Supporting Information

Disulfide Bonds and Disorder in Granulin-3: Unusual Handshake Between Structural Stability and Plasticity.

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SI Materials and Methods

Expression and purification of GRN-3 for ^{15}N labelling.

For uniform labeling of the backbone and side chain amide nitrogens with ^{15}N the cells were grown in M9 minimal medium with $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Inc., MA) as the sole nitrogen source. 10x M9 medium was prepared by adding 60g of Na_2HPO_4, 30g KH_2PO_4, 5 g of NaCl, and 5 g of $^{15}NH_4Cl$ to 1 liter of H_2O and sterilized by autoclaving at 121 °C, 15 psi for 20 mins. The 10% (v/v) of 10x M9 medium was supplemented with 1% (v/v) of 100x trace elements solution, 2% (v/v) of 20 % (w/v) Glucose, 0.1% (v/v) of 1M MgSO4, 0.1% (v/v) of 1M CaCl2, 0.1% (v/v) of 1 mg mL^{-1} biotin, 0.1% (v/v) of 1 mg mL^{-1} thiamine and 100 μg mL^{-1} of ampicillin and the final volume was made upto 1000 ml. All the stock solutions, except for 20 % Glucose, were filter sterilized using 0.2 μm filter; glucose was sterilized by autoclaving at 121 °C, 15 psi for 20 mins. The 100x trace elements solution was prepared by adding 5 g of EDTA, 0.83 g of $FeCl_3$ x $6H_2O$, 84 mg of $ZnCl_2$, 13 mg of $CuCl_2$ x 2 H_2O, 10 mg of $CoCl_2$ x 6 H_2O, 10

mg H_3BO_3, and 1.6 mg of $MnCl_2$ x 6 H_2O to 1 liter of sterilized H_2O and filter sterilized using a 0.2 μm filter. ^{15}N-labeled GRN-3-trxA was expressed and purified using the same protocol as detailed above. The ^{15}N-labeling was confirmed using MALDI-ToF by assessing the increase in the molecular weight

Expression and purification of GRN-3 in Human Embryonic Kidney (HEK) cells.

hGRN-3 was expressed and purified from human embryonic kidney cells by Dr. Kukar's lab at the Emory university. The DNA sequences encoding granulin 3 (AKA B) was codon optimized and custom synthesized by GenScript (Piscataway, NJ). The amino acid sequence for human GRN-3 including the linker region at the carboxyl-terminal end was identified based on the Universal Protein Resource database (P28799l; GRN_HUMAN). The endogenous PGRN signal peptide (SP) sequence followed by a twin-Strep and FLAG tag was added to the amino-terminus of GRN-3. Synthetic GRN gene constructs were designed to add a 5' HindIII (AAGCTT) site, a Kozak sequence (GCCACC) before the ATG start codon, a 3' Stop codon, and a XhoI (TGACTCGAG) site. Following synthesis, each gene was inserted into the pcDNA3.1 (+) vector using a HindIII/XhoI cloning strategy. All constructs were verified using DNA sequencing, restriction digests and PCR amplification. Subsequently, primers were designed to remove the linker region of GRN-3; each construct was amplified via PCR, and subcloned into pcDNA3.1 (+) vector, and verified using DNA sequencing. Amino acid sequence for Granulin-3 without linker 4 (GRN-3):

MWTLVSWVALTAGLVAGSAWSHPQFEKGGGSGGGSGGSAWSHPQFEKGASDYKDDD
DKSVMCPDARSRCPDGSTCCELPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSKCL
S. (MW 12.17 KDa)

HEK Expi293 cells were transfected with the pcDNA3.1 GRN-3 constructs and conditioned media was collected following the manufacturer's protocol (Thermo-Fisher; Cat# A14635). GRNs were affinity-purified from conditioned media over Strep-Tactin XT Superflow resin using a slightly modified protocol as described by the manufacturer (IBA GmbH; Göttingen, Germany). The elutions containing recombinant GRNs were concentrated and desalted into PBS using Vivaspin 500 Protein Concentrators (molecular weight cut-off 50 kDa; GE Healthcare Life Sciences). The purity of recombinant GRNs was assessed by SDS-PAGE followed by colloidal coomassie dye G-250 protein stain (GelCode Blue; Thermo-Fisher) or silver stain (cat #24600; Thermo-Fisher) and estimated to be >95% pure.

Mass spectrometry.

The purity of protein preparation was routinely analyzed by MALDI-ToF mass spectrometry (Bruker Inc). The sinnapinic acid (SA) matrix was prepared by resuspending 10 mg of SA (Sigma) in 1 mL of 1:1 acetonitrile:water with 0.1% TFA. One μL of protein sample was mixed with 1 μL of SA matrix such that the final protein concentration was in the range of 0.01-0.1 nM. The protein-matrix mixture was then spotted in duplicates (1 μL) on a MSP 96 microchip target (Bruker Daltonics Inc). The laser intensity and the detector gain were kept constant at 80-90% laser and 3x detector gain. For alkylation of GRN-3 using Iodoacetamide, a 2000 molar excess of iodoacetamide was incubated with GRN-3 and mGRN-3 in presence of 1000 molar excess of guanidine hydrochloride overnight in dark. The samples were subsequently spotted on MALDI-ToF MSP 96 microchip target as described above.

Circular dichroism (CD).

Far-UV CD spectra were obtained on a Jasco J-815 CD spectrometer. For protein samples with concentrations ≤100 μM, a 1 mm path length quartz cuvette (Hellma) was used. For temperature melt experiments, the samples were first scanned at 25 °C, prior to addition of 1 % SDS, using the continuous scan mode from 260 to 195 nm with a scanning speed of 50 nm min^{-1} with a data integration time of 8 seconds, 1 nm bandwidth, and a data pitch of 0.1 nm. Each data set represents an average of 3 scans with appropriate blank subtraction. After adding SDS, the samples were scanned immediately over the temperature range of 10 °C to 90 °C with an interval of 10 °C and a ramp rate of 10 °C/min using the temperature interval scan method. Rest of the parameters were same as continuous scanning mode. The scans were subjected to smoothening via the Savitzky-Golay algorithm (provided by the manufacturer) with the convolution width of 9 using the Jasco spectrum analysis program, were blank subtracted, zero corrected, and normalized.

Structure prediction.

The structure prediction of GRN-3 was carried out using the free structure predicting tool called I-TASSER (Iterative Threading ASSEmbly Refinement) available at http://zhanglab.ccmb.med.umich.edu/I-TASSER/. The primary amino acid sequence of GRN-3 was submitted in the FASTA format. Briefly, I-TASSER identifies similar structural templates from PDB (protein data bank) using multiple threading approach LOMETS (local meta threading server). The server then uses the iterative template fragment assembly simulations to generate the full-length atomic models. Each model is assigned a confidence score (c-score) which determined the validity the predictions and higher the c-score, better is the prediction validity.

Supplementary Figures

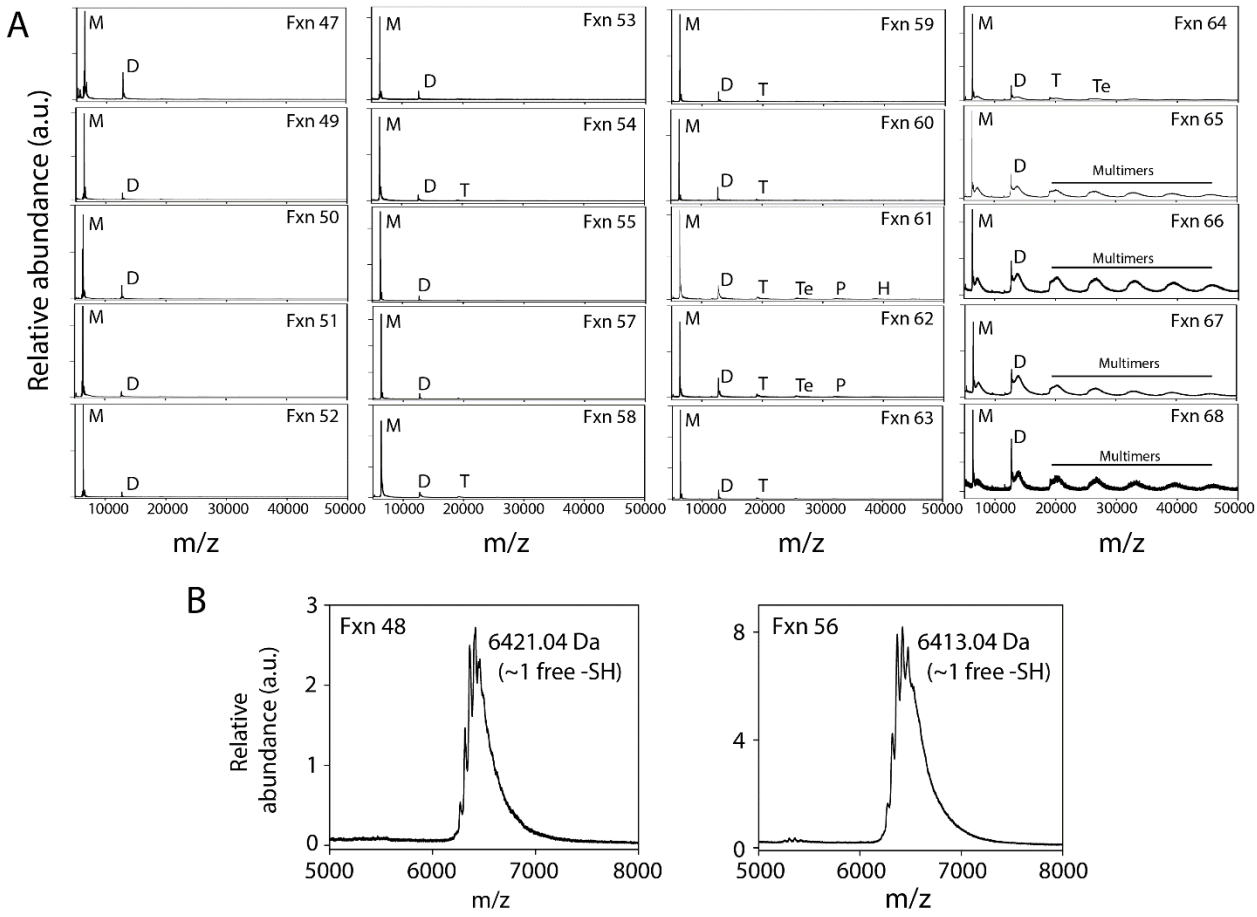

Fig. S1. MALDI-ToF spectra of GRN-3 isoforms and iodoacetamide alkylation of fractions 48 and 56. (A) MALDI-ToF spectra of fractions 47, 49 – 55, and 57 – 68 (GRN-3 isoforms) showing signals for dimeric (D), trimeric (T), tetrameric (Te), and pentameric (P) GRN-3 along with monomeric (M) confirming the presence of inter-molecular disulfide bonded multimers. (B) Alkylation of fractions 48 and 56 using iodoacetamide showing an increase in the molecular weight corresponding to approximately one free sulfhydryl.

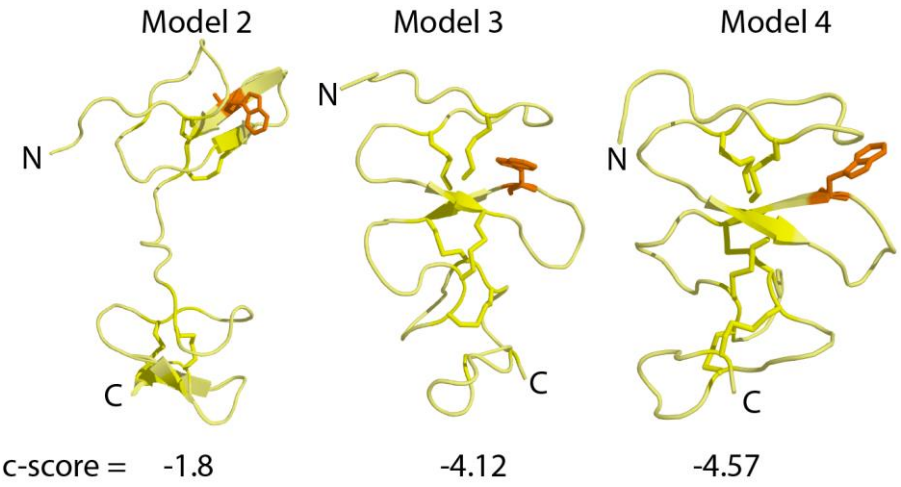

Fig. S2. The other possible structures of GRN-3 predicted using I-TASSER with negative c-score.

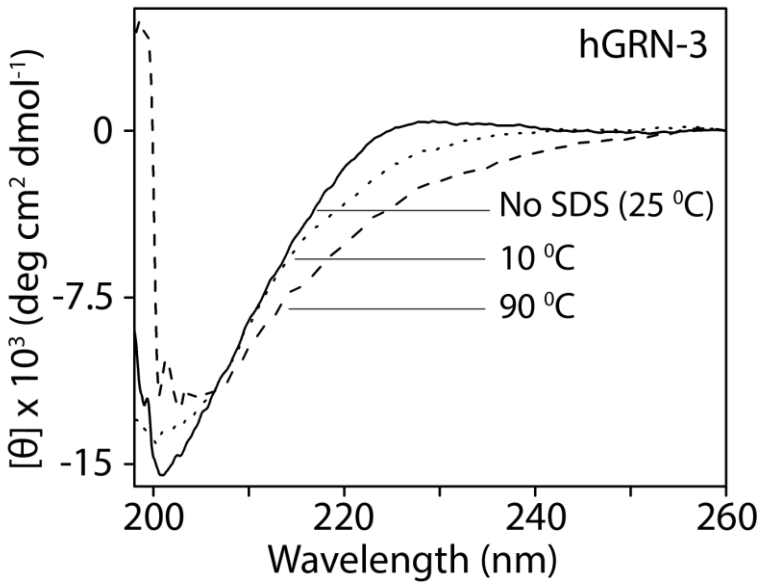

Fig. S3. SDS-induced temperature stability of hGRN-3. Temperature stability of hGRN-3 in presence of SDS monitored using far-UV CD, showing that hGRN-3, like GRN-3 from *E. coli*, resists SDS-induced thermal denaturation.

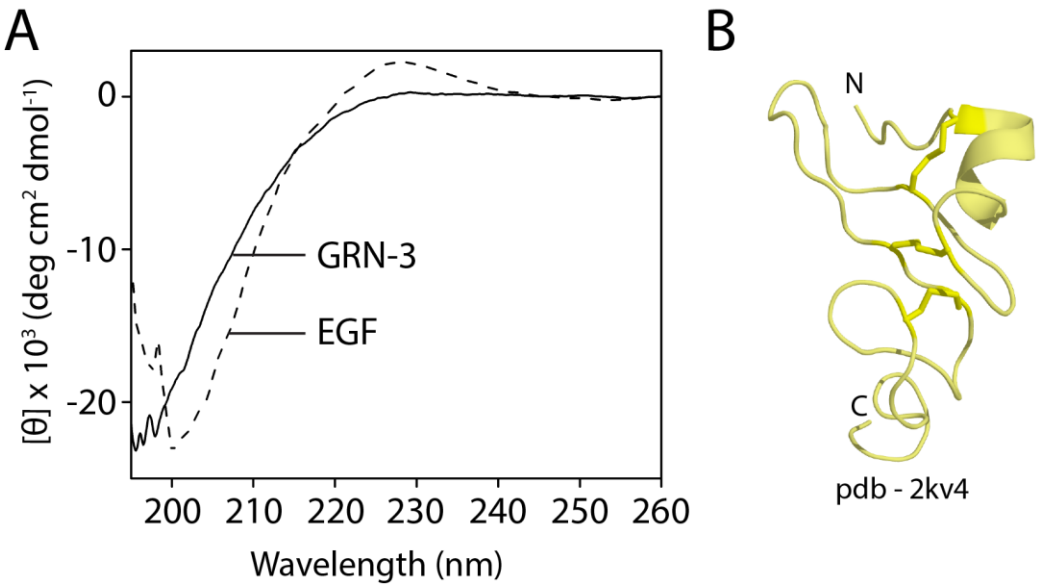

Fig. S4. (A) Far-UV CD of EGF (dotted line) and GRN-3 (solid line). (B) Solution NMR structure of human epidermal growth factor (EGF; pdb – 2kv4) plotted using PyMol with disulfide bonds indicated in yellow.

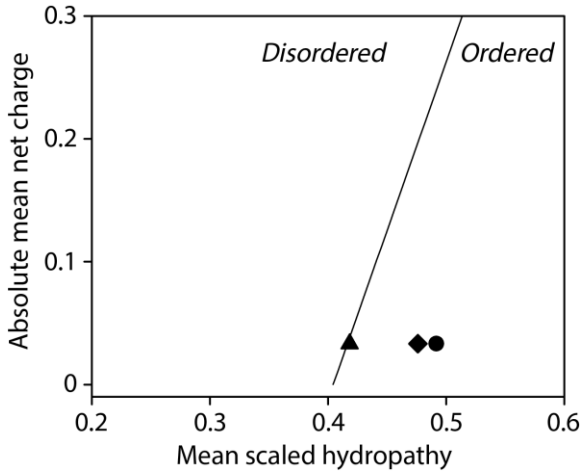

Fig. S5. Uversky plots.The charge-hydropathy plot for GRN-3 (●), alaGRN-3 (♦), and serGRN-3 (▲) was plotted using PONDR tool. The line indicates the boundary between disordered and ordered regions.