red. *F–I*, FACS plots of single- or dual-labeled cell populations for monitoring expression or both expression and binding, respectively. *F*, unstained cells (control). *G*, cells stained with anti-cMyc antibody and anti-chicken PE antibody to test for the expression of HTB1 and its variants. *H*, cells incubated with 20 nM biotinylated human IgG and NeutrAvidin PE to test for binding to human IgG. *I*, first set of sorting (*S*₁) of cell populations from the HTB1 library using 10 μ M SOD1^{G93A}. The sorting gate is marked in black. The *S*₁ library was further sorted using 5 μ M SOD1^{G93A}, which resulted in the *S*₂ library; the latter library was sorted using 3 μ M SOD1^{G93A}, which resulted in the *S*₃ library. The *S*₃ library was finally sorted using 2 μ M SOD1^{G93A} to give *S*₄ HTB1 sorted clones. *J–M*, double staining analysis using 10 μ M SOD1^{G93A} for *S*₁ (*J*), *S*₂ (*K*), *S*₃ (*L*), and *S*₄ (*M*).

Affinity maturation of the HTB1 library

HTB1 and the mutated HTB1 library were expressed on the yeast surface as cMyc–HTB1 fusion proteins. The high level of expression of wild-type HTB1 was confirmed by using anti-cMyc antibodies (supplemental Fig. S1B), and its proper folding was confirmed by using human IgG antibody, a natural binder for HTB1 (45) (Fig. S1C). As expected, the displayed wild-type HTB1 scaffold did not show any affinity for SOD1^{G93A} (supplemental Fig. S1D).

Initial sorting of the HTB1-focused library based on the expression of the C terminus c-Myc epitope produced the *S*₀ library, comprising only highly expressed, intact HTB1 clones

(Fig. 1G). The *S*₀ library was then subjected to four sequential rounds of sorting for high-affinity binders, starting with 10 μ M of fluorescently labeled SOD1^{G93A} (Fig. 1I) and decreasing the concentration of the soluble SOD1^{G93A} protein in each sorting step as described in the legend for Fig. 1, *J–M*. Single clones selected from the final sorting were propagated, and genomic DNA was extracted and sequenced, yielding a single HTB1 clone, designated HTB1_M, with an affinity of 2.8 μ M for SOD1^{G93A} when HTB1_M was displayed on yeast (supplemental Fig. S2A). This HTB1_M mutant had 12 point mutations in the randomized regions and one additional mutation, K15I, added during PCR amplification.

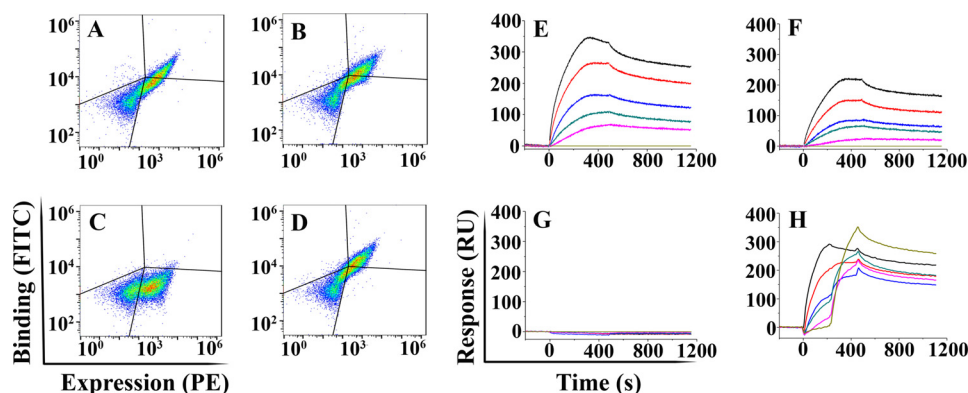


Figure 2. YSD FACS plots and SPR binding sensorgrams for the interactions between HTB1_M and SOD1 proteins. A–D, binding of YSD HTB1_M to soluble SOD1 variants was analyzed by FACS using a dual staining method (PE for expression and FITC for binding). To detect expression, the cells were stained with anti cMyc antibody (ab19233) followed by an anti-chicken PE antibody (sc3730). To detect binding, YSD HTB1_M was incubated with SOD1^{G93A} (A), SOD1^{G85R} (B), SOD1^{WT} (C), and heat-treated SOD1^{WT} (D), followed by NeutrAvidin-PE staining for detection by FACS. E–H, dose–response SPR sensorgrams illustrating the interaction of HTB1_M with the SOD1 analytes at different concentrations (dark yellow, magenta, dark cyan, blue, and black represent 0, 39, 78.1, 312.5, and 625 nM, respectively). Sensorgrams are shown for purified HTB1_M (immobilized ligand) interacting with the following analytes: SOD1^{G93A} (E), SOD1^{G85R} (F), and SOD1^{WT} (G). In the competitive binding experiment (H), SOD1^{G85R} competes for binding with HTB1_M in the presence of SOD1^{G93A}. In this experiment, SOD1^{G93A} was first allowed to flow for 3 min on a sensor chip covered with HTB1_M, and then SOD1^{G85R} was injected, and the binding sensorgram was monitored.

HTB1_M binds SOD1^{G93A} and SOD1^{G85R} but not folded wild-type SOD1

HTB1_M was expressed on the yeast surface and tested for binding to 2 μ M soluble SOD1^{G93A}, SOD1^{G85R}, or wild-type SOD1 (SOD1^{WT}) (Fig. 2, A–C). The two SOD1 mutants bound to HTB1_M to a similar extent, whereas SOD1^{WT} did not bind (Fig. 2, A–C). When SOD1^{WT} was heated at 94 °C for 30 min and cooled down to room temperature (without causing precipitation), this thermally treated SOD1^{WT} bound very strongly to HTB1_M but not to HTB1 (Fig. 2D). To determine whether this effect was specific to SOD1 and not caused by nonspecific interactions with exposed hydrophobic patches resulting from thermal denaturation, we tested the binding of HTB1_M to similarly treated bovine serum albumin or ovalbumin (chicken egg white). No binding to either of these proteins was detected (supplemental Fig. S3). The above findings show that HTB1_M binds to a specific region that is buried in SOD1^{WT} but becomes exposed because of thermal denaturation or mutation (G93A or G85R (46, 47)) of this protein. It is possible that HTB1_M binds to structurally destabilized SOD1 mutants or thermally denatured SOD1 through the exposed dimer interface. To test this idea, we incubated SOD1 mutant proteins with SPC-206 (48), a monoclonal antibody that specifically binds to the dimeric interface of SOD1, prior to SOD1 binding to HTB1_M. As shown in supplemental Fig. S2, B and C, the SPC-206 mAb did indeed compete with HTB1_M for binding to SOD1 mutants, thereby supporting our hypothesis that HTB1_M interacts with the exposed dimeric interface of SOD1.

To further elucidate the binding interaction of HTB1 and HTB1_M with SOD1, both HTB1 and HTB1_M were purified from *Escherichia coli* strain BL21 DE3. The molecular masses (obtained by MALDI-TOF) of pure HTB1 and HTB1_M monomers were 8.7 and 8.9 kDa, respectively, which matched the theoretical molecular mass of these proteins (supplemental Fig. S4, B and C). Size-exclusion chromatography analysis under native conditions revealed that purified HTB1 was a monomer, whereas HTB1_M existed as oligomer (supplemental Fig. S4A). Both proteins had a tryptophan fluorescence emission maxi-

mum at around 337 nm, indicating that they are folded under physiological conditions (supplemental Fig. S4D). (We note that tryptophan fluorescence spectra are an accepted tool for studying protein folding (49, 50).)

The binding of purified HTB1 and HTB1_M proteins to SOD1^{WT} or the SOD1 mutants SOD1^{G93A} and SOD1^{G85R} was determined with surface plasmon resonance (SPR) spectroscopy. As expected, correctly folded HTB1 efficiently bound IgG (supplemental Fig. S5A) but not SOD1^{WT}, SOD1^{G93A}, or SOD1^{G85R} (supplemental Fig. S5, B–D). Unlike HTB1, HTB1_M did not show any affinity for IgG (supplemental Fig. S6A), probably because of the mutations in its α -helix domain that harbor the IgG binding epitope (45). HTB1_M, however, efficiently bound SOD1^{G93A} and SOD1^{G85R} with calculated equilibrium binding constants of 300 and 674 nM, respectively (Fig. 2, E and F). No detectable binding of HTB1_M to SOD1^{WT} was observed up to a concentration of 600 nM (Fig. 2G).

To determine whether the same epitope of HTB1_M is used for interaction with the two SOD1 mutants, SOD1^{G93A} and SOD1^{G85R}, we performed a competitive binding assay. Saturation with SOD1^{G93A} prevented binding of SOD1^{G85R} and vice versa, indicating that the two SOD1 mutants compete for the same (overlapping) binding site on HTB1_M (Fig. 2H). The binding specificity of HTB1_M for the two SOD1 mutants was further validated by measuring its binding to two other amyloidogenic proteins, α -synuclein and amyloid β -42. Neither showed any significant interaction with HTB1_M up to a concentration of 5 μ M (supplemental Fig. S6, B and C). The binding of HTB1_M to SOD1^{G93A} was also verified in a motor neuron–like cell line, NSC-34. In this experiment, HA-tagged HTB1_M was co-expressed with SOD1^{G93A}–enhanced GFP (EGFP), followed by immunoprecipitation (IP) with anti-SOD1 antibody and Western blotting with anti-HA antibody (supplemental Fig. S7).

Docking analysis of HTB1_M with wild-type SOD1 and SOD1 mutants

To acquire insight into the binding mode of HTB1_M to SOD1, we used computational docking analysis. The structure

of HTB1_M was generated by homology modeling based on the published structure of the B1 domain of protein G (supplemental Fig. S8, A–C) (51). We used the homology modeling under the assumption that the inability of HTB1_M to bind IgG was due to the mutations in the α -helix IgG binding domain of the former and not due to global structural perturbations. The HTB1_M modeled structure was then docked with SOD1^{G93A} and SOD1^{G85R}.

Selection of the best docking solutions was made on the basis of ZRANK score, cluster size, nature of the interface, and type of interactions in each complex together with the available biological information (see “Experimental Procedures”). ZDOCK docking poses solutions were clustered according to the ligand position, and analysis of the top poses of the largest clusters indicated the preferred interfaces (supplemental Fig. S8E). Based on the experimental finding indicating that HTB1_M binds at the SOD1 native dimer interface, we focused on the cluster that shares the same interface with the SOD1^{WT} homodimer.

A comparison between the docking results for HTB1_M with the two SOD1 mutants showed that the HTB1_M/SOD1^{G93A} complex gave a better docking score and ranking than the docking solution to SOD1^{G85R} (supplemental Table S1), in keeping with the experimental results; the various types of HTB1_M–SOD1 interactions are shown in supplemental Tables S2 and S3. In addition, the cluster size and cluster density of the HTB1_M–SOD1^{G93A} complex were higher, indicating that more docking solutions were obtained for this interface (supplemental Table S1). These differences in the number of docking solutions might reflect the fact that there are certain differences in the conformers of SOD1 side chains at the complex interface in SOD1^{G93A} versus SOD1^{G85R} (supplemental Fig. S8F and Table S2). In G93A and G85R, the mutations are not located in the predicted interface HTB1_M–SOD1 binding site, and, hence, their influence on the binding is minor, if any.

Our detailed analysis of the mutated HTB1_M residues positioned on the putative SOD1^{G93A}–HTB1_M complex interface revealed that Arg-29 in HTB1_M makes the highest contribution to the polar contact surface area and that Phe-44 and Ile-33 contribute to the nonpolar contact surface area (Fig. 3A). Although Arg-29 and Ile-33 mutations are located on the α -helix domain of HTB1_M, the Phe-44 mutation is located on the β -strand. Thus, it appears that both the α -helical and β -sheet structural domains of HTB1_M contribute to the interaction with SOD1. To validate our docking model, we made three single point mutants, HTB1_M^{R29E}, HTB1_M^{I33K}, and HTB1_M^{F44E} (positions 29, 33, and 44 were changed back to the residues HTB1 has in those positions), and studied the binding of three mutants to SOD1^{G93A} and SOD1^{G85R} in YSD (Fig. 3, B and C). The HTB1_M^{R29E} mutant showed slightly higher binding than HTB1_M, as expected from the docking model in which Arg-29 is in close proximity to Lys-9 of SOD1, and replacing Arg with Glu may create a salt bridge that facilitates binding (Fig. 3A, left panel). The other two mutants showed lower affinity for SOD1 mutants. Replacing Ile-33 with Lys (HTB1_M^{I33K} mutant) may cause repulsion between Lys-33 of HTB1_M^{I33K} and Lys-9 of SOD1, and this repulsion could account for the lower binding of HTB1_M^{I33K} to the SOD1 mutant (Fig. 3A, left panel, B, and C).

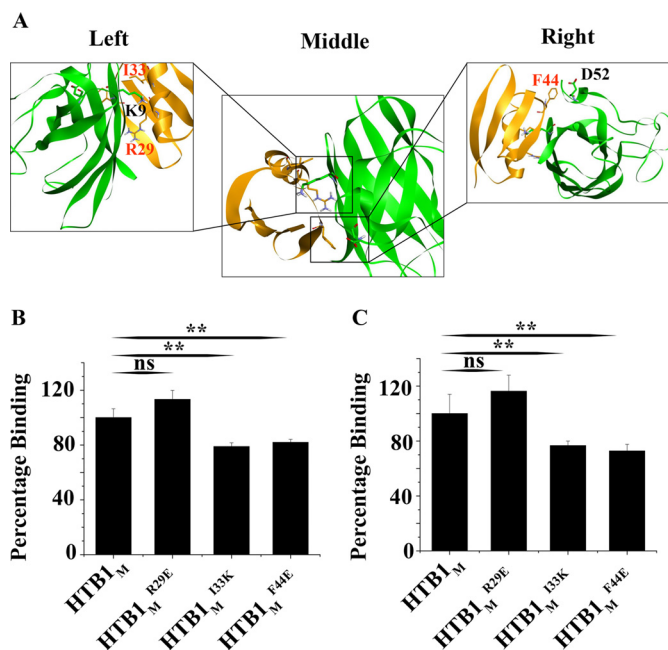


Figure 3. A, center panel, docking result between SOD1^{G93A} and HTB1_M. SOD1 is depicted as a green ribbon and HTB1_M as an orange ribbon. Left panel, focusing the interface between the HTB1_M α -helix domain and SOD1-K9. Right panel, focusing the interface of HTB1_M β -sheet domain and the trio of hydrophobic SOD1 residues. B and C, binding of YSD expressed HTB1_M and its single point mutants to 0.8 μ M biotinylated SOD1^{G93A} (B) and SOD1^{G85R} (C). The fluorescence intensity of the nutravidine FITC (which was used to detect biotinylated SOD1) was normalized to the fluorescence intensity of anti-chicken PE (which was used as a secondary antibody to detect the expression level of HTB1_M). These values were then divided by the ratio of binding to expression (i.e. FITC intensity to PE intensity) of HTB1_M and multiplied by 100. Quantitative analysis ($n = 3$) was performed by unpaired Student's t test. **, $p < 0.01$; ns, not significant. Error bars indicate the standard deviation of independent experiments performed in triplicate.

Phe-44 is situated in close proximity to Asp-52 of SOD1 (Fig. 3A, right panel). The outcome of replacing Phe-44 with a negatively charged residue Glu would be electrostatic repulsion between Glu-44 of HTB1_M^{F44E} and Asp-52 of SOD1, which is probably responsible for the lower binding of HTB1_M^{F44E} to the SOD1 mutant (Fig. 3, B and C).

HTB1_M inhibits amyloid-like aggregation of SOD1 mutants in vitro

We next investigated the effect of HTB1_M on mutant SOD1 amyloid-like aggregate formation. To this end, we allowed SOD1^{G85R} or SOD1^{G93A} (50 μ M) to aggregate in the presence of different concentrations of HTB1_M. Aggregate formation was followed by monitoring the fluorescence of thioflavin T (ThT) dye, which specifically interacts with amyloid-like aggregates (52). Our results showed that HTB1_M inhibited aggregation of the SOD1 mutants in a concentration-dependent manner (Fig. 4). Notably, a substoichiometric amount of HTB1_M was able to inhibit the aggregation of both SOD1^{G93A} and SOD1^{G85R} (Fig. 4, A and B). In contrast, HTB1 did not inhibit aggregation of the SOD1 mutants (Fig. 4, A and B). In addition, HTB1 and HTB1_M, when tested alone, did not show any significant ThT response, thereby indicating a lack of amyloid-like fibril formation under aggregation-promoting conditions (Fig. 4B).

Transmission electron microscopy (TEM) showed that fibrils formed by SOD1^{G93A} and SOD1^{G85R} in the presence or

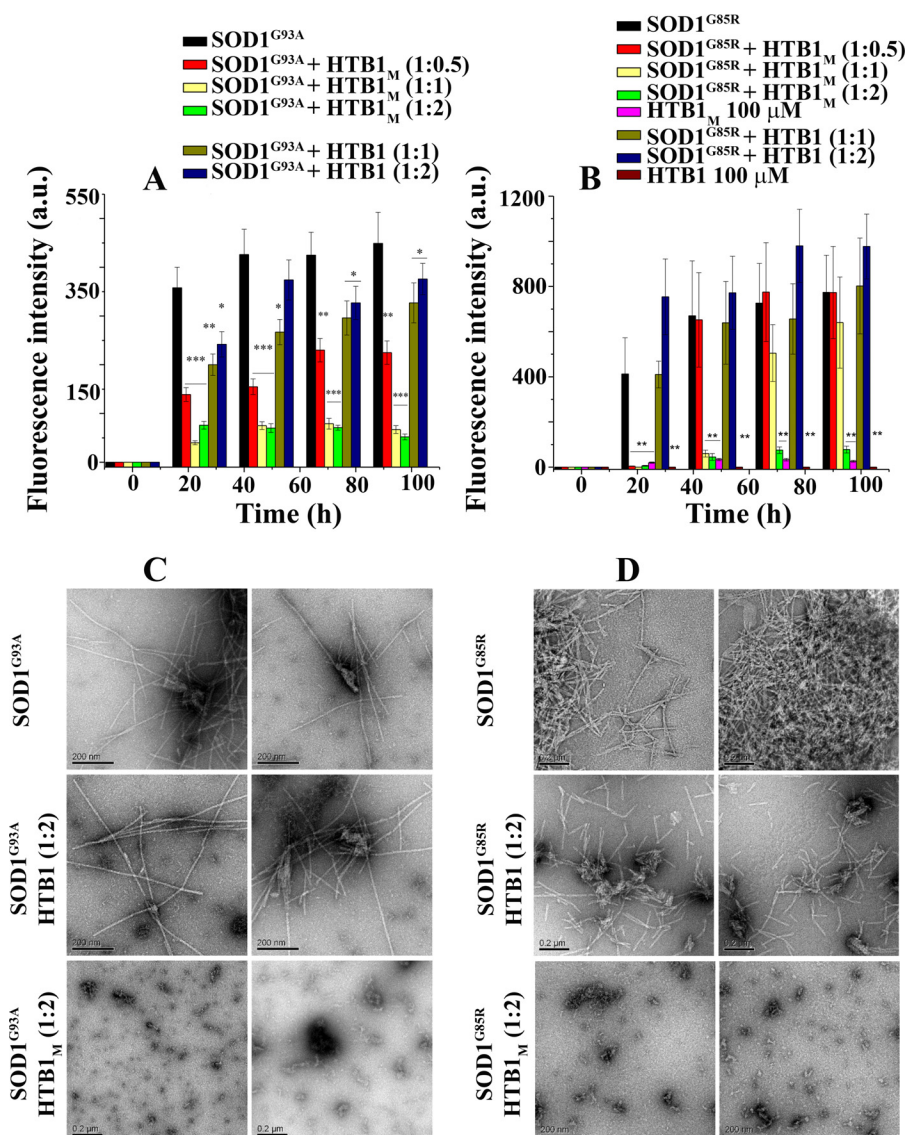


Figure 4. Amyloid-like aggregation of SOD1^{G93A} and SOD1^{G85R} in the presence and absence of HTB1 and HTB1_M. A and B, thioflavin T binding assay of SOD1^{G93A} (A) and SOD1^{G85R} (B) in the presence and absence of HTB1 and HTB1_M. Quantitative analysis ($n = 3$) was performed by unpaired Student's *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate the standard deviation of independent experiments performed in triplicate. C and D, transmission electron microscope images of SOD1^{G93A} (C) and SOD1^{G85R} (D) incubated for 4 days at 37 °C with shaking at 600 rpm in the presence and absence of HTB1 and HTB1_M.

absence of HTB1 were of similar width (10–12 nm), indicating that HTB1 did not have any effect on the morphology of the aggregates that were formed (Fig. 4, C and D). In contrast, no such fibrils were observed for the SOD1 mutants incubated with HTB1_M in a 1:2 molar ratio (SOD1:HTB1_M) (Fig. 4, C and D). However, even though HTB1_M prevented the formation of ordered amyloid-like fibrils, it could not prevent the appearance of some amorphous aggregates of the SOD1 mutants (Fig. 4, C and D).

HTB1_M suppresses misfolding of SOD1^{G93A} in the cytosol of motor neuron-like NSC-34 cells

To examine whether HTB1_M can attenuate the misfolding of the SOD1^{G93A} mutant in cells, we co-expressed SOD1^{G93A}-EGFP and HTB1_M-IRES-EBFP proteins (in 1:0.5 and 1:1 SOD1^{G93A}:HTB1_M DNA concentration ratios) in motor neuron-like NSC-34 cells. Comparable transfection efficiencies were

verified by measuring the fluorescence intensity of both EGFP and EBFP by FACS; as shown, high expression levels were found for both SOD1^{G93A} and HTB1_M (supplemental Fig. S9). In the absence of HTB1_M, misfolded SOD1^{G93A} accumulated in the cytosol, as identified by IP with B8H10 antibody, which specifically recognizes the misfolded SOD1 conformation (3, 53), followed by Western blotting with a C-17 antibody for SOD1. HTB1_M co-transfected with SOD1^{G93A} reduced the levels of misfolded SOD1^{G93A} in a dose-dependent manner without affecting the overall expression levels of SOD1^{G93A} (Fig. 5). At a 1:1 SOD1^{G93A}:HTB1_M DNA ratio, the accumulation of misfolded SOD1^{G93A} was around 60% of the amount observed in the absence of HTB1_M (Fig. 5).

HTB1_M reduces SOD1^{G93A}-induced toxicity in NSC-34 cells

Misfolded or aggregated SOD1 mutants are believed to be major cause of motor neuron degeneration in ALS. Our obser-

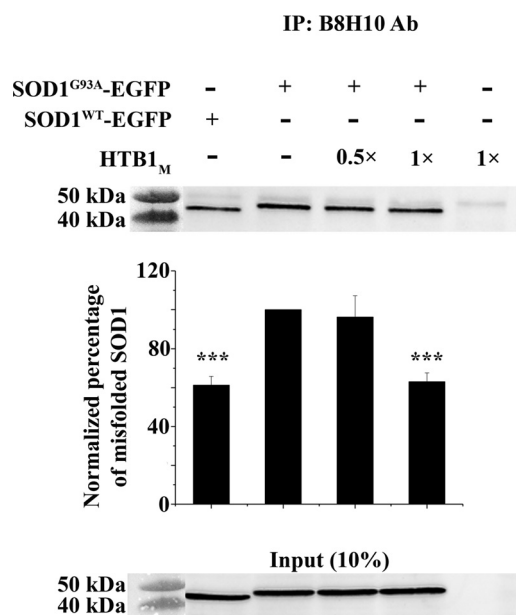


Figure 5. IP experiment for misfolded SOD1 in the ALS NSC-34 cell culture model using B8H10 and C-17 antibodies. SOD1^{G93A}-EGFP was expressed in the presence and absence of HTB1_M in NSC-34 cells. After 48 h, the cells were harvested, and IP was performed with B8H10 antibody. IP samples were analyzed using Western blotting with C-17 antibody (Ab). The Western blot images were analyzed using National Institutes of Health ImageJ software (<https://imagej.nih.gov/ij/>). Error bars indicate the standard deviation of independent experiments performed in triplicate. Quantitative analysis ($n = 3$) was performed by unpaired Student's t test; ***, $p < 0.001$.

vation that HTB1_M attenuated both misfolding and aggregation of SOD1^{G93A} implies that this protein may have the potential to reduce SOD1^{G93A}-induced cytotoxicity. The effect of HTB1_M on cell viability was tested in NSC-34 motor neuron-like cells expressing both SOD1^{G93A} and HTB1_M. Fig. 6 shows that SOD1^{WT} had a minimum effect on cell survival, but SOD1^{G93A} reduced cell survival by ~40%. Co-expression with HTB1_M (in a 1 μ g:0.5 μ g SOD1^{G93A}:HTB1_M DNA ratio) had no significant protective effect on cell viability. Nonetheless, at 1 μ g:0.7 μ g and 1 μ g:1 μ g SOD1^{G93A}:HTB1_M DNA ratios, the toxic effect of SOD1^{G93A} was abolished (Fig. 6). HTB1_M alone had no effect on cell survival.

Discussion

Protein misfolding and aggregation have been linked to various neurodegenerative disorders, including ALS (7, 54–57). Misfolded and aggregated mutant SOD1 proteins have been found in the neuron and glial cells of ALS patients and in tissues of an ALS mouse model (11, 54, 58, 59). Even though the role of mutant SOD1 aggregates in ALS is still a subject for debate, recent studies have shown that these aggregates may be the major cause of the damage to motor neurons (60, 61). In particular, these aggregates may elicit cellular toxicity in fALS patients by hampering the nuclear export and import system (19, 53, 60, 62). Mutant SOD1 is, therefore, a potential target in the search for fALS therapeutics. In this study, we developed a novel inhibitor, HTB1_M, that exhibits specificity toward SOD1 mutants and prevents their misfolding and aggregation both *in vitro* and in cells, thus providing a foundation for the development of new fALS therapeutics.

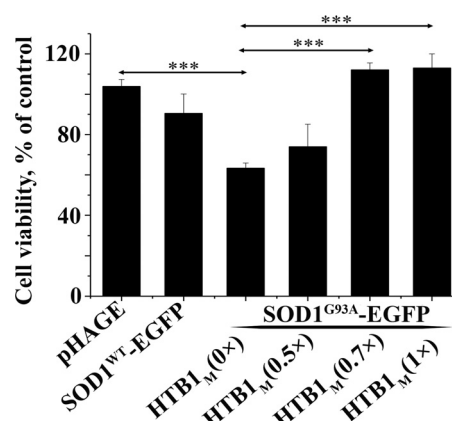


Figure 6. NSC-34 cells were transfected to express human wild-type SOD1 (SOD1^{WT}-EGFP) or human mutant SOD1^{G93A}-EGFP with co-transfection of HTB1_M. Cell viability analysis was performed using an XTT assay kit. Quantitative analysis ($n = 3$) was performed by Student's t test; ***, $p < 0.001$. Error bars indicate the standard deviation of independent experiments performed in triplicate.

To maximize the likelihood of achieving desirable properties for the YSD-engineered protein, in terms of both binding affinity and selectivity, we used a focused library, the construction of which was guided by a computational analysis of the HTB1 surface. We focused our library on the HTB1 residues that constitute the surface stability patches (clusters of static residues). We have demonstrated previously that stability patches represent a characteristic feature of protein surfaces involved in intermolecular interactions (35, 36). The presence of interfacial stability patches may facilitate complex formation by multiple mechanisms, including moderating the conformational entropy of the unbound state, therefore diminishing the enthalpy–entropy compensation effect upon binding, and augmenting the favorable entropy of desolvation (32). Thus, because of a diminished potential for enthalpy–entropy compensation, the enthalpy contribution to the binding free energy (both positive and negative) is expected to be maximal for contact residues located within existing stability patches, and optimization of this contribution by side-chain mutagenesis is expected to improve binding affinity (32). The use of a focused library also enabled a reduction of the library size to $\sim 10^5$ variants, which is sufficiently large to cover a considerable portion of the sequence space for the randomized positions but small enough to be tractable by the YSD technique.

According to our molecular docking results, among the residues randomized in HTB1, Arg-29 and Ile-33 in the α -helix domain and Phe-44 in the β -sheet domain are predicted to play a central role in the interaction of HTB1_M with SOD1 (Fig. 3). Smith *et al.* (41) evolved the same scaffold (HTB1; in their case, to bind amyloid β -40 peptide), but they mutated only the β -sheet domain. In contrast, assuming that both the α -helix and β -sheet domains may contribute to the interaction with SOD1, we mutated both domains. In addition, position 31 was mutated from isoleucine in HTB1 to proline in HTB1_M. This mutation is predicted to cause a break in the α -helix secondary structure of HTB1_M (supplemental Fig. S8, C and D), which might be one of the reasons why HTB1_M does not interact with human IgG but HTB1 does. The results of both docking and competitive YSD binding experiments are consistent with the

idea that HTB1_M uses the same binding epitope (predicted to comprise both the α -helix and β -sheet domains) to interact with the two structurally and functionally different mutants, SOD1^{G93A} and SOD1^{G85R} (Figs. 2H and 3).

Under normal physiological conditions, SOD1 is a homodimeric protein (32 kDa), but under certain conditions, such as elevated temperature and/or a mutation, the protein monomer–dimer equilibrium may be shifted toward the monomeric state, exposing the dimer interface (47, 63). Indeed, the results of a competitive binding experiment using the SPC-206 mAb, whose binding epitope is located within the native SOD1 dimer interface, and *in silico* HTB1_M–SOD1 docking suggest that HTB1_M binds to the native dimer interface of SOD1, which appears to be accessible in the SOD1 mutants but is hidden in SOD1^{WT}, thereby providing the basis for the HTB1_M binding selectivity. Our experimental results indicate that HTB1_M binding thermodynamically stabilizes mutant SOD1 proteins (supplemental Fig. S10 and Table S4), which may lead to their enhanced solubility and a reduced propensity to form potentially toxic amyloid aggregates. It is also possible that interaction with HTB1_M sterically interferes with the formation of aberrant SOD1 complexes.

One of the main problems in developing treatments for CNS diseases is delivery of the therapeutic agent to the CNS. However, recent progress in vector-mediated drug delivery has made it possible to exploit this type of biotechnology in the treatment of various CNS diseases (64). Of particular relevance to our study are clinical trials showing a significant safety margin for the adeno-associated virus (AAV) vector in delivery into the brain of therapeutics for Alzheimer's, Parkinson's, Canavan, and Batten diseases (65–67). AAV, with its inherent advantage of long-term expression of the desired gene without causing any deleterious inflammatory response, would be our choice of vehicle to test HTB1_M as a therapeutic agent. Previous work on AAV-mediated delivery of a single-chain variable fragment in an ALS mouse model has shown sustained expression of the antibody in the spinal cord, leading to an extension of around 28% of the life span of the mice (68).

In conclusion, we demonstrated that HTB1_M acts as a specific inhibitor that reduces the extent of both misfolding and aggregation of SOD1 ALS mutants in stoichiometric concentrations and decreases SOD1^{G93A}-induced toxicity to motor neuron-like cells. We also showed that the HTB1 scaffold is well suited for the design of high-affinity and specificity precursors for ALS therapeutics. Our unique approach of combining computational and directed evolution methods for protein engineering can potentially be extended to other proteins that form amyloids, thereby increasing the availability of anti-fibril therapeutics.

Experimental procedures

SMD analysis and selection of HTB1 positions for randomization and library construction

The solution NMR structure (model 1) of HTB1 (PDB code 1GB4) was solvated in a water box (using 8-Å padding) with sodium/chlorine for charge neutralization by using the VMD program (69). The system was minimized and equilibrated

by a molecular dynamics simulation, with spatial constraints applied first to the entire protein (water equilibration, 200 ps), then only to the backbone atoms (100 ps), and finally without any spatial constraints (100 ps). These and subsequent simulations were performed in an isothermal–isobaric (NPT) ensemble at 310 K using the NAMD 2.9 program and the CHARMM27 force field for proteins (70, 71).

The surface-exposed residues of the equilibrated HTB1 structure were subjected to SMD analysis, essentially as described previously (35), using a flexible hemisphere of 13-Å radius surrounding the SMD atoms. For each residue, the SMD simulation was repeated 12 times, and applied forces computed from the trajectories were plotted *versus* distances traveled by the dummy atoms. The average slopes were calculated by applying linear regression analysis (with Prism 6, GraphPad Software, Inc.) and were referred to as the resistance coefficients (RCs) (35). The total scale of RCs (expressed in piconewton (pN) per angstrom) was divided into equal intervals to which numbers 1 to 8 were assigned, corresponding to the highest and lowest backbone mobility, respectively. The positions harboring static residues (RC ≥ 6) were selected for randomization to yield a stability-patch-focused library of HTB1 variants to be displayed on the yeast surface.

Generation of a combinatorially based HTB1 library

The HTB1 gene, codon-optimized for *Saccharomyces cerevisiae*, was purchased from Integrated DNA Technologies and inserted into *S. cerevisiae* strain EBY-100 via homologous recombination as described previously (72, 73). Generation of the rational and combinatorial HTB1 library is described in detail in the supplemental Experimental Procedures. In brief, 12 residue positions were selected based on the SMD simulation, as described above, and the library was created by assembly PCR with primers having NNS (N = Ala, Cys, Thr, or Gly; S = Cys or Gly) at the specific positions. The resulting DNA library was amplified and transformed into *S. cerevisiae* strain EBY-100 through homologous recombination. The size of the HTB1 focused library was about 3×10^5 , with 12 mutations per clone.

Flow cytometry and cell sorting

The yeast-displayed HTB1 library and individual HTB1 variants were grown in SDCAA-selective medium (as for SDCAA plates but without agar, see supplemental Experimental Procedures) and induced for expression with galactose medium (as for SDCAA but with galactose instead of dextrose), according to established protocols (73, 74). In the first step, $\sim 1 \times 10^6$ cells were incubated with different concentrations of biotinylated target SOD1^{G93A} for 2 h together with a 1:100 dilution of chicken anti-c-Myc IgY antibody (Abcam) in PBST buffer (1× PBS, 50 mM NaCl, and 0.05% Tween 20). Thereafter, cells were washed with PBST and incubated with FITC-conjugated NeutrAvidin (Thermo Fisher Scientific, 1:800 dilution) and phycoerythrin (PE)-conjugated goat anti-chicken IgY (Santa Cruz Biotechnology, 1:100 dilution) for 1 h. Cells were washed again and analyzed by dual-color flow cytometry (Accuri C6, BD Biosciences). Cell sorting was carried out with a BD FACSAria III (Ilse Katz Institute for Nanoscale Science and Technology, Ben-

Gurion University of the Negev (BGU)). In brief, $\sim 1 \times 10^8$ cells were first sorted to select for high-expressing clones (positive for anti-c-Myc antibody). Sorted cells were then grown in selective medium, and several colonies were sequenced DNA Microarray and Sequencing Unit (DMSU), National Institute for Biotechnology in the Negev (NIBN), BGU). Following each sorting, the number of yeast cells used for the subsequent sorting was at least 10-fold in excess of the number of sorted cells. Several clones from each round of sorting were sequenced (DMSU, NIBN, BGU) using a protocol established previously (72). Selected clones were expressed and purified from BL21 DE3 *E. coli* bacterial cells as described in the [supplemental Experimental Procedures](#).

SPR spectroscopy

The constants for binding of HTB1_M to SOD1^{G93A} and SOD1^{G85R} were determined by SPR spectroscopy on a ProteOn XPR36 (Bio-Rad) as follows. HTB1_M and HTB1 were immobilized on the surface of the chip by using the amine coupling reagents sulfo-NHS (0.1 M *N*-hydroxysuccinimide) and EDC (0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Bio-Rad). The proteins (1 μ g) were each covalently immobilized on the chip in 10 mM sodium acetate buffer (pH 4.0), to give 2100 and 2204 response units for HTB1_M and HTB1, respectively. BSA (3 μ g, 4016 response units) was immobilized on the chip as a negative control. Unbound esters were deactivated with 1 M ethanolamine HCl at pH 8.5. Before each binding assay, the temperature was set at 25 °C. Soluble human SOD1 mutants and wild-type SOD1 (the analytes) were then allowed to flow over the surface-bound HTB1_M and HTB1 separately at concentrations of 39, 78.1, 156.3, 312.5 and 625 nM and a flow rate of 50 μ l/min. While the analyte was flowing over the surface (for 8 min), the interactions between HTB1_M and SOD1 proteins were determined. The next step was to examine the dissociation of the proteins while allowing PBST to flow over the surface for 11 min at 50 μ l/min. After each run, a regeneration step was performed with 50 mM NaOH at a flow rate of 100 μ l/min. For each protein complex, a sensorgram was generated from the response units measured during the course of the protein–protein interaction minus the values of the BSA background channel. The dissociation constant (K_D) was determined from the sensorgram of the equilibrium binding phase.

Molecular modeling

The human homology models of HTB1_M were prepared with the Discovery Studio 4.5 Homology Model module (Biovia, Dassault Systemes, San Diego, CA) using the published structures of HTB1 (PDB codes 1GB4, 2ON8, and 2ONQ) as the template. The HTB1_M sequence was aligned to the template structures using the Align Sequence to Templates module, and the Built Homology Model module was built based on this structural alignment. The ψ and ϕ torsion angles of the predicted model were computed by using Ramachandran plots, as updated by Richardson and co-workers (75). The models were verified with the Verify Protein (MODELLER) module. The discrete optimized protein energy (DOPE) score and normalized DOPE score were calculated for each structure. In addition, the probability density function (PDF), PDF total energy, and PDF

physical energy were taken into consideration. The final model exhibited the highest profile 3D score (76) and the lowest number of Ramachandran violations (75). The models were eventually refined by energy minimization using the CHARMM force field (77).

Molecular protein–protein interaction docking

Molecular protein–protein docking of the HTB1_M structure to two SOD1 mutant proteins (G93A protein (PDB code 3GZO) and G85R protein (PDB code 2ZKW)) was carried out in Discovery Studio 4.5 (Biovia, Dassault Systemes) with ZDOCK, which is a rigid-body docking program that predicts the structure of a number of protein complexes. ZDOCK uses a simple shape complementarity method known as pairwise shape complementarity. The ZDOCK program integrates the pairwise shape complementarity scoring function for identifying docked conformations and scores hits based on atomic contact energies (higher scores are better predictions for the protein–protein complexes) (78). The ZDOCK search scheme exhaustively searched all rotational and translational spaces for HTB1_M relative to SOD1 mutant protein, in which the latter is fixed at its starting orientation (from its PDB coordinates). Next, HTB1_M orientations were sorted by ZRANK score method, which is used for rapidly and accurately reranking the docked protein complexes predicted by ZDOCK with the aim to pick out near-native structures (most negative score, thus favorable to binding) (79). Clustering the top 2000 orientations into groups according to their spatial proximity by using a root mean square deviation cutoff of 6 and an interface cutoff of 9 assisted us to select the promising near-native structures docking solutions to be analyzed (80, 81). The cluster size indicates the size of the cluster to which the docked protein orientation belongs. The cluster density indicates the number of docked protein poses that fall within the root mean square deviation cutoff specified for clustering. This number represents the number of neighbors used in the first stage of clustering.

ThT aggregation assay for SOD1 mutants

Prior to the aggregation assay, all mixtures containing the protein were filtered through a 4-mm Millex-GV (PVDF) syringe filter with a 0.2- μ m pore size (Millipore). SOD1^{G93A} or SOD1^{G85R} (50 μ M) was incubated with or without HTB1_M (25–100 μ M) in 200 μ l of 20 mM Na⁺/phosphate buffer (pH 7.4), 0.1 M NaCl, 5 mM EDTA, and 1 mM tris(2-carboxyethyl)phosphine at 37 °C with 600 rpm orbital shaking. At each time point (every 24 h), 20 μ l of reaction mixture was removed and stored at 4 °C for further analysis. At the end of the experiment, all samples were analyzed for their ability to bind ThT (Sigma), an amyloid-specific fluorophore, using Infinite M1000 (Tecan) plate reader with fluorescence excitation and emission wavelengths set at 440 nm and 485 nm, respectively. Each experiment was performed in triplicate. All data points were analyzed using an unpaired *t* test to verify that the differences between the samples tested were statistically significant.

TEM analysis of SOD1 fibril formation

Samples for TEM imaging were prepared as described elsewhere (82). Briefly, at the end of the aggregation assay, 2.5- μ l

samples (diluted 5-fold in Milli-Q water) were deposited on a carbon-coated copper 300 mesh. After 1 min, the excess liquid was carefully blotted onto filter paper, and the grid was held at ambient temperature for 1 min. Uranyl acetate (5 μ l, 2%) was added to the grid, and, after 1 min, the excess of the salt solution was carefully removed with filter paper. The imaging was performed using a Tecnai G2 12 BioTWIN (FEI) (Ilse Katz Institute for Nanoscale Science and Technology, BGU) transmission electron microscope with an acceleration voltage of 120 kV. Different magnifications were used for visualization, depending on the size of the aggregates.

Cell culture

The NSC-34 neuronal cell line was a generous gift from Dr. Adrian Israelson (Faculty of Health Sciences, BGU). Cells were grown at 37 °C and 5% CO₂ in DMEM supplemented with 10% tetracycline-free FBS, L-glutamine (2 mM), and penicillin (100 units/ml)/streptomycin (0.1 mg/ml) (Biological Industries). Transfection was performed by using TurboFect (Thermo) according to the protocol of the manufacturer. When co-transfections were performed, empty plasmids were transfected as controls.

Immunoprecipitation

The SOD1-EGFP plasmid (pEGFP-N3) was a generous gift from Dr. Adrian Israelson (Faculty of Health Sciences, BGU). We cloned the HTB1_M gene into the pHAGE vector with the IRES-EBFP reporter for cell culture experiments. SOD1-EGFP-transfected NSC-34 whole-cell extracts (in the absence or presence of HTB1_M co-transfection, 100 μ g of the whole-cell extract) were solubilized in IP buffer (20 mM Tris-Cl (pH 7.6), 200 mM NaCl, 0.72 mM EDTA, 10% glycerol, 7 mM DTT, protease inhibitor mixture (Sigma, added fresh before use at a ratio of 1:200), and Triton X-100 (0.15%)). The solubilized fraction was incubated overnight with B8H10 (MediMabs) antibody (selective for misfolded SOD1) cross-linked previously to Dynabeads protein G (Invitrogen) according to the instructions of the manufacturer. The beads were magnetically isolated and washed three times with PBS. Samples were eluted by boiling with sample buffer. Proteins were separated on 12% SDS-PAGE gel, transferred to PVDF membranes (Trans-BlotTM Turbo Midi PVDF Transfer Packs, Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad), and probed with goat anti-SOD1 (C-17, Santa Cruz Biotechnology), rabbit anti HA antibody (to detect HTB1_M, Abcam), and rabbit anti β -tubulin antibodies (Abcam). Horseradish peroxidase-conjugated anti-goat and anti-rabbit IgG (Abcam), respectively, were used as secondary antibodies, and their signal was detected by ECL (GE Biosciences). The experiment was performed in triplicate, and quantitative analysis ($n = 3$) was performed using unpaired Student's t test, where the level of significance was taken as *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$.

Cell viability

Cell viability was determined with NSC-34 cells transfected with SOD1-EGFP by using an XTT-based kit (Sigma-Aldrich) according to the protocol of the manufacturer (83). The level of transfection was calculated by FACS. SOD1^{WT} and empty vec-

tor pHAGE served as controls for the experiment. Data for each sample were normalized according to its transfection efficiency and later normalized for the empty vector control. The data are shown as percentage control. The experiment was performed in triplicate, and quantitative analysis ($n = 3$) was performed by unpaired Student's t test, where the level of significance was taken as *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$.

Author contributions—V. B. and N. P. designed the research. V. B. performed and analyzed the experiments shown in Figs. 1, 2, and 4 and supplemental Figs. S1–S6 and S10 and Table S4. O. O. performed and analyzed the experiments shown in Fig. 6 and supplemental Figs. S7 and S9. V. B. and O. O. together performed and analyzed the experiment shown in Figs. 3, B and C, and Fig. 5. E. B. Z. performed and analyzed the experiments shown in Fig. 3A and supplemental Fig. S8 and Tables S1–S3. V. B., O. O., E. B. Z., R. T., S. E., and N. P. analyzed data. V. B. and N. P. wrote the paper. All authors edited the manuscript and approved the final version.

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