Supplementary Figures, Tables, Notes and Methods

Structural and molecular insights into the mechanism of action of human angiogenin-ALS variants in neurons

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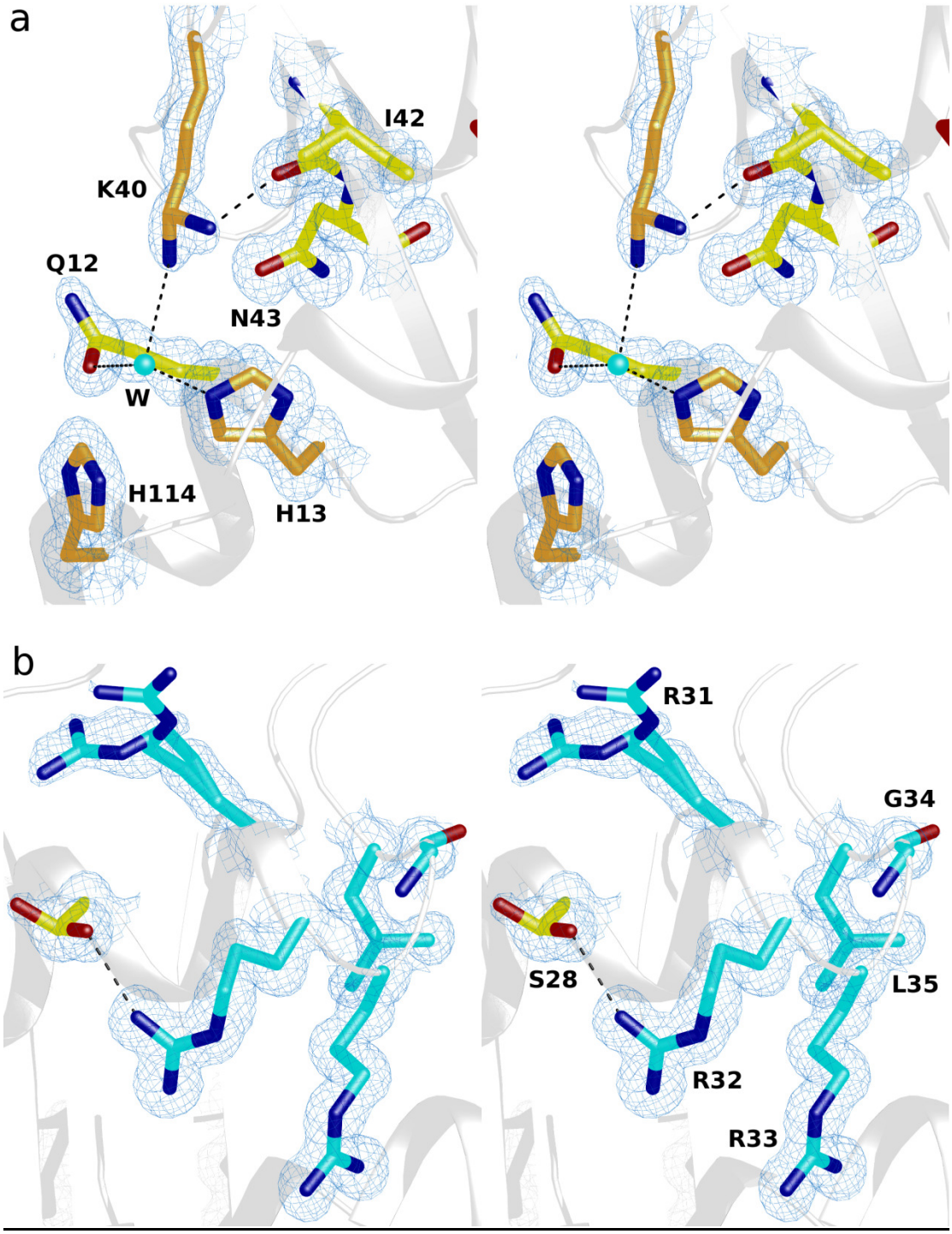
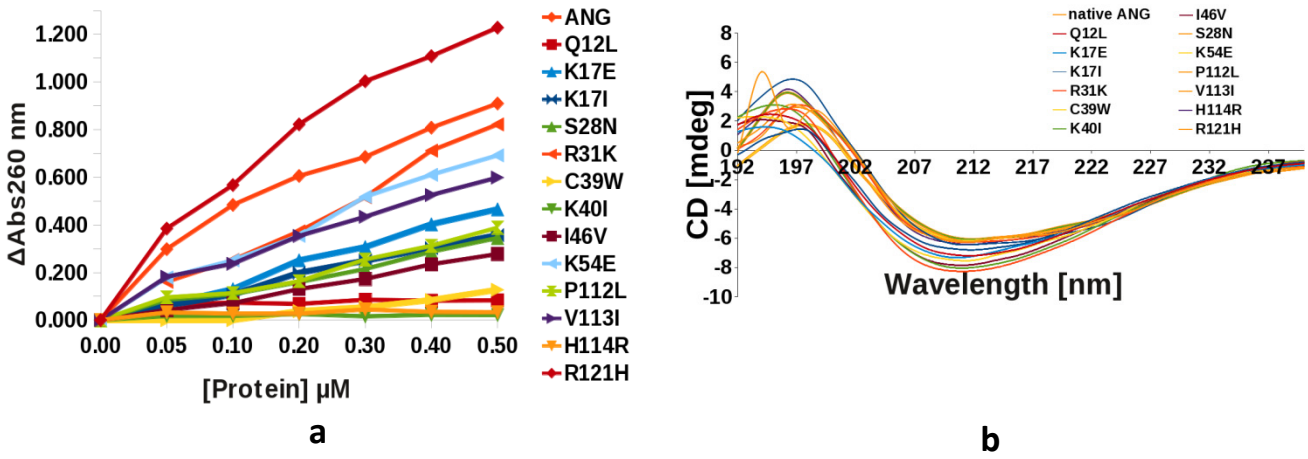


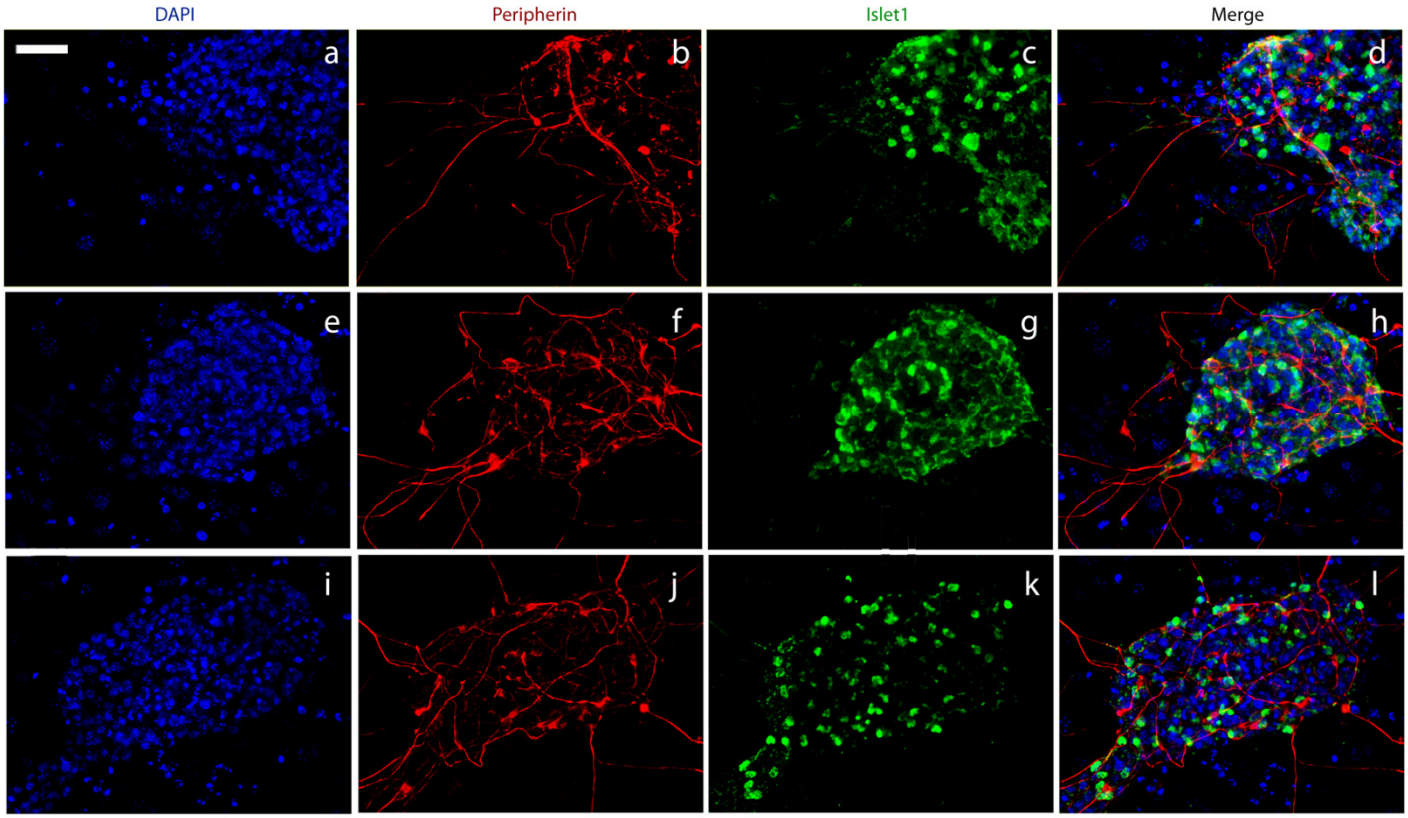
Supplementary Figure S1. Environment of selected active site residues and part of the nuclear localization sequence of hANG

(a) Active site residues of hANG in the high resolution structure are shown in gold colour, **(b)** Nuclear Localization Sequence (NLS) residues are shown in cyan colour. Interacting residues are shown in yellow colour. Nitrogen and oxygen atoms are coloured in blue and red respectively. Electron density map (2Fo-Fc) contoured at 1.0 σ is shown around the residues. Dual conformation for Lys40, Ser28 and Arg31 are also shown.

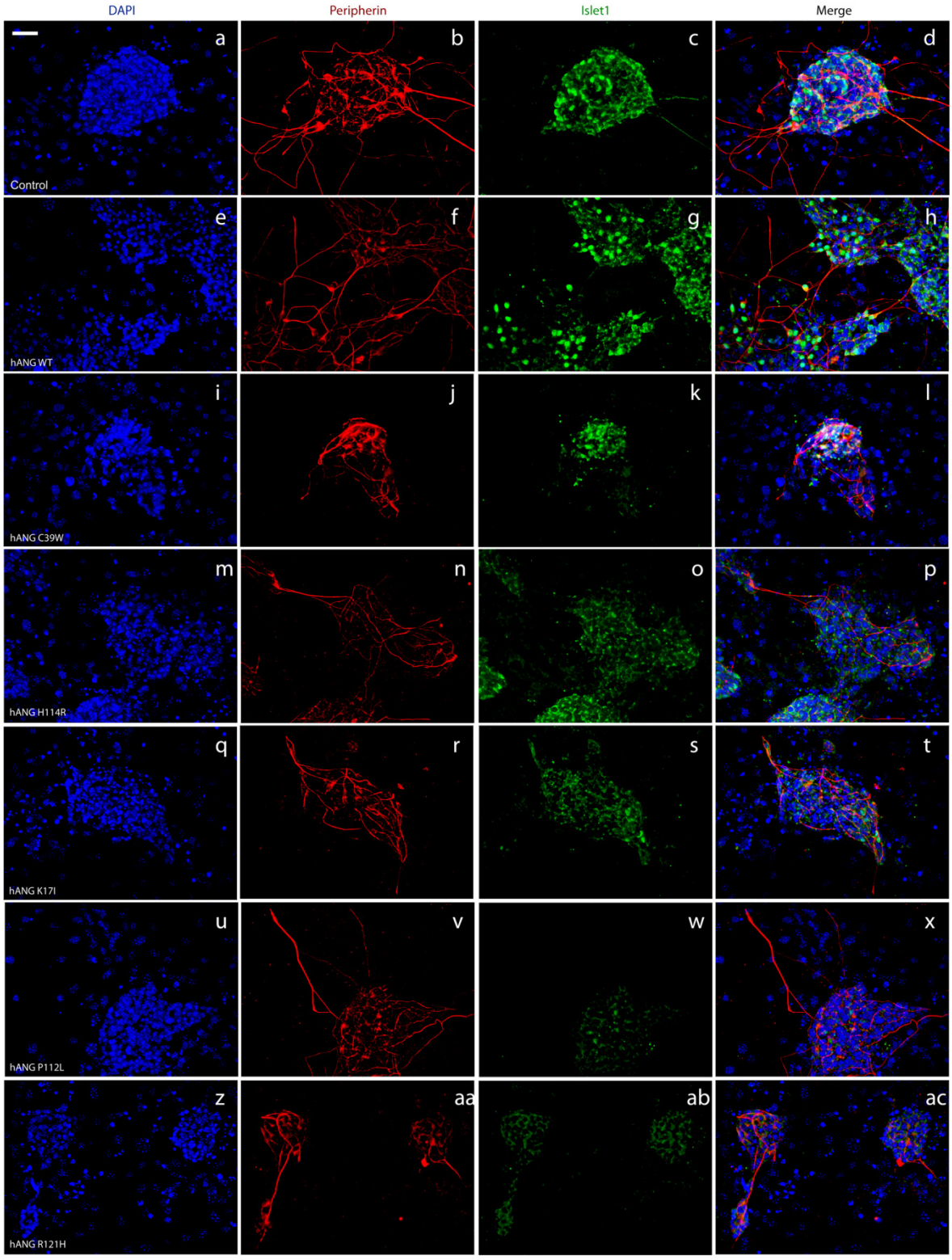


Supplementary Figure S2. Properties of hANG and ANG-ALS variants

(a) Ribonucleolytic activities of hANG and variants toward tRNA. Assays measure the production of perchloric-acid soluble fragments (**see methods- main paper**). ΔA_{260} is the difference between the A_{260} values measured in the presence and absence of test sample. Each data point represents the mean of 3 measurements. In all cases, the standard deviation is less than 2% of the mean. The activities listed on the figure are the means of the values calculated for 0.3, 0.4 and 0.5 µM variant. **(b) CD spectra analysis for wild-type hANG and variants performed at 20°C**. Samples of wild-type ANG and the variants were dissolved at a concentration of 0.1 mg/ml in 20 mM sodium acetate (pH 6.0) containing 0.1 M sodium chloride. Wavelength scans were performed at 20°C from 195-250 nm at a scan rate of 50 nm/min.

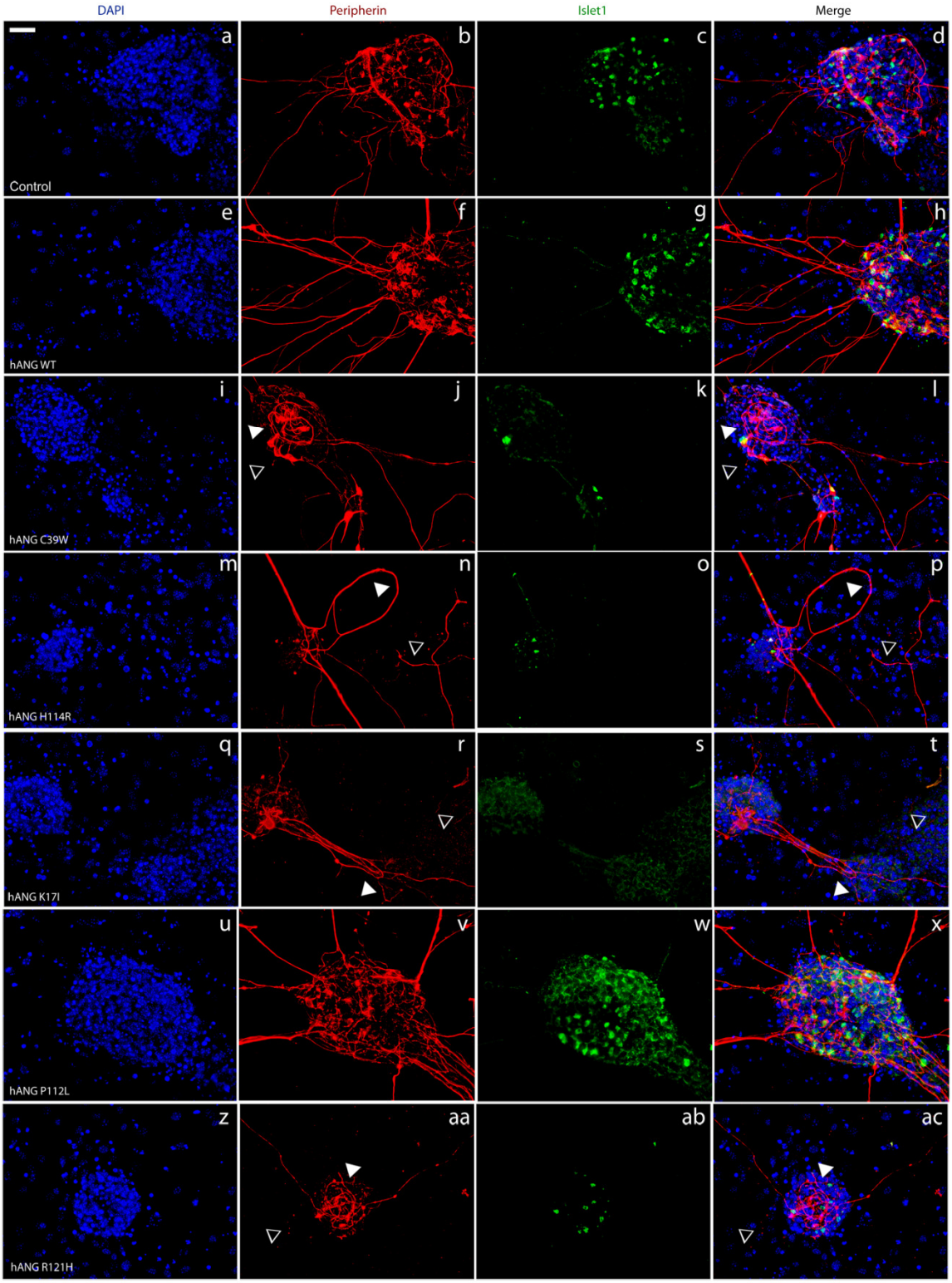


Supplementary Figure S3. Differentiation time course of P19 cells to MNs (day four to six).
P19 cells were induced to differentiate by plating on PA6 feeder cells in the presence of RA. Cells were fixed on day four, five and six, and stained for Peripherin (red) and Islet 1 (green). Day four **a-d**; Day five **e-h**; Day six **i-l**. Well extended neurites form by day six. Co-expression of these markers indicates the presence of P19 derived post mitotic motor neurons. Scale bars 50µM.



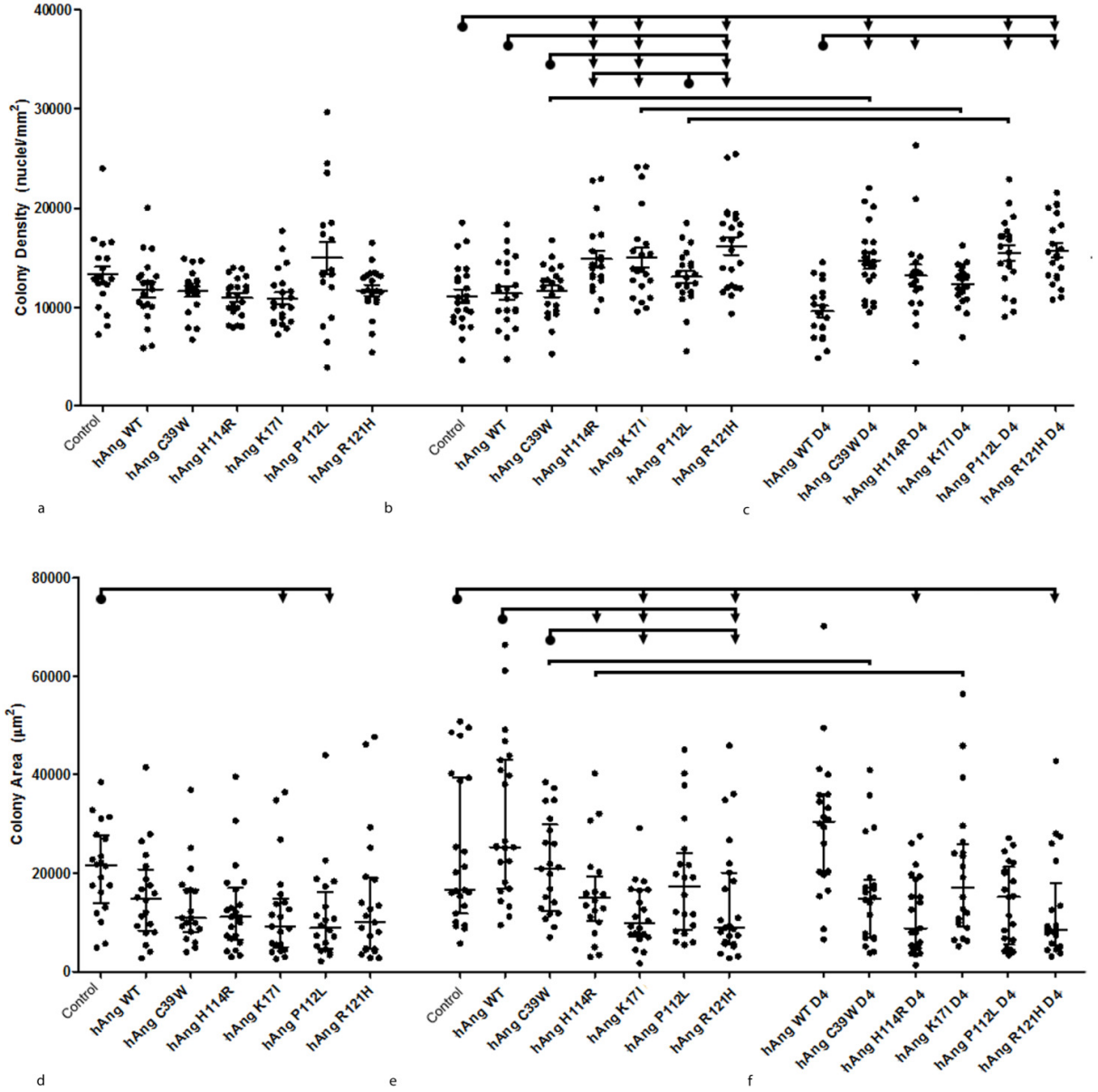
Supplementary Figure S4. Effects of hANG and hANG-ALS variants on MNs on day five of differentiation.

P19 cells were induced to differentiate to MNs by plating on PA6 feeder cells in the presence of RA for five days. Cells were differentiated in the absence of ANG (control) **(a-d)** or in the presence of an ANG variant throughout: WT **(e-h),** C39W **(i-l),** H114R **(m-p)**, K17I **(q-t),** P112L **(u-x)** and R121H **(z-ac).** Cells fixed on day five were stained for Peripherin (red) and Islet 1 (green) to identify postmitotic MNs and counterstained with DAPI (blue). Scale bars 50µM.



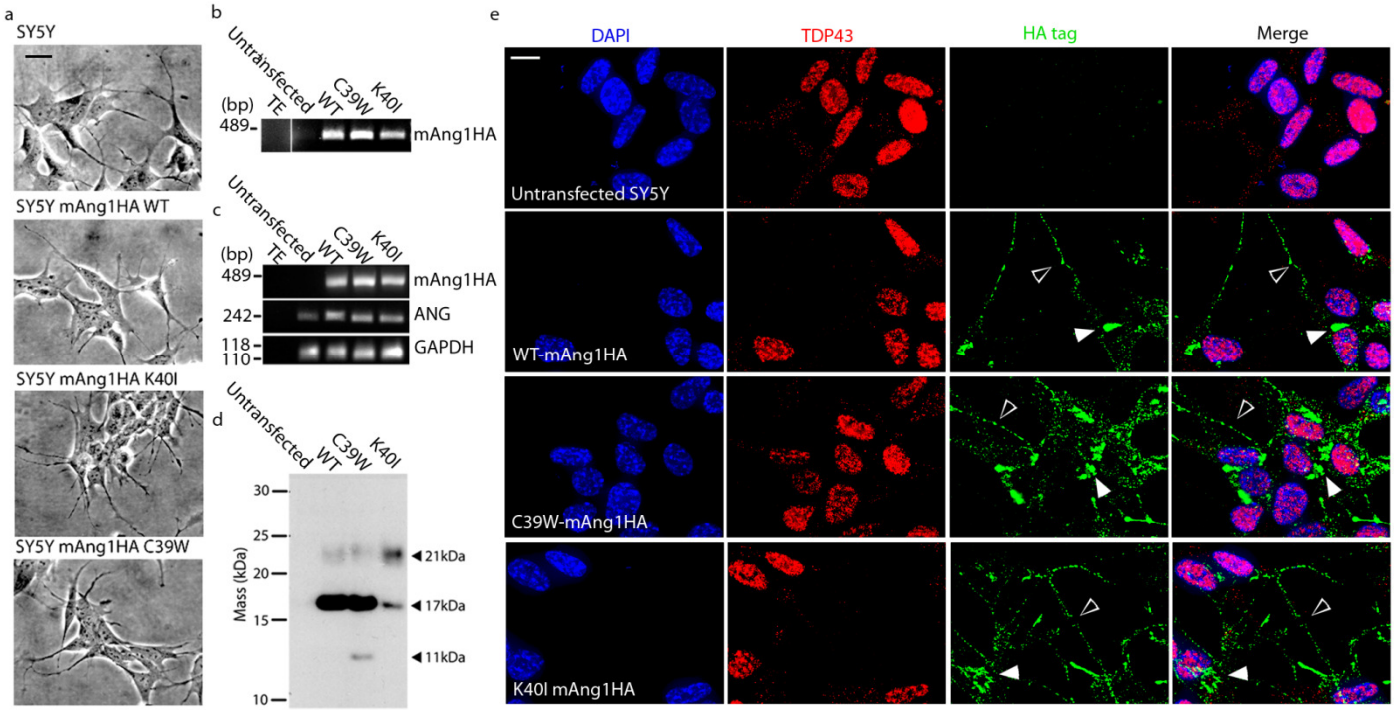
Supplementary Figure S5. Effect of hANG and hANG-ALS variants on MNs and neurite extension (48h treatment from day four).

P19 cells were induced to differentiate to MNs by plating on PA6 feeder cells in the presence of RA. Cells were differentiated for four days and then incubated for a further 48 hr either in the absence of hANG (control) **(a-d)** or in the presence of an hANG variant: WT **(e-h),** C39W **(i-l),** H114R **(m-p),** K17I **(q-t),** P112L **(u-x)** and R121H **(z-ac).** Cells were fixed on day six and stained with Peripherin (red) and Islet 1 (green) to identify post mitotic MNs. Cells were counterstained with DAPI (blue). Scale bars 50µM.



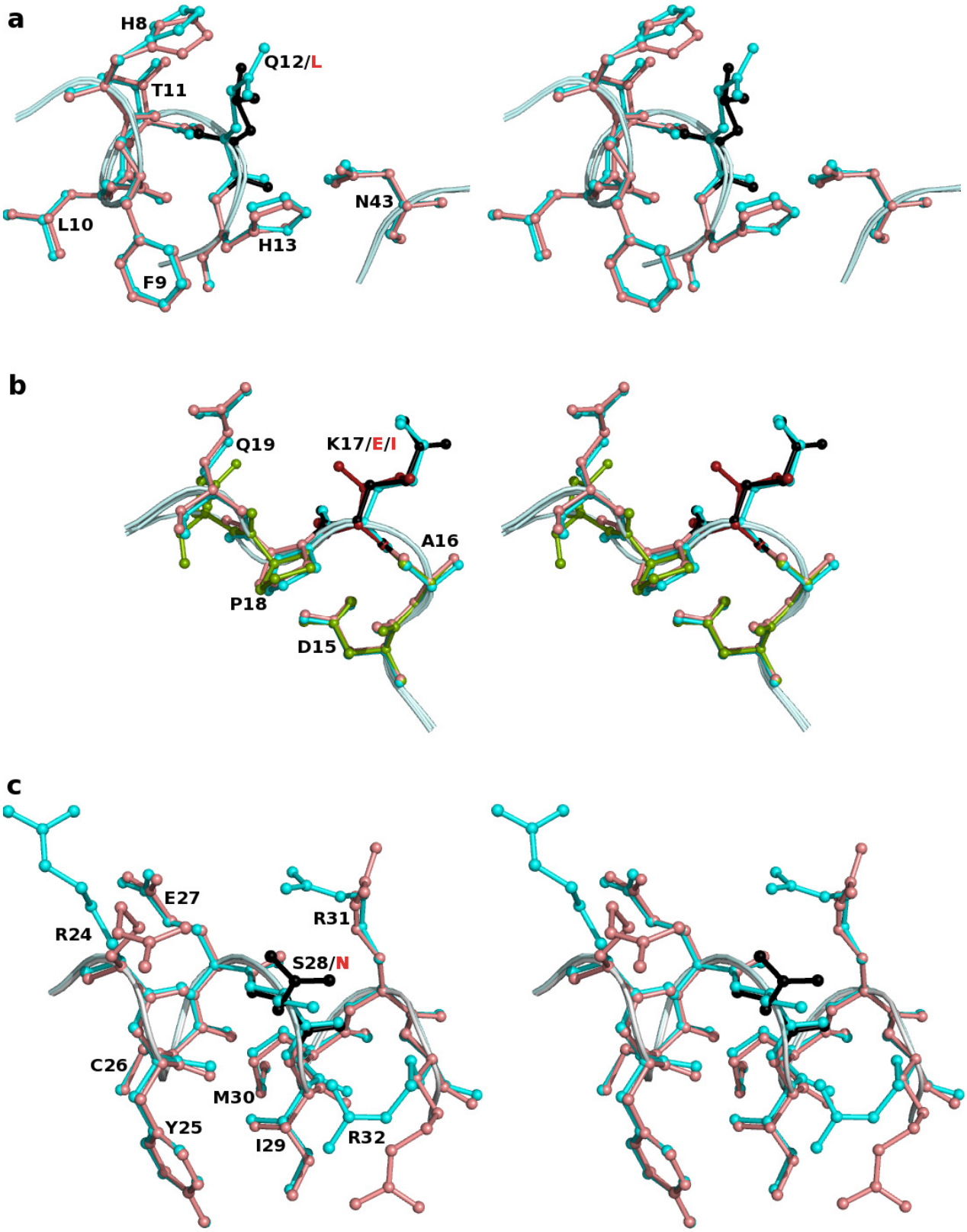
Supplementary Figure S6. Effect on hANG and hANG-ALS variants proteins on cell densities of neuronal colonies.

The density of nuclei within P19 colonies after five or six days of differentiation was calculated from colony area and total number of DAPI stained nuclei. Cells were differentiated in presence or absence of human ANG variants from day zero for five days **(a)** and six days **(b)**, or from day four to six only **(c)**. Data were collected from two experiments, n≥10 from each experiment. Mean bars ±SEM. Ball and arrow bars show where multiple conditions are significantly different from a single condition while solid bars show significant difference between two conditions ($p<0.05$, compared by ANOVA with Tukey's *post hoc*). The total area of colonies was determined after differentiation in presence or absence of hANG variants from day zero for five days **(d)** and six days **(e)**, or from day four to six only **(f)**. Data were collected from two experiments, n≥10 from each experiment. Median bars with interquartile range. Ball and arrow bars show where multiple conditions are significantly different from a single condition while solid bars show significant difference between two conditions ($P<0.05$ Log_e transformed data compared by ANOVA with Tukey's *post hoc*).



Supplementary Figure S7. Expression of HA-tagged mAng1 and mAng1 ALS variants by stably transfected SH-SY5Y cell lines.

(a) Phase-contrast images of transfected cell lines. mAng1-ALS variants expressing cell lines show some aggregation. **(b)** PCR for a 459bp mAng1HA in DNA from transfected SY5Y clones shows successful integration of the expression vector. **(c)** RT-PCR showing the presence of mAng1HA and variant transcripts (459bp). Endogenous human ANG transcripts (231bp) are also present in untransfected SY5Y and all three transgenic cell lines. GAPDH (112bp) used as loading control. **(d)** Western Blot of whole cell lysates for the HA-tag shows the presence of mAng1HA variant protein in each transfected cell line with bands at the expected size of 17kDa and additional bands at 21kDa in all cases and an extra 11kDa in SY5Y mAng1HA C39W. A larger proportion of 21kDa versus 17kDa is seen in SY5Y mAng1HA K40I. **(e)** Immunostaining for the HA-tagged WT, C39W and K40I-mAng1 protein in the SY5Y clones shows a consistent punctate distribution within the cell body and neurites (open arrows) with a large accumulation adjacent to the nucleus (closed arrows). TDP-43 immunostaining shows a nuclear distribution and no co-localisation with HA staining. No differences in intensity or distribution seen between the untransfected and transfected undifferentiated cell lines. Scale bar 25µm.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Q12/L
T11
N43
L10
H13
F9
b
Q19
K17/E/I
A16
P18
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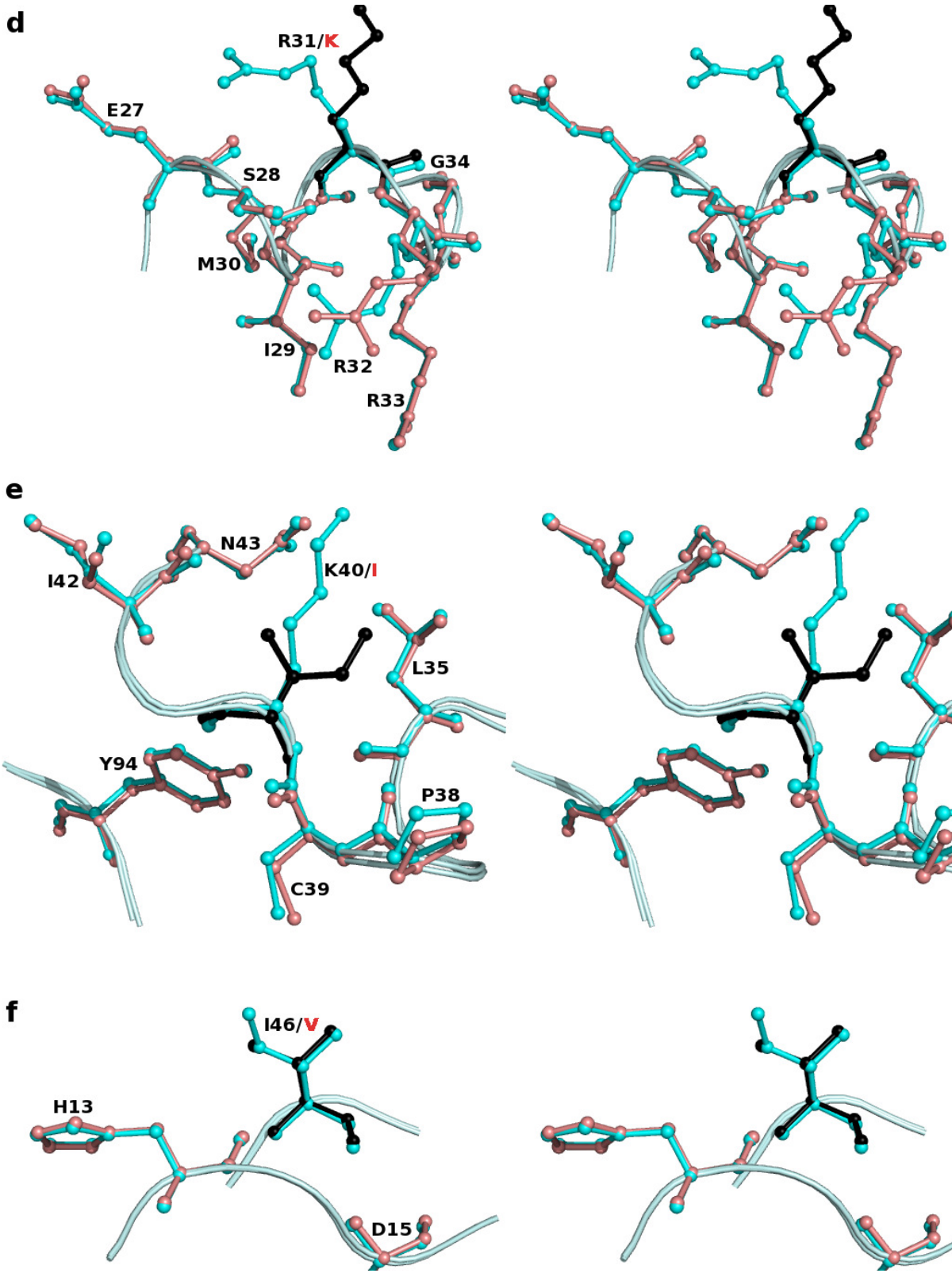
R24
S28/N
C26
M30
Y25
I29
R32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E27
S28
G34
M30
I29
R32
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e
N43
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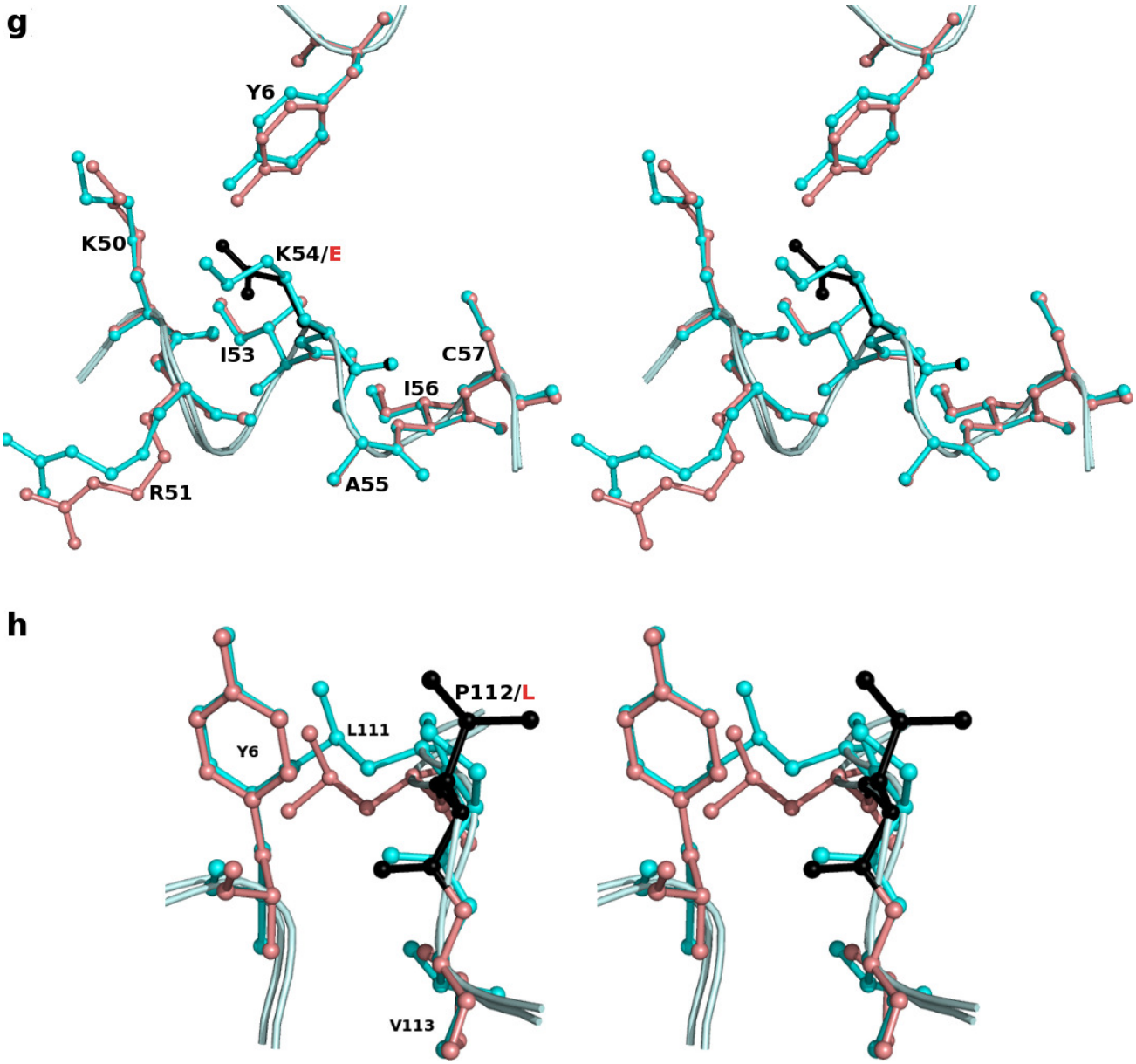
C39
f
I46/V
H13
D15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R51
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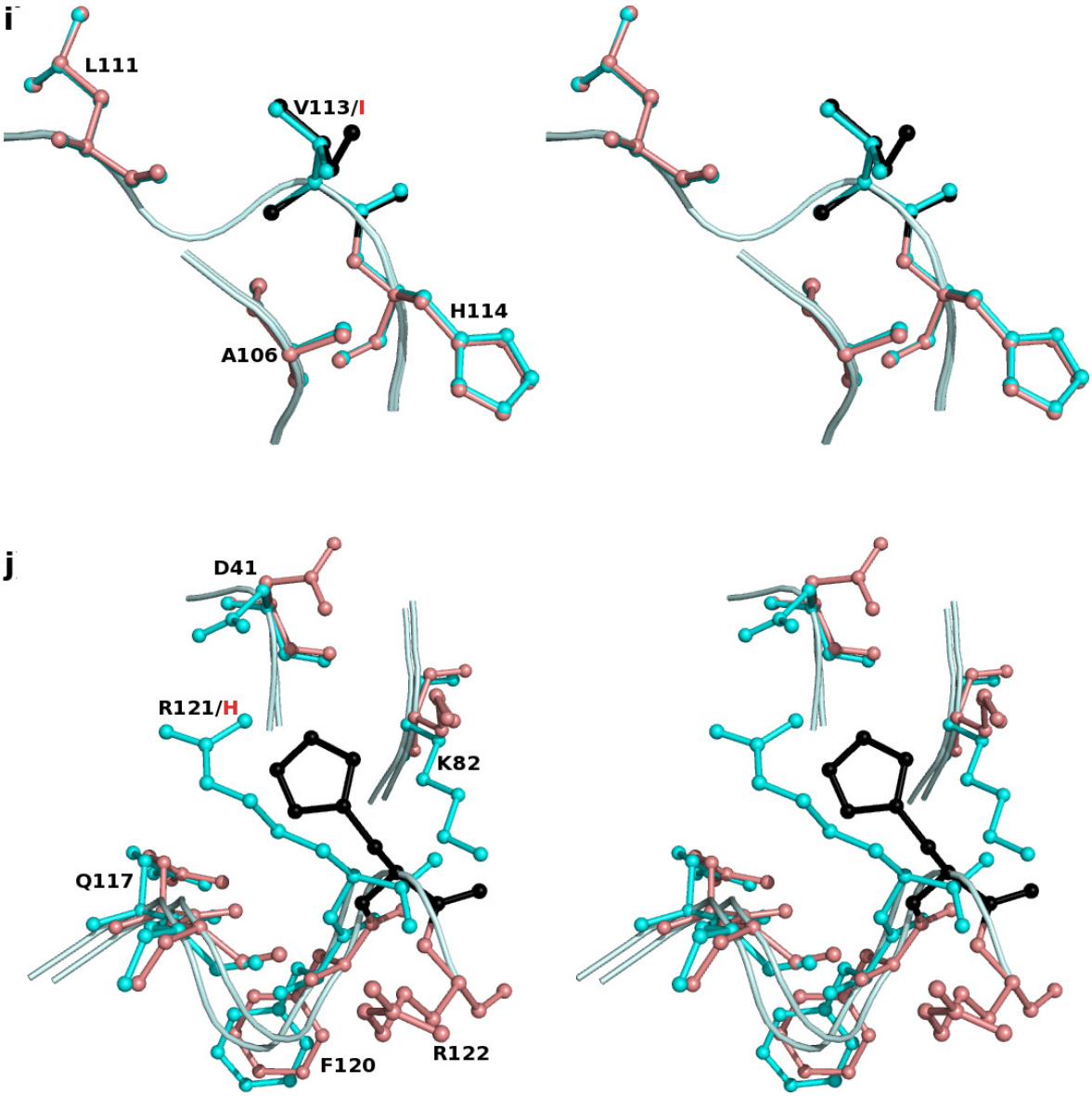
h
P112/L
L111
Y6
V113



Supplementary Figure S8. Structural comparison of hANG and ALS-associated variants (stereo diagram).

Superposition of native hANG (in cyan) and hANG-ALS (in salmon except for the mutant in black) variants. Residues that exhibit hydrogen bonding interactions with the hANG-ALS residues both in native hANG and mutant structures are also shown. K17/I is shown in firebrick colour while the hydrogen bonding residues are shown in pea colour. **(a) Q12L, (b) K17E/I, (c) S28N, (d) R31K, (e) K40I, (f) I46V, (g) K54E, (h) P112L, (i) V113I, (j) R121H.**

Supplementary Table S1. X-ray crystallographic data statistics for native hANG and hANG-ALS variants

	Native	***Q12L***	***K17E***	***K17I***	***S28N***	***R31K***	***K40I***
Crystallisation condition	20 % PEG 4000, 0.1 M Hepes pH 7.0, 0.05 M Na/K tartrate, 0.1 M NaCl	20 % PEG 4000, 0.1 M Hepes pH 7.0, 0.05 M Na/K tartrate, 0.1 M NaCl	30 % PEG 8000, 0.1 M sodium cacodylate pH 6.5, 0.2 M $(NH_4)_2SO_4$	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.2 M Na/K tartrate
Space group	$P2_12_12$	$P2_1$	$P2_12_12$	$C222_1$	$C222_1$	$C222_1$	$C222_1$
Cell dimensions	a = 85.6, b = 37.5, c = 37.5 Å, α = β = γ = 90.0°	a = 33.4, b = 38.0, c = 85.9 Å, α = γ = 90.0°, β = 90.08°	a = 37.4 , b = 82.9 , c = 42.9 Å, α = β = γ = 90.0°	a = 82.0 Å, b = 117.1 Å, c = 37.3 Å, α = β = γ = 90.0°	a = 81.2 Å, b = 119.8 Å, c = 37.4 Å, α = β = γ = 90.0°	a = 82.9 Å, b = 116.3 Å, c = 37.4 Å, α = β = γ = 90.0°	a = 83.2 Å, b = 118.7 Å, c = 37.6 Å, α = β = γ = 90.0°
Resolution range (Å)	50 – 1.04	50 – 2.47	50 – 2.11	50 – 2.07	50 – 2.45	50 – 2.5	50 – 2.8
R_{symm}[a] (outer shell)	0.061 (0.430)	0.084 (0.173)	0.057 (0.151)	0.081 (0.491)	0.089 (0.330)	0.086 (0.299)	0.089 (0.183)
$I/\sigma I$ (outer shell)	34.1 (1.9)	13.3 (5.4)	20.9 (7.2)	12.2 (0.8)	15.9 (4.3)	12.9 (2.3)	18.6 (5.8)
Completeness (outer shell) %	82.7 (37.3)	82.6 (64.7)	92.7 (92.4)	75.2 (16.5)	95.1 (77.7)	96.6 (77.8)	82.8 (39.4)
Total no. of reflections measured	1,164,220	159,607	71,016	43,320	300,936	94,973	272,953
Number of unique reflections	58,714	8,834	8,151	8,519	7,062	6,595	4,870
Redundancy (outer shell)	11.2 (2.8)	3.4 (2.5)	3.7 (3.6)	3.7 (1.5)	6.1 (4.7)	4.6 (2.2)	7.5 (5.9)
Wilson *B*-factor (Å^2)	9.7	35.8	21.1	34.8	51.9	49	46.6
R_{cryst}[b]/R_{free}[c]	16.9/18.8	23.7/24.9	21.7/28.5	22.5/29.9	23.4/27.5	20.1/24.2	21.3/27.8
Average *B*-factor (Å^2)							
Overall	18.0	35.3	21.8	43.3	58.9	34.3	48.2
Protein	16.0	35.2	21.0	43.1	58.7	34.1	48.4
Ligand	26.2	-	39.7 (SO_4)	48.9 (Tartrate)	77.8 (Tartrate)	48.6 (Tartrate)	-
Solvent	30.1	39.5	25.6	44.9	56.5	35.4	35.8
RMSD							
bond length (Å)	0.006	0.008	0.007	0.007	0.009	0.007	0.009
bond angle (°)	1.136	1.4	1.04	1.1	1.35	1.2	1.3
Ramachandran plot statistics (%)							
Favoured	97.5	91.8	94.5	93.2	92.2	94.9	88.8
Additionally allowed	2.5	8.2	5.5	6.8	7.8	5.1	11.2
PDB codes	**4AOH**	**4AHD**	**4AHE**	**4AHF**	**4AHG**	**4AHH**	**4AHI**

	I46V	***K54E***	***P112L***	***V113I***	***R121H***
Crystallisation condition	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M sodium citrate pH 5.5, 0.4 M Na/K tartrate	20 % PEG 4000, 0.1 M Hepes pH 7.0, 0.05 M Na/K tartrate, 0.1 M NaCl	20 % PEG 4000, 0.1 M Hepes pH 7.0, 0.05 M Na/K tartrate, 0.1 M NaCl
Space group	$C222_1$	C2	$C222_1$	$C222_1$	$C222_1$
Cell dimensions	a = 82.7 , b = 117.4 , c = 37.3 Å, α = β = γ = 90.0°	a = 124.4 , b = 37.3 , c = 83.2 Å, α = γ = 90.0°, β = 119.9°	a = 75.3 , b = 109.8 , c = 34.5 Å, α = 90.0°, β = 90.0°, γ = 90.0°	a = 82.3 , b = 118.3 , c = 37.2 Å, α = β = γ = 90.0°	a = 82.7 , b = 116.7 , c = 37.4 Å, α = β = γ = 90.0°
Resolution range (Å)	50 – 2.03	50 – 1.97	50 – 2.05	50 – 1.96	50 – 2.97
R_{symm}[a] (outer shell)	0.060 (0.165)	0.078 (0.233)	0.102 (0.377)	0.054 (0.185)	0.107 (0.247)
$I/\sigma I$ (outer shell)	21.7 (11.8)	7.3 (2.9)	16.1 (2.5)	29.0 (7.1)	10.1 (3.9)
Completeness (outer shell) %	95.4 (99.7)	88.8 (72.9)	93.0 (60.9)	92.1 (69.0)	92.6 (76.9)
Total no. of reflections measured	292,605	160,912	234,721	276,978	81,524
Number of unique reflections	12,089	23,796	9,350	13,478	3,976
Redundancy (outer shell)	6.7 (6.9)	2.1 (2.0)	5.6 (2.8)	7.5 (6.6)	4.1 (2.9)
Wilson *B*-factor (Å^2)	40	26.5	30.4	25.4	62
R_{cryst}[b]/R_{free}[c]	23.0/28.0	21.4/27.8	24.1/30.1	19.8/22.1	20.5/28.4
Average *B*-factor (Å^2) Overall Protein Ligand Solvent	 49.1 48.9 56.4 (Tartrate) - 52.1	 40.5 40.0 48.2 (Tartrate) - 45.4	 32.5 32.4 39.1 (Tartrate) - 34.7	 34.5 33.8 37.7 - 40.4	 35.5 35.5 40.6 (Cl^-) - 40.6
RMSD bond length (Å) bond angle (°)	 0.007 1.0	 0.006 1.0	 0.018 1.99	 0.007 1.05	 0.009 1.33
Ramachandran plot statistics (%) Favoured Additionally allowed	 96.6 3.4	 95.7 4.3	 89.7 10.3	 97.5 2.5	 87.1 12.9
PDB codes	**4AHJ**	**4AHK**	**4AHL**	**4AHM**	**4AHN**

<u>Supplementary Table S2.</u> Conservation of amino acid residues across species based on ANG variants implicated in ALS.

Sequence Analysis - All the sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and their accession numbers are Human - 18307851, Mouse angiogenin 1 - 113874, Mouse angiogenin 2 - 112818586, Mouse angiogenin 3 - 183979962, Mouse angiogenin 4 - 157388977, Mouse angiogenin 5 - 148702048, Mouse angiogenin 6 - 156255137, Bovine - 12043712, Rat - 55742836, Horse - 126352544, Rabbit - 399039, Pig - 113205746, Baboon - 20454800, Gorilla - 18150520, Chimpanzee - 57114182, Orangutan - 20454803, Langur - 28629464, Guereza - 62510449, Talapoin - 20454801, Vervet monkey - 20454802 and Squirrel monkey - 20454796. Multiple sequence alignment was performed using ClustalW tool from www.ebi.ac.uk. Amino acid residues are colour coded based on their property. Red – polar and uncharged; Blue – positively charged; Black – hydrophobic.

Angiogenin source (RNase activity %)	**hANG variants reported in Amyotrophic Lateral Sclerosis (RNase activity %)**												
	***Q12L (2.7)**	***K17E (19.0)**	***K17I (13.1)**	**S28N (21.1)**	***R31K (91.1)**	***C39W (4.3)**	***K40I (0.7)**	***I46V (9.3)**	**K54E (80.3)**	**P112L (28.0)**	**V113I (75.0)**	**H114R (1.6)**	**R121H (155.5)**
Human (100)	Q	K	K	S	R	C	K	I	K	P	V	H	R
Mouse_Ang1 (23)	Q	K	K	R	K	C	K	I	K	P	V	H	S
Mouse_Ang2 (84)	Q	K	K	S	V	C	K	I	K	P	V	H	S
Mouse_Ang3 (30)	Q	K	K	S	K	C	K	I	K	P	V	H	S
Mouse_Ang4	Q	K	K	S	K	C	K	I	R	P	V	H	S
Mouse_Ang5	Q	K	K	S	K	C	K	I	K	P	V	H	S
Mouse_Ang6	Q	K	K	C	K	C	K	I	K	P	V	H	S
Bovine	Q	K	K	N	K	C	K	I	K	P	V	H	T
Rat	Q	K	K	S	R	C	K	I	K	P	V	H	S

Horse	Q	K	K	S	V	C	K	I	K	P	V	H	R
Rabbit	Q	K	K	T	K	C	K	V	K	P	V	H	Q
Pig	Q	K	K	S	K	C	K	I	K	P	V	H	I
Baboon	K	T	T	S	R	C	K	I	N	P	V	H	R
Gorilla	Q	K	K	S	R	C	K	I	K	P	V	H	R
Chimpanzee	Q	K	K	S	R	C	K	I	K	P	V	H	R
Orangutan	Q	K	K	S	R	C	K	I	K	P	V	H	R
Langur	K	T	T	S	R	C	K	I	K	P	V	H	R
Guereza	K	T	T	S	R	C	K	I	K	P	V	H	R
Talapoin	K	R	R	S	R	C	K	I	R	P	V	H	R
Vervet_monkey	K	T	T	S	R	C	K	I	K	P	V	H	R

Supplementary Note 1

Structural implications for the new hANG-ALS variants. Recently four new hANG-ALS variants- T80S (ref. 8), F100I (ref. 8), V103I (ref. 7) and R121C (ref. 9) were reported (Table 1). Based on our current knowledge of ANG and ANG-ALS variant structures we can predict the likely structural features. In the native structure, T80 participates in an H-bond with T44 (part of the B_1 site). In a previous study it has been shown that mutation of T80 to Ala selectively weakens the interaction between T44 and N3 of cytosine (ref. 51). Modeling results suggest that the T80S variant may still retain its H-bond contact with T44, but would have lost a significant number of van der Waals contacts surrounding residue T44 which may result in reduced RNase activity. Both F100I and V103I variants may have slightly reduced RNase activity due to the loss of van der Waals interactions with S75 and I56 respectively. It is possible that similar to the R121H variant, the R121C variant could also open-up the obstructive C-terminal portion of the molecule thereby acquiring enhanced RNase activity. A detailed biochemical and structural characterization of these variants are currently underway.

Supplementary Methods

Oligonucleotide primers used for the generation of ANG-ALS variants

Mutation		Primers (5'-3')
Q12L	sense	GTATACACATTTCCTGACCCTGCACTATGACGCTAAACCG
	antisense	CGGTTTAGCGTCATAGTGCAGGGTCAGGAAATGTGTATAC
K17I	sense	CACTATGACGCTATACCGCAGGGCCG
	antisense	CGGCCCTGCGGTATAGCGTCATAGTG
K17E	sense	CACTATGACGCTGAACCGCAGGGCC
	antisense	GGCCCTGCGGTTCAGCGTCATAGTG
S28N	sense	GATCGTTACTGCGAAAATATTATGAGACGCCG
	antisense	CGGCGTCTCATAATATTTTCGCAGTAACGATC
R31K	sense	CGAATCGATTATGAAACGCCGTGGGTTAAC
	antisense	GTTAACCCACGGCGTTTCATAATCGATTCG
C39W	sense	GGGTTAACTAGTCCGTGGAAAGATATCAACACTTTC
	antisense	GAAAGTGTTGATATCTTTCCACGGACTAGTTAACCC
K40I	sense	GGGTTAACTAGTCCGTGCATCGATATCAACACTTTCATCC
	antisense	GGATGAAAGTGTTGATATCGATGCACGGACTAGTTAACCC
I46V	sense	GATATCAACACTTTCGTCCATGGTAACAAGCG
	antisense	CGCTTGTTACCATGGACGAAAGTGTTGATATC
K54E	sense	GCGTTCTATCGAAGCCATCTG
	antisense	CAGATGGCTTCGATAGAACGC
P112L	sense	CGGTCTGCTAGTCCATCTAG
	antisense	CTAGATGGACTAGCAGACCG
V113I	sense	CGGTCTGCCAATCCATCTAG
	antisense	CTAGATGGATTGGCAGACCG
H114R	sense	CGGTCTGCCAGTCCGTCTAG
	antisense	CTAGACGGACTGGCAGACCG
R121H	sense	CAGTCTATCTTCCATAGGCC
	antisense	GGCCTATGGAAGATAGACTG

Authentification of purified ANG and ANG-ALS variant proteins. ***Mass spectrometry*** **-** All purified protein masses were experimentally confirmed using the electrospray ionisation mass spectrometry (ESMS). Protein solutions were prepared by dilution of a desalted standard stock solution of protein in water (1 mg/ml, ≈70 μM) in a 50:50 ratio with HPLC grade acetonitrile containing 0.1% formic acid. ESMS was performed in positive ion mode on a QStar XL System (Applied Biosystems), fitted with a NanoMate (Advion Biosciences) automated nanospray source. Transformed mass spectra were calculated using the Bayesian protein reconstruct algorithm provided as part of the MDS Sciex Analyst 1.1 software package. ***Ribonucleolytic activity assay*** **-** Activity toward tRNA was determined by measuring the formation of acid soluble fragments as described by Shapiro et al[29]. Assay mixtures contained 2mg ml^{-1} yeast tRNA (Sigma), 0.1 mg ml^{-1} bovine serum albumin (BSA) and 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 μM test protein in 33 mM Na-Hepes, 33 mM NaCl, pH 7.0. After 2h of incubation at 37°C, reactions were terminated by the addition of 2.3 vol ice-cold 3.4% perchloric acid, the mixtures were centrifuged at 13000 g for 10 min at 4°C, and the absorbance of the supernatants were measured at 260 nm (Supplementary Fig. S2a). ***Circular dichroism studies*** **-** Circular dichroism (CD) studies were performed using a Jasco J-600 spectropolarimeter with a 2-mm rectangular cell. Samples of native ANG and variants were dissolved at a concentration of 0.1 mg/ml in 20 mM sodium acetate (pH 6.0) containing 0.1 M sodium chloride. Wavelength scans were performed at 20°C from 195-250 nm at a rate of 50 nm/min (Supplementary Fig. S2b).

MN differentiation of P19 cells and treatment with variant ALS-ANG proteins. PA6 cells were plated on gelatinized glass cover slips in 24-well plates at a cell density of 2×10^4 cells/ml, two days prior to seeding with P19 EC cells. P19 cells (5×10^3 cells/ml) were plated on the PA6 feeder in differentiation medium (alpha-MEM medium with glutamax, 0.5% (v/v) KOSR (Invitrogen), 1% (v/v) NEAA, and 5×10^{-7} M RA (Sigma). Cell morphology was monitored daily using a Leica DMIL microscope and images acquired using a DFC 496 camera. Differentiating P19 cells were treated with ANG and ANG-ALS variants at 200 ng/ml on day of plating. Medium was replaced every 48h and cultures fixed in 4% buffered PFA (Sigma) at 24h intervals over 144h. For some experiments P19 cells were allowed to differentiate for 4 days in differentiation medium and then the appropriate purified proteins were added and the cultures incubated for a further 48h.

Western blot. Cells were lysed in reducing SDS-PAGE loading buffer [2% SDS (Sigma), 10% Glycerol (BDH), 60mM Tris (Sigma), 100mM DTT (Sigma)] and protein concentration determined using Biorad Protein Assay. 50µg of each sample was denatured by boiling for 5 min then run on a 10% Tris-Tricine gel alongside Fermentas PageRuler Unstained Low Range Protein Ladder. Proteins were transferred to PVDF membrane (Millipore) was performed at 30V for 1h. The protein blot was blocked in 5% Marvel, 0.1% Tween20 (Sigma) in PBS for 1h RT then incubated with mouse anti-HA (Covance) 1:5000 overnight at 4°C. The unbound primary antibody was removed by washing (4 x 5min) with PBST and incubated with HRP conjugated anti-mouse (Sigma) 1:500 for 2h at RT. The unbound secondary washed with PBST (4 x 5min) and incubated with ECL reagents and exposed to Amersham Hyperfilm MP.

RNA isolation and RT PCR Cells were lysed in Trizol reagent (Invitrogen) and the RNA isolated following manufacturer's instruction. Nucleic acid concentrations were determined using a NanoDrop spectrophotometer. RNA quality was determined by 260/280 ratio, 260/230 ratio and by running denatured samples on 1.5% Agarose-TBE gels. RNA pretreated with DNAse1 from each sample **(**1µg) was reverse transcribed using a RevertAid™ H Minus Reverse Transcriptase kit (Fermentas). Duplicate reactions substituting the RT enzyme for DEPC H_2O were also performed as above for each sample. RT reactions were performed at 42°C for 60min and inactivated by 5min at 70°C. Each reaction was diluted to 10ng/µl of starting material in H_2O and stored at -20°C. RT-PCR. reactions(20ul) were set up using 2XBiomix (Bioline) and each +/- RT reaction contained cDNA which was the equivalent of 10ng of starting total RNA. Cycling conditions were denaturation at 94°C (3min) followed by 30 cycles of annealing (30s at the appropriate annealing temperature) extension (30s at 72°C) and denaturation (94°C, 30s). An annealing temperature of 53°C was used for hANG and hGAPDH reactions while 58°C was used for mAng1HA reactions. PCR reactions were analysed on a 1.5% Agarose TBE gel along with low molecular weight DNA weight markers.

Primers for RT PCR:

hGapdh F	TCCTGTTCGACAGTCAGCCGCA
hGapdh R	GCGCCCAATACGACCAAATCCGT
hANG F	TGGGCCTGGGCGTTTTGTTGT
hANG R	TGGCCTTGATGCTGCGCTTGT
mAng1 F	CGACAGATACTGTGAACGTAT

HA R AGCGTAGTCTGGGACGTCGTATGGGTA

Cell viability assay. SY5Y cells were plated at $2.5x10^4$ cells /cm^2 in complete medium either on coverslips in 24 well plates in 0.5ml for immunostaining, or in triplicate on 96 well plates in 50µl for viability testing. Media was changed to sodium arsenate (Sigma) and incubated for 2.5h. Cells on 24 well plates on coverslips were fixed in 4% PFA. Viability after exposure was determined using the cells in 96 well plates using CellTiter 96® Non-Radioactive Cell Proliferation Assay and following the manufacturer's instructions. Data from two experiments of identical design were pooled by normalising datasets from each cell line to the untreated from each series.

Image analysis and statistical methods for effects on P19 derived neurons. Ten or more randomly selected colonies stained for ISL1, Peripherin and counterstained with DAPI per experiment (from two independent sets of experiments) were analysed. For each colony, the number of $ISL1^+$ nuclei, the total number of nuclei, the density of the colony (nuclei/mm2) and the total area of the colony were determined using Photoshop CS3 v10.0 (Adobe) or ImageJ v1.42q (Rasband, W.S., ImageJ, National Institutes of Health, http://rsb.info.nih.gov/ij/, 1997-2004.). The statistical significance of these data was determined using GraphPad Prism v5.00 for Windows (GraphPad Software, www.graphpad.com). Data collected were highly skewed so were transformed by natural log (Transformed Y=ln (Y+1)). Transformed data subsequently passed the D'Agostino and Pearson omnibus normality test. No significant differences in variance were found between data sets after transformation. Transformed data from untreated and treated sets were compared by one-way ANOVA and Tukey's Multiple Comparison Test within time points. Transformed data from sets treated with native ANG or ANG variants from day zero and day four were compared by unpaired two-tail Student's t-tests. Representative images were acquired using a Leica DM55 microscope, Z-stacked and deconvoluted using the LAS software.

Estimation of cell death. SY5Y cells treated with the Ang proteins for 10 days were for stained for cleaved Caspase 3 and counterstained with DAPI. The total number of cells (DAPI positive) and the proportion of cleaved Caspase 3 positive SY5Y cells were counted from six independent fields (650µm by 480µm) from two sets of differentiation experiments. Each field contained a mean of 448 ±225 cells. Differentiated and undifferentiated data were compared separately by ANOVA using the untransfected SY5Y dataset as a reference for Dunnett's post-hoc comparison.