Mutations in *ELA2*, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis

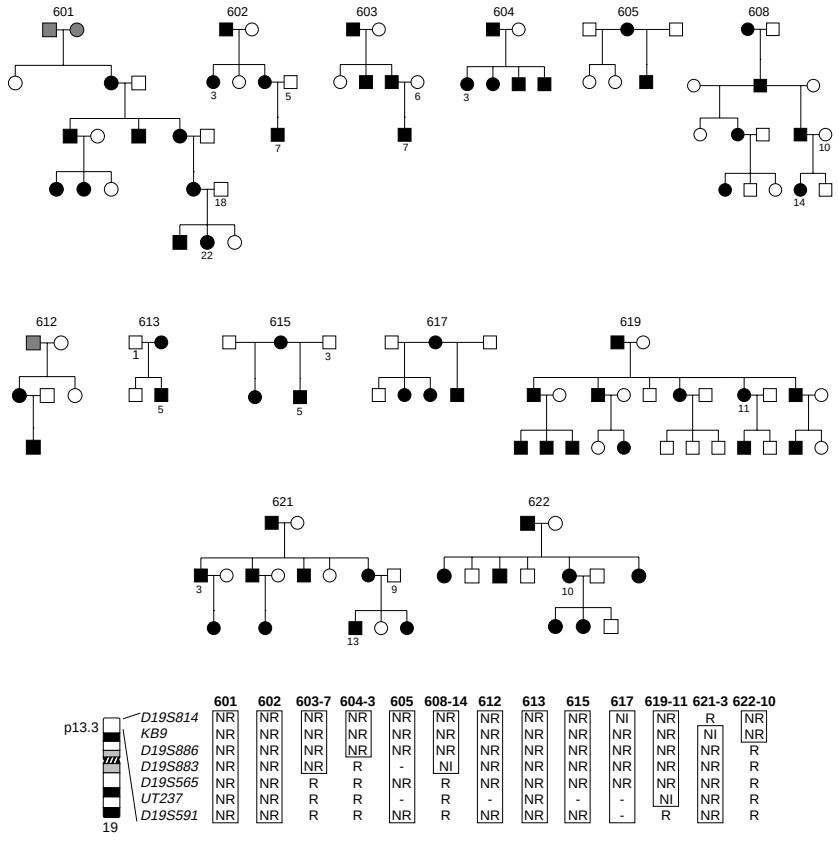
Marshall Horwitz[1*], Kathleen F. Benson[1*], Richard E. Person[2*], Andrew G. Aprikyan[2] & David C. Dale[2]

*These authors contributed equally to this work.

Human cyclic haematopoiesis (cyclic neutropenia, MIM 162800) is an autosomal dominant disease in which blood-cell production from the bone marrow oscillates with 21-day periodicity[1,2]. Circulating neutrophils vary between almost normal numbers and zero. During intervals of neutropenia, affected individuals are at risk for opportunistic infection[3]. Monocytes, platelets, lymphocytes and reticulocytes also cycle with the same frequency. Here we use a genome-wide screen and positional cloning to map the locus to chromosome 19p13.3. We identified 7 different single-base substitutions in the gene (*ELA2*) encoding neutrophil elastase (EC 3.4.21.37, also known as leukocyte elastase, elastase 2 and medullasin), a serine protease of neutrophil and monocyte granules, on unique haplotypes in 13 of 13 families as well as a new mutation in a sporadic case. Neutrophil elastase (a 240-aa mature protein predominantly found in neutrophil granules[4]) is the target for protease inhibition by α_1-antitrypsin, and its unopposed release destroys tissue at sites of inflammation. We hypothesize that a perturbed interaction between neutrophil elastase and serpins or other substrates may regulate mechanisms governing the clock-like timing of haematopoiesis.

We assembled 13 pedigrees (Fig. 1), performed a genome-wide screen with families 619 and 601, and found evidence for linkage to chromosome 19p13.3. After genotyping all families for markers in this region, the peak two-point lod score reached 13.11 at θ=0 with newly identified marker *KB9*. Haplotype analysis determined a minimum genetic interval of 0 cM, corresponding to a physical distance of approximately 320 kb, defined by recombination centromerically with *D19S886* and telomerically with *D19S814*. This region contains a family of genes encoding chymotrypsin-like S1 serine proteases. Mutational analysis of genomic DNA revealed 7 different single-base substitutions (Fig. 2 and Table 1) in *ELA2* (which has five exons), none of which appeared in at least 125 control chromosomes.

Substitution of arginine with glutamine at residue 191 (R191Q; numbering from the first residue after the pre-signal peptide has been cleaved[5]) results from a G→A transition at the second position in the codon and is present in families 619, 622 and 613. R191 is conserved in mouse neutrophil elastase (81% identity[6]) and pig pancreatic elastase (43% identity[7]), but is a differentiating feature of elastases that is not conserved in other S1 leukocyte proteases (azurocidin, proteinase 3 or cathepsin G). Replacement of the normal leucine with a phenylalanine at residue 177 (L177F), resulting from a G→T transversion at the wobble position of the codon, is present in families 608 and 617. L177 is located four residues carboxy terminal from the active site serine and is a conserved feature of mouse neutrophil elastase and pig pancreatic elastase. Family 621 contains a substitution of the normal alanine with a valine at amino acid 32 (A32V), resulting from a C→T transition in the middle of the codon. A32 is



	601	602	603-7	604-3	605	608-14	612	613	615	617	619-11	621-3	622-10
D19S814	NR	NR	NR	NR	NR	NR	NR	NR	NR	NI	NR	R	NR
KB9	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NI	NR
D19S886	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	R
D19S883	NR	NR	NR	R	-	NI	NR	NR	NR	NR	NR	NR	R
D19S565	NR	NR	R	R	NR	R	NR	NR	NR	NR	NR	NR	R
UT237	NR	NR	R	R	-	R	-	NR	-	-	NI	NR	R
D19S591	NR	NR	R	R	NR	R	NR	NR	NR	-	R	NR	R

Fig. 1 Pedigrees and haplotype summary. Only individuals referred to elsewhere are numbered. The common haplotype shared by affecteds is boxed for each family; the specified individuals were recombinant for the indicated marker. Filled symbols, affected individuals; open symbols, unaffected; grey symbols, unknown phenotype. NR, non-recombinant; R, recombinant; NI, not informative; –, not determined.

Divisions of [1]Medical Genetics and [2]Hematology, Department of Medicine, University of Washington School of Medicine, Seattle, Washington, USA. Correspondence should be addressed to M.H. (e-mail: horwitz@u.washington.edu).

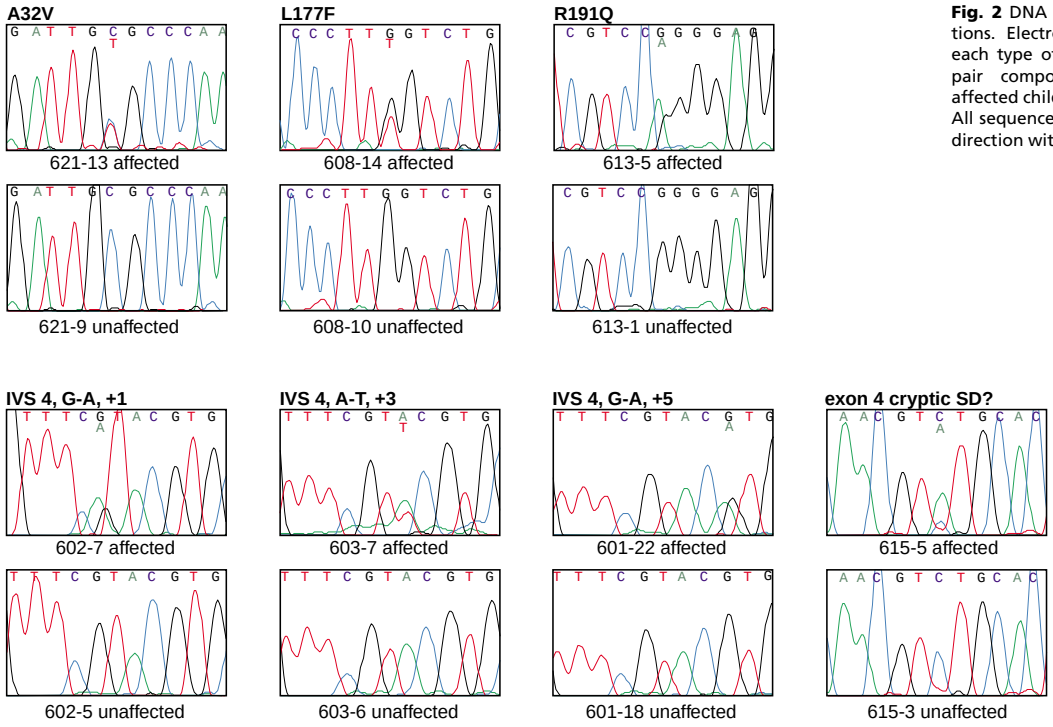


Fig. 2 DNA sequence analysis of mutations. Electropherograms documenting each type of mutation are shown in a pair composed of a representative affected child and an unaffected parent. All sequences shown are in the forward direction with respect to *ELA2* mRNA.

conserved in mouse neutrophil elastase, but not in other chymotryptic leukocyte granule proteases.

All splice-site mutations cluster in the splice-donor region of intron 4. Transition of the invariant guanine to an adenine at the +1 splice-donor site (IVS4, G→A, +1) occurs in families 602 and 605. This mutation also arose in a sporadic case of cyclic haematopoiesis, X1, whose parents are not affected and do not carry the mutation, and whose paternity is confirmed. An A→T transversion at the highly conserved +3 position (IVS4, A→T, +3) in which a purine is found in 96% of all genes[8], was found in family 603. A G→A transition at the conserved +5 position (IVS4, G→A, +5), where a guanine is present in 84% of cases[8], exists in families 601, 604 and 612. Family 615 has an exon 4 silent C→A transversion at the wobble position of the V157 codon. This substitution may create a cryptic splice-donor site (from GUCUGC to GUAUGC).

To determine the consequences of splice-donor mutations in intron 4, we performed RT–PCR using bone marrow aspirated from sporadic case X1 and an unaffected control, and HL60 cells (a human leukaemia line expressing neutrophil elastase[9]). Agarose gel electrophoresis indicated that X1 yields both the expected 678-bp fragment and a shorter product absent from control bone marrow or HL60 cells (Fig. 3*c*). Sequencing across the abnormal splice junction in the cDNA derived from both alleles (Fig. 3*a*,*d*) showed that the G→A transition at +1 of intron 4 results in the use of a cryptic splice-donor site (GUGAGG) 30 bases upstream, causing in-frame deletion of the last 10 residues (V161–F170) from exon 4. The normal transcript (Fig. 3*b*) is homogenous for the S173 polymorphism unique to the normal maternal chromosome, indicating that the allele with the +1 splice-site substitution cannot produce the normal transcript. Presumably, the mutations at the +3 and + 5 positions of intron 4 act similarly, although the unavailability of bone marrow prevents verification. The effect of the substitution in family 615 is similarly indeterminable, but if a cryptic splice-donor site were activated at this position, it would delete the last 14 amino acids (V161–F170) of exon 4.

Neutrophil elastase is a monomeric glycoprotein of approximately 25 kD (refs 4,10) that is synthesized during the differentiation of promyelocytes and promonocytes and packaged in cytoplasmic granules. Tissue destruction promoted by neutrophil elastase, which is extracellularly released from neutrophils migrating to sites of inflammation, has a role in emphysema, cystic fibrosis, the adult respiratory distress syndrome and rheumatoid arthritis. Neutrophil elastase is inhibited by the serpins α_1-antitrypsin and neutrophil elastase inhibitor and the non-serpins elafin and human secretory leukocyte protease inhibitor.

The mutations (Fig. 4) affect residues encircling the crevice formed between the two β-barrel cylindrical domains encompassing the catalytic site[11,12]. R191 participates in substrate specificity at the P_4 position of the active site[10]. V161–F170 defines a surface loop, conserved among all serine proteases, responsible for substrate specificity. Engineered mutations within this loop convert trypsin into a derivative with elastase activity[13]. The V161–F170 loop also interfaces with serpins[14]. L177 is conserved

Table 1 • Mutations

Families	Location	Effect	Base change
621	exon 2	A32V	C→T
608, 617	exon 5	L177F	G→T
613, 619, 622	exon 5	R191Q	G→A
602, 605, sporadic X1	intron 4	+1 splice donor Del V161–F170	G→A
603	intron 4	+3 splice donor	A→T
601, 604, 612	intron 4	+5 splice donor	G→A
615	exon 4	cryptic splice donor activation? Del V157–F170?	C→A

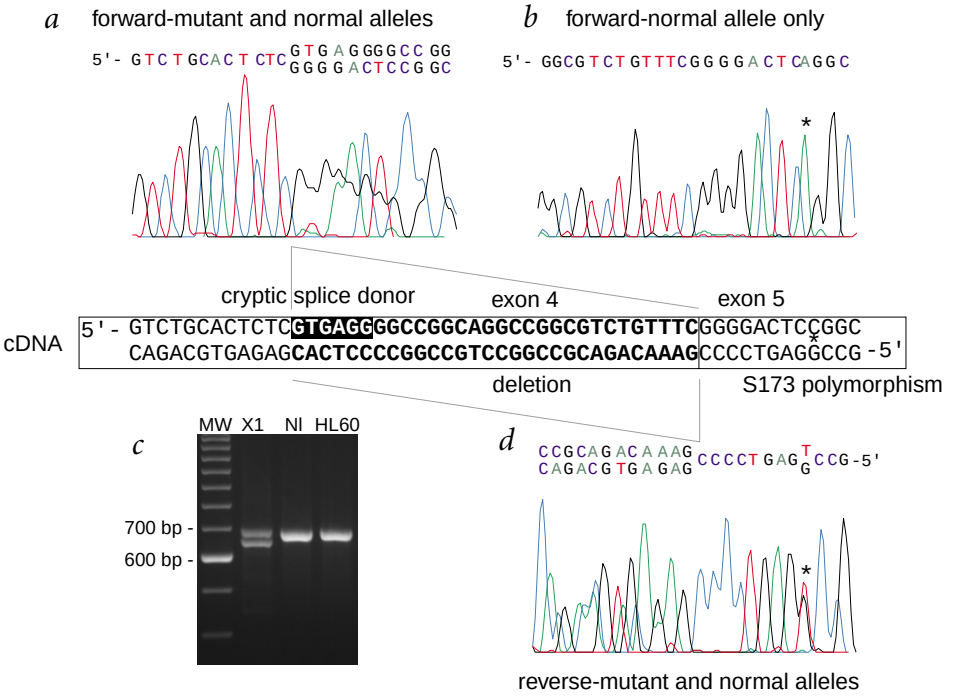


Fig. 3 RT–PCR analysis from sporadic patient X1 bone marrow. The middle of the figure lists the double-stranded cDNA sequence of the exon 4-exon 5 splice junction site with flanking sequences for *ELA2* (ref. 9). The cryptic splice-donor site is indicated in reverse type, and the deleted segment resulting from the intron 4 splice-donor mutation is indicated in bold. The lines above and below the sequence indicate the aberrant splicing observed in the transcript from the mutant allele. ***a***, Simultaneous sequencing of both RT–PCR products from the sporadic patient using a forward primer from the upstream region of exon 4. The sequence trace is aligned with the cDNA sequence in the centre of the figure. Note that the sequence diverges at the cryptic splice site and then represents a superimposition of the remainder of exon 4 and the beginning of exon 5. ***b***, Specific amplification of the normal allele with a forward primer hybridizing to the deleted segment of the mutant transcript. Note that this transcript is homogeneous for the S173 polymorphism, indicating that the normal length transcript results exclusively from expression from the normal allele. ***c***, Ethidium-stained agarose gel electrophoresis of the RT–PCR product from bone marrow from the sporadic patient (X1), bone marrow from an unaffected control (NI) and HL60 cells. The molecular weight size standard is a 100-bp ladder. ***d***, Simultaneous sequencing of both RT–PCR products from the sporadic patient using a reverse primer from the downstream region of exon 5. A mirror-image reversal of the original electrophoretic tracing is shown so that the sequence can be aligned with the antisense strand of the cDNA sequence in the centre of the figure. Note heterozygosity for the S173 polymorphism, whose position is indicated (asterisk).

in most S1 enzymes, although phenylalanine replaces it in thrombin, hepsin and complement components C1r and C1s (ref. 15), where, at least for thrombin[16], it confers trypsin-like activity and liability to antithrombin III.

Targeted homologous recombination of *Ela2* produces no phenotype in heterozygous mice[17]. *Ela2*$^{-/-}$ mice do not have deficient neutrophil numbers, but demonstrate impaired intracellular killing of bacteria by neutrophils and are vulnerable to Gram-negative sepsis. In contrast, individuals with cyclic neutropenia are not unusually susceptible to infection during non-neutropenic phases of the cycle. Given the dominant inheritance and the different phenotype of *Ela2*$^{-/-}$ mice, human cyclic haematopoiesis likely results from gain-of-function mutations requiring the integrity of much of the protein.

We hypothesize that the effects of the mutations disrupt interaction with serpins. α_1-Antitrypsin binds the catalytic site of neutrophil elastase and undergoes proteolysis[18]. Thus, both substrate recognition and proteolytic activity of neutrophil elastase are important for serpin inhibition. A recent report indicated that neutrophil elastase cleaves tyrosyl-tRNA synthetase[19], yielding cytokine domains that induce neutrophil chemotaxis. Similarly, neutrophil elastase causes the release of the neutrophil chemoattractant interleukin-8 from endothelial cells[20]. Neutrophil elastase may promote a feedback circuit to recruit neutrophils in the inflammatory response. Theoretical considerations of cyclic haematopoiesis suggest that a defect in a feedback loop might give rise to periodic cycling[1]. Conceivably, perturbations between neutrophil elastase and its inhibitors may disrupt normal controls in a similar circuit operating during haematopoiesis in the bone marrow.

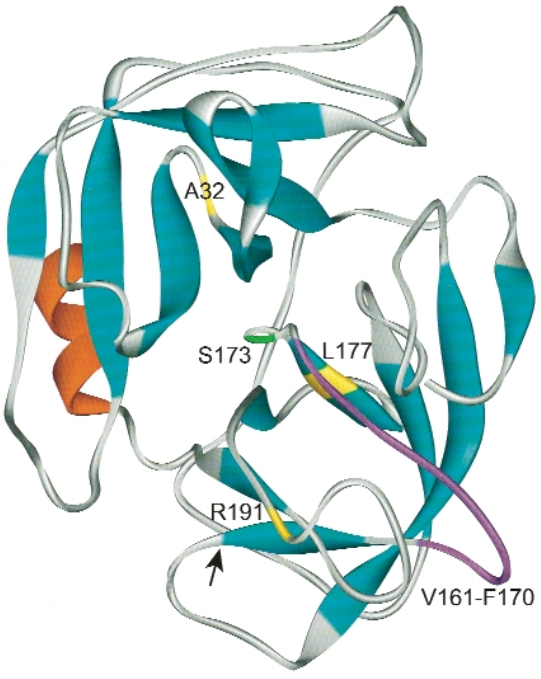


Fig. 4 Position of mutations in the tertiary structure of neutrophil elastase. The missense mutations (yellow), the deletion resulting from the intron 4 splice-donor mutation (magenta) and the catalytic S173 (green) residue are shown. β-Pleated sheets (blue) and α-helices (red) are indicated. The arrow indicates the amino-terminal extension of the deletion to V157 that is predicted for the potential cryptic splice-donor activation mutation in family 615. The image was prepared from the X-ray crystallographic coordinates[12] taken from the Molecular Modeling DataBase (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.html).

Methods

Patients. Clinical details of some families have been described[2]. Phenotypes were assigned on the basis of serial absolute neutrophil counts demonstrating recurring episodes of severe neutropenia (<200 μl^{-1}) and a history of infection with 21-day periodicity.

Genetic linkage analysis. We performed a 10-cM density genome-wide screen for linkage with the fluorescent ABI/PE Biosystems PRISM Linkage Mapping Panel 2 with capillary electrophoretic analysis on an ABI/PE Biosystems 310 machine. The genetic model consisted of autosomal dominant inheritance, with penetrance of 0.9, a gene frequency of 0.005 and allele frequencies taken from The Genome Database[21] when possible. We performed lod score analysis with FASTLINK (ref. 22). Haplotypes parsimonious for recombination were calculated with GENEHUNTER (ref. 23).

Identification of new markers. We used the Lawrence Livermore National Laboratory Genome Center DNA sequence map (http://www-bio.llnl.gov/sequence-bin/seq_status?id=25). New polymorphic markers were identified in the chromosome 19p13.3 critical region by visually scanning genomic sequence and picking appropriate PCR primers. Potentially informative markers were ^{32}P γ-end-labelled, and PCR products sized by denaturing polyacrylamide gel electrophoresis with autoradiography. The marker *KB9* amplifies a complex repeat and is contained within cosmid R33586. The forward primer 5′ end is at position 31,029 (5′–TGCAAAGGCTTGGAGGGCTGATG–3′); the reverse primer 5′ end is complementary to position 31,180 (5′–ATCTCGGACAACAGCAGGCCTCG–3′).

Mutational analysis. We directly sequenced PCR-amplified genomic DNA with the ABI/PE Biosystems PRISM Big Dye terminator chemistry on an ABI/PE Biosystems 310 machine. Each exon of *ELA2*, along with at least 20 bases of flanking intron or untranslated region, was sequenced from both directions with corroborating results in each individual.

RT–PCR. *ELA2* is specifically expressed in promyelocytes and promonocytes[5], and attempts to amplify transcripts from EBV-immortalized lymphoblastoid cells derived from patients proved unsuccessful. We therefore extracted RNA from freshly aspirated bone marrow and HL60 cells using RNeasy kits (Qiagen). Control bone marrow was from a patient with a sarcoma. RT–PCR used Omniscript reverse transcriptase (Qiagen) with subsequent PCR and direct DNA sequencing. The primers are from exons 2 and 5 of *ELA2*.

Acknowledgements

We thank the families for their help; A.-A. Bolyard, R. Oosterwyk, M. Lieschke and participating physicians of the Severe Chronic Neutropenia Registry; E. Lynch, G. Stamatoyannopoulos and M.-C. King for advice; and R. Livingston, S. Palmer, C. Bos, L. Keith and K. Stephens for contributions to an earlier phase of this study. This work was supported by the Markey Molecular Medicine Center and the Doris Duke Charitable Foundation grant T98006 (M.H.) and funding of the Registry by Amgen and NIH grant DK18951 (D.C.D.).

Received 15 September; accepted 27 October 1999.

1. Haurie, C., Dale, D.C. & Mackey, M.C. Cyclical neutropenia and other periodic hematological disorders: a review of mechanisms and mathematical models. *Blood* **92**, 2629–2640 (1998).
2. Palmer, S.E., Stephens, K. & Dale, D.C. Genetics, phenotype, and natural history of autosomal dominant cyclic hematopoiesis. *Am. J. Med. Genet.* **66**, 413–422 (1996).
3. Dale, D.C. & Hammond, W.P.T. Cyclic neutropenia: a clinical review. *Blood Rev.* **2**, 178–185 (1988).
4. Bieth, J.G. Leukocyte elastase. in *Handbook of Proteolytic Enzymes* (eds Barrett, A.J., Rawlings, N.D. & Woessner, J.F.) 54–60 (Academic Press, San Diego, 1998).
5. Takahashi, H., Nukiwa, T., Basset, P. & Crystal, R.G. Myelomonocytic cell lineage expression of the neutrophil elastase gene. *J. Biol. Chem.* **263**, 2543–2547 (1988).
6. Nuchprayoon, I. *et al.* PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2 β/CBF β proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. *Mol. Cell. Biol.* **14**, 5558–5568 (1994).
7. Sinha, S. *et al.* Primary structure of human neutrophil elastase. *Proc. Natl Acad. Sci. USA* **84**, 2228–2232 (1987).
8. Ohno, K., Brengman, J.M., Felice, K.J., Cornblath, D.R. & Engel, A.G. Congenital end-plate acetylcholinesterase deficiency caused by a nonsense mutation and an A→G splice-donor-site mutation at position +3 of the collagenlike-tail-subunit gene (COLQ): how does G at position +3 result in aberrant splicing? *Am. J. Hum. Genet.* **65**, 635–644 (1999).
9. Takahashi, H. *et al.* Structure of the human neutrophil elastase gene. *J. Biol. Chem.* **263**, 14739–14747 (1988).
10. Bode, W., Meyer, E. Jr & Powers, J.C. Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate specificity, and mechanism-based inhibitors. *Biochemistry* **28**, 1951–1963 (1989).
11. Navia, M.A. *et al.* Structure of human neutrophil elastase in complex with a peptide chloromethyl ketone inhibitor at 1.84-A resolution. *Proc. Natl Acad. Sci. USA* **86**, 7–11 (1989).
12. Cregge, R.J. *et al.* Inhibition of human neutrophil elastase. 4. Design, synthesis, X-ray crystallographic analysis, and structure-activity relationships for a series of P2-modified, orally active peptidyl pentafluoroethyl ketones. *J. Med. Chem.* **41**, 2461–2480 (1998).
13. Hung, S.H. & Hedstrom, L. Converting trypsin to elastase: substitution of the S1 site and adjacent loops reconstitutes esterase specificity but not amidase activity. *Protein Eng.* **11**, 669–673 (1998).
14. Whisstock, J., Lesk, A.M. & Carrell, R. Modeling of serpin-protease complexes: antithrombin-thrombin, α 1-antitrypsin (358Met→Arg)-thrombin, α 1-antitrypsin (358Met→Arg)-trypsin, and antitrypsin-elastase. *Proteins* **26**, 288–303 (1996).
15. Rawlings, N.D. & Barrett, A.J. Families of serine peptidases. *Methods Enzymol.* **244**, 19–61 (1994).
16. Le Bonniec, B.F. *et al.* Characterization of the P2′ and P3′ specificities of thrombin using fluorescence-quenched substrates and mapping of the subsites by mutagenesis. *Biochemistry* **35**, 7114–7122 (1996).
17. Belaaouaj, A. *et al.* Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nature Med.* **4**, 615–618 (1998).
18. Perlmutter, D.H., Glover, G.I., Rivetna, M., Schasteen, C.S. & Fallon, R.J. Identification of a serpin-enzyme complex receptor on human hepatoma cells and human monocytes. *Proc. Natl Acad. Sci. USA* **87**, 3753–3757 (1990).
19. Wakasugi, K. & Schimmel, P. Two distinct cytokines released from a human aminoacyl-tRNA synthetase. *Science* **284**, 147–151 (1999).
20. Berger, S.P. *et al.* Proteinase 3, the major autoantigen of Wegener's granulomatosis, enhances IL-8 production by endothelial cells in vitro. *J. Am. Soc. Nephrol.* **7**, 694–701 (1996).
21. Fasman, K.H., Letovsky, S.I., Li, P., Cottingham, R.W. & Kingsbury, D.T. The GDB Human Genome Database Anno 1997. *Nucleic Acids Res.* **25**, 72–81 (1997).
22. Cottingham, R.W. Jr, Idury, R.M. & Schaffer, A.A. Faster sequential genetic linkage computations. *Am. J. Hum. Genet.* **53**, 252–263 (1993).
23. Kruglyak, L., Daly, M.J., Reeve-Daly, M.P. & Lander, E.S. Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am. J. Hum. Genet.* **58**, 1347–1363 (1996).