The molecular basis for Pompe disease

revealed by the structure of human acid α -glucosidase

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Abstract

Pompe disease results from a defect in human acid α -glucosidase (GAA), a lysosomal enzyme that cleaves terminal α 1-4 and α 1-6 glucose from glycogen. In Pompe disease (also known as Glycogen Storage Disorder type II), the accumulation of undegraded glycogen in lysosomes leads to cellular dysfunction, primarily in muscle and heart tissues. Pompe disease is an active candidate of clinical research, with pharmacological chaperone therapy tested and enzyme replacement therapy approved. Despite production of large amounts of recombinant GAA annually, the structure of GAA has not been reported until now. Here, we describe the first structure of GAA, at 1.7Å resolution. Three structures of GAA complexes reveal the molecular basis for the hundreds of mutations that lead to Pompe disease and for pharmacological chaperoning in the protein. The GAA structure reveals a surprising second sugar-binding site 34Å from the active site, suggesting a possible mechanism for processing of large glycogen substrates. Overall, the structure will assist in the design of next-generation treatments for Pompe disease.

Introduction

The lysosomal storage disorder Pompe disease (also known as Glycogen Storage Disorder type II) is caused by loss of activity in the acid α -glucosidase (GAA) enzyme (Fukuda et al., 2007, Lim et al., 2015, Raben et al., 2002). GAA is an exo-hydrolase cleaving both α 1-4 and α 1-6 glucose linkages in glycogen, releasing α -glucose as a product (Fig. 1A). In the absence of functional GAA, glycogen accumulates in the lysosomes, leading to cellular dysfunction and the onset of Pompe disease symptoms, including muscle atrophy, liver enlargement, and heart defects (Hirschhorn and Reuser, 2001). The age of onset of Pompe disease correlates with GAA activity, where infantile, juvenile, and adult onset forms have an average of 0%, 5%, and 8% residual GAA activity respectively (Hirschhorn and Reuser, 2001).

Pompe disease can be treated by enzyme replacement therapy, by infusion of recombinant GAA, an approach approved by the FDA in 2006 (Lim et al., 2014). Because of the number of cells requiring enzyme and inefficient uptake of recombinant enzyme, massive doses of purified recombinant GAA (up to 20 mg/kg biweekly) are required to treat Pompe disease patients, making enzyme replacement therapy expensive (Schoser et al., 2008). Additionally, large doses can provoke immune responses in Pompe disease patients (Bigger et al., 2015, Doerfler et al., 2016). Thus, GAA remains an active research target, and alternative approaches are in preclinical and clinical development. For example, current approaches include modification of the glycans on GAA to improve its uptake into the lysosome (Zhu et al., 2009, Zhu et al., 2005), addition of insulin-like growth factor II tags to improve uptake (Maga et al., 2013), gene therapy using adeno-associated-virus vectors (Doerfler et al., 2016), pharmacological chaperoning with small-molecule stabilizers, and combination therapy using both enzyme replacement and pharmacological chaperoning approaches (Khanna et al., 2014).

Pharmacological chaperones bind to the active site of their targets and thus stabilize the folded state of the protein (Fan et al., 1999). For Pompe disease, the iminosugar glucose analog 1-deoxynojirimycin (DNJ) can increase GAA activity, improving lysosomal localization of some GAA variants (Flanagan et al., 2009). Co-administration of pharmacological chaperone with recombinant GAA enzyme can increase enzyme activity in the lysosome (Khanna et al., 2012, Khanna et al., 2014). While DNJ can chaperone GAA, it also inhibits multiple glucosidases, potentially leading to unwanted effects. The identification of more specific chaperones has been hindered by a lack of structural information on GAA.

GAA is synthesized as a 952-residue zymogen and undergoes at least nine proteolytic processing steps during maturation (Hoefsloot et al., 1988, Moreland et al., 2005, Wisselaar et al., 1993). After cleavage of the 27-residue signal sequence and glycosylation in the ER, the 110 kDa GAA protein (residues 28-952) is trafficked to the Golgi, where glycans are further modified and proteolytic cleavage yields residues 57-952 (Moreland et al., 2005). Multiple cleavage events in the late endosome and lysosome produce the mature protein, containing four polypeptides with approximate masses of 3, 10, 70, and 19 kDa corresponding to residues 78-113, 122-200, 204-782, and 792-952 respectively (Moreland et al., 2005) (Fig. 1E). The mature enzyme shows improved kinetics against glycogen (with a 10-fold lower K_M and a 2-fold higher k_{cat}), but the molecular basis of the effect is not well understood (Bijvoet et al., 1998, Wisselaar et al., 1993).

To better understand the molecular basis of Pompe disease, we determined the three-dimensional structure of the mature human GAA by X-ray crystallography. Additionally, to reveal substrate recognition and pharmacological chaperoning in GAA, we determined complexes of GAA with the catalytic product glucose, the pharmacological chaperone DNJ, a disaccharide, and a trisaccharide. The structures reveal a surprising second sugar-binding site in GAA distal

from the active site, suggesting a mechanism for processive catalysis of glycogen polymers. The structure allows for modeling of the locations of Pompe disease variants onto the structure, indicating that the disease is primarily a protein folding disorder, because mutations tend to disrupt the hydrophobic core of the protein.

Results and Discussion

Structure determination

Extensive crystallization trials with the intact GAA (residues 70-952) resulted in crystals that diffracted to 10Å resolution at best. To reduce conformational heterogeneity, we developed an *in vitro* limited protease digestion to mimic the proteolytic maturation *in vivo* and to remove loops hindering crystallization. Limited proteolysis cleaved the protein into fragments, including a doublet of 70-80 kDa and a fragment of approximately 20 kDa (Fig. 1 supplement A). The fragments migrate as a single species during size exclusion chromatography, indicating they remain associated post cleavage. Crystallization of the protease-treated GAA selected for the more highly processed fragments (Fig. 1 supplement A), leading to crystals that diffracted to 1.7Å resolution (Table 1).

Overall GAA structure

The human GAA structure contains five domains, seven N-linked glycans, five disulfide bonds, three unpaired cysteines, and three missing loops (consistent with their removal by protease). The five domains include an N-terminal P-type trefoil domain (N1), a β-sandwich domain (N2), a (β/α)₈ barrel containing the catalytic site, a proximal β-sandwich domain (C1), and a distal C-terminal β-sandwich domain (C2) (Fig. 1 and Fig. 1 supplement B). The N1 domain spanning residues 80-136 contains little secondary structure other than a short α-helix (100-106) and is maintained with three disulfide bonds. The N2 domain spanning residues 137-349 has a β-sandwich topology with 16 antiparallel β-strands in two β-sheets. The third domain

encompasses residues 347-726 and has the characteristic $(\beta/\alpha)_8$ topology found in the catalytic domain of family 31 glycoside hydrolases. C-terminal to the catalytic $(\beta/\alpha)_8$ domain, the GAA structure contains two additional β -sandwich domains. Domain C1 spans residues 727-820 and is composed of seven antiparallel β -strands in 5- and 2-stranded β -sheets. The C2 domain has a β -sandwich topology and is composed of 10 β -strands in two 5-stranded β -sheets, with two small α -helices inserted (Fig. 1 and Fig. 1 supplement B).

The GAA structure reveals extensive post-translational modifications of the polypeptide. The N-linked glycosylation sites show clear electron density at six of the seven reported sites. The 13 cysteine residues form five disulfide bonds, three in domain N1 (82-109, 92-108, and 103-127) and two in the catalytic domain (533-558 and 647-655). The disulfide arrangement in N1 matches the 1-5, 2-4 and 3-6 connectivity found in other P-type trefoil domains (Sim et al., 2008, Wright et al., 1997). Three remaining cysteines are unpaired, including C374 near the active site and the surface-exposed C938 and C952 in the C2 domain (Fig. 1).

Proteolytic treatment prior to crystallization mimics proteolytic maturation in the late endosome and lysosome. Electron density is missing for loops 116-122, 199-205, and 782-792. Presumably, the flexible loops in the intact protein limited the resolution of the intact GAA crystals. After cleavage of the three loops, the remaining fragments remain associated by non-covalent interactions and a disulfide bond in N1 and by non-covalent interactions in domains N2 and C1. Extensive surface area remains buried in the mature GAA: 1105 Å² (and the C103-C127 disulfide bond) between the 80-115 fragment and the rest of the protein; 2944 Å² between the 123-200 fragment and the rest of the protein; and 2901 Å² between the 793-952 fragment and the rest of the protein (Fig. 1 supplement C). Because of the large surface area buried between proteolytic fragments, the GAA subunits remain associated after proteolytic maturation (Moreland et al., 2005).

Active site and ligand binding

The active site of GAA is an acidic pocket at the base of a conical funnel lined by hydrophobic residues in the $(\beta/\alpha)_8$ barrel catalytic domain (Fig. 1, Fig. 2, and Fig. 2 supplement A). The funnel and active site are formed by loops at the C-terminal ends of each of the eight β -strands forming the β -barrel. Additionally, loop 281-286 from the N2 domain contributes to substrate recognition in the active site (Fig. 2 supplement A). Thirteen residues (D282, W376, D404, L405, I441, W481, W516, D518, M519, R600, D616, W618, F649, L650, H674) define the active site within 5Å of the glucose ligand (Fig. 2).

GAA uses a double-displacement reaction mechanism found in family 31 glycoside hydrolases, leading to overall retention of the anomeric configuration of the α -glucoside substrate (Bourne and Henrissat, 2001, Koshland, 1953). The nucleophile D518 falls 3.1Å from the C1 position of glucose, in position to make a nucleophilic attack on the carbon. The acid/base D616 falls on the opposite side of the cleaved glycosidic linkage, 2.9Å from the O1 of the ligand, in position to protonate the leaving group during catalysis (Fig. 2). Fitting the pH-dependence curve for GAA activity (Fig. 2 supplement B) yields two pKa values (3.6 and 5.3), which we interpret as the pKa's for D518 and D616 respectively, consistent with the double-displacement reaction mechanism.

To better understand substrate recognition in GAA, we determined complexes of the enzyme with isomaltose and maltotriose (di- and tri-saccharide fragments of glycogen) at 2.0 and 1.9Å resolution, respectively. The structures show glucose present in the active site with no clear electron density for the remaining portion of the substrates (Fig. 2). We interpret the density as catalytic product in the active site, despite the limited GAA activity at pH 7.5 (Fig. 2 supplement B). The glucose density appears as a mixture of α and β anomers. Because of the close

proximity of active site residues D282, M519, and R600, β -linked substituents beyond a hydroxyl are sterically blocked, and thus we conclude that the β -glucose appears in the active site after cleavage of α -linked substrate and subsequent ring opening of the α -glucose product. Residues D404, D616, and H674 confer glucose substrate specificity by hydrogen bonding to hydroxyls on the ligand (Fig. 2). Hydrophobic residues W376, L405, I441, W481, W516, M519, W613 and F649 contribute to active site architecture and substrate binding.

We also determined the complex of GAA bound to 1-deoxynojirimycin (DNJ) (Fig. 2), a pharmacological chaperone investigated for the treatment of Pompe disease. DNJ, a glucose analog, binds in the active site and acts as a competitive inhibitor of GAA. A global fit of substrate vs. inhibitor vs. velocity data leads to a K_i for DGJ of 150 $\pm$ 8 nM (Fig. 2 supplement C).

Second ligand binding site

Surprisingly, the maltotriose and isomaltose structures reveal a second sugar binding site, a shallow cleft in the N1 domain 34Å away from the active site (Fig. 3 and Fig. 3 supplement A). In the second site, the non-reducing ends of isomaltose and maltotriose bind identically, with hydrogen bonds between the glucose hydroxyls and the side chain of D91 and the main chain of G123, W126 and C127 (Fig. 3 and Fig. 3 supplement A). In the second site, the penultimate sugars of the two substrate fragments bind differently, where the maltotriose stacks directly over W126 and the isomaltose shifts closer to the main chain of I98. The second binding site falls close to a loop that is removed during proteolytic maturation of GAA, which might explain how glycogen affinity and enzymatic activity increase in mature GAA.

Many eukaryotic family 31 glycoside hydrolases are multi-domain proteins, but the functions of the non-catalytic domains are poorly understood. The identification of a second carbohydrate-

binding site in GAA suggests a functional role for the P-type trefoil domain in domain N1 as a carbohydrate-binding module (CBM). The presence of a CBM in the N-terminal domain explains the observation that the D91N variant of GAA binds carbohydrate less effectively and exhibits faster electrophoretic mobility on a starch gel (Martiniuk et al., 1990).

Glycogen spanning the two ligand-binding sites on GAA

The second binding site offers a potential explanation of for the observation that mature GAA shows increased enzymatic activity for larger substrates. To better understand the interaction of GAA with glycogen, we modeled the GAA:glycogen interaction, guided by the two ligand-binding sites seen crystallographically (Figure 4). We computationally minimized a glycogen fragment containing 26 glucose residues with one branch point, and then manually docked the glycogen model into the GAA structure. The glycogen model fits into the two GAA binding sites with only minor adjustments of two dihedrals. The model immediately suggests a mechanism of action of GAA on glycogen, where the cleavage of the glycogen in the active site is followed by release of product from the active site coincident with glycogen binding in the second site, leading to a high effective concentration of glycogen at all stages.

Pompe disease mutations

More than 550 polymorphisms in human GAA have been identified (Kroos et al., 2012), and over 450 mutations are thought to be pathogenic for Pompe disease (Table 2). Of the Pompe disease-associated mutations, 245 are missense mutations leading to substitutions of single amino acids. To better understand genotype-phenotype correlations in Pompe disease, we mapped the 245 reported missense mutations onto the structure (Fig. 1 supplement B). 64% of all pathological point mutations map to the catalytic domain, 22% map to the N2 domain, and the remaining 14% map to the other three domains. Changes in buried residues are found more often than in surface residues: 23% of all buried residues have a Pompe disease-associated

mutation, while only 5% of surface residues do (Fig. 4 supplement A). Because missense mutations in buried residues are most common, Pompe disease is primarily a disorder of protein misfolding.

Materials and Methods

Protease treatment of GAA

Human GAA was expressed in Chinese hamster ovary cells (Moreland et al., 2005). GAA (in 50mM sodium phosphate pH 6.2, 4% mannitol and 0.01% Tween 80) was incubated with proteinase K at a molar ratio of 280:1 at 37°C for 20 hours. The protease was removed by size exclusion chromatography on a Superdex 75 10/300 GL column (GE) equilibrated with 50mM sodium phosphate pH 6.2 and 250mM NaCl.

X-ray crystallography

Protease-treated GAA was concentrated to 7-20mg/ml in 20mM Bis-Tris, pH 6. The protein was mixed 1:1 to 1:3 with crystallization solution containing 100mM sodium HEPES, pH 7.5, 2% (v/v) PEG 400 and 2M ammonium sulfate. Crystals took two to six months to grow in sitting drop vapor diffusion experiments. Crystals were transferred into harvest buffer (100mM sodium HEPES, pH 7.5, 3% (v/v) PEG 400, and 2.2M ammonium sulfate) and flash cooled in harvest buffer with 20% glycerol added. To form complexes, crystals were soaked in harvest buffer supplemented with ligand, either 2mM DNJ for 12 hours, 10% (w/v) isomaltose for 2-10 minutes, or 10% (w/v) maltotriose for 2-10 minutes. Prior to cooling, the ligand soaks were shifted to harvest buffer containing 2mM DNJ and 20% glycerol or harvest buffer containing 20% saccharide. X-ray diffraction data were collected on beam line 14-1 of Stanford Synchrotron Radiation Lightsource at 1.1808Å wavelength. Diffraction data were indexed and integrated using iMosflm, and scaled and reduced in space group $P2_12_12_1$ using Aimless (Evans and Murshudov, 2013) and Ctruncate (Stein and Ballard, 2009). Molecular replacement was

performed in AMoRe (Navaza, 1994) from the CCP4 suite (Cowtan et al., 2011) using the 44% identical N-terminal subunit of human maltase-glucoamylase (PDB code 2QLY) (Sim et al., 2008), resulting in an initial R-factor of 46%. Subsequent building and refinement was performed in Coot (Emsley and Cowtan, 2004) and Refmac5 (Murshudov et al., 2011), respectively. Figures were constructed in Pymol (Schrödinger, 2015) and PovScript (Fenn et al., 2003).

Mutation analysis

Accessible surface area per residue was calculated using Areaimol in CCP4 (Cowtan et al., 2011) and then ported to Excel for further analysis. Residues with an accessible surface area per side chain atom possessing less than 2 Å², between 2-9 Å², and above 9 were considered buried, partially buried or exposed respectively.

Modeling and docking of glycogen fragment

A model of branched glycogen containing 26 glucose residues molecules was energy minimized using SWEET2 (Bohne et al., 1998). Docking of the minimized fragment was performed manually and was guided by electron density at both binding sites.

Enzyme Kinetics

Kinetic parameters were determined with and without the inhibitors DNJ and glucose. 380nM GAA was incubated with 0-11μM DNJ or 0-600mM glucose in reaction buffer (100mM sodium phosphate citric acid, pH 4.5, and 100mM NaCl) at 37°C before the addition of an equal volume of 0-33mM 4-nitrophenyl-α-D-glucopyranoside substrate in reaction buffer. Aliquots of the reaction were removed every 2.5 minutes for 12.5 minutes and diluted 30-fold into 200mM sodium borate, pH 10.2. The amount of product was determined by measuring the absorbance of *para*-nitrophenylate at 400nM using an extinction coefficient of 18.2mM⁻¹cm⁻¹. Enzymatic

parameters K_m , K_i , and V_{max} were calculated in Prism Pro (GraphPad) using a global fit of the Michaelis-Menten equation for competitive inhibition:

$$V_0 = \frac{(V_{max} \cdot [S])}{K_m(1 + \frac{[I]}{K_i}) + [S]}$$

A small K_i for DNJ from the above global fit indicated DNJ was a tight binder, so we refit the data in Prism Pro using the Morrison equation for tight binding inhibitors:

$$V_i = V_0 \cdot \left(1 - \frac{\left(\left([E]_T + [I]_T + K_i \left(1 + \frac{[S]}{K_m} \right) \right) - \left(\left(\left([E]_T + [I]_T + K_i \left(1 + \frac{[S]}{K_m} \right) \right)^2 - 4 \cdot [I]_T \cdot K_i \left(1 + \frac{[S]}{K_m} \right) \right)^{0.5} \right)}{2 \cdot [E]_T} \right)}{\right)}$$

The GAA enzyme activity was measured as a function of pH, using 6.6mM 4-nitrophenyl- α -D-glucopyranoside in 100 mM sodium phosphate citric acid buffer with pH values ranging from 3-7 using 190nM GAA. Amount of product was measured as stated above. The data was fit in Prism Pro using the following equation where K_1 and K_2 describe the K_a values for the catalytic nucleophile and acid/base, respectively (Cornish-Bowden, 2004).

$$V = \frac{V_{max}}{[H^+]/K_1 + 1 + [H^+]/K_2}$$

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Author contributions

D.D. and S.C.G. designed experiments, performed experiments, analyzed data, and wrote the manuscript. K.L., T.M., R.R.W., and T.E. produced recombinant GAA and suggested experiments. All authors read and commented on the manuscript.

Conflict of Interest

D.D. declares no conflict of interest. S.C.G has received travel and speaking honoraria from Genzyme-Sanofi and Amicus. K.L., T.M., R.R.W., and T.E. are present or former employees of Sanofi.

Data Deposition

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.pdb.org) under accession codes 5KZR, 5KZW, and 5KZX.

References

- Alcántara-Ortigoza MA, Gonzalez-del Angel A, Barrientos-Rios R, Cupples C, Garrido-Garcia LM, de Leon-Bojorge B, Alva-Chaire Adel C. 2010. Screening of late-onset Pompe disease in a sample of Mexican patients with myopathies of unknown etiology: identification of a novel mutation in the acid alpha-glucosidase gene. *J Child Neurol* **25**:1034-7. doi:10.1177/0883073809356035
- Alejaldre A, Díaz-Manera J, Ravaglia S, Tibaldi EC, D'Amore F, Morís G, Muelas N, Vélchez JJ, García-Medina A, Usón M, García FAM, Illa I, Pichiecchio A. 2012. Trunk muscle involvement in late-onset Pompe disease: Study of thirty patients. *Neuromuscular Disorders* **22**, **Supple**:S148-S154. doi:10.1016/j.nmd.2012.05.011
- Amartino H, Pinceira D, Pomponio RJ, Niizawa G, Sabio Paz V, Blanco M, Chamoles N. 2006. Two clinical forms of glycogen-storage disease type II in two generations of the same family. *Clinical Genetics* **69**:187-188. doi:10.1111/j.1399-0004.2005.00557.x
- Anneser JM, Pongratz DE, Podskarbi T, Shin YS, Schoser BG. 2005. Mutations in the acid α -glucosidase gene (M. Pompe) in a patient with an unusual phenotype. *Neurology* **64**:368-70. doi:10.1212/01.WNL.0000149528.95362.20
- Ayca A, Huseyin O, Melis K, Ebru EC, Emin K, Mahmut C, Ferda O, *Two novel mutations in acid α -glucosidase gene in two patients with Pompe disease*. 2014. p. 1265-1265.
- Bali DS, Goldstein JL, Banugaria S, Dai J, Mackey J, Rehder C, Kishnani PS. 2012. Predicting cross-reactive immunological material (CRIM) status in Pompe disease using GAA mutations: Lessons learned from 10 years of clinical laboratory testing experience. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics* **160C**:40-49. doi:10.1002/ajmg.c.31319
- Bali DS, Tolun AA, Goldstein JL, Dai J, Kishnani PS. 2011. Molecular analysis and protein processing in late-onset Pompe disease patients with low levels of acid α -glucosidase activity. *Muscle Nerve* **43**:665-70. doi:10.1002/mus.21933
- Becker JA, Vlach J, Raben N, Nagaraju K, Adams EM, Hermans MM, Reuser AJ, Brooks SS, Tiffet CJ, Hirschhorn R, Huie ML, Nicolino M, Plotz PH. 1998. The African origin of the common mutation in African American patients with glycogen-storage disease type II. *Am J Hum Genet* **62**:991-4. doi:10.1086/301788
- Bigger BW, Saif M, Linthorst GE. 2015. The role of antibodies in enzyme treatments and therapeutic strategies. *Best Pract Res Clin Endocrinol Metab* **29**:183-94. doi:10.1016/j.beem.2015.01.006
- Bijvoet AG, Kroos MA, Pieper FR, Van der Vliet M, De Boer HA, Van der Ploeg AT, Verbeet MP, Reuser AJ. 1998. Recombinant human acid α -glucosidase: high level production in mouse milk, biochemical characteristics, correction of enzyme deficiency in GSDII KO mice. *Hum Mol Genet* **7**:1815-24.
- Bodamer OA, Haas D, Hermans MM, Reuser AJ, Hoffmann GF. 2002. l-alanine supplementation in late infantile glycogen storage disease type {II}. *Pediatric Neurology* **27**:145-146. doi:10.1016/S0887-8994(02)00413-7
- Bohne A, Lang E, von der Lieth C-W. 1998. W3-SWEET: Carbohydrate modeling my internet. *Molecular modeling annual* **4**:33-43. doi:10.1007/s008940050068

- Bourne Y, Henrissat B. 2001. Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* **11**:593-600.
- Case LE, Koeberl DD, Young SP, Bali D, DeArme SM, Mackey J, Kishnani PS. 2008. Improvement with ongoing Enzyme Replacement Therapy in advanced late-onset Pompe disease: A case study. *Molecular Genetics and Metabolism* **95**:233-235. doi:10.1016/j.ymgme.2008.09.001
- Castro-Gago M, Eirís-Puñal J, Rodríguez-Núñez A, Pintos-Martínez E, Benlloch-Marín T, Barros-Angueira F. 1999. Severe form of juvenile type II glycogenosis in a compound-heterozygous boy (Tyr-292-> Cys/Arg-854->Stop). *Revista de Neurologia* **29**:46-49.
- Chien Y-H, Lee N-C, Chen C-A, Tsai F-J, Tsai W-H, Shieh J-Y, Huang H-J, Hsu W-C, Tsai T-H, Hwu W-L. 2015. Long-term prognosis of patients with infantile-onset Pompe disease diagnosed by newborn screening and treated since birth. *The Journal of Pediatrics* **166**:985-991.e2. doi:10.1016/j.jpeds.2014.10.068
- Chien Y-H, Lee N-C, Huang H-J, Thurberg BL, Tsai F-J, Hwu W-L. 2011. Later-onset Pompe disease: early detection and early treatment initiation enabled by newborn screening. *The Journal of Pediatrics* **158**:1023-1027.e1. doi:10.1016/j.jpeds.2010.11.053
- Cornish-Bowden A, Fundamentals of Enzyme Kinetics. 3rd ed. 2004, London: Portland Press.
- Cowtan K, Emsley P, Wilson KS. 2011. From crystal to structure with CCP4. *Acta Crystallogr D Biol Crystallogr* **67**:233-4. doi:10.1107/S0907444911007578
- Ding J, Huang Y, Yang H, Zhang Q, Hou X, Liu X, Yang H, Xiong Y. 2015. Analysis of clinical features of 6 patients with infantile type glycogen storage disease type II. *Zhonghua Er Ke Za Zhi* **33**:1161-1165.
- Dlamini N, Jan W, Norwood F, Sheehan J, Spahr R, Al-Sarraj S, Hulse JA, Hughes D, Champion MP, Jungbluth H. 2008. Muscle MRI findings in siblings with juvenile-onset acid maltase deficiency (Pompe disease). *Neuromuscular Disorders* **18**:408-409. doi:10.1016/j.nmd.2008.02.006
- Doerfler PA, Nayak S, Corti M, Morel L, Herzog RW, Byrne BJ. 2016. Targeted approaches to induce immune tolerance for Pompe disease therapy. *Mol Ther Methods Clin Dev* **3**:15053. doi:10.1038/mtm.2015.53
- Doerfler PA, Todd AG, Clement N, Falk DJ, Nayak S, Herzog RW, Byrne BJ. 2016. Copackaged AAV9 vectors promote simultaneous immune tolerance and phenotypic correction of Pompe disease. *Hum Gene Ther* **27**:43-59. doi:10.1089/hum.2015.103
- Dou W, Peng C, Zheng J, Gu X, Fu L, Martiniuk F, Sheng HZ. 2006. A novel missense mutation in the acid alpha-glucosidase gene causing the classic infantile form of Pompe disease. *Clin Chim Acta* **374**:145-6. doi:10.1016/j.cca.2006.04.007
- Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**:2126-32. doi:10.1107/S0907444904019158
- Evans PR, Murshudov GN. 2013. How good are my data and what is the resolution? *Acta Crystallogr D Biol Crystallogr* **69**:1204-14. doi:10.1107/s0907444913000061
- Fan JQ, Ishii S, Asano N, Suzuki Y. 1999. Accelerated transport and maturation of lysosomal α -galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* **5**:112-5.

- Fenn TD, Ringe D, Petsko GA. 2003. POVScript+: a program for model and data visualization using persistence of vision ray-tracing. *J. Appl. Cryst.* **36**:944-947.
- Fernandez-Hojas R, Huie ML, Navarro C, Dominguez C, Roig M, Lopez-Coronas D, Teixeira S, Anyane-Yeboah K, Hirschhorn R. 2002. Identification of six novel mutations in the acid α -glucosidase gene in three Spanish patients with infantile onset glycogen storage disease type II (Pompe disease). *Neuromuscul Disord* **12**:159-66.
- Flanagan JJ, Rossi B, Tang K, Wu X, Mascioli K, Donaudy F, Tuzzi MR, Fontana F, Cubellis MV, Porto C, Benjamin E, Lockhart DJ, Valenzano KJ, Andria G, Parenti G, Do HV. 2009. The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid α -glucosidase. *Hum Mutat* **30**:1683-92. doi:10.1002/humu.21121
- Fu L, Qiu W, Yu Y, Guo Y, Zhao P, Zhang X, Liu C, Li F, Huang H, Huang M, Chen S. 2014. Clinical and molecular genetic study of infantile-onset Pompe disease in Chinese patients: Identification of 6 novel mutations. *Gene* **535**:53-59. doi:10.1016/j.gene.2013.10.066
- Fujimoto S, Manabe Y, Fujii D, Kozai Y, Matsuzono K, Takahashi Y, Narai H, Omori N, Adachi K, Nanba E, Nishino I, Abe K. 2013. A novel mutation of the *GAA* gene in a patient with adult-onset Pompe disease lacking a disease-specific pathology. *Internal Medicine* **52**:2461-2464. doi:10.2169/internalmedicine.52.0311
- Fukuda T, Roberts A, Plotz PH, Raben N. 2007. Acid α -glucosidase deficiency (Pompe disease). *Curr Neurol Neurosci Rep* **7**:71-7.
- GAA Database Consortium, *Acid α -glucosidase mutations*. 2016.
- Gort L, Coll MJ, Chabás A. 2007. Glycogen storage disease type II in Spanish patients: High frequency of c.1076-1G>C mutation. *Molecular Genetics and Metabolism* **92**:183-187. doi:10.1016/j.ymgme.2007.05.011
- Hermans MM, De Graaff E, Kroos MA, Mohkamsing S, Eussen BJ, Joosse M, Willemsen R, Kleijer WJ, Oostra BA, Reuser AJ. 1994. The effect of a single base pair deletion (Δ T525) and a C1634T missense mutation (pro545leu) on the expression of lysosomal α -glucosidase in patients with glycogen storage disease type II. *Hum Mol Genet* **3**:2213-8.
- Hermans MM, de Graaff E, Kroos MA, Wisselaar HA, Oostra BA, Reuser AJ. 1991. Identification of a point mutation in the human lysosomal α -glucosidase gene causing infantile glycogenosis type II. *Biochem Biophys Res Commun* **179**:919-26.
- Hermans MM, de Graaff E, Kroos MA, Wisselaar HA, Willemsen R, Oostra BA, Reuser AJ. 1993. The conservative substitution Asp-645->Glu in lysosomal α -glucosidase affects transport and phosphorylation of the enzyme in an adult patient with glycogen-storage disease type II. *Biochem J* **289 (Pt 3)**:687-93.
- Hermans MM, Kroos MA, de Graaff E, Oostra BA, Reuser AJ. 1993. Two mutations affecting the transport and maturation of lysosomal α -glucosidase in an adult case of glycogen storage disease type II. *Hum Mutat* **2**:268-73. doi:10.1002/humu.1380020406
- Hermans MM, van Leenen D, Kroos MA, Beesley CE, Van Der Ploeg AT, Sakuraba H, Wevers R, Kleijer W, Michelakakis H, Kirk EP, Fletcher J, Bosshard N, Basel-Vanagaite L, Besley G, Reuser AJ. 2004. Twenty-two novel mutations in the lysosomal α -glucosidase gene (*GAA*) underscore the

genotype-phenotype correlation in glycogen storage disease type II. *Hum Mutat* **23**:47-56. doi:10.1002/humu.10286

Herzog A, Hartung R, Reuser AJ, Hermanns P, Runz H, Karabul N, Gökce S, Pohlenz J, Kampmann C, Lampe C, Beck M, Mengel E. 2012. A cross-sectional single-centre study on the spectrum of Pompe disease, German patients: molecular analysis of the *GAA* gene, manifestation and genotype-phenotype correlations. *Orphanet Journal of Rare Diseases* **7**:1-14. doi:10.1186/1750-1172-7-35

Hirschhorn R, Reuser AJ. 2001. Glycogen storage disease type II: acid α -glucosidase (acid maltase) deficiency, in *The Metabolic and Molecular Bases of Inherited Disease*, C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, Editors. McGraw-Hill: New York. p. 3389–3420.

Hoefsloot LH, Hoogeveen-Westerveld M, Kroos MA, van Beeumen J, Reuser AJ, Oostra BA. 1988. Primary structure and processing of lysosomal α -glucosidase; homology with the intestinal sucrase-isomaltase complex. *EMBO J* **7**:1697-704.

Hoefsloot LH, Hoogeveen-Westerveld M, Reuser AJ, Oostra BA. 1990. Characterization of the human lysosomal α -glucosidase gene. *Biochem J* **272**:493-7.

Hu J, Sun L, Ren W, Xu M. 2015. Two novel mutations in the gene that codes for acid α -glucosidase in a baby with Pompe disease. *Eur Heart J* **36**:883. doi:10.1093/eurheartj/ehu454

Huie ML, Chen AS, Brooks SS, Grix A, Hirschhorn R. 1994. A de novo 13 nt deletion, a newly identified C647W missense mutation and a deletion of exon 18 in infantile onset glycogen storage disease type II (GSDII). *Human Molecular Genetics* **3**:1081-1087. doi:10.1093/hmg/3.7.1081

Huie ML, Hirschhorn R, Chen AS, Martiniuk F, Zhong N. 1994. Mutation at the catalytic site (M519V) in glycogen storage disease type II (Pompe disease). *Human Mutation* **4**:291-293. doi:10.1002/humu.1380040410

Huie ML, Tsujino S, Sklower Brooks S, Engel A, Elias E, Bonthron DT, Bessley C, Shanske S, DiMauro S, Goto YI, Hirschhorn R. 1998. Glycogen storage disease type II: identification of four novel missense mutations (D645N, G648S, R672W, R672Q) and two insertions/deletions in the acid α -glucosidase locus of patients of differing phenotype. *Biochem Biophys Res Commun* **244**:921-7.

Ishigaki K, Yoshikawa Y, Kuwatsuru R, Oda E, Murakami T, Sato T, Saito T, Umezū R, Osawa M. 2012. High-density CT of muscle and liver may allow early diagnosis of childhood-onset Pompe disease. *Brain and Development* **34**:103-106. doi:10.1016/j.braindev.2011.05.013

Joshi PR, Gläser D, Schmidt S, Vorgerd M, Winterholler M, Eger K, Zierz S, Deschauer M. 2008. Molecular diagnosis of German patients with late-onset glycogen storage disease type II. *Journal of Inherited Metabolic Disease* **31**:261-265. doi:10.1007/s10545-008-0820-2

Khanna R, Flanagan JJ, Feng J, Soska R, Frascella M, Pellegrino LJ, Lun Y, Guillen D, Lockhart DJ, Valenzano KJ. 2012. The pharmacological chaperone AT2220 increases recombinant human acid α -glucosidase uptake and glycogen reduction in a mouse model of Pompe disease. *PLoS One* **7**:e40776. doi:10.1371/journal.pone.0040776

Khanna R, Powe AC, Jr., Lun Y, Soska R, Feng J, Dhulipala R, Frascella M, Garcia A, Pellegrino LJ, Xu S, Brignol N, Toth MJ, Do HV, Lockhart DJ, Wustman BA, Valenzano KJ. 2014. The pharmacological chaperone AT2220 increases the specific activity and lysosomal delivery of

- mutant acid α -glucosidase, and promotes glycogen reduction in a transgenic mouse model of Pompe disease. *PLoS One* **9**:e102092. doi:10.1371/journal.pone.0102092
- Kishnani PS, Nicolino M, Voit T, Rogers RC, Tsai AC, Waterson J, Herman GE, Amalfitano A, Thurberg BL, Richards S, Davison M, Corzo D, Chen YT. 2006. Chinese hamster ovary cell-derived recombinant human acid α -glucosidase in infantile-onset Pompe disease. *J Pediatr* **149**:89-97. doi:10.1016/j.jpeds.2006.02.035
- Ko T-M, Hwu W-L, Lin Y-W, Tseng L-H, Hwa H-L, Wang T-R, Chuang S-M. 1999. Molecular genetic study of Pompe disease in Chinese patients in Taiwan. *Human Mutation* **13**:380-384. doi:10.1002/(SICI)1098-1004(1999)13:5<380::AID-HUMU6>3.0.CO;2-A
- Koshland DE. 1953. Stereochemistry and the mechanism of enzymatic reactions. *Biol. Rev. Cambridge Philos. Soc.* **28**:416-436.
- Kroos M, Hoogeveen-Westerveld M, Michelakakis H, Pomponio R, Van der Ploeg A, Halley D, Reuser A, GAA Database Consortium, Augoustides-Savvopoulou P, Ausems M, Llona JB, Bautista Lorite J, van der Beek N, Bonafe L, Cuk M, D'Hooghe M, Engelen B, Farouk A, Fumic K, Garcia-Delgado E, Herzog A, Hurst J, Jones S, Kariminejad MH, Küçükçongar A, Lissens W, Lund A, Majoor-Krakauer D, Kumamoto S, Maravi E, Marie S, Mengel E, Mavridou I, Munteis Olivas E, Najmabadi H, Okumiya T, Peric S, Paschke E, Plecko B, Robberecht W, Serdaroglu P, Shboul M, Tansek MZ, Tarnutzer A, Stojanovic VR, Tylki-Szymanska A, Venâncio M, Verhoeven K. 2012. Update of the Pompe disease mutation database with 60 novel GAA sequence variants and additional studies on the functional effect of 34 previously reported variants. *Human Mutation* **33**:1161-1165. doi:10.1002/humu.22108
- Kroos M, Manta P, Mavridou I, Muntoni F, Halley D, Van der Helm R, Zaifeiriou D, Van der Ploeg A, Reuser A, Michelakakis H. 2006. Seven cases of Pompe disease from Greece. *J Inherit Metab Dis* **29**:556-63. doi:10.1007/s10545-006-0280-5
- Kroos M, Pomponio RJ, van Vliet L, Palmer RE, Phipps M, Van der Helm R, Halley D, Reuser A, GAA Database Consortium. 2008. Update of the Pompe disease mutation database with 107 sequence variants and a format for severity rating. *Hum Mutat* **29**:E13-26. doi:10.1002/humu.20745
- Kroos MA, Kirschner J, Gellerich FN, Hermans MM, Van Der Ploeg AT, Reuser AJ, Korinthenberg R. 2004. A case of childhood Pompe disease demonstrating phenotypic variability of p.Asp645Asn. *Neuromuscul Disord* **14**:371-4. doi:10.1016/j.nmd.2004.02.012
- Kroos MA, van Leenen D, Verbiest J, Reuser AJ, Hermans MM. 1998. Glycogen storage disease type II: identification of a dinucleotide deletion and a common missense mutation in the lysosomal α -glucosidase gene. *Clin Genet* **53**:379-82.
- Labrousse P, Chien Y-H, Pomponio RJ, Keutzer J, Lee N-C, Akmaev VR, Scholl T, Hwu W-L. 2010. Genetic heterozygosity and pseudodeficiency in the Pompe disease newborn screening pilot program. *Molecular Genetics and Metabolism* **99**:379-383. doi:10.1016/j.ymgme.2009.12.014
- Laforêt P, Nicolino M, Eymard PB, Puech JP, Caillaud C, Poenaru L, Fardeau M. 2000. Juvenile and adult-onset acid maltase deficiency in France: genotype-phenotype correlation. *Neurology* **55**:1122-8.

- Lam CW, Yuen YP, Chan KY, Tong SF, Lai CK, Chow TC, Lee KC, Chan YW, Martiniuk F. 2003. Juvenile-onset glycogen storage disease type II with novel mutations in acid α -glucosidase gene. *Neurology* **60**:715-7.
- Lim JA, Kakhlon O, Li L, Myerowitz R, Raben N. 2015. Pompe disease: Shared and unshared features of lysosomal storage disorders. *Rare Dis* **3**:e1068978. doi:10.1080/21675511.2015.1068978
- Lim JA, Li L, Raben N. 2014. Pompe disease: from pathophysiology to therapy and back again. *Front Aging Neurosci* **6**:177. doi:10.3389/fnagi.2014.00177
- Lin CY, Shieh JJ. 1995. Identification of a de novo point mutation resulting in infantile form of Pompe's disease. *Biochemical and Biophysical Research Communications* **208**:886-893. doi:10.1006/bbrc.1995.1418
- Lin HY, Tj W, Hsu JH, Hc Y, Chuang CK, Huang CH, Niu DM. 2010. Novel human pathological mutations. Gene symbol: GAA. Disease: glycogen storage disease 2. *Human Genetics* **127**:464-464.
- Liu Q, Zhao J, Wang ZX, Zhang W, Yuan Y. 2013. [Clinical features and acid α -glucosidase gene mutation in 7 Chinese patients with glycogen storage disease type II]. *Zhonghua Yi Xue Za Zhi* **93**:1981-5.
- Liu X, Wang Z, Jin W, Lv H, Zhang W, Que C, Huang Y, Yuan Y. 2014. Clinical and GAA gene mutation analysis in mainland Chinese patients with late-onset Pompe disease: identifying c.2238G > C as the most common mutation. *BMC Medical Genetics* **15**:1-9. doi:10.1186/s12881-014-0141-2
- Loureiro Neves F, Garcia PC, Madureira N, Araújo H, Rodrigues F, Estêvão MH, Lacerda L, Diogo Matos LM. 2013. Juvenile Pompe disease: retrospective clinical study. *Acata Medica Portuguesa* **26**:361-370.
- Maga JA, Zhou J, Kambampati R, Peng S, Wang X, Bohnsack RN, Thomm A, Golata S, Tom P, Dahms NM, Byrne BJ, LeBowitz JH. 2013. Glycosylation-independent lysosomal targeting of acid α -glucosidase enhances muscle glycogen clearance in pompe mice. *J Biol Chem* **288**:1428-38. doi:10.1074/jbc.M112.438663
- Maimaiti M, Takahashi S, Okajima K, Suzuki N, Ohinata J, Araki A, Tanaka H, Mukai T, Fujieda K. 2009. Silent exonic mutation in the acid- α -glycosidase gene that causes glycogen storage disease type II by affecting mRNA splicing. *J Hum Genet* **54**:493-6. doi:10.1038/jhg.2009.66
- Manwaring V, Prunty H, Bainbridge K, Burke D, Finnegan N, Franses R, Lam A, Vellodi A, Heales S. 2012. Urine analysis of glucose tetrasaccharide by HPLC; a useful marker for the investigation of patients with Pompe and other glycogen storage diseases. *Journal of Inherited Metabolic Disease* **35**:311-316. doi:10.1007/s10545-011-9360-2
- Martiniuk F, Bodkin M, Tzall S, Hirschhorn R. 1990. Identification of the base-pair substitution responsible for a human acid α -glucosidase allele with lower "affinity" for glycogen (GAA 2) and transient gene expression in deficient cells. *Am J Hum Genet* **47**:440-5.
- Martiniuk F, Mehler M, Tzall S, Meredith G, Hirschhorn R. 1990. Sequence of the cDNA and 5'-flanking region for human acid α -glucosidase, detection of an intron in the 5' untranslated leader sequence, definition of 18-bp polymorphisms, and differences with previous cDNA and amino acid sequences. *DNA Cell Biol* **9**:85-94. doi:10.1089/dna.1990.9.85

- 614 McCready ME, Carson NL, Chakraborty P, Clarke JTR, Callahan JW, Skomorowski MA, Chan AKJ,
615 Bamforth F, Casey R, Rupar CA, Geraghty MT. 2007. Development of a clinical assay for
616 detection of GAA mutations and characterization of the GAA mutation spectrum in a Canadian
617 cohort of individuals with glycogen storage disease, type II. *Molecular Genetics and Metabolism*
618 **92**:325-335. doi:10.1016/j.ymgme.2007.07.006
619
- 620 Montagnese F, Barca E, Musumeci O, Mondello S, Migliorato A, Ciranni A, Rodolico C, De Filippi P,
621 Danesino C, Toscano A. 2015. Clinical and molecular aspects of 30 patients with late-onset
622 Pompe disease (LOPD): unusual features and response to treatment. *Journal of Neurology*
623 **262**:968-978. doi:10.1007/s00415-015-7664-0
624
- 625 Montalvo AL, Cariatì R, Deganuto M, Guerri V, Garcia R, Ciana G, Bembi B, Pittis MG. 2004.
626 Glycogenosis type II: identification and expression of three novel mutations in the acid α -
627 glucosidase gene causing the infantile form of the disease. *Mol Genet Metab* **81**:203-8.
628 doi:10.1016/j.ymgme.2003.11.011
629
- 630 Montalvo ALE, Bembi B, Donnarumma M, Filocamo M, Parenti G, Rossi M, Merlini L, Buratti E, De Filippi
631 P, Dardis A, Stroppiano M, Ciana G, Pittis MG. 2006. Mutation profile of the GAA gene in 40
632 Italian patients with late onset glycogen storage disease type II. *Human Mutation* **27**:999-1006.
633 doi:10.1002/humu.20374
634
- 635 Moreland RJ, Jin X, Zhang XK, Decker RW, Albee KL, Lee KL, Cauthron RD, Brewer K, Edmunds T,
636 Canfield WM. 2005. Lysosomal acid α -glucosidase consists of four different peptides processed
637 from a single chain precursor. *J Biol Chem* **280**:6780-91. doi:10.1074/jbc.M404008200
638
- 639 Müller-Felber W, Horvath R, Gempel K, Podskarbi T, Shin Y, Pongratz D, Walter MC, Baethmann M,
640 Schlotter-Weigel B, Lochmüller H, Schoser B. 2007. Late onset Pompe disease: Clinical and
641 neurophysiological spectrum of 38 patients including long-term follow-up in 18 patients.
642 *Neuromuscular Disorders* **17**:698-706. doi:10.1016/j.nmd.2007.06.002
643
- 644 Muraoka T, Murao K, Imachi H, Kikuchi F, Yoshimoto T, Iwama H, Hosokawa H, Nishino I, Fukuda T,
645 Sugie H, Adachi K, Nanba E, Ishida T. 2011. Novel mutations in the gene encoding acid α -1,4-
646 glucosidase in a patient with late-onset glycogen storage disease type II (Pompe disease) with
647 impaired intelligence. *Intern Med* **50**:2987-91.
648
- 649 Murshudov GN, Skubak P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, Vagin
650 AA. 2011. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D*
651 *Biol Crystallogr* **67**:355-67. doi:10.1107/S0907444911001314
652
- 653 Nascimbeni AC, Fanin M, Tasca E, Angelini C. 2008. Molecular pathology and enzyme processing in
654 various phenotypes of acid maltase deficiency. *Neurology* **70**:617-626.
655 doi:10.1212/01.wnl.0000299892.81127.8e
656
- 657 Navaza J. 1994. AMoRe: an automated package for molecular replacement. *Acta Crystallographica*
658 **A50**:157-163.
659
- 660 Nilsson MI, Kroos MA, Reuser AJ, Hatcher E, Akhtar M, McCready ME, Tarnopolsky MA. 2014. Novel
661 GAA sequence variant c.1211 A>G reduces enzyme activity but not protein expression in infantile
662 and adult onset Pompe disease. *Gene* **537**:41-5. doi:10.1016/j.gene.2013.12.033
663
- 664 Niño MY, Mateus HE, Fonseca DJ, Kroos MA, Ospina SY, Mejia JF, Uribe JA, Reuser AJ, Laissue P.
665 2013. Identification and Functional Characterization of GAA Mutations in Colombian Patients
666 Affected by Pompe Disease. *JIMD Rep* **7**:39-48. doi:10.1007/8904_2012_138

- Oba-Shinjo SM, da Silva R, Andrade FG, Palmer RE, Pomponio RJ, Ciociola KM, Carvalho MS, Gutierrez PS, Porta G, Marrone CD, Munoz V, Grzesiuk AK, Llerena JC, Jr., Berditchevsky CR, Sobreira C, Horovitz D, Hatem TP, Frota ER, Pecchini R, Kouyoumdjian JA, Werneck L, Amado VM, Camelo JS, Jr., Mattaliano RJ, Marie SK. 2009. Pompe disease in a Brazilian series: clinical and molecular analyses with identification of nine new mutations. *J Neurol* **256**:1881-90. doi:10.1007/s00415-009-5219-y
- Palmer RE, Amartino HM, Niizawa G, Blanco M, Pomponio RJ, Chamoles NA. 2007. Pompe disease (glycogen storage disease type II) in Argentineans: Clinical manifestations and identification of 9 novel mutations. *Neuromuscular Disorders* **17**:16-22. doi:10.1016/j.nmd.2006.09.004
- Pardo J, García-Sobrino T, López-Ferreiro A. 2015. Gastrointestinal symptoms in late-onset Pompe disease: Early response to enzyme replacement therapy. *Journal of the Neurological Sciences* **353**:181-182. doi:10.1016/j.jns.2015.04.012
- Park J-S, Kim H-G, Shin J-H, Choi Y-C, Kim D-S. 2015. Effect of enzyme replacement therapy in late onset Pompe disease: open pilot study of 48 weeks follow-up. *Neurological Sciences* **36**:599-605. doi:10.1007/s10072-014-2000-5
- Park Y-E, Park K-H, Lee C-H, Kim C-M, Kim D-S. 2006. Two new missense mutations of GAA in late onset glycogen storage disease type II. *Journal of the Neurological Sciences* **251**:113-117. doi:10.1016/j.jns.2006.09.012
- Pipo JR, Feng J-H, Yamamoto T, Ohsaki Y, Nanba E, Tsujino S, Sakuragawa N, Martiniuk F, Ninomiya H, Oka A, Ohno K. 2003. New GAA mutations in Japanese patients with GSDII (Pompe disease). *Pediatric Neurology* **29**:284-287. doi:10.1016/S0887-8994(03)00267-4
- Pittis MG, Donnarumma M, Montalvo AL, Dominissini S, Kroos M, Rosano C, Stroppiano M, Bianco MG, Donati MA, Parenti G, D'Amico A, Ciana G, Di Rocco M, Reuser A, Bembi B, Filocamo M. 2008. Molecular and functional characterization of eight novel GAA mutations in Italian infants with Pompe disease. *Hum Mutat* **29**:E27-36. doi:10.1002/humu.20753
- Pittis MG, Montalvo ALE, Miocic S, Martini C, Deganuto M, Candusso M, Ciana G, Bembi B. 2003. Identification of four novel mutations in the α -glucosidase gene in five Italian patients with infantile onset glycogen storage disease type II. *American Journal of Medical Genetics Part A* **121A**:225-230. doi:10.1002/ajmg.a.20164
- Qiu JJ, Wei M, Zhang WM, Hp S. 2007. Clinical and molecular genetic study on two patients of the juvenile form of Pompe disease in China. *Zhonghau Er Ke Za Zhi* **45**:760-764.
- Quenardelle V, Bataillard M, Bazin D, Lannes B, Wolff V, Echaniz-Laguna A. 2015. Pompe disease presenting as an isolated generalized dilative arteriopathy with repeated brain and kidney infarcts. *Journal of Neurology* **262**:473-475. doi:10.1007/s00415-014-7582-6
- Raben N, Nichols RC, Boerkoel C, Plotz P. 1995. Genetic defects in patients with glycogenosis type II (acid maltase deficiency). *Muscle and Nerve supplemental* **3**:S70-74.
- Raben N, Plotz P, Byrne BJ. 2002. Acid α -glucosidase deficiency (glycogenosis type II, Pompe disease). *Curr Mol Med* **2**:145-66.
- Ravaglia S, De Filippi P, Pichiecchio A, Ponzio M, Saeidi Garaghani K, Poloni GU, Bini P, Danesino C. 2012. Can genes influencing muscle function affect the therapeutic response to enzyme

- replacement therapy (ERT) in late-onset Type II Glycogenosis? *Molecular Genetics and Metabolism* **107**:104-110. doi:10.1016/j.ymgme.2012.05.016
- Remiche G, Ronchi D, Magri F, Lamperti C, Bordoni A, Moggio M, Bresolin N, Comi GP. 2014. Extended phenotype description and new molecular findings in late onset glycogen storage disease type II: a northern Italy population study and review of the literature. *Journal of Neurology* **261**:83-97. doi:10.1007/s00415-013-7137-2
- Reuser AJ, Kroos MA, Hermans MM, Bijvoet AG, Verbeet MP, Van Diggelen OP, Kleijer WJ, Van der Ploeg AT. 1995. Glycogenosis type II (acid maltase deficiency). *Muscle Nerve Supplement* **3**:S61-69.
- Sacconi S, Bocquet JD, Chanalet S, Tanant V, Salviati L, Desnuelle C. 2010. Abnormalities of cerebral arteries are frequent in patients with late-onset Pompe disease. *Journal of Neurology* **257**:1730-1733. doi:10.1007/s00415-010-5618-0
- Sampaolo S, Esposito T, Farina O, Formicola D, Diodato D, Gianfrancesco F, Cipullo F, Cremone G, Cirillo M, Del Viscovo L, Toscano A, Angelini C, Di Iorio G. 2013. Distinct disease phenotypes linked to different combinations of GAA mutations in a large late-onset GSDII sibship. *Orphanet Journal of Rare Diseases* **8**:159-159. doi:10.1186/1750-1172-8-159
- Schneider I, Hanisch F, Müller T, Schmidt B, Zierz S. 2013. Respiratory function in late-onset Pompe disease patients receiving long-term enzyme replacement therapy for more than 48 months. *Wiener Medizinische Wochenschrift* **163**:40-44. doi:10.1007/s10354-012-0153-5
- Schoser B, Hill V, Raben N. 2008. Therapeutic approaches in glycogen storage disease type II/Pompe Disease. *Neurotherapeutics* **5**:569-78. doi:10.1016/j.nurt.2008.08.009
- Schrödinger, LLC, *The PyMOL Molecular Graphics System, Version 1.8*. 2015.
- Sim L, Quezada-Calvillo R, Sterchi EE, Nichols BL, Rose DR. 2008. Human intestinal maltase-glucoamylase: crystal structure of the N-terminal catalytic subunit and basis of inhibition and substrate specificity. *J Mol Biol* **375**:782-92. doi:10.1016/j.jmb.2007.10.069
- Spada M, Porta F, Vercelli L, Pagliardini V, Chiadò-Piat L, Boffi P, Pagliardini S, Remiche G, Ronchi D, Comi G, Mongini T. 2013. Screening for later-onset Pompe's disease in patients with paucisymptomatic hyperCKemia. *Molecular Genetics and Metabolism* **109**:171-173. doi:10.1016/j.ymgme.2013.03.002
- Stein N, Ballard C. 2009. Intensity to amplitude conversion using CTRUNCATE. *Acta Crystallographica Section A* **65**:s161. doi:doi:10.1107/S0108767309096706
- Tsujino S, Huie M, Kanazawa N, Sugie H, Goto Y-i, Kawai M, Nonaka I, Hirschhorn R, Sakuragawa N. 2000. Frequent mutations in Japanese patients with acid maltase deficiency. *Neuromuscular Disorders* **10**:599-603. doi:10.1016/S0960-8966(00)00142-5
- Tsunoda H, Ohshima T, Tohyama J, Sasaki M, Sakuragawa N, Martiniuk F. 1996. Acid α -glucosidase deficiency: identification and expression of a missense mutation (S529V) in a Japanese adult phenotype. *Hum Genet* **97**:496-9.
- Turaça LT, de Faria DOS, Kyosen SO, Teixeira VD, Motta FL, Pessoa JG, e Silva MR, de Almeida SS, D'Almeida V, Rojas MVM, Martins AM, Pesquero JB. 2015. Novel GAA mutations in patients with Pompe disease. *Gene* **561**:124-131. doi:10.1016/j.gene.2015.02.023

- 773
- 774 Van den Hout JM, Kamphoven JH, Winkel LP, Arts WF, De Klerk JB, Loonen MC, Vulto AG, Cromme-
775 Dijkhuis A, Weisglas-Kuperus N, Hop W, Van Hirtum H, Van Diggelen OP, Boer M, Kroos MA,
776 Van Doorn PA, Van der Voort E, Sibbles B, Van Corven EJ, Brakenhoff JP, Van Hove J, Smeitink
777 JA, de Jong G, Reuser AJ, Van der Ploeg AT. 2004. Long-term intravenous treatment of Pompe
778 disease with recombinant human α -glucosidase from milk. *Pediatrics* **113**:e448-57.
779
- 780 Wan L, Lee CC, Hsu CM, Hwu WL, Yang CC, Tsai CH, Tsai FJ. 2008. Identification of eight novel
781 mutations of the acid α -glucosidase gene causing the infantile or juvenile form of glycogen
782 storage disease type II. *J Neurol* **255**:831-8. doi:10.1007/s00415-008-0714-0
783
- 784 Wisselaar HA, Kroos MA, Hermans MM, van Beeumen J, Reuser AJ. 1993. Structural and functional
785 changes of lysosomal acid α -glucosidase during intracellular transport and maturation. *J Biol*
786 *Chem* **268**:2223-31.
787
- 788 Wright NA, Hoffmann W, Otto WR, Rio MC, Thim L. 1997. Rolling in the clover: trefoil factor family (TFF)-
789 domain peptides, cell migration and cancer. *FEBS Letters* **408**:121-123. doi:10.1016/S0014-
790 5793(97)00424-9
791
- 792 Yang C-F, Liu H-C, Hsu T-R, Tsai F-C, Chiang S-F, Chiang C-C, Ho H-C, Lai C-J, Yang T-F, Chuang S-
793 Y, Lin C-Y, Niu D-M. 2014. A large-scale nationwide newborn screening program for Pompe
794 disease in Taiwan: Towards effective diagnosis and treatment. *American Journal of Medical*
795 *Genetics Part A* **164**:54-61. doi:10.1002/ajmg.a.36197
796
- 797 Zhong N, Martiniuk F, Tzall S, Hirschhorn R. 1991. Identification of a missense mutation in one allele of a
798 patient with Pompe disease, and use of endonuclease digestion of PCR-amplified RNA to
799 demonstrate lack of mRNA expression from the second allele. *Am J Hum Genet* **49**:635-45.
800
- 801 Zhu Y, Jiang JL, Gumlaw NK, Zhang J, Bercury SD, Ziegler RJ, Lee K, Kudo M, Canfield WM, Edmunds
802 T, Jiang C, Mattaliano RJ, Cheng SH. 2009. Glycoengineered acid α -glucosidase with improved
803 efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of
804 Pompe disease. *Mol Ther* **17**:954-63. doi:10.1038/mt.2009.37
805
- 806 Zhu Y, Li X, McVie-Wylie A, Jiang C, Thurberg BL, Raben N, Mattaliano RJ, Cheng SH. 2005.
807 Carbohydrate-remodelled acid α -glucosidase with higher affinity for the cation-independent
808 mannose 6-phosphate receptor demonstrates improved delivery to muscles of Pompe mice.
809 *Biochem J* **389**:619-28. doi:10.1042/BJ20050364
810
811
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Figure Legends

Figure 1. Overall Structure of GAA. (A) GAA cleaves terminal α 1-4- and α 1-6-linked glucose from glycogen polymers. (B) Electrostatic surface potential with the reaction product α -glucose (yellow) bound to the active site. (C-D) Ribbon diagram of GAA colored by domain, with N-linked glycans shown in grey and glucose (yellow) bound to the active site. Dotted lines indicate residues missing in electron density. (E) Schematic of the GAA polypeptide colored by domain showing the location of disulfide bonds, N-linked glycans and free cysteines. Gaps represent residues cleaved during maturation.

Figure 2. Active site and ligand binding. (A) The reaction product glucose (yellow) bound to the active site. (B) Diagram of binding interaction between GAA and glucose. (A-B) Electron density contoured at 1.0σ . (C) The pharmacological chaperone DNJ bound to the active site. (D) Comparison of the binding interaction between the heterocyclic atom of the ligand and nucleophile. (E) Proposed α -retaining reaction mechanism.

Figure 3. GAA contains a second substrate binding site. (A) Structure of GAA with glucose (yellow) bound to the active site and maltotriose (yellow) bound to the N-terminal domain. (B) Surface representation of the CBM in the N-terminal domain with maltotriose bound (yellow). 2Fo-Fc electron density contoured at 1.0σ . (C) Identification of key residues involved in substrate binding with polar contacts highlighted by grey dashed lines. Dotted lines represent residues missing in electron density.

Figure 4. Model of GAA bound to glycogen fragment. (A) Front view of GAA bound to a glycogen fragment (yellow) showing one non-reducing end in the active site and the other non-reducing end bound the N-terminal CBM. Full length N-linked glycans shown in grey. (B) Model viewed from the back.

Figure 1 Supplement A- Protease treatment of rhGAA leads to crystals. (A) Reducing SDS-PAGE shows protease treatment resulted in two new bands at molecular weight 70-80 kDa and a third new band at 20 kDa. The predicted 10 and 3 kDa fragments presumably migrated off the 12% gel. (B) Reducing SDS-PAGE of dissolved crystals shows the crystallization process selected for cleaved GAA.

Figure 1 Supplement B - Sequence and structure of GAA. The primary sequence of human GAA is shown (colored by domain as in figure 1), along with secondary structure assignment (cylinders and arrows), disulfides (in grey) and glycans (as cartoons).

Figure 1 Supplement C. Pathological point mutations. Pathological point mutations are identified as surface exposed, buried, or active site and mapped onto the topology of GAA.

Figure 1 Supplement D. Noncovalent subunit association after proteolytic cleavage. (A,C) Surface representation of GAA colored by subunit. (B,D) Separated polypeptides with the buried interfaces colored in grey. The table shows the amino acid residues in each fragment and the buried surface area in each interface.

Figure 2 Supplement A. GAA contains a funnel-shaped active site. (A) Top view showing α -glucose (yellow) bound at the bottom of the active site funnel. The catalytic domain (green) and the N2 domain (cyan) are shown. A collection of hydrophobic and

charged residues (shown as sticks) line the active site cavity. (B-C) Cutaway views from the sides.

Figure 2 Supplement B. pH profile of GAA activity. Profile shows maximal activity around pH 4.5. Data were fit to equation 3, with revealing pKa values of 3.6 and 5.3 for the two ionizable groups. Error bars show the standard deviation of two replicates.

Figure 2 Supplement C. Inhibition of GAA by glucose and DNJ. (A) Inhibition of GAA by glucose, using a global fit to equation 1, the Michaelis-Menten equation for a competitive inhibitor. (B-C) Inhibition of GAA by DNJ. Data were initially fit using equation 1, then refit using the Morrison equation (Equation 2) because DNJ is a tight-binding inhibitor. Error bars show the standard deviation of two replicates.

Figure 2 Supplement D. Both α and β anomers of glucose bind to the active site. Electron density reveals both α and β anomers of glucose monosaccharide bind to the active site. Larger β -linked adducts are sterically prevented from binding. 2Fo-Fc electron density is contoured at 1σ .

Figure 3 Supplement A. Binding of glycogen fragments to the second binding site. (A) Binding of isomaltose to the N-terminal domain with 2Fo-Fc electron density is contoured at 1σ . Dotted lines show residues absent in the electron density. (B) Key residues in ligand binding are labeled, with polar contacts highlighted by grey dashed lines. (C) Comparison of isomaltose and maltotriose bound to the second binding site.

Figure 4 Supplement A. Most pathological point mutations occur in buried residues. (A) The accessible surface area per side chain atom was calculated and residues plotted in ascending order. (B) Using the same residue order as panel A, point mutations causing Pompe disease were plotted. Mutations affecting the active site are shown in red.

Table 1: Crystallographic data

PDB Code	5KZR	5KZW	5KZX
Ligand Soak	DNJ	isomaltose	maltotriose
Active site ligand	DNJ	glucose	glucose
Second site ligand	-	isomaltose	maltotriose
cell lengths, Å	96.6, 102.9, 128.7	97.1, 102.6, 128.6	97.1, 102.6, 128.5
Resolution, Å*	50-1.7 (1.73-1.7)	50-2.0 (2.04-2.0)	50-1.9 (1.93-1.9)
Observations*	870,867 (41,948)	326,654 (21,225)	658,306 (17,008)
Unique observations*	141,597 (6,895)	73,011 (4,969)	90,718 (2,389)
Completeness, %*	100.0 (99.9)	84.0 (93.3)	89.2 (47.8)
Multiplicity*	6.2 (6.1)	4.5 (4.3)	7.3 (7.1)
R _{sym} , %*	7.6 (47.2)	7.3 (36.7)	8.7 (34.8)
<I/σ _i >*	11.8 (3.0)	13.7 (3.5)	12.9 (5.9)
R _{work} / R _{free} , %	13.7 / 15.6	15.4 / 18.5	15.5 / 17.9
Atoms	7,828	7,803	7,827
Protein	6,734	6,742	6,726
Carbohydrate	87	218	229
Water	836	792	816
Other	171	51	56
Average B-factor (Å ²)	22.0	26.1	17.8
Protein	19.6	23.9	14.9
Carbohydrate	49.9	57.0	51.6
Water	33.1	36.2	32.1
Ramachandran plot, %			
Favored	97.6	97.3	97.4
Allowed	2.4	2.7	2.6
Outlier	0	0	0
RMS deviations			
Bonds, Å	0.012	0.010	0.009
Angles, °	1.55	1.47	1.41

*Highest resolution shell appears in parentheses

Table 2: Pompe disease mutations

Mutation	Percent wild type activity*	Accessible surface area per side chain atom (Å ²)	Mutation Category	Proposed effect	Reference
R11Q	115	ND	Signal sequence		(Kroos et al., 2008)
S46P	100.2 ^a	ND	Signal sequence		(Oba-Shinjo et al., 2009)
D67N	ND	ND	Pro sequence		(Maimaiti et al., 2009)
R74H	103	ND	ND		(Kroos et al., 2012)
R89H	43.5	3.2	Partially buried	Loss of hydrogen bonding network	(Kroos et al., 2012)
D91N	ND	9.1	Surface exposed	Mild; conservative substitution of surface-exposed residue	(Martiniuk et al., 1990)
C103G	8.2 ^a	0	Buried	Loss of disulfide to C127	(Hermans et al., 2004)
C103R	0	0	Buried	Loss of disulfide to C127	(Kroos et al., 2012)
C108S	ND	0	Buried	Loss of disulfide to C92	(Liu et al., 2014)
C127F	1.5	0.3	Buried	Loss of disulfide to C103	(Kroos et al., 2012)
L141M	ND	11.3	Surface exposed	Less room for larger Met	(Wan et al., 2008)
R154P	ND	0	Buried	Proline not favored in β-strand; loss of H-bonding	(Nascimbeni et al., 2008)
R168P	ND	15.5	Surface exposed	Proline not favored in β-strand; loss of ion pair to D170	(Liu et al., 2013)
R168Q	ND	15.5	Surface exposed	Mild; loss of ion pair to D170	(Liu et al., 2013)
L169P	ND	0.1	Buried	Proline not favored in β-sheet; loss of buried nonpolar interactions	(Gort et al., 2007)
R178C	134	3.9	Partially buried	Loss of hydrogen bonding; loss of ion pair to E174	(Kroos et al., 2008)
R178H	ND	3.9	Partially buried	Less room for imidazole ring	(Chien et al., 2011)
R190H	3.9 ^a	2.7	Partially buried	Loss of hydrogen bonding network; loss of ion pair to D243	(Kroos et al., 2008)
Y191C	0.6	2	Partially buried	Loss of hydrogen bonding with S315; loss of hydrophobic packing	(Pittis et al., 2008)
R199H	ND	ND	ND	*Not present in structure; processing affected?	(Martiniuk et al., 1990)
L208P	ND	12.9	Surface exposed	Loss of main chain H-bond	(Laforêt et al., 2000)

P217L	13.3 ^a	7	Partially buried	Proline favored in β -turn ; less room for Pro	(Oba-Shinjo et al., 2009)
G219R	ND	0	Buried	No room for larger side chain	(Fernandez-Hojas et al., 2002)
V220L	100	0.5	Buried	Less room for larger Leu	(Kroos et al., 2012)
V222M	98	0	Buried	Less room for larger Met	(Kroos et al., 2012)
H223R	ND	5	Partially buried	Mild	(Reuser et al., 1995)
R224P	0 ^a	1.2	Buried	Proline not favored in β -strand; loss of H-binding network and ion pair with E302	(Labrousse et al., 2010)
R224Q	0.7	1.2	Buried	Loss of H-binding network and ion pair with E302	(Kroos et al., 2012)
R224W	ND	1.2	Buried	No room for aromatic Trp	(Pittis et al., 2003)
T234K	2.7	0.4	Buried	No room for larger Lys	(Kroos et al., 2012)
T234R	1.6	0.4	Buried	No room for larger Arg	(Kroos et al., 2012)
A237V	ND	0.1	Buried	No room for larger Val	(Anneser et al., 2005)
F240S	ND	0.5	Buried	Loss of hydrophobic packing	(Sacconi et al., 2010)
A242V	14	0.4	Buried	Little room for larger Val	(Kroos et al., 2008)
L246R	ND	0.1	Buried	No room for larger Arg in hydrophobic pocket	(Spada et al., 2013)
L248P	0	2.2	Partially buried	Proline not favored in β -strand; loss of hydrophobic packing	(Kroos et al., 2008)
L248R	ND	2.2	Partially buried	No room for larger Arg in hydrophobic pocket	(Sacconi et al., 2010)
S251L	ND	13	Surface exposed	Mild	(Wan et al., 2008)
S254L	ND	5.2	Partially buried	Loss of H-bonding to main chain	(Wan et al., 2008)
G259V	0	0.7	Buried	Little room for larger Val, Gly-specific phi/psi	(Kroos et al., 2008)
E262K	ND	0.1	Buried	No room for larger Lys, loss of buried H-bond network	(Fernandez-Hojas et al., 2002)
P266S	18 ^b	25.6	Surface exposed	Mild	(Park et al., 2006)
T271A	83.6 ^a	3.3	Partially buried	Loss of hydrogen bonding with Q255	(Labrousse et al., 2010)
D282H	ND	6.5	Partially buried	Loss of hydrogen bonding might network in active site	(Pardo et al., 2015)
P285R	ND	0.3	Buried	Little room for larger Arg	(Bodamer et al., 2002)
P285S	4.8 ^a	0.3	Buried	Mild	(Bali et al., 2011)

N290D	71	0.1	Buried	Change in buried hydrogen bonding network	(Kroos et al., 2012)
L291F	0.7 ^a	0.2	Buried	No room for larger Phe	(Kroos et al., 2008)
L291H	2	0.2	Buried	Less room for larger His	(Kroos et al., 2008)
L291P	0.7 ^a	0.2	Buried	Phi/psi angles outside of proline range; loss of main chain H-bond	(Kroos et al., 2008)
Y292C	ND	0	Buried	Loss of hydrophobic packing near active site	(Castro-Gago et al., 1999)
G293R	ND	0	Buried	No room for larger Arg	(Hermans et al., 2004)
H295Q	ND	0	Buried	Loss of buried H-bonding network	(Joshi et al., 2008)
Y298S	ND	0	Buried	Loss of hydrophobic packing	(Wan et al., 2008)
L299P	0	0.4	Buried	Proline not favored in β -strand; loss of main chain H-bond; loss of hydrophobic packing	(Kroos et al., 2008)
L299R	ND	0.4	Buried	No room for larger Arg	(Raben et al., 1995)
H308L	ND	0.1	Buried	Loss of buried hydrogen bonding network	(Laforêt et al., 2000)
H308P	ND	0.1	Buried	Proline not favored in β -strand; loss of main chain H-bond; loss of buried H-bonding network	(Hermans et al., 2004)
G309R	ND	0	Buried	No room for larger Arg	(Kroos et al., 1998)
V310G	ND	0.5	Buried	Loss of hydrophobic packing	(Kroos et al., 2012)
L312R	ND	0.3	Buried	No room for larger Arg; loss of hydrophobic packing	(Hermans et al., 2004)
N316I	0	0.1	Buried	Loss of hydrogen bonding network; no room for β -branched side chain	(Kroos et al., 2012)
M318K	3.2 ^a	0.2	Buried	Loss of buried hydrophobic interactions	(Kroos et al., 2008)
M318T	ND	0.2	Buried	Loss of buried hydrophobic interactions	(Zhong et al., 1991)
P324L	ND	7.2	Partially buried	Proline favored in β -turn	(Laforêt et al., 2000)
W330G	ND	0.9	Buried	Loss of buried hydrophobic packing	(Dou et al., 2006)
T333K	ND	0.2	Buried	No room for larger side chain	(Ding et al., 2015)
G334S	ND	2.1	Partially buried	Little room for larger side chain	(Manwaring et al., 2012)
G335E	0.2	1.5	Buried	No room for larger side chain; Gly-specific phi/ psi angles	(Kroos et al., 2012)

G335R	0.8 ^a	1.5	Buried	No room for larger side chain; Gly-specific phi/ psi angles	(Kroos et al., 2008)
P347R	1	0.1	Buried	N-cap of α -helix; no room for larger Arg	(Kroos et al., 2008)
V350M	ND	0	Buried	Little room for larger Met	(Quenardelle et al., 2015)
L355P	ND	0.2	Buried	Proline kinks α -helix; loss of main chain H-bond	(Hermans et al., 2004)
G359R	54.3 ^a	10.8	Surface exposed	Glycine specific phi/psi angles	(Müller-Felber et al., 2007)
P361L	3.9 ^a	0.3	Buried	Little room for Leu	(Lam et al., 2003)
W367R	ND	0.5	Buried	Loss of hydrophobic packing	(Palmer et al., 2007)
L369P	0.2	0.2	Buried	No room for Pro; loss of main chain H-bond	(Niño et al., 2013)
H372L	ND	0	Buried	Loss of buried H-bonding network	(Van den Hout et al., 2004)
L373R	ND	0.2	Buried	No room for larger side chain	(Bali et al., 2012)
C374R	ND	0	Buried	No room for larger Arg; disrupts active site	(Hermans et al., 2004)
R375H	ND	3.4	Partially buried	Loss of H-bonding network; disrupts active site	(Bali et al., 2011)
R375L	0.3	3.4	Partially buried	Loss of H-bonding network; disrupts active site	(Pittis et al., 2008)
G377R	0 ^b	5.2	Partially buried	Glycine specific phi/psi angles	(Kishnani et al., 2006)
M391V	ND	0	Buried	Mild; perturbs hydrophobic core	(Kroos et al., 2012)
P397L	0	1.6	Buried	Mild; less room for Leu	(Kroos et al., 2012)
Q401R	5	0.1	Buried	Little room for larger Arg	(Kroos et al., 2008)
W402R	ND	0	Buried	Loss of hydrophobic packing	(Hoefsloot et al., 1990)
N403K	ND	0	Buried	No room for larger Lys; loss of H-bonding network	(GAA Database Consortium, 2016)
D404G	ND	2	Partially buried	Loss of substrate recognition	(Nilsson et al., 2014)
D404N	ND	2	Partially buried	Loss of Hydrogen bond acceptor, might affect substrate recognition	(Amartino et al., 2006)
L405P	ND	1.1	Buried	Disrupts hydrogen bonding network in active site; perturbs active site geometry	(Hermans et al., 2004)

M408V	ND	0	Buried	little room for β -branched side chain	(Fernandez-Hojas et al., 2002)
S410F	111	25.3	Surface exposed	Mild	(Kroos et al., 2008)
D413E	ND	0.4	Buried	No room for larger Glu	(Dlamini et al., 2008)
D419V	21.3	27.5	Surface exposed	Mild	(Kroos et al., 2012)
M427T	ND	0.6	Buried	Loss of hydrophobic packing	(Ding et al., 2015)
Q429R	ND	18.2	Surface exposed	Mild	(Chien et al., 2015)
Q433K	ND	22.3	Surface exposed	Mild	(Ravaglia et al., 2012)
R437C	ND	6.6	Partially buried	loss of H-bonding network; loss of hydrophobic packing	(Lam et al., 2003)
R437H	ND	6.6	Partially buried	loss of H-bonding network; less room for aromatic His	(Turaça et al., 2015)
M439K	ND	0	Buried	loss of hydrophobic packing	(Park et al., 2006)
V442M	ND	0.9	Buried	Less room for larger Met	(Chien et al., 2011)
P444R	ND	0.8	Buried	No room for larger Arg	(Remiche et al., 2014)
A445P	ND	2.6	Partially buried	Mild; less room for Pro	(Montalvo et al., 2006)
Y455C	ND	1.3	Buried	Loss of hydrophobic packing; loss of main chain H-bond	(Gort et al., 2007)
Y455F	ND	1.3	Buried	Loss of main chain H-bond	(Hermans et al., 2004)
P457H	0	2.7	Partially buried	Little room for larger His	(Kroos et al., 2012)
P457L	10	2.7	Partially buried	Little room for larger Leu	(Kroos et al., 2008)
Y458C	45	4.2	Partially buried	Loss of hydrophobic packing	(Kroos et al., 2012)
D459N	ND	7.4	Partially buried	Mild; some H-bonding effect	(Wan et al., 2008)
G461S	0	0	Buried	No room for larger side chain	(Kroos et al., 2008)
L462P	ND	17.1	Surface exposed	Loss of main chain H-bond; Pro causes kink in α -helix	(Hu et al., 2015)
V466F	0	0	Buried	No room for larger Phe	(Kroos et al., 2008)
V466G	ND	0	Buried	Loss of hydrophobic packing	(Palmer et al., 2007)
G478R	ND	0	Buried	No room for larger Arg	(Reuser et al., 1995)
K479N	ND	14.6	Surface exposed	Mild	(Wan et al., 2008)

W481R	ND	6	Partially buried	Loss of hydrophobic packing	(58)
P482L	ND	1.7	Buried	Proline favored in β -turn; no room for larger Leu	(Gort et al., 2007)
P482R	0 ^b	1.7	Buried	Proline favored in β -turn; no room for larger Arg	(Kroos et al., 2008)
G483R	0	20.7	Surface exposed	Glycine-specific phi/psi angles	(Kroos et al., 2008)
G483V	0 ^a	20.7	Surface exposed	Glycine-specific phi/psi angles	(Kroos et al., 2008)
A486P	0 ^a	0	Buried	Little room for larger Pro; loss of main chain H-bond	(Oba-Shinjo et al., 2009)
F487S	0	0.1	Buried	Loss of hydrophobic packing	(Kroos et al., 2008)
D489N	ND	1.3	Buried	Loss of H-bond network, loss of main chain H-bond	(Montalvo et al., 2006)
D489Y	ND	1.3	Buried	No room for larger Tyr; loss of H-bond network	(Fu et al., 2014)
D489G	ND	1.3	Buried	loss of H-bond network	(Bali et al., 2012)
P493L	ND	21.6	Surface exposed	Mild, Pro initiates α -helix	(Schneider et al., 2013)
W499R	ND	0	Buried	Loss of hydrophobic packing	(Joshi et al., 2008)
M502V	ND	0.9	Buried	Mild; less room for β -branched side chain	(Montalvo et al., 2006)
G514R	ND	0	Buried	No room for larger Arg	(McCready et al., 2007)
M515K	ND	0.3	Buried	Introduction of charge into hydrophobic pocket	(Fujimoto et al., 2013)
M519T	ND	2.6	Partially buried	Disrupts hydrophobic packing in active site	(Reuser et al., 1995)
M519V	ND	2.6	Partially buried	Mild active site perturbation	(Huie et al., 1994)
E521K	ND	0.1	Buried	No room for larger Lys; loss of ion pair with R600 in the active site	(Hermans et al., 1991)
E521Q	0 ^b	0.1	Buried	loss of ion pair w/ R600 in the active site	(Kroos et al., 2008)
E521V	ND	0.1	Buried	loss of ion pair w/ R600 in the active site	(Liu et al., 2013)
P522A	0	0	Buried	Loss of hydrophobic packing	(Müller-Felber et al., 2007)
P522S	0.8	0	Buried	Loss of hydrophobic packing	(Kroos et al., 2008)
P522T	ND	0	Buried	Loss of hydrophobic packing	(McCready et al., 2007)
S523Y	0	1.4	Buried	Little room for larger Tyr; near active site	(Kroos et al., 2012)
F525Y	13.4 ^a	8.6	Partially buried	Less room for larger Tyr; perturbs active site	(Labrousse et al., 2010)
S529V	ND	2.3	Partially buried	Loss of main chain H-bond; less room for β -branched side chain	(Tsunoda et al., 1996)

P545L	ND	0	Buried	No room for larger Leu	(Hermans et al., 1994)
V548F	ND	13.2	Surface exposed	Mild; some loss of hydrophobic packing	(Case et al., 2008)
G549R	ND	21.7	Surface exposed	Mild; glycine specific phi/psi angles	(Hermans et al., 2004)
L552P	ND	0.2	Buried	Loss of hydrophobic packing	(Bodamer et al., 2002)
T556A	ND	0.6	Buried	Mild; some loss of H-bonding	(Loureiro Neves et al., 2013)
I557F	2.7 ^a	0	Buried	No room for larger Phe	(Labrousse et al., 2010)
C558S	2.1 ^a	3.2	Partially buried	Loss of disulfide with C533	(Alcántara-Ortigoza et al., 2010)
S566P	ND	6.5	Partially buried	Phi/psi angle outside of proline range	(Hermans et al., 1994)
H568L	ND	0	Buried	loss of H-bonding network	(Reuser et al., 1995)
H568Q	ND	0	Buried	Mild; some changes in H-bonding network	(Alejandre et al., 2012)
N570K	0.9 ^a	5.1	Partially buried	Loss of some hydrogen bonding	(Kroos et al., 2008)
H572Q	0 ^a	0	Buried	Mild; loss of pi stacking with W279	(Kroos et al., 2008)
N573H	0	0.4	Buried	Mild; less room for larger His	(Kroos et al., 2008)
N573K	ND	0.4	Buried	Little room for larger Lys	(Bali et al., 2012)
Y575C	2	0.1	Buried	Loss of hydrophobic packing	(Kroos et al., 2012)
Y575S	ND	0.1	Buried	Loss of hydrophobic packing	(Hermans et al., 2004)
G576D	0	0	Buried	No room for larger Asp	(Kroos et al., 2008)
G576R	3.8	0	Buried	No room for larger Arg	(Kroos et al., 2012)
G576S	ND	0	Buried	No room for larger Ser	(Reuser et al., 1995)
E579K	ND	0	Buried	Less room for larger Lys	(Hermans et al., 2004)
S583F	0	0	Buried	No room for larger Phe; loss of H-bond to W499	(Kroos et al., 2008)
R585K	94	12.9	Surface exposed	Mild; conservative substitution of surface-exposed residue	(Kroos et al., 2008)
R585M	ND	12.9	Surface exposed	Mild	(Laforêt et al., 2000)
R591W	ND	2.8	Partially buried	Loss of ion pair to D513	(Montagnese et al., 2015)
R594H	0.1	2.1	Partially buried	Loss of ion pair to D860; loss of hydrogen bonding network	(Kroos et al., 2012)

R594P	1.2 ^a	2.1	Partially buried	Loss of ion pair to D860; loss of hydrogen bonding network	(Kroos et al., 2008)
S599Y	0	0	Buried	No room for larger Tyr; loss of H-bond to N520	(Kroos et al., 2008)
R600C	ND	0.8	Buried	Active site residue; loss of ion pair with E521; loss of H-bonding network	(Tsuji et al., 2000)
R600H	ND	0.8	Buried	Active site residue; loss of ion pair with E521; loss of H-bonding network	(Ko et al., 1999)
R600L	ND	0.8	Buried	Active site residue; loss of ion pair with E521; loss of H-bonding network	(McCreedy et al., 2007)
S601L	0.4	0.1	Buried	Less room for larger Leu; loss of H-bond with N280	(Kroos et al., 2012)
S601W	ND	0.1	Buried	No room for larger Trp; loss of H-bond with N280	(Palmer et al., 2007)
T602A	5.8	0	Buried	Mild; loss of some hydrophobic packing	(Kroos et al., 2012)
G605D	ND	4.7	Partially buried	No room for larger Asp; Gly-specific phi/psi	(Muraoka et al., 2011)
G607D	ND	0.3	Buried	No room for larger Asp	(Hermans et al., 2004)
A610V	5 ^b	0	Buried	No room for larger Val	(Müller-Felber et al., 2007)
G611D	ND	0	Buried	No room for larger Asp	(Kroos et al., 2012)
H612Q	ND	0	Buried	Mild; loss of ion pair with E262	(Montalvo et al., 2006)
H612Y	0 ^a	0	Buried	Mild; loss of ion pair with E262; loss of H-bonding network	(Oba-Shinjo et al., 2009)
T614K	1.7 ^a	0.3	Buried	Little room for larger Lys	(Kroos et al., 2008)
G615R	ND	0	Buried	No room for larger Arg; Gly-specific phi/psi; near active site	(Ko et al., 1999)
D616N	ND	8.8	Partially buried	Active site residue critical for mechanism	(Lin et al., 2010)
V617A	65	1.2	Buried	Mild; loss of hydrophobic packing	(Kroos et al., 2008)
S619N	ND	0.1	Buried	Mild; less room for larger Asn	(Kroos et al., 2006)
S619R	ND	0.1	Buried	Less room for larger Arg	(Pipo et al., 2003)
S627F	ND	1.1	Buried	No room for larger Phe	(Case et al., 2008)
S627P	0.5	1.1	Buried	No room for larger Pro; loss of main chain H-bond; Pro causes kink in α -helix	(Kroos et al., 2012)
P629L	49	1.5	Buried	Mild; Pro creates kink in α -helix	(Kroos et al., 2012)

N635K	0 ^a	0	Buried	No room for larger Lys; loss of H-bonding network	(Oba-Shinjo et al., 2009)
G638V	ND	0.5	Buried	No room for larger Val	(Van den Hout et al., 2004)
G638W	ND	0.5	Buried	No room for larger Trp	(Joshi et al., 2008)
L641V	ND	0.2	Buried	Mild; less room for larger Leu	(Turaça et al., 2015)
G643R	ND	0	Buried	No room for larger Arg	(Hermans et al., 1993)
A644P	ND	0.4	Buried	Little room for larger Pro	(Gort et al., 2007)
D645E	1.9	0.2	Buried	Less room for larger Asp; perturbs active site	(Hermans et al., 1993)
D645H	ND	0.2	Buried	Little room for larger Asp; perturbs active site	(Lin and Shieh, 1995)
D645N	ND	0.2	Buried	Mild	(Huie et al., 1998)
D645Y	0 ^b	0.2	Buried	Little room for larger Asp; perturbs active site	(Gort et al., 2007)
C647W	ND	0	Buried	Loss of disulfide to C658	(Huie et al., 1994)
G648D	0	0	Buried	No room for larger Asp; Gly-specific phi/psi	(Kroos et al., 2012)
G648S	ND	0	Buried	Little room for larger Asp; Gly-specific phi/psi	(Huie et al., 1998)
T653N	ND	0.5	Buried	Less room for larger Asn; perturbs 647-658 disulfide	(Chien et al., 2011)
S654P	ND	22.2	Surface exposed	Mild; less room for larger Pro; loss of main chain H-bond	(Wan et al., 2008)
R660C	ND	0.1	Buried	Loss of hydrogen bonding network	(Palmer et al., 2007)
R660H	ND	0.1	Buried	Loss of hydrogen bonding network	(Pipo et al., 2003)
W661G	2	0	Buried	Loss of hydrophobic packing	(Kroos et al., 2008)
G665R	ND	0	Buried	No room for larger Arg	(Fu et al., 2014)
M671R	ND	0	Buried	No room for larger Arg	(Kishnani et al., 2006)
M671K	ND	0	Buried	Little room for larger Lys; loss of hydrophobic packing	(Ding et al., 2015)
R672Q	ND	0.1	Buried	Loss of ion pair to D645; perturbs active site	(Huie et al., 1998)
R672W	ND	0.1	Buried	Loss of ion pair to D645; perturbs active site	(Huie et al., 1998)
Q682R	ND	0.4	Buried	Less room for larger Arg	(Ayca et al., 2014)
E689K	ND	20	Surface exposed	Mild	(Huie et al., 1994)
R702C	ND	0	Buried	Loss of hydrogen bonding network	(Montalvo et al., 2004)

R702H	13 ^b	0	Buried	Perturbs hydrogen bonding network	(Qiu et al., 2007)
R702L	0.4	0	Buried	Loss of hydrogen bonding network	(Kroos et al., 2012)
L705P	ND	0.5	Buried	Loss of main chain H-bond; Pro causes kink in α -helix	(Turaça et al., 2015)
T711R	86 ^a	1.1	Buried	little room for larger Arg	(Qiu et al., 2007)
L712P	8	5.4	Partially buried	Loss of main chain H-bond; Pro causes kink in α -helix	(Kroos et al., 2008)
V718I	127	10.1	Surface exposed	Mild; room for additional methyl group	(Kroos et al., 2012)
V723M	ND	0	Buried	Less room for larger Met	(Qiu et al., 2007)
A724D	ND	0	Buried	Less room for larger Asp in hydrophobic pocket	(Park et al., 2015)
R725P	ND	0.9	Buried	Loss of H-bonding network; loss of main chain H-bond; unfavorable phi/psi angles for Pro	(Wan et al., 2008)
R725W	ND	0.9	Buried	Loss of H-bonding network; loss of ion pair with E730; less room for aromatic Trp	(Hermans et al., 1993)
P726R	ND	0	Buried	No room for larger Arg	(Ishigaki et al., 2012)
T737N	0 ^a	0	Buried	Less room for larger Asn	(Kroos et al., 2008)
Q743K	4.1	1.2	Buried	Less room for larger Lys; loss of H-bonding network	(Kroos et al., 2012)
Q743R	0.8	1.2	Buried	Less room for larger Arg; loss of H-bonding network	(Kroos et al., 2008)
W746C	29.4 ^c	0	Buried	Loss of hydrophobic packing	(Wan et al., 2008)
W746G	2.1 ^c	0	Buried	Loss of hydrophobic packing	(Labrousse et al., 2010)
W746R	3.5 ^c	0	Buried	Loss of hydrophobic packing	(Niño et al., 2013)
W746S	40.1 ^c	0	Buried	Loss of hydrophobic packing	(Kroos et al., 2008)
G759A	ND	28.7	Surface exposed	Mild	(Sampaolo et al., 2013)
E762K	ND	20.2	Surface exposed	Mild	(Lin et al., 2010)
Y766C	ND	0.7	Buried	Loss of hydrophobic packing; loss of hydrogen bond with D734	(Herzog et al., 2012)
P768L	ND	0.3	Buried	Less room for larger Leu	(Yang et al., 2014)
P768R	ND	0.3	Buried	Less room for larger Arg	(Reuser et al., 1995)
W772R	5	0.2	Buried	Loss of hydrophobic packing	(Kroos et al., 2008)

I780V	ND	6.4	Partially buried	Mild; loss of some hydrophobic packing	(Martiniuk et al., 1990)
H799D	ND	16.9	Surface exposed	Mild	(Montagnese et al., 2015)
V816I	ND	1	Buried	Mild; less room for extra methyl group	(Hermans et al., 1993)
R819P	0.4	6.7	Partially buried	Loss of main chain H-bond	(Kroos et al., 2012)
A880D	0	0.2	Buried	Less room for larger Asp in hydrophobic pocket	(Hermans et al., 2004)
L901Q	ND	0	Buried	loss of hydrophobic packing	(Kroos et al., 2004)
P913R	ND	0.1	Buried	No room for larger Arg	(Herzog et al., 2012)
V916F	0	0	Buried	Little room for larger Phe	(Kroos et al., 2012)
S924P	167 ^b	29.2	Surface exposed	Mild	(Kishnani et al., 2006)
T927I	ND	24.4	Surface exposed	Mild	(Hermans et al., 1993)
L935P	0.5 ^a	0	Buried	Loss of main chain H-bond	(Kroos et al., 2008)
V949D	ND	0.1	Buried	No room for larger Asp	(Becker et al., 1998)

* References for activity and identification of mutants is the same unless indicated by superscript: (a) Kroos et al. 2012, (b) Kroos et al. 2008, (c) Niño et al. 2013.
ND, not determined.

Deming et al., Figure 1

Deming et al., Figure 3

A

B

C

Deming et al., Figure 4

A

B

Subunit:	N-terminal	C-terminal
Residues:	80-115, 123-198	793-952
Mass (kDa):	13.2 (3.9+10.3)	19.4
Interface area with catalytic domain (Å ²):	5045	5803

A

B

C

180°

A

B

C