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Ribosomal Protein SA Haploinsufficiency in Humans with Isolated Congenital Asplenia

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Isolated congenital asplenia (ICA) is characterized by the absence of a spleen at birth in individuals with no other developmental defects. The patients are prone to life-threatening bacterial infections. The unbiased analysis of exomes revealed heterozygous mutations in *RPSA* in 18 patients from eight kindreds, corresponding to more than half the patients and over one third of the kindreds studied. The clinical penetrance in these kindreds is complete. Expression studies indicated that the mutations carried by the patients - a nonsense, a frameshift duplication and five different missense - cause autosomal dominant ICA by haploinsufficiency. *RPSA* encodes ribosomal protein SA, a component of the small subunit of the ribosome. This discovery establishes an essential role for RPSA in human spleen development.

Patients with isolated congenital asplenia (ICA) are born without a spleen and display no other known developmental anomalies (MIM 271400) (*1*–*3*). Only 73 patients from 48 kindreds have been reported to date (*1*, *3*–*6*). We recruited an international cohort of 33 ICA patients from 23 kindreds (fig. S1, table S1). Most patients with ICA, particularly the index cases, died in childhood from invasive bacterial disease (*1*). Due to the high proportion of familial cases (*1*), we hypothesized that ICA might result from single-gene inborn errors of spleen development. Moreover, ICA seems to segregate as an autosomal dominant (AD) trait in five multiplex kindreds (A-E in fig. S1). We have reported a candidate heterozygous mutation in *NKX2-5* in one kindred with AD ICA (*7*), but the genetic etiology of ICA remains essentially unknown. We therefore set out to decipher the main genetic etiology of ICA, both to cast light on the development of the human spleen and to guide clinical care and genetic counseling in families with ICA.

Given the apparent clinical homogeneity of the ICA patients, we hypothesized that there would be at least some genetic homogeneity among the 23 kindreds studied. We therefore sequenced one exome (*8*–*11*) from each of the 23 kindreds, including the kindred bearing the *NKX2-5* mutation, and analyzed them together (fig. S1, table S2). We hypothesized that the disease-causing variants would be very rare, due to the rarity of ICA (*1*). We also gave priority to coding mutations predicted not to be silent (non-synonymous). We found that 764 genes in at least two ICA kindreds carried very rare and non-synonymous mutations (table S3). We performed the same analysis on 508 control exomes sequenced in-house (table S4), to identify the best candidate morbid gene for ICA. We then used the results of these two analyses (comparison of ICA and controls) to test the null hypothesis that mutations in a given gene were not specific to ICA, by calculating the *p*-value for each gene in Fisher's exact test. *RPSA* had a highly significant *p*-value of 2.89×10^{-11} (Fig. 1A), indicating that mutations in this gene were specific to the ICA cohort. The coding region of *RPSA* carried very rare non-synonymous variants in eight of 23 ICA kindreds and in only one of the 508 control exomes. No other gene had a statistically significant *p*-value (table S5).

RPSA encodes the ribosomal protein (RP) SA. The genes encoding RPs have numerous pseudogenes (*12*), which can hinder their sequencing. RPSA has 61 processed pseudogenes (table S6) (*12*). We thus Sanger sequenced all coding exons of *RPSA* in all 33 ICA patients, using pri-

mers mapping to the introns of *RPSA*, which cannot amplify *RPSA* pseudogenes (*13*). Eighteen of the 33 patients (55%) had *RPSA* mutations (Fig. 1B, fig. S2). Altogether, we identified seven mutations in eight kindreds: one frameshift duplication (p.P199SfsX25), one nonsense (p.Q9X) and five missense mutations, including the recurrent p.R180G mutation (table S7). A missense mutation, p.M185V, was identified in one control exome from a patient displaying severe viral infection, but not ICA. The seven ICA mutations were not present in more than 10,000 alleles reported in public databases (table S8). Moreover, the five ICA-associated missense mutations affected residues strictly conserved in mammals, vertebrates, and even yeast (fig. S3). All ICA patients in these eight kindreds carried a mutation in *RPSA* and all individuals carrying *RPSA* mutations displayed ICA (Fig. 1B).

Strikingly, neither of the two parents carried an *RPSA* mutation in kindreds F, O and T, although a mutation was found in the two affected siblings in kindred F and in the sporadic patients in kindreds O and T (Fig. 1B). Microsatellite analysis confirmed the parental relationships of the samples (table S9, fig. S4). Thus, mutations in kindreds O and T appeared de novo and resulted from a germline mosaicism in kindred F. Moreover, a comparison of the haplotypes at the *RPSA* locus between patients from families A and D showed that the p.R180G mutation was not inherited from a common ancestor (a founder effect), but had instead occurred twice, independently (fig. S5). This is consistent with the complete penetrance of *RPSA* mutations for ICA and the high mortality of ICA, because a founder effect would require the existence of multiple generations of healthy *RPSA* heterozygotes (fig. S5), before the advent of antibiotics. Collectively, these genetic results suggest that heterozygous coding mutations in *RPSA* underlie most cases of ICA, with apparently complete clinical penetrance. In particular, heterozygous coding mutations in *RPSA* were found to underlie ICA in all multiplex kindreds displaying an AD pattern of inheritance studied, including the kindred with the heterozygous mutation in *NKX2-5* (ICA-B, (*7*)).

Our identification of two mutations resulting in a premature termination codon (p.Q9X and p.P199SfsX25) led us to hypothesize that haploinsufficiency at the *RPSA* locus might underlie AD ICA. TA-cloning of cDNA generated from activated T cells of three patients from family C showed that less than 10% (12/160) of the transcripts carried the P199SfsX25 mutation (Fig. 2A), suggesting that the mRNAs generated from the mutated allele were subject to nonsense-mediated mRNA decay (fig. S6). *RPSA* mRNA levels in activated T cells from these patients were only half those in their healthy relative (Fig. 2B). We then investigated the missense mutations, by overproducing the N-terminally FLAG-tagged mutant and WT proteins in HEK293T cells. The mutant proteins were produced in much smaller amounts than the WT protein (Fig. 2C). We next determined whether *RPSA* was under purifying selection in the general population. *RPSA* is at the 2.8th percentile with respect to a metric of purifying selection (Fig. 2D) (*14*) among ~15,000 genes exome-sequenced by the 1,000 Genomes Project (*15*). These data suggest that heterozygosity for null *RPSA* alleles underlies AD ICA, possibly accounting for the strong purifying selection acting on these alleles in the population.

It is surprising that germline mutations in *RPSA* cause a spleen-specific developmental defect. *RPSA* is ubiquitously expressed. RPSA is involved in pre-rRNA processing (*16*), and is part of the small subunit of the ribosome (*17*). *RPSA* was not known to be involved in spleen development, which is controlled by a cascade of transcription factors (e.g., *Tlx1*, *Nkx2-5*, *Wt1*) in mice (*7, 18*). Moreover, haploinsufficiency of any of 10 other RPs, including RPS19, is associated with Diamond-Blackfan anemia (DBA), which is characterized by bone marrow failure and a broad range of developmental defects, ranging from craniofacial defects to thumb abnormalities (*19–21*). Patients with *RPSA* mutations present none of these phenotypes (table S10). Conversely, DBA patients mutated in other RPs display no spleen abnormalities. At the cellular level, there was no pre-rRNA processing defect in activated lymphocytes from RPSA-mutated ICA patients (fig. S7), contrasting with the pre-rRNA processing defects observed in all RP-mutated DBA patients (*20*). Lastly, heterozygosity for a null *Rpsa* allele in the mouse is not associated with ICA (fig. S8, table S11). We do not yet understand the pathogenesis of ICA. However, the emerging idea that ribosomes can be "specialized" might account for the narrow phenotype caused by mutations in *RPSA* (*22*). Alternatively, an extra-ribosomal function of RPSA (*23*) may explain the phenotype. The surprising connection between RPSA and spleen development in humans calls for explorations of the underlying mechanisms.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1234864/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 to S11
References (*24*–*39*)

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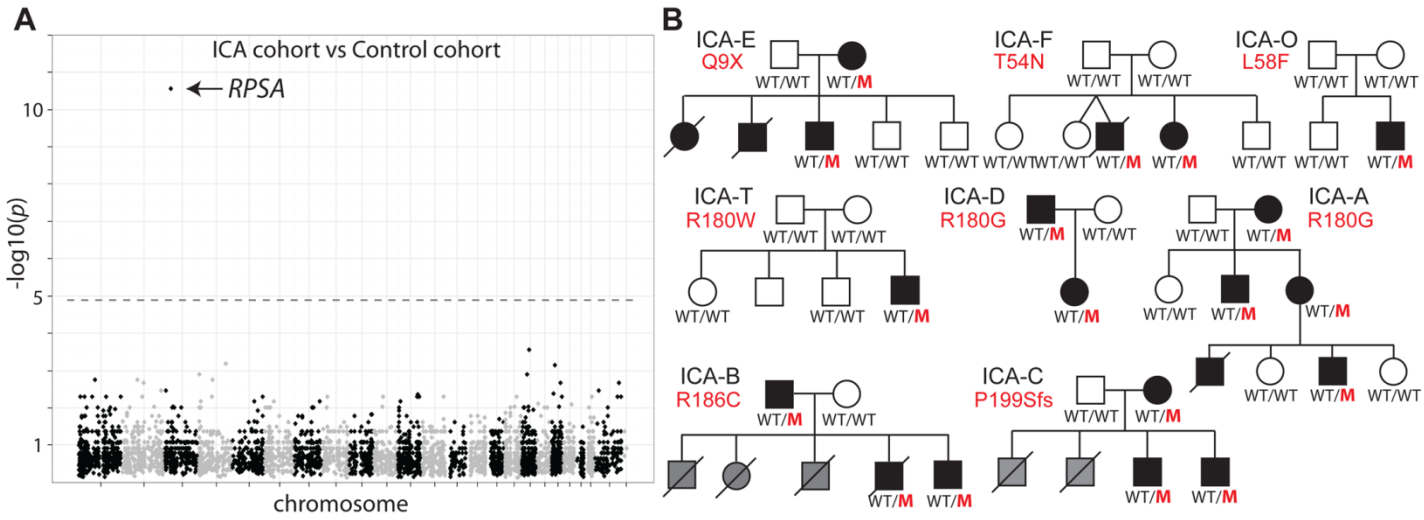

Fig. 1. *RPSA* heterozygous coding mutations are the most frequent genetic etiology of ICA. (**A**) Manhattan plot showing the *p*-value for tests of the hypothesis that "mutations in a given gene were not specific to the ICA cohort". Each dot represents one gene. *X* axis: physical position of each gene on the chromosome. *Y* axis: -log10(*p*). *p* was calculated for Fisher's exact test comparing 23 exomes from 23 ICA kindreds and 508 exomes from patients with phenotypes other than invasive bacterial disease. The gray dashed line indicates threshold for statistical significance ($0.05/4{,}222=1.2\times10^{-5}$) (**B**) Familial segregation of all *RPSA* coding mutations. Mutations are described in red. Capital letters represent the kindred code. When available, the genotype of *RPSA* is indicated under each symbol. WT, wild-type; M, mutant. Black, ICA; gray, probable ICA.

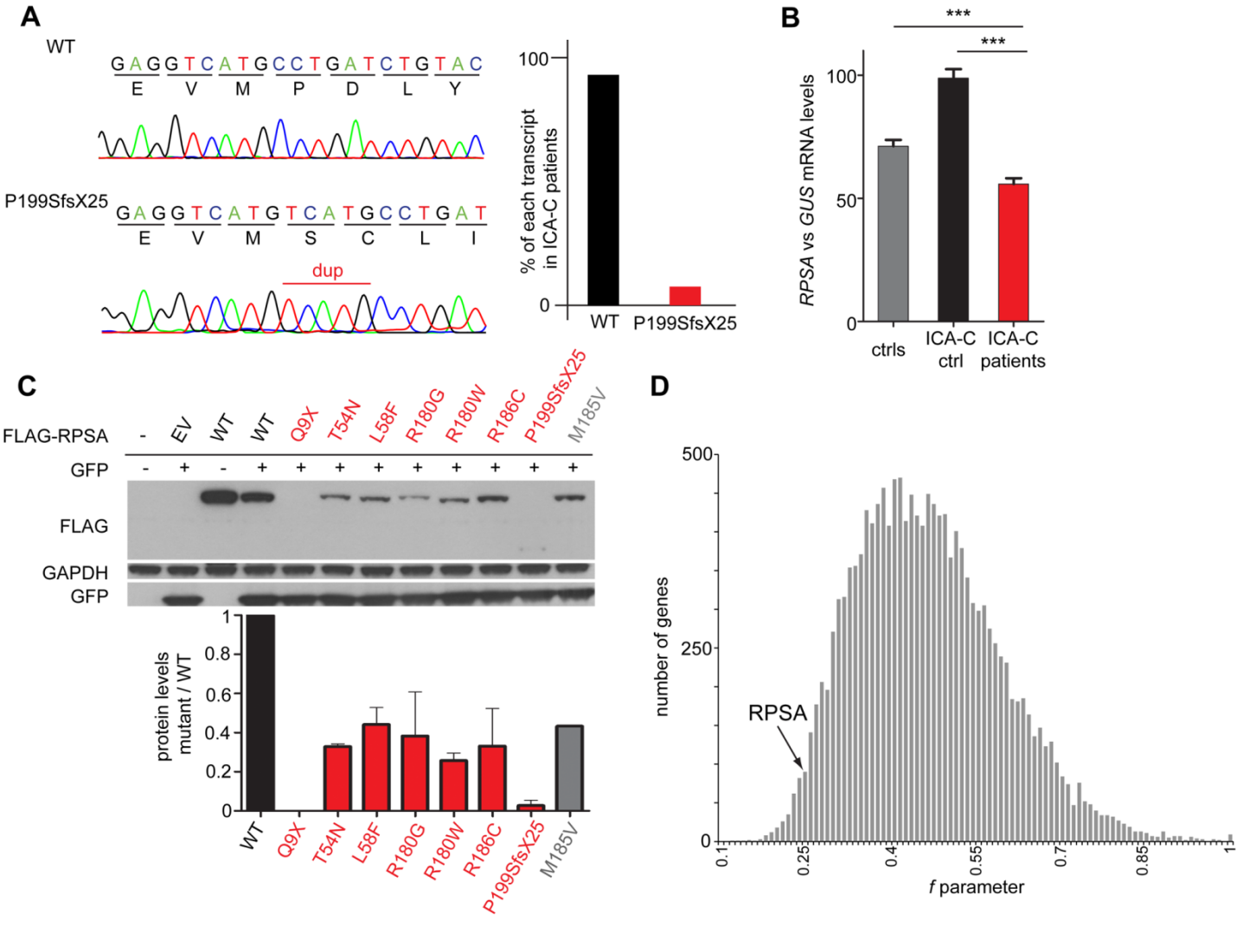

Fig. 2. Haploinsufficiency at the *RPSA* locus. (**A**) *RPSA* cDNA was obtained from activated T cells of patients ICA-C-I.2, ICA-C-II.3 and ICA-C-II.4. Sequences of WT and mutant cDNA are shown. The deduced frequency of each mRNA is indicated in the diagram on the right. (**B**) Relative levels of *RPSA* mRNA in activated T cells from three patients, a healthy member of kindred C (ICA-C-I.1), and four unrelated healthy controls. PBMCs were activated with PHA for 5 days. A mean of four independent experiments is shown. Error bars indicate the SEM. ***$p < 0.001$. (**C**) Immunoblot showing the levels of the WT and mutant RPSA proteins following overproduction in HEK293T cells. GAPDH, loading control; GFP, transfection control. The blot shown is representative of 4 independent experiments. Below: intensity of the bands corresponding to the FLAG antibody normalized with respect to the band from the GFP immunoblot. Error bars indicate the SEM. (**D**) Genome-wide distribution of the strength of purifying selection acting in 14,993 human genes. A low *f* estimate (*13*) indicates that the gene is particularly constrained.