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Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutières syndrome at the *AGS1* locus

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Aicardi-Goutières syndrome (AGS) presents as a severe neurological brain disease and is a genetic mimic of the sequelae of transplacentally acquired viral infection[1,2]. Evidence exists for a perturbation of innate immunity as a primary pathogenic event in the disease phenotype[3]. Here, we show that *TREX1*, encoding the major mammalian 3′→5′ DNA exonuclease[4], is the *AGS1* gene, and AGS-causing mutations result in abrogation of TREX1 enzyme activity. Similar loss of function in the *Trex1*$^{-/-}$ mouse leads to an inflammatory phenotype[5]. Our findings suggest an unanticipated role for TREX1 in processing or clearing anomalous DNA structures, failure of which results in the triggering of an abnormal innate immune response.

Aicardi-Goutières syndrome (OMIM 225750) is a severe genetically determined encephalopathy, characterized by calcification of the basal ganglia and white matter, demyelination and raised levels of lymphocytes in the cerebrospinal fluid (CSF)[1,6]. These features mimic those of acquired *in utero* viral infection, so that AGS is sometimes mistaken for the sequelae of congenital infection[2], and the exclusion of common perinatal infections is a diagnostic criterion for AGS. This diagnostic distinction has obvious importance, given that AGS is an autosomal recessive disorder whose true nature is often only recognized upon the birth of a second affected child.

Much evidence suggests that the pathogenesis of AGS is related to an aberrant immune response. We and others have drawn attention to the immunological characteristics of AGS, including the consistent finding of raised levels of interferon alpha (IFN-α) in the CSF and serum[7] and the observation of sterile pyrexias, a polygammaglobulinaemia and Coombs' positive hemolytic anemia in some affected individuals[3,6]. Reports have also highlighted the phenotypic overlap of AGS with the autoimmune syndrome systemic lupus erythematosus (SLE)[8–10]. In this regard, it is of note that intrathecal levels of IFN-α are raised in cerebral lupus[11], and serum levels of IFN-α are elevated in SLE[12] but not in other autoimmune disorders[13]. Intracranial calcification with a predilection for the basal ganglia occurs in up to 30% of individuals with cerebral SLE[14], and tubuloreticular inclusions, ultrastructural features related to the presence of circulating IFN-α, are found in SLE[15] as well as in AGS[6]. Additionally, the vasculitic skin lesions observed in AGS[16] (**Supplementary Figure 1** online), with IgM deposition at the dermal/epidermal junction, also suggest an underlying immune pathology.

AGS is a genetically heterogeneous disorder. We have previously localized[17] and subsequently refined[3] the position of a gene causing AGS on chromosome 3p21 (*AGS1*) and demonstrated that it is allelic to Cree encephalitis, a severe infantile neurodegenerative disorder found among the Canadian Cree tribe[18]. Subsequently, we defined, again by linkage analysis in consanguineous families, a second disease

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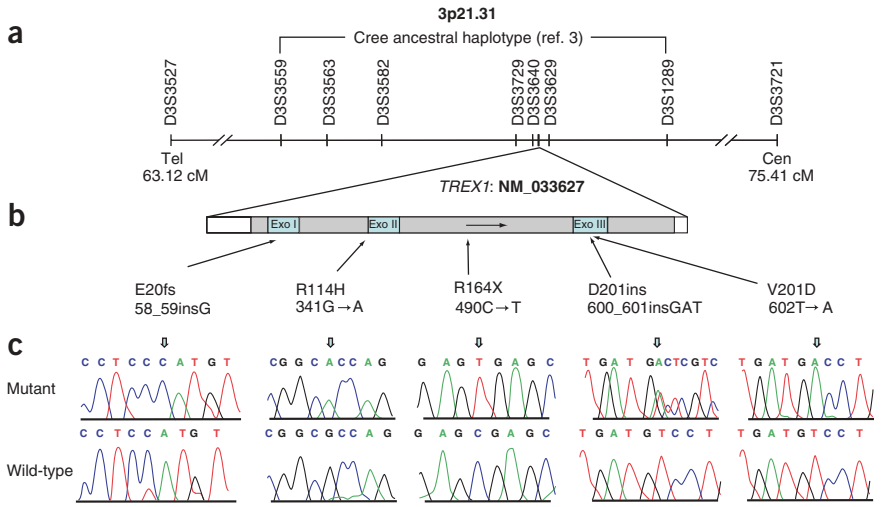

Figure 1 Schematic of the *AGS1* critical interval and *TREX1* gene depicting the location of identified mutations. (**a**) Genetic map of chromosome 3p21.31. The *AGS1* critical interval was previously defined by the markers D3S3527 and D3S3721 with a smaller ancestral haplotype identified in children affected by Cree encephalitis between D3S3559 and D3S1289. (**b**) *TREX1* comprises a single exon with an ORF encoding a 314-amino acid protein. Gray filled bars represent coding sequence. Three exonuclease motifs, containing Mg^{2+}-coordinating aspartic acid residues required for catalysis, are indicated[25]. The locations of mutations are indicated by arrows, with mutation position enumerated relative to the translational start site and the corresponding amino acid change shown in bold. (**c**) Sequence electropherograms showing *TREX1* mutations (reverse sequence shown for 58_59insG).

locus (*AGS2*) on chromosome 13q14-21 (ref. 19). Recently, we have identified mutations in the three non-allelic components of the RNase H2 protein complex in individuals with AGS[20]. However, none of these genes corresponds to the original *AGS1* locus.

The 3′→5′ DNA exonuclease TREX1 is the only annotated nuclease in the *AGS1* critical region (May 2004 assembly, University of California Santa Cruz (UCSC) Genome Browser). Furthermore, *Trex1*$^{-/-}$ mice show inflammatory myocarditis, suggesting a possible role for this enzyme in immune regulation[5]. Similarly, mice deficient in the lysosomal DNA endonucleases DNase I and II do not clear DNA derived from apoptotic cells, leading to an activation of innate immunity[21,22]; additionally, mutations in the gene encoding DNase I have been found in individuals with SLE[23]. For these reasons, we considered *TREX1* to be an attractive *AGS1* gene candidate.

The *TREX1* gene comprises a single exon with an ORF encoding a 314–amino acid protein that is expressed in all mammalian tissues examined and acts as a homodimeric 3′→5′ exonuclease[24,25]. We screened the *TREX1* gene in a panel of affected individuals satisfying diagnostic criteria for AGS as previously described[17] (**Supplementary Note** online).

We identified five distinct *TREX1* mutations in ten AGS families (**Fig. 1** and **Supplementary Table 1** online). A 341G→A transition was seen in both the homozygous and compound heterozygous state in seven European pedigrees (**Table 1**). This results in a nonconservative R→H substitution at a residue predicted to be involved in protein dimerization[26]. A second mutation, insGAT, duplicates a catalytic site aspartate residue involved in divalent cation binding. In a Turkish family, we found a homozygous missense mutation, 602T→A, in this same exonuclease (Exo) III motif. We also identified two protein-truncating mutations. The first of these was a homozygous frameshift, 58_59insG, in a Pakistani family. The second was a homozygous nonsense mutation at codon 164. This mutation was found in individuals with Cree encephalitis, confirming our previous conclusion, from genetic linkage studies, that this disorder is allelic to AGS[3]. All of these *TREX1* mutations segregated with the disease in the families investigated, and we found all tested parents to be heterozygous for the mutations. None of the mutations was present in 160 control alleles of European descent.

We measured the TREX1 exonuclease activity in cell extracts with poly(dA) as substrate. We used a simple one-step purification of the extract on single-stranded DNA-cellulose to remove unspecific phosphodiesterase activity[5]. Positive controls of extracts of a lymphoblastoid cell line and two fibroblast lines contained readily detectable TREX1 activity, with the enzyme present at a higher level in lymphoblastoid cells than in fibroblasts (**Fig. 2a**). In contrast, cell lines of lymphoblastoid and fibroblast origin derived from Dutch individuals

Table 1 Mutations in *TREX1* in individuals with Aicardi-Goutières syndrome

Family	Ancestry	Nucleotide alterations	Amino acid alterations	Segregation	Parental consanguinity
F39A	Dutch	341G→A	R114H	Hom, P	Yes
F39B	Dutch	[341G→A]+[600_601insGAT]	[R114H]+[D201ins]	Het, M, P	No
F40	Cree Indian	490C→T	R164X	Hom, M, P	Yes
F41	Pakistani	58_59insG	E20fs	Hom, nps	Yes
F42	British, of European descent	341G→A	R114H	Hom, nps	No
F43	German, of European descent	341G→A	R114H	Hom, M, P	No
F44	Mixed European/Afro-Caribbean	[341G→A]+[600_601insGAT]	[R114H]+[D201ins]	Het, nps	No
F45	Irish	341G→A	R114H	Hom, M, P	Yes
F46	Dutch, of European descent	341G→A	R114H	Hom, M, P	No
F47	Turkish	602T→A	V201D	Hom, nps	Yes

cDNA and amino acid numbering was determined using the TREX1 protein transcript AAK07616 and the nucleotide sequence NM_033627 (with the A at 2986 as the first base of the initiating ATG codon). ins, insertion; fs, frameshift. Hom, homozygous in affected individual; Het, compound heterozygous in affected individual; M, mutation identified in mother; P, mutation identified in father; nps, no parental samples. All mutations were absent from 160 control alleles of European descent.

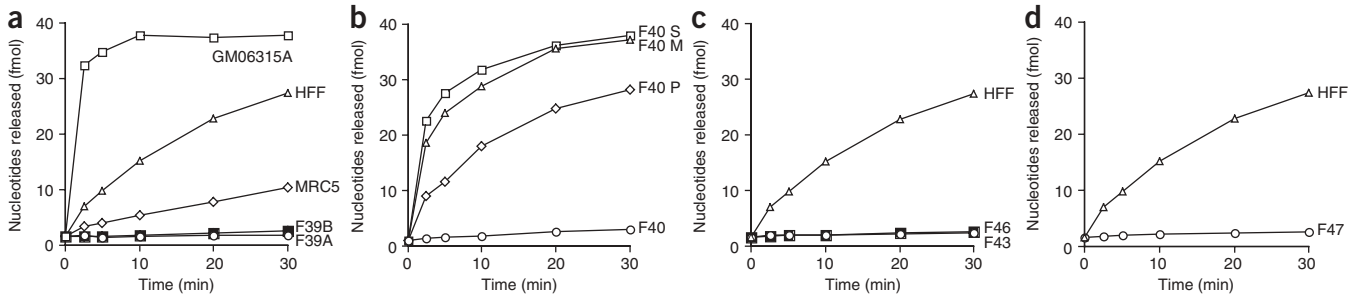

Figure 2 DNA 3′→5′ exonuclease activity in AGS cells. Cell-free protein extracts were assayed for 3′ DNA exonuclease activity with a 3′-end-labeled poly(dA) substrate after partial purification by column chromatography on single-stranded DNA cellulose. The proband of a family, or unaffected heterozygous mother (M), father (P) or sibling (S), are designated as in **Table 1**. GM06315A (NIGMS) is a normal lymphoblastoid control, and MRC5 (American Type Culture Collection) and HFF (human foreskin) are normal fibroblast controls.

with AGS (F39A, homozygous for the common R114H amino acid substitution (**Table 1**), and F39B) showed no detectable TREX1 activity (**Fig. 2a**).

A lymphoblastoid line from an individual with Cree encephalitis with a protein-truncating mutation (F40; **Table 1**) also had no detectable TREX1 activity, whereas extracts of cell lines from the heterozygous parents and an unaffected sibling showed normal levels of TREX1 activity in enzyme assays (**Fig. 2b**). Moreover, the independently derived AGS fibroblast cell lines from individuals F43 and F46, carrying the same *TREX1* mutation as F39A, and from F47, carrying a different, catalytic-site mutation (**Table 1**), showed no detectable TREX1 activity (**Fig. 2c**,**d**). The data show a consistent correlation between mutations in the *TREX1* gene that are likely to lead to loss of function and the absence of detectable TREX1 DNA 3′-exonuclease activity. The same experimental approach has been used previously to verify the absence of detectable TREX1 enzyme activity in extracts of mouse *Trex1*-null cells[5].

TREX1, previously called DNase III, is the major DNA-specific 3′-exonuclease measured in mammalian cells[4]. To explore a possible DNA editing role for TREX1 during lagging-strand DNA synthesis or a gap-filling role during DNA repair, we had previously generated *Trex1*$^{-/-}$ mice by targeted disruption of the *Trex1* gene in embryonic stem cells[5]. We were surprised to find that these mice showed no increase in spontaneous mutation frequency or cancer incidence. This is now seen to be consistent with the fact that individuals with AGS do not show an increased cancer risk, despite the known survival of several affected individuals into their third decade (Y.J.C., personal observation). Rather, *Trex1* knockout mice demonstrated an inflammatory myocarditis with progressive dilated cardiomyopathy, which was not consequent upon an increased susceptibility to cardiotoxic viral infection. Of interest, a cardiomyopathy has been reported in AGS on at least one occasion[2], and another affected child known to us had such a severe prenatal-onset cardiomegaly that a clinical diagnosis of glycogen storage disease II (Pompe's disease) was made.

Although there was a markedly increased morbidity of *Trex1*-null mice post-weaning, these mice did not show any obvious neurological defects. This observation indicates differences between individuals with AGS and *Trex1*$^{-/-}$ mice in expression of a pathogenic phenotype associated with the common molecular defect. Similarly, loss of peripheral tolerance is associated with mutations in the *PDCD1* gene in individuals with SLE but with either autoimmune myocarditis with dilated cardiomyopathy or a lupus-like disease in *Pdcd1*$^{-/-}$ mice, depending on the strain background[27].

At the level of its clinical presentation, AGS is a notable mendelian mimic of the sequelae of congenital viral infection[1,2]. These resemblances are so strong as to suggest the likelihood of common mechanisms involved in the pathogenesis of the inherited and the infectious syndromes[3]. Consistent with this notion, both AGS and a number of placentally acquired viral infections are characterized by the production of high levels of IFN-α (refs. 7,28). That this IFN-α may have a pathogenic role in the disease is indicated by the observation that astrocyte-specific chronic overproduction of IFN-α in transgenic mice recapitulates the neuropathological findings seen in AGS[29]. How, then, would dysfunction of TREX1 (or RNase H2) lead to this immunological induction? One possibility concerns the innate immune response to viral infection, which depends on the cell's ability to recognize and respond to viral nucleic acid while ensuring that recognition of endogenous DNA does not normally occur[30]. Our finding, that mutations in the gene encoding the major cellular 3′→5′ DNA exonuclease and in the apparently unrelated RNase H2 complex underlie AGS, suggests an intriguing possibility: that the two enzymes function in the same DNA processing pathway such that failure of these nuclease activities may result in the survival and accumulation of intracellular DNA repair and replication intermediates, which then trigger an inappropriate viral-like innate immune response.

METHODS

Affected individuals and families. All affected individuals included in this study fulfilled diagnostic criteria for Aicardi-Goutières syndrome, with neurological features of an early onset encephalopathy, negative investigations for common prenatal infections, intracranial calcification in a typical distribution, a CSF lymphocytosis >5 cells/mm^3 and/or >2 international units (IU)/ml of IFN-α in the CSF. With consent, blood samples were obtained from affected children, their parents and unaffected siblings. Genomic DNA was extracted from peripheral blood leukocytes by standard methods. The study was approved by the Leeds Teaching Hospitals Research Ethics Committee, the Multicentre Research Ethics Committee (reference number 04:MRE00/19); the Eeyou Awaash Foundation and the Cree Board of Health and Social Services of James Bay, Canada.

Mutation detection. Primers were designed to amplify the coding exon of *TREX1*. Purified PCR amplification products were sequenced using dye-terminator chemistry and electrophoresed on a MegaBace500 (Amersham Pharmacia) capillary sequencer.

Preparation of cell extracts and enzyme assay. Cell-free extracts were prepared from exponentially growing cultures (10^7 cells; 40–50 μl packed cell volume). Frozen cell pellets were thawed and incubated on ice for 30 min in ten volumes of lysis buffer (50 mM Tris-HCl pH 8.0, 125 mM NaCl, 1% Nonidet P-40,

2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride). After centrifugation (14,000*g*, 10 min), the supernatant was further purified by column chromatography on denatured DNA cellulose (GE Healthcare) equilibrated in 50 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol (15 µg protein per 1 µl bed volume). The column was washed in buffer containing 400 mM NaCl, bound protein was eluted with three column volumes 1.5 M NaCl, and fractions were assayed for 3′ DNA exonuclease activity, as described previously[5]. Briefly, reaction mixtures (100 µl) contained 2 µl protein eluate, 0.01 µg of radiolabeled poly(dA) substrate (3′ end-labeled by the incorporation of [^{32}P]dAMP by terminal transferase) in 50 mM Tris-HCl (pH 8.5), 4 mM $MgCl_2$, 1 mM DTT, 10 µg bovine serum albumin. After incubation at 37 °C for the times indicated, the substrate was ethanol precipitated, and the quantity of nucleotides released (in fmol) was determined by scintillation counting of ethanol-soluble radioactive material.

Accession codes. GenBank: cDNA and amino acid numbering was determined using the TREX1 protein transcript AAK07616 and the nucleotide sequence NM_033627 (with the A at 2986 as the first base of the initiating ATG codon).

URLs. The UCSC Genome Browser is available at http://genome.ucsc.edu/. The Marshfield chromosome 3 genetic map is found at http://research.marshfieldclinic.org/genetics.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y.J.C. was responsible for the development of the project and wrote the paper with D.T.B., A.P.J., D.E.B. and T.L. B.E.H., R.P., M.A., A.L. and A.P.J. performed mutation screening of patients and controls. P.R. performed the TREX1 enzyme assays. All other authors provided clinical samples and data.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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