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Authors

Das, Debanu
Finn, Robert D
Abdubek, Polat
[et al.](#)

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The crystal structure of a bacterial Sufu-like protein defines a novel group of bacterial proteins that are similar to the N-terminal domain of human Sufu

Debanu Das,^{1,2} Robert D. Finn,³ Polat Abdubek,^{1,4} Tamara Astakhova,^{1,5} Herbert L. Axelrod,^{1,2} Constantina Bakolitsa,^{1,6} Xiaohui Cai,^{1,5} Dennis Carlton,^{1,7} Connie Chen,^{1,4} Hsiu-Ju Chiu,^{1,2} Michelle Chiu,^{1,4} Thomas Clayton,^{1,7} Marc C. Deller,^{1,7} Lian Duan,^{1,5} Kyle Ellrott,^{1,6} Carol L. Farr,^{1,7} Julie Feuerhelm,^{1,4} Joanna C. Grant,^{1,4} Anna Grzechnik,^{1,7} Gye Won Han,^{1,7} Lukasz Jaroszewski,^{1,5,6} Kevin K. Jin,^{1,2} Heath E. Klock,^{1,4} Mark W. Knuth,^{1,4} Piotr Kozbial,^{1,6} S. Sri Krishna,^{1,5,6} Abhinav Kumar,^{1,2} Winnie W. Lam,^{1,2} David Marciano,^{1,7} Mitchell D. Miller,^{1,2} Andrew T. Morse,^{1,5} Edward Nigoghossian,^{1,4} Amanda Nopakun,^{1,7} Linda Okach,^{1,4} Christina Puckett,^{1,4} Ron Reyes,^{1,2} Henry J. Tien,^{1,7} Christine B. Trame,^{1,2} Henry van den Bedem,^{1,2} Dana Weekes,^{1,6} Tiffany Wooten,^{1,4} Qingping Xu,^{1,2} Andrew Yeh,^{1,2} Jiadong Zhou,^{1,4} Keith O. Hodgson,^{1,8} John Wooley,^{1,5} Marc-André Elsliger,^{1,7} Ashley M. Deacon,^{1,2} Adam Godzik,^{1,5,6} Scott A. Lesley,^{1,4,7} and Ian A. Wilson^{1,7*}

¹Joint Center for Structural Genomics, <http://www.jcsg.org>

²Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, Menlo Park, California 94025

³Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton CB10 1SA, United Kingdom

⁴Protein Sciences Department, Genomics Institute of the Novartis Research Foundation, San Diego, California 92121

⁵Center for Research in Biological Systems, University of California, San Diego, La Jolla, California 92093

⁶Program on Bioinformatics and Systems Biology, Sanford-Burnham Medical Research Institute, La Jolla, California 92037

⁷Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

⁸Photon Science, SLAC National Accelerator Laboratory, Menlo Park, California 94025

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Abstract: Sufu (Suppressor of Fused), a two-domain protein, plays a critical role in regulating Hedgehog signaling and is conserved from flies to humans. A few bacterial Sufu-like proteins have previously been identified based on sequence similarity to the N-terminal domain of eukaryotic Sufu proteins, but none have been structurally or biochemically characterized and their function in bacteria is unknown. We have determined the crystal structure of a more distantly related Sufu-like homolog, NGO1391 from *Neisseria gonorrhoeae*, at 1.4 Å resolution, which provides the first biophysical characterization of a bacterial Sufu-like protein. The structure revealed a striking similarity to the N-terminal domain of human Sufu (r.m.s.d. of 2.6 Å over 93% of the NGO1391 protein), despite an extremely low sequence identity of ~15%. Subsequent sequence analysis

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*Correspondence to: Dr. Ian A. Wilson, JCSG, The Scripps Research Institute, BCC206, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: wilson@scripps.edu

revealed that NGO1391 defines a new subset of smaller, Sufu-like proteins that are present in ~200 bacterial species and has resulted in expansion of the SUFU (PF05076) family in Pfam.

Keywords: *Neisseria gonorrhoeae*; NGO1391; UniProt Q5F6Z8; Pfam PF05076; suppressor of fused; sufu-like; structural genomics

Introduction

The Hedgehog (Hh) and Wingless (Wnt) signaling pathways are critical for animal development.^{1–4} They are important in embryonic growth in vertebrates and stem cell differentiation. Disruption of these pathways can lead to disruption of stem cell function and cancer, for example.^{5,6} Recent studies have addressed how these pathways may have evolved.^{7–9} The human Sufu (Suppressor of Fused) protein is a key transcriptional regulator in the Hh pathway. Until recently, 20 eukaryotic, two-domain proteins of ~500 residues [N-terminal domain: Pfam SUFU family (PF05076) and C-terminal domain: Pfam SUFU_C family (PF12470)] and 13 bacterial Sufu-like single-domain proteins of unknown function (~250 residues, with ~25–40% sequence identity to the N-terminal Sufu domain of eukaryotic proteins) were classified in Pfam (version 23.0).¹⁰ The Sufu_C domain interacts with the N-terminal domain of Gli transcription factors, whereas the Sufu domain is believed to interact with the C-terminal tail of Gli.^{11,12} Apart from general classification of these bacterial proteins as Sufu-like, none had been investigated experimentally and their roles were consequently unclear, since bacteria do not use Hedgehog signaling.

As part of our efforts to characterize novel protein sequence space, we identified a set of proteins in *Neisseria* species that are distantly related to members of PF05076. This similarity was only detectable using profile–profile sequence comparison methods.¹³ NGO1391 (UniProt id Q5F6Z8) from *Neisseria gonorrhoea* FA1090 was selected as a representative of this set for structure determination, using the semiautomated, high-throughput pipeline of the Joint Center for Structural Genomics (JCSG; <http://www.jcsg.org>) as part of the National Institute of General Medical Sciences' Protein Structure Initiative.

Results and Discussion

Overall structure

NGO1391 from *Neisseria gonorrhoeae* FA 1090 was cloned, expressed, purified, and crystallized according to JCSG protocols as described in Materials and Methods. The crystal structure (Fig. 1) was determined by single-wavelength anomalous diffraction (SAD) phasing to a resolution of 1.40 Å. Data collection, model, and refinement statistics are summarized in Table I.¹⁴ The final model contains one monomer consisting of Gly0 (from the purification tag) and residues 1–181 of NGO1391 (the full-length pro-

tein is 182 residues), 265 waters, 4 sulfates, and 1 glycerol molecule in the asymmetric unit (ASU). Residue 182 was disordered and was not modeled. A monomer is the likely oligomeric form in solution as judged from crystal lattice packing and assembly analysis using PISA, and the monomeric form is supported by analytical size-exclusion chromatography. The Matthews' coefficient (V_M)¹⁵ is 2.5 Å³/Da, with an estimated solvent content of 50%. The Ramachandran plot produced by Molprobit¹⁶ shows that 97.8% of the amino acids are in the favored regions with one outlier, Asp132, which is in a region of good density, but with alternate main-chain and side-chain conformations.

NGO1391 is a compact molecule with approximate dimensions of 50 Å × 40 Å × 30 Å and has a tapered appearance due to a bulkier N-terminal region. It consists of a central, slightly S-shaped, anti-parallel β-sheet (β1–β7) flanked by α- and 3₁₀-helices (H1–H8) (Fig. 1). Some of the loops are long and extend out from the protein core. The C-terminus consists of a long tail with no secondary structure (residues 165–181). A glycerol molecule from the cryoprotectant binds in a relatively hydrophobic cleft formed by Ser119, Tyr122, Trp138, Leu140, Glu159, Phe162, Asp163, Ile167, and Tyr169 near the C-terminus. Most of these residues are conserved in the newly identified Sufu-like proteins (see Sequence Analysis) and three are also conserved in human Sufu (Ser119, Leu140 and Ile167 correspond to human Sufu Thr180, Val201, and Ile228, respectively).

Comparison to human Sufu

A search for other proteins of similar structure using sequence and structure-based methods was carried out using FFAS,¹³ DALI,¹⁷ FATCAT,¹⁸ and SSM.¹⁹ In all cases, the only significant hit (for example, the *e*-value of the top FATCAT hit is 3.95e-10 and the next hit is 4.35e-03; the top DALI hit has a Z-score of 16.7 and the next hit has 5.2, the top FFAS hit has a score of –49.4 and the next hit is –5.7) was to the N-terminal domain (NTD, 236 out of 484 residues) of the human Sufu protein (PDB accession code 1m11,¹² UniProt Q9UMX1). NGO1391 can be superimposed onto the NTD of Sufu with an r.m.s.d. of 2.6 Å over 170 Cα atoms (93% of the NGO1391 protein) with structure-based (DALI) and sequence-based (FFAS) sequence identities of 15% (Fig. 2). The notable structural differences between NGO1391 and the NTD of Sufu include: short helices H2 and H3 in NGO1391 correspond to long loops in Sufu; longer

Table I. Summary of Crystal Parameters, Data Collection, and Refinement Statistics for PDB 3k5j

Space group	$P3_221$		
Unit cell parameters	$a = 50.72 \text{ \AA}, b = 50.72 \text{ \AA}, c = 143.60 \text{ \AA}$		
Data collection	λ_1 SAD-Se		
Wavelength (Å)	0.9789		
Resolution range (Å)	27.7–1.40		
Number of observations	365,215		
Number of unique reflections	43,173		
Completeness (%)	99.7 (98.6) ^a		
Mean I/σ (I)	17.2 (1.8) ^a		
R_{sym} on I (%)	6.0 (76.1) ^a		
Highest resolution shell (Å)	1.45–1.40		
Model and refinement statistics			
Resolution range (Å)	27.7–1.40	Data set used in refinement	λ_1 SAD-Se
Number of reflections (total)	43,107 ^b	Cutoff criteria	$ F > 0$
Number of reflections (test)	2169	R_{cryst}	0.134
Completeness (% total)	99.8	R_{free}	0.163
Stereochemical parameters			
Restraints (RMSD observed)			
Bond angle (°)	1.60		
Bond length (Å)	0.015		
Average protein isotropic B-value (Å ²)	20.3 ^c		
ESU based on R_{free} (Å)	0.05		
No. of protein residues/atoms	182/1593		
No. of water/sulfate/glycerol molecules	265/4/1		

ESU = Estimated overall coordinate error.¹⁴

$R_{\text{sym}} = \sum |I_i - \langle I_i \rangle| / \sum I_i$, where I_i is the scaled intensity of the i th measurement and $\langle I_i \rangle$ is the mean intensity for that reflection.

$R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.

R_{free} = as for R_{cryst} , but for 5.0% of the total reflections chosen at random and omitted from refinement.

^a Highest resolution shell.

^b Typically, the number of unique reflections used in refinement is slightly less than the total number that were integrated and scaled. Reflections are excluded due to negative intensities and rounding errors in the resolution limits and cell parameters.

^c This value represents the total B.

proteins. Seven residues contribute to a more negatively charged region on one side of Sufu: Glu106, Asp111, Glu152, Asp159, Glu181, Glu221, and Asp262 (Fig. 4). These residues are not conserved across bacterial Sufu-like proteins. Both sides of NGO1391 appear to have a similar number of negatively charged residues (Fig. 4). Some other residues are more strongly conserved between the eukaryotic and bacterial forms of the protein with several on the protein surface (NGO1391 Thr24, Glu49, Trp51, Tyr53, Pro106, Ser111, and His115) that could be functionally important.

Sequence analysis and genomic context

At the time of the NGO1391 structure determination, Pfam (version 23.0) SUFU family (PF05076) contained 33 proteins from 27 species, 20 of which were eukaryotic including the N-terminal domain of human Sufu and 13 were bacterial. The eukaryotic Sufu proteins (primarily from chordates and insects) all contained ~500 residues, and consisted of two domains of approximately equal size (Sufu and Sufu_C). The bacterial proteins, on the other hand, were uncharacterized proteins of only around 250 residues.

A search against the UniProt database using PSI-BLAST revealed that NGO1391 (Q5F6Z8) had

several sequence homologs of ~92% sequence identity in other *Neisseria* species: *N. gonorrhoeae* 1291 (C1HYY6), *N. gonorrhoeae* NCCP11945 (B4RNC6), *N. flavescens* NRL30031/H210 (C0EME3), *N. meningitidis* serogroup C/serotype 2a (A1KSR8), *N. cinerea* ATCC14685 (C0DN13), and *N. lactamica* ATCC23970 (C0F5L2). The next nearest hits were to proteins with ~33–39% sequence identity (B5HWZ3 from *Streptomyces sviveus* ATCC 29083, C2A7G8 from *Thermomonospora curvata* DSM 43183, C1YUI8 from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111 and A6A6Q5 from *Vibrio cholerae* MZO-2). However, none of these proteins (Fig. 3) were included in PF05076.

Three rounds of iterative PSI-BLAST, including sequence similarities down to a threshold of 0.1, identified ~300 additional bacterial proteins, in addition to the 33 vertebrate and bacterial Sufu proteins already present in PF05076. Pairwise comparisons revealed that the bacterial proteins already included in PF05076 had sequence identities of ~25–40% to the eukaryotic Sufu proteins, but less than 16% identity to the additional Sufu-like proteins identified here. A multiple sequence alignment of a representative set of these new Sufu-like proteins identified residues that are invariant or similar in these proteins (Fig. 3) and their distribution in the structure (Fig. 5).

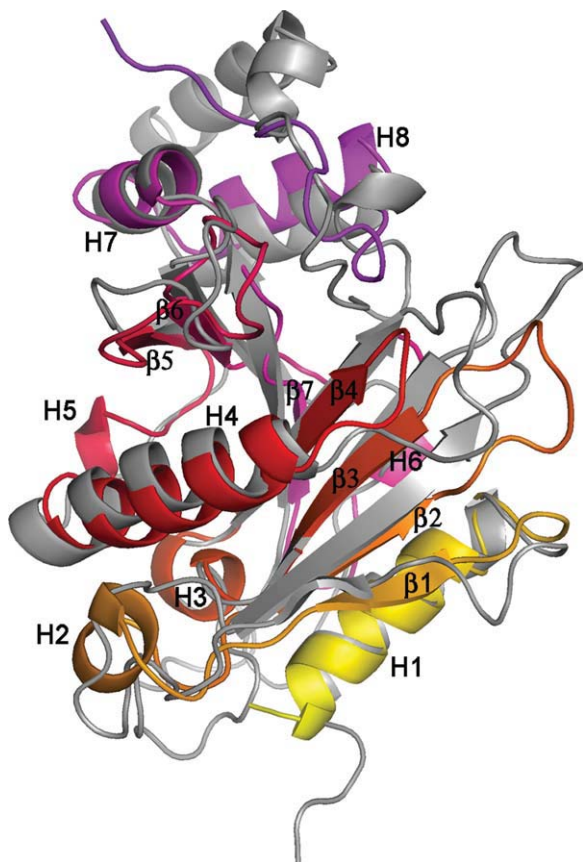


Figure 2. Structural comparison of NGO1391 and human Sufu protein. The structures of NGO1391 and the N-terminal domain (NTD) of human Sufu protein (gray) are highly similar and superimpose with an r.m.s.d. of 2.6 Å over 170 C α atoms.

Based on this analysis, Pfam PF05076 was independently revised to include these proteins using the HMMER3 software (<http://hmmmer.janelia.org>). Thus, PF05076 in the current release of Pfam (v. 24.0, October 2009) now contains 341 proteins from 31 eukaryotic and 220 bacterial species. Inspection of the residue conservation scores in the multiple sequence alignment for this revised protein family on the Pfam website (<http://pfam.sanger.ac.uk/family/PF05076>), reveals that the only residues that are conserved across the entire protein family (341 proteins) are Val134, Phe136, Leu139, Ile142, Glu146, and Leu158 in NGO1391. Of these residues, only Val134 is surface exposed whereas the rest are in the protein core.

The genomic neighbors of NGO1391 are putative MafB-like (NGO1392) and MafA2 (NGO1393) proteins that are predicted to be functional partners of NGO1391 with high scores of 0.87 and 0.86, respectively.²² The Maf proteins are believed to have adhesion capabilities.^{23,24} Interestingly, this predicted association is similar to that of the β -catenin in the Wnt signaling pathway, where β -catenin can form a complex with the cadherin cell-adhesion molecules. β -catenin also functions as a transcriptional regulator. However, no sequence or structural similarity is

found between NGO1391 and β -catenin and this genomic context does not appear to be conserved across other members of this Pfam.

The crystal structure and sequence analysis presented here provides the first molecular characterization of a bacterial Sufu-like protein that may be a prokaryotic homolog of modern Sufu proteins involved in embryonic development in vertebrates. This study has also led to identification of a large number of other bacterial Sufu-like proteins. Mechanistic and mutagenesis experiments can help to elucidate which residues are important for molecular function and substrate specificity and aid in understanding the function of NGO1391 and related proteins. Additional information about the proteins described in this study is available from TOPSAN^{25,26} at <http://www.topsan.org/explore?PDBid=3k5j>.

Materials and Methods

Protein expression, purification, and crystallization

Clones were generated using the Polymerase Incomplete Primer Extension (PIPE) cloning method.²⁷ The gene encoding NGO1391 (GenBank: YP_208451, gi|59801739, UniProt: Q5F6Z8) was amplified by polymerase chain reaction (PCR) from *Neisseria gonorrhoeae* genomic DNA using *PfuTurbo* DNA polymerase (Stratagene) and I-PIPE (Insert) primers (forward primer: 5'-ctgtacttccagggc ATGGACTATAA CCAAACGTGTTTTATCTC3', reverse primer: 5'-aat taagtcgctgta TCCTGCCTGCCAGACAGTACTCGC ACG-3', target sequence in upper case) that included sequences for the predicted 5' and 3' ends. The expression vector, pSpeedET, which encodes an amino-terminal tobacco etch virus (TEV) protease-cleavable expression and purification tag (MGSDK IHHHHHHENLYFQ/G), was PCR amplified with V-PIPE (Vector) primers (forward primer: 5'-taacgc gactaattaactcgtttaaacggtctccagc-3', reverse primer: 5'-gccctggaagtacaggttttcgtgatgatgatgatg-3'). V-PIPE and I-PIPE PCR products were mixed to anneal the amplified DNA fragments together. *Escherichia coli* GeneHogs (Invitrogen) competent cells were transformed with the I-PIPE/V-PIPE mixture and dispensed on selective LB-agar plates. The cloning junctions were confirmed by DNA sequencing. Expression was performed in a selenomethionine-containing medium at 37°C. Selenomethionine was incorporated via inhibition of methionine biosynthesis, which does not require a methionine-auxotrophic strain.²⁸ At the end of fermentation, lysozyme was added to the culture to a final concentration of 250 μ g/mL, and the cells were harvested and frozen. After one freeze/thaw cycle the cells were sonicated in lysis buffer [50 mM HEPES pH 8.0, 50 mM NaCl, 10 mM

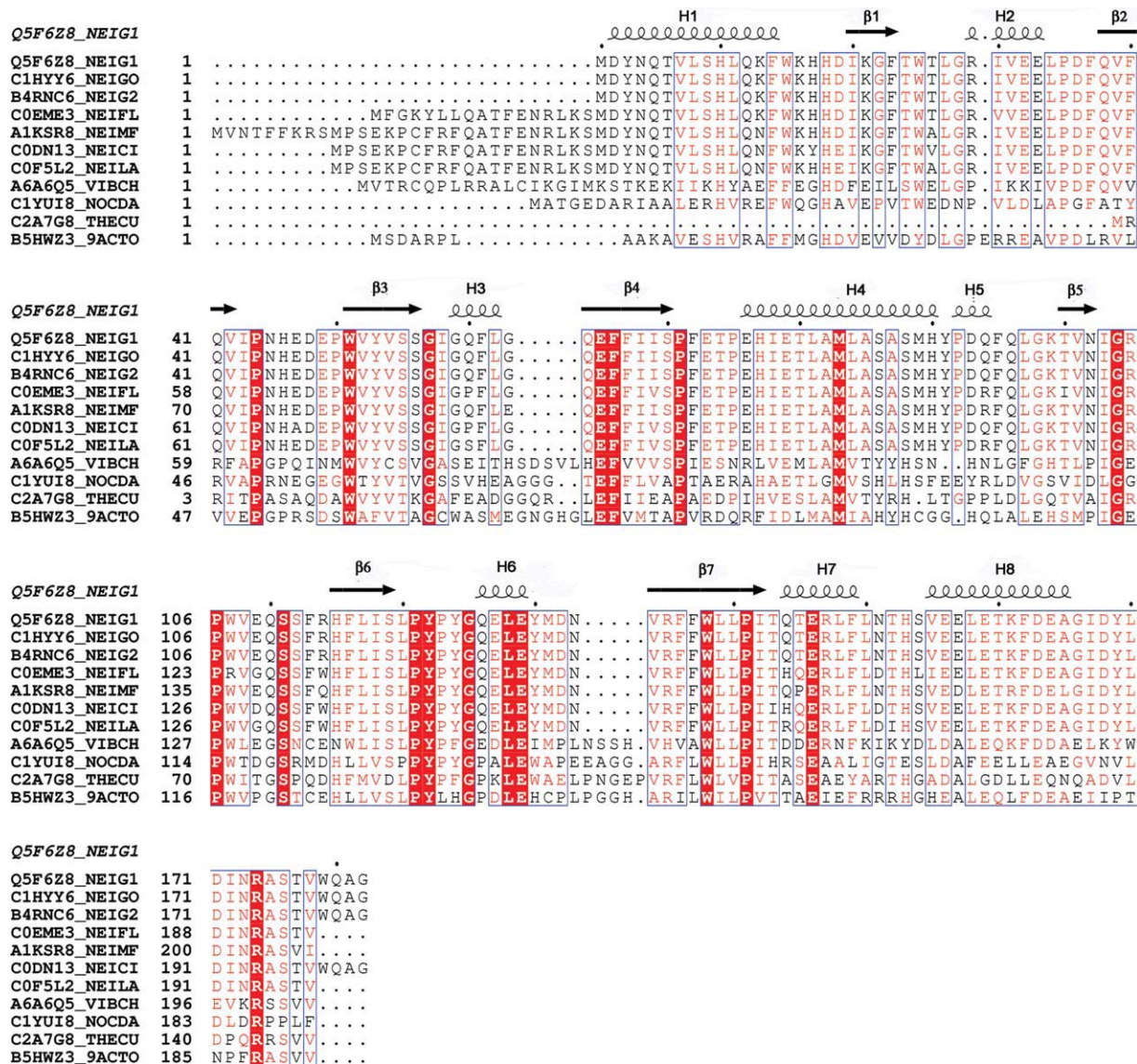


Figure 3. Multiple sequence alignment of Sufu-like proteins. The *Neisseria* proteins with UniProt codes Q5F6Z8 (NGO1391) from *N. gonorrhoeae* FA1090, C1HY6 from *N. gonorrhoeae* 1291, B4RNC6 from *N. gonorrhoeae* NCCP11945, C0EME3 from *N. flavescens* NRL30031/H210, A1KSR8 from *N. meningitidis* serogroup C/serotype 2a, C0DN13 from *N. cinerea* ATCC14685 and C0F5L2 from *N. lactamica* ATCC23970 share ~92% sequence identity. More distant homologs with ~33–39% sequence identity are B5HWZ3 from *Streptomyces svuceus* ATCC 29083, C2A7G8 from *Thermomonospora curvata* DSM 43183, C1YUI8 from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111, and A6A6Q5 from *Vibrio cholerae* MZO-2. The alignment was generated using the CLUSTALW web-server.²⁰

imidazole, 1 mM Tris(2-carboxyethyl)phosphine-HCl (TCEP)] and the lysate was clarified by centrifugation at 32,500 × g for 30 minutes. The soluble fraction was passed over nickel-chelating resin (GE Healthcare) pre-equilibrated with lysis buffer, the resin was washed with wash buffer [50 mM HEPES pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% (v/v) glycerol, 1 mM TCEP], and the protein was eluted with elution buffer [20 mM HEPES pH 8.0, 300 mM imidazole, 10% (v/v) glycerol, 1 mM TCEP]. The eluate was buffer exchanged with TEV buffer [20 mM HEPES pH 8.0, 200 mM NaCl, 40 mM imidazole, 1 mM TCEP] using a PD-10 column (GE Healthcare),

and incubated with 1mg of TEV protease per 15 mg of eluted protein. The protease-treated eluate was passed over nickel-chelating resin (GE Healthcare) pre-equilibrated with HEPES crystallization buffer [20 mM HEPES pH 8.0, 200 mM NaCl, 40 mM imidazole, 1 mM TCEP] and the resin was washed with the same buffer. The flow-through and wash fractions were combined and concentrated for crystallization trials to 13 mg/mL by centrifugal ultrafiltration (Millipore). NGO1391 was crystallized by mixing 200 nL protein solution with 200 nL crystallization solution in a sitting drop format over a 50 μL reservoir volume using the nanodroplet vapor

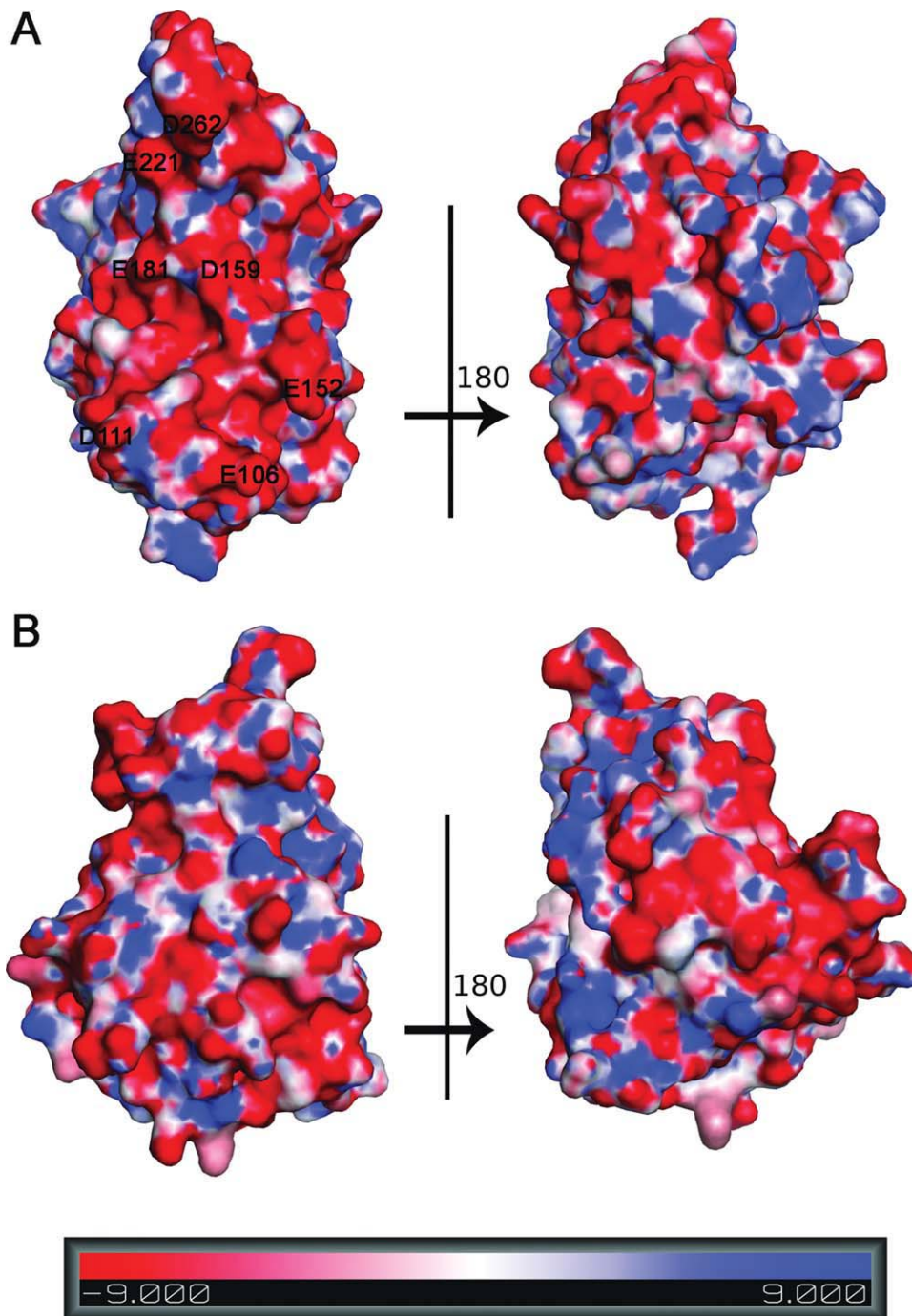


Figure 4. Electrostatic surface potential representations of human Sufu and NGO1391. (a) Surface for the NTD of human Sufu shows a greater accumulation of negatively charged residues on one side (left panel) of the protein due to surface residues Glu106, Asp111, Glu152, Asp159, Glu181, Glu221, and Asp262. (b) Surface for the NGO1391 indicates that charged residues are equally distributed on both sides of the protein. The color scale is in units of kT/e from -9 to $+9$. The figure in the right panel represents a 180° rotation around the vertical axis compared to the left panel.

diffusion method²⁹ with standard JCSG crystallization protocols.³⁰ The crystallization reagent consisted of 1.6M ammonium sulfate, and 0.1M MES pH 6.0. Glycerol was diluted using the reservoir solution and then added 1:1 to the drop to a final concentration 20% (v/v) as a cryoprotectant prior to harvesting. A cubic-shaped crystal of approximate size 100

$\mu \times 100 \mu \times 100 \mu$ was harvested after 15 days at 277 K for data collection. Initial screening for diffraction was carried out using the Stanford Automated Mounting system (SAM)³¹ at the Stanford Synchrotron Radiation Lightsource (SSRL, SLAC National Accelerator Laboratory, Menlo Park, CA). The diffraction data were indexed in trigonal space

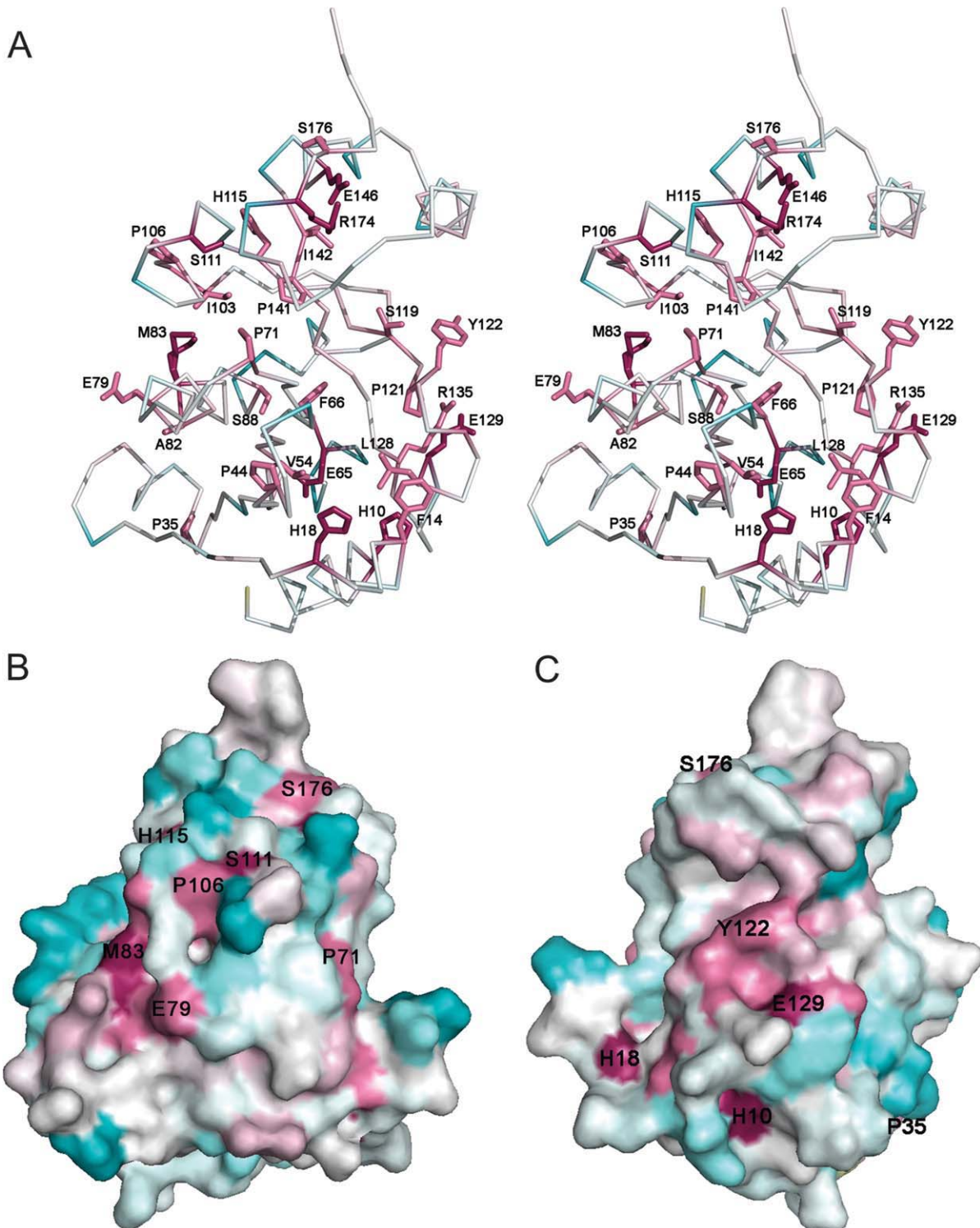


Figure 5. Sequence conservation plot of NGO1391. The Sufu-like proteins from Figure 3 have been used for the calculation of conservation scores²¹ (a) A stereo stick representation in which the most conserved residues are represented in dark pink, the light pink residues are less conserved and residues in cyan are least conserved. (b) and (c) Two views of some of the most conserved surface exposed residues in the same color scheme. The molecular orientations in the top and bottom panels are different to facilitate visualization using PyMOL.

group $P3_221$. Protein concentrations were determined using the Coomassie Plus assay (Pierce). To determine its oligomeric state in solution, NGO1391 was analyzed using a $1 \times 30 \text{ cm}^2$ Superdex 200 size-exclusion

column (GE Healthcare). The mobile phase consisted of 20 mM Tris pH 8.0, 150 mM NaCl, and 0.02% (w/v) sodium azide. The molecular weight was calculated using ASTRA 5.1.5 software (Wyatt Technology).

Data collection, structure solution, and refinement

SAD data were collected at 100 K using a MarMosaiC 325 CCD detector (Rayonix) at the SSRL on beamline 11-1 at the selenium peak (λ_1) wavelength using the BLU-ICE³² data collection environment. The SAD data were integrated and reduced using XDS³³ and XSCALE, respectively. All other programs for data manipulation were from the CCP4 suite.³⁴ The heavy atom substructure and phasing calculations were performed using SHELXD³⁵ and autoSHARP³⁶, respectively, and ARP/wARP³⁷ was used for automatic model building to 1.40 Å resolution. Model completion and crystallographic refinement were performed using COOT³⁸ and REFMAC5,³⁹ respectively. The refinement protocol included the experimental phase restraints in the form of Hendrickson–Lattman coefficients from autoSHARP. Data and refinement statistics are summarized in Table I.

Validation and deposition

The quality of the crystal structure was analyzed using the JCSG Quality Control server (<http://smb.slac.stanford.edu/jcsg/QC>). This server automatically processes the coordinates and data through a variety of validation tools including AutoDepInputTool,⁴⁰ MolProbity,¹⁶ WHATIF 5.0,⁴¹ agreement between the atomic model and the data using SFcheck 4.0⁴² and RESOLVE,⁴³ the protein sequence using CLUSTALW,⁴⁴ atomic occupancies using MOLEMAN2,⁴⁵ consistency of NCS pairs and evaluates difference in $R_{\text{cryst}}/R_{\text{free}}$, expected $R_{\text{free}}/R_{\text{cryst}}$ and maximum/minimum B-factors by parsing the refinement log-file and PDB header. Protein quaternary structure analysis was performed using the PISA server,⁴⁶ Figure 1(B) was adapted from an analysis using PDBsum,⁴⁷ and all others were prepared with PyMOL.⁴⁸ Figure 3 was prepared using the PDB2PQR⁴⁹ server and the APBS^{49,50} module in PyMOL. Atomic coordinates and experimental structure factors for NGO1391 have been deposited in the PDB under the accession code 3k5j.

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necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. Genomic DNA from *Neisseria gonorrhoeae* FA 1090 (ATCC Number: 700825D) was obtained from the American Type Culture Collection (ATCC).

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