Mutations in signal recognition particle \textit{SRP54} cause syndromic neutropenia with Shwachman-Diamond–like features

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Shwachman-Diamond syndrome (SDS) (OMIM #260400) is a rare inherited bone marrow failure syndrome (IBMFS) that is primarily characterized by neutropenia and exocrine pancreatic insufficiency. Seventy-five to ninety percent of patients have compound heterozygous loss-of-function mutations in the Shwachman-Bodian-Diamond syndrome (\textit{sbds}) gene. Using trio whole-exome sequencing (WES) in an \textit{sbds}-negative SDS family and candidate gene sequencing in additional \textit{SBDS}-negative SDS cases or molecularly undiagnosed IBMFS cases, we identified 3 independent patients, each of whom carried a de novo missense variant in \textit{srp54} (encoding signal recognition particle 54 kDa). These 3 patients shared congenital neutropenia linked with various other SDS phenotypes. 3D protein modeling revealed that the 3 variants affect highly conserved amino acids within the GTPase domain of the protein that are critical for GTP and receptor binding. Indeed, we observed that the GTPase activity of the mutated proteins was impaired. The level of \textit{SRP54} mRNA in the bone marrow was 3.6-fold lower in patients with \textit{SRP54}-mutations than in healthy controls. Profound reductions in neutrophil counts and chemotaxis as well as a diminished exocrine pancreas size in a \textit{SRP54}-knockdown zebrafish model faithfully recapitulated the human phenotype. In conclusion, autosomal dominant mutations in \textit{SRP54}, a key member of the cotranslation protein-targeting pathway, lead to syndromic neutropenia with a Shwachman-Diamond–like phenotype.

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Shwachman-Diamond syndrome (SDS) (OMIM #260400) is a rare inherited bone marrow failure syndrome (IBMFS) that is primarily characterized by neutropenia and exocrine pancreatic insufficiency. Seventy-five to ninety percent of patients have compound heterozygous loss-of-function mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene. Using trio whole-exome sequencing (WES) in an SBDS-negative SDS family and candidate gene sequencing in additional SBDS-negative SDS cases or molecularly undiagnosed IBMFS cases, we identified 3 independent patients, each of whom carried a de novo missense variant in SRP54 (encoding signal recognition particle 54 kDa). These 3 patients shared congenital neutropenia linked with various other SDS phenotypes. 3D protein modeling revealed that the 3 variants affect highly conserved amino acids within the GTPase domain of the protein that are critical for GTP and receptor binding. Indeed, we observed that the GTPase activity of the mutated proteins was impaired. The level of SRP54 mRNA in the bone marrow was 3.6-fold lower in patients with SRP54-mutations than in healthy controls. Profound reductions in neutrophil counts and chemotaxis as well as a diminished exocrine pancreas size in a SRP54-knockdown zebrafish model faithfully recapitulated the human phenotype. In conclusion, autosomal dominant mutations in SRP54, a key member of the cotranslation protein-targeting pathway, lead to syndromic neutropenia with a Shwachman-Diamond–like phenotype.

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Introduction

Shwachman-Diamond syndrome (SDS) (OMIM #260400), an inherited bone marrow failure syndrome (IBMFS), is a rare multisystem disorder that was first recognized more than 50 years ago (1–4). Its cardinal features include exocrine pancreatic insufficiency and hematological abnormalities in which neutropenia is para-
mount and can be life threatening. Skeletal abnormalities, neurocognitive deficits, and a variety of other diverse symptoms are also associated with the disease (5). Like many other IBMFSs, patients with SDS have an increased risk of myelodysplasia and malignant transformation, especially acute myeloid leukemia (6). Approximately 75% to 90% of SDS patients have compound heterozygous loss-of-function mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene (7–9). Since the identification of SBDS in 2003, only 2 other causal SDS genes, DNAJC21 (10, 11) and EFL1 (12), have been reported, in contrast to other IBMFSs, for which the situation is the opposite (e.g., >20 loci in Fanconi anemia, >10 loci in Diamond-Blackfan anemia, and several loci in severe congenital neutropenia) (13).

The SBDS protein plays a central role in ribosome biogenesis, as it is involved in the maturation of the 60S subunit (as are DNAJC21 and EFL1) prior to the assembly of the actively translating 80S ribosome (14, 15). During the biogenesis and initiation of protein translation, the ribosome associates with many partner molecules, and of these, the signal recognition particle (SRP) is a major one. SRP is a ribonucleoprotein (RNP) complex that mediates the targeting of nascent signal sequence–carrying polypeptides (including both membrane and secretory proteins) to the translocon at the surface of the ER (16). The mammalian SRP is composed of a single RNA molecule (the ~300-nucleotide-long 7SL RNA) and the following 6 polypeptides: SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72 (17). SRP54 plays a central role in this RNP complex. With its M domain, SRP54 binds to the SRP19-bound 7SL RNA and associates with the signal peptide of the nascent polypeptide chain. Then, via its N and G domains, SRP54 binds to the SRP receptor (SR) on the ER membrane (18, 19). Ultimately, SRP54 forms a link between the SRP and the ER membrane and allows insertion of the signal peptide–carrying polypeptide into the translocation channel. In addition to the SRP RNA, SRP54 is the only other universally conserved SRP component. Here, we demonstrate that mutations in SRP54 can lead to syndromic neutropenia with SDS-like features.

Results
Clinical characteristics of patients. The clinical features of the 3 patients are summarized in Supplemental Table 1 (supplemental material available online with this article; https://doi.org/10.1172/JCI92876DS1). Patient AII.1 was a white male and the second child of healthy nonconsanguineous parents (Figure 1A). The patient was born after a full-term, uneventful pregnancy and presented with neonatal transitory respiratory distress, hypotonia, and central apneas. Morphological examination of the newborn revealed small size (-1.5 SD), low weight (-1.5 SD), an occipito-frontal circumference (OFC) of +1.5 SD, low-set, asymmetric ears, thinning hair, frontal angioma, mandibular microretrognathism, a high-arched palate, small teeth, and a pectus carinatum—a series of symptoms that are all uncommon in typical SDS patients. Complete blood counts in the first week of life showed severe neutropenia (0.28 × 10^9/L and 0 × 10^9/L). Hemoglobin (15.8 g/dl) and platelet (265 × 10^9/L) counts were normal. He received granulocyte-CSF (G-CSF) injections, with no effect on neutrophil counts. A bone marrow smear was hypolobular and showed hypoplasia in the neutrophil lineage with retarded granulocyte maturation. Moreover, numerous cytoplasmic vacuoles were observed in myeloblasts, promyelocytes, and apoptotic cells of the granulocyte lineage (Supplemental Figure 1A). Of note, none of the erythroblasts contained vacuoles, and Perl’s staining revealed no ring sideroblasts, thus excluding Pearson syndrome. Furthermore, iron was detected in macrophages using the Prussian blue reaction. To definitively exclude a mitochondrial DNA disorder (i.e., Pearson syndrome), whole mitochondrial DNA analysis was performed using next-generation sequencing, which revealed no pathogenic mutations or rearrangements (Supplemental Figure 2).

The patient’s lymphocyte immunophenotype results and serum Ig levels were normal. There were no alloimmunizations against neutrophils or granulocyte-specific antigens. Cardiac ultrasound showed large interauricular and interventricular septal defects. The cerebral MRI was normal. At 6 months of age, the patient had feeding difficulties and diarrhea. His weight, height, and OFC were 6.5 kg (-1.5 SD), 63 cm (-1.5 SD), and 45 cm (+1.5 SD), respectively. Stool analysis revealed very low elastase levels (<15 μg/g of stool; reference value: >200 μg/g of stool). Fatty infiltration of the pancreas was documented by CT (Supplemental Figure 3A). Blood levels of the fat-soluble vitamins A, D, E, and K were very low. At 11 months of age, the patient’s ventricular and atrial septal defects required surgery. Severe neutropenia led to infections including acute ethmoiditis and perianal abscesses. At 4 years of age, the patient underwent genodentical allogeneic hematopoietic cell transplantation (HCT) without major complications. His hematologic reconstitution was uneventful. The patient showed psychomotor developmental delay and autistic behavior. At 6 years of age, he continues to receive lipase substitution (see Supplemental Table 1). Direct sequencing of the SBDS gene was performed using genomic DNA isolated from pretransplantation peripheral blood cells and failed to identify any mutations, as did panel sequencing of all known IBMFS genes. Comparative genome hybridization (CGH) also excluded pathogenic structural variants and copy number variations (CNVs) throughout the genome, including inside and in the vicinity of SBDS.

Patient BII.1 was a 1-year-old white girl who was the only child of healthy nonconsanguineous parents (Figure 1A). At birth, her weight, height, and OFC were 3.26 kg (0 SD), 45.5 cm (-2 SD), and 32.5 cm (-1 SD), respectively. She had no dysmorphic features. Repeated blood counts revealed severe neutropenia (e.g., 0.02 × 10^9/L), while her other blood parameters were normal, including fetal hemoglobin, cupremia, lymphocyte immunophenotyping, and Ig levels. No alloimmunization against granulocytes was found. Cerebral MRI as well as abdominal and cardiac ultrasound imaging results were normal. Her fecal elastase level was very low (<15 μg/g of stool; reference value: >200 μg/g of stool). Abdominal CT showed a fatty infiltration of the pancreas (Supplemental Figure 3B). At 6 months of age, the patient presented with facial cellulitis and ethmoiditis. She was given G-CSF without success. At 1 year of age, she underwent unrelated cord blood transplantation. Pretransplantation clinical examination revealed small stature (69 cm; ~2 SD), low weight (8.5 kg; -0.5 SD), and an OFC of 48 cm (+1 SD) (Supplemental Table 1). No overt developmental delay or psychomotor retardation was observed before she passed away at 16 months of age (see below). A blood cell count revealed a total absence of neutrophils. Her other blood count parameters
Patient CII.1 was a Hispanic boy and the second child of healthy nonconsanguineous parents (Figure 1A), who was born after a full-term, uneventful pregnancy. His birth weight was 3.24 kg (25th percentile), and no dysmorphic features or bony abnormalities were visible. He did not have any feeding or gastrointestinal problems. At 2 years of age, he was diagnosed with language delay and autism spectrum disorder. At the same time, he was noted as having anemia (hemoglobin 10.5 g/dl) with normal iron stores and neutropenia (0.76 × 10⁹/l). At 4 years of age, the patient was referred to the hematology clinic for evaluation of mild anemia (hemoglobin 9.4–10.5 g/dl), intermittent neutropenia (0.13 × 10⁹/l to 1.88 × 10⁹/l), fevers, and recurrent episodes of mucositis, otitis media, and cellulitis (Supplemental Table 1). Serial complete blood were normal. Anomalies similar to those in patient AII.1 were observed in the bone marrow smear of this patient, who also had no ring sideroblasts and no mitochondrial DNA anomalies (Supplemental Figure 1B and Supplemental Figure 2). Her ferritin level was normal (37 μg/l). Other cell lineages were normal. HCT and hematopoietic reconstitution were uneventful. Two months after transplantation, the patient developed multiorgan failure, which rapidly led to death. Postmortem pathology showed severe veno-occlusive disease. Direct sequencing of the SBDS gene (and panel sequencing of all other IBMFS genes) using genomic DNA isolated from pretransplantation peripheral blood cells failed to identify any mutations. No pathogenic structural variants were detected via CGH array, as was the case with the previous patient.

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counts showed a cyclical pattern of neutropenia ($0 \times 10^9/l$ to $2.92 \times 10^9/l$) lasting 13–15 days. He tested negative for anti-neutrophil antibodies as well as for mutations in an IBMFS gene panel that included $SBDS$ (the analysis was performed using genomic DNA extracted from peripheral blood) (Supplemental Table 1). His initial bone marrow evaluation at 4 years of age showed 80% cellularity with a maturation arrest at the myelocyte stage, while other cell lineages were normal (as reported in the patient’s medical records; images not available). At 8 years of age, he was started on G-CSF at 5 mcg/kg every other day, with improvement in anemia, neutrophil counts, mucositis, and infections. His early growth parameters showed height and weight in the 13th percentile, and these improved, reaching the 30th percentile with G-CSF therapy. He continues to do well on G-CSF therapy and is now 18 years of age.

In summary, all 3 patients presented with neutropenia associated with a host of various other symptoms, including exocrine pancreatic deficiency (2 of 3 patients) and/or autistic behavior (documented in 2 of 3 patients; not assessable in the third, patient BII.1, due to early death), along with a host of other morphological abnormalities and/or bone marrow morphology that are not typically observed in classical SDS (Supplemental Table 1). All 3 patients were negative for any mutations in all known IBMFS genes, including $SBDS$.

**Identification of a candidate gene.** Prior to exome sequencing, $SBDS$ single point mutations were excluded in all 3 cases using Sanger sequencing of all coding exons. $SBDS$ CNVs/structural variants were also excluded using CGH microarray analysis, as were mutations in other IBMFS genes using panel sequencing.

were included from the Hacettepe Bone Marrow Failure Center (Ankara, Turkey). Of these, 10 had neutropenia and/or bone marrow failure, while 8 had no cytopenias. Steatorrhea or abnormal exocrine pancreatic function was noted in 18 of these patients, and failure to thrive was noted in 16 of them. (b) Twenty $SBDS$-negative patients were included from the North American Shwachman-Diamond Syndrome Registry and local US Bone Marrow Failure Registries, including thirty-three with neutropenia and/or bone marrow failure and one with no cytopenias. Steatorrhea or abnormal exocrine pancreatic function was noted in 10 patients, and failure to thrive was noted in 8. (c) Thirty cases were included from a mix of French and European case-by-case referrals. Of the latter cohort, the only available information was the presence of severe congenital neutropenia and/or a clinical SDS phenotype. During the course of this screening, we identified a second family (family B) that harbored a second de novo $c.343A>G$ (p.T115A) missense mutation in the $SBDS$ gene. The variant filtering strategy and the identified variants are summarized in Supplemental Figure 4 and Supplemental Table 2, respectively. All 3 mutations affected highly conserved amino acids located in the GTPase domain of the protein (Figure 1, C and D). The mutations have deleterious predictive values, as indicated by various bioinformatics analyses (Table 1). In addition, none of the 3 variants was found in the 1000 Genomes database (http://www.internationalgenome.org/1000-genomes-browsers/), the Exome Aggregation Consortium (ExAC) Browser (http://exac.broadinstitute.org/), our internal exome database, or various other databases that altogether contain more than 100,000 exomes. All 3 mutations and their de novo nature were validated using Sanger sequencing in all probands and their parents. Finally, the mutations

| Table 1. Details of SRP54 sequence alterations in the 3 patients |
|--------------------------|--------------------------|--------------------------|
|                         | Patient AII.1            | Patient BII.1            | Patient CII.1            |
| Nucleotide alteration    | Chr14:35482592G>A        | Chr14:35476576A>G        | Chr14:35476582,35476584del|
| Exon                     | 8                        | 4                        | 4                        |
| Coding sequence alteration| c.677G>A                  | c.343A>G                  | c.349_351del             |
| Predicted causal effect   | Disease causing           | Disease causing           | Disease causing           |
| GERP++_NRI               | 5.11                     | 5.5                       | NA                       |
| Polyphen (HumDiv)        | 0.793                    | 0.999                     | NA                       |
| Mutation Assessor        | 3.89                     | 4.66                      | NA                       |
| SIFT_score               | 0                        | 0                         | 0                        |
| LRT_score                | 0                        | 0                         | NA                       |
| GERP++_NR                | 5.11                     | 5.5                       | NA                       |

ClinVar accession no. SCV000583969 SCV000583970 SCV000583971

Predictive analyses were performed using MutationTaster (57), Polyphen-2 v2.2.2r398 (58), and the GATK analysis toolkit (59). Positions refer to Hg19 (GRCh37). Positions refer to GenBank transcript NM_003136.3. The closer the phastCon value is to 1, the more probable the nucleotide is conserved. Values varied between -14 and +6. Sites predicted to be fast evolving are assigned negative scores. Scores not available for deletions (indels are not supported). Function prediction tool. Variants with scores between 0.85 and 1.0 are highly confidently predicted to be damaging. Function prediction tool. Deleterious threshold: >0.85. Function prediction tool based on protein sequence conservation among homologs. Variants with scores between 0 and 0.05 are considered deleterious. $DNA$ sequence evolutionary model expressed as a $P$ value. $DNA$ sequence conservation score. Deleterious threshold: >4.4.
in SRP54 and G233, T232, and D43 in the receptor, without major rearrangements of the local protein fold (Figure 2B). This was confirmed by the modification of the electrostatic surface induced by this mutation (Supplemental Figure 5). The mutation p.T115A in family B most likely directly affects GTP binding, because T115 forms an important hydrogen bond with GTP that is lost when the residue is mutated to alanine (Figure 2C). The mutation p.T117del in family C might also impact GTP binding. Residues 115–117 correspond to a threonine triplet. When 1 of these threonines is deleted, the helix structure at Gly113 starts earlier than expected, while the 112–115 region forms extensive H-bonds with GTP. However, because the region before G113 is a loop region, it is difficult to determine the impact of this mutation on GTP binding (Figure 2D). The p.T117del mutation is therefore likely to have a milder effect than the other mutations (see also below).

In vivo and in vitro functional impact of the mutations. The level of SRP54 mRNA in (pretransplantation) bone marrow mononuclear cells from patients AII.1 and kidney (patient BII.1), thereby confirming the germline nature of these mutations.

Figure 2. Structural impact of SRP54 mutations. (A) Molecular model of the N and G domains of SRP54 (PDB 2j37 chain W) and its SR (FtsY, PDB 1rj9 chain B). SRP54 and SR are in green and pink, respectively. The positions of SRP GTPase-specific motifs and the conserved nucleotide-binding elements (G1–G5) are indicated in orange and blue, respectively. The mutated residues are shown in red. Each partner binds a GTP molecule. (B) Close-up of the WT residue G226 and side-chain modeling of mutant E226. The very short distances between oxygens of E226 of SRP54 (dark red) and D43, T232, and G233 of the receptor (pink) induce repulsive electrostatic forces that perturb the stability of the complex. Assuming a given protein backbone fold, the observed distances of 1.9 to 2.5 Å are indeed lower than those of standard hydrogen bonds between a hydroxyl group and an oxygen atom (2.8 Å). (C) Close-up of the WT residue T115 and side-chain modeling of mutant A115. According to the model, the hydrogen bond between the hydroxyl side chain of T115 and GTP is lost when mutated to A115. This modification most likely impairs GTP binding. (D) Close-up of the WT and structural model of deletion in T117. As residues 115–117 are all threonine the deletion of threonine introduces an earlier start of the helix structure at G113. According to the structural model, residues 113–116 form extensive hydrogen bonds with GTP. The deletion of T117 may affect GTP binding through rearrangements of residues 113–116, but this is difficult to predict.

were also validated using Sanger sequencing on genomic DNA extracted from nonhematopoietic cells, i.e., buccal swabs (patient AII.1) and kidney (patient BII.1), thereby confirming the germline nature of these mutations.

3D modeling of srp54 mutations. We analyzed the spatial location and predicted functional impact of the 3 identified mutations (p.T115A, p.T117del and p.G226E) using 3D modeling. Figure 2A shows the locations of the 3 affected residues on the structure of SRP54 associated with the SR. The mutated residues are all located around the GTP-binding domain in highly conserved regions (G domain) (20–22). The amino acid change p.G226E, which we observed in family A, is predicted to affect receptor binding, because it would lead to extremely short contacts between E226 and G233, T232, and D43 in the receptor, without major rearrangements of the local protein fold (Figure 2B). This was confirmed by the modification of the electrostatic surface induced by this mutation (Supplemental Figure 5). The mutation p.T115A in family B most likely directly affects GTP binding, because T115 forms an important hydrogen bond with GTP that is lost when the residue is mutated to alanine (Figure 2C). The mutation p.T117del in family C might also impact GTP binding. Residues 115–117 correspond to a threonine triplet. When 1 of these threonines is deleted, the helix structure at Gly113 starts earlier than expected, while the 112–115 region forms extensive H-bonds with the GTP. However, because the region before G113 is a loop region, it is difficult to determine the impact of this mutation on GTP binding (Figure 2D). The p.T117del mutation is therefore likely to have a milder effect than the other mutations (see also below).
in 2 cell lines (HeLa and HL60), it had no impact on the expression of 2 other proteins known to be mutated in SDS (i.e., SBDS and DNAJC21) (Supplemental Figure 6) or on the mTOR pathway, which was previously linked to SDS pathophysiology (23, 24).

**Zebrafish model.** The zebrafish is an established model for studying hematopoiesis and development (25, 26). At 26 hours post fertilization (hpf), expression data indicated ubiquitous staining for the zebrafish homolog srp54 (bottom image, Supplemental Figure 7A). Later in development, srp54 expression was more pronounced in the anterior part of the zebrafish (top image, Supplemental Figure 7A). A similar expression pattern has been shown for the zebrafish homolog of the SBDS gene at these developmental time points (27).

To investigate the role of srp54 in zebrafish embryonic development, we performed in vivo loss-of-function experiments by treating zebrafish embryos with 2 different antisense morpholino oligonucleotides (MOs), to inhibit srp54 pre-mRNA splicing. Both MOs were designed to induce intron retention. We found that transcripts in morphants were indeed misspliced (Supplemental Figure 7B).

To analyze the neutrophil numbers in developing zebrafish embryos, control and srp54 MO–injected transgenic Tg(mpisk:eGFP) and Tg(lyz:DsRed) fish were first analyzed using flow cytometry, which revealed respectively fewer mpx+ and lyz+ neutrophils in dissociated srp54 morphants than in the control-injected fish, which could be partially rescued by coinjection of human SRP54 mRNA (Figure 4) (28). This suggests a conserved functional identity between the 2 orthologs, which is consistent with their high evolutionary conservation (96% sequence identity between zebrafish and human proteins) (Supplemental Figure 8). Following tail fin injury, we further observed fewer numbers of neutrophils migrating to the site of injury in srp54 morphants than in the embryos injected with the control...
MO (Figure 5, A and B), which again could be rescued by coinjection of human SRP54 mRNA. Time-lapse imaging in double-transgenic embryos further documented both the quantitative reduction in neutrophils — visualized as migrating lyz:DsRed/mpx:eGFP cells — and the chemotaxis defect in response to injury (Figure SC and Supplemental Videos 1 and 2). Pancreas development was also impaired upon srp54 knockdown, as shown by altered ptf1a expression at 72 hpf, indicating exocrine pancreatic defects (Figure 6A), while the endocrine pancreas appeared unaltered as monitored by whole-mount ISH (WISH) of insulin-a (insa) (Figure 6B). Both the neutrophil and the pancreas phenotypes were confirmed by mpx and trypsin WISH, which showed reduced numbers of neutrophils (Figure 7A and Supplemental Figure 9A) and incompletely surrounded islets (Figure 7B) in srp54 morphants versus control MO-injected embryos.
embryos. We obtained similar results for both phenotypes with a second MO (Supplemental Figure 10). Interestingly, while injection with human SRP54 mRNA was able to rescue the number of neutrophils and their migration capacity (Figure 4, Figure 5, Figure 7A, and Supplemental Figure 9, A and B) as well as pancreas development (Figure 6 and Figure 7B), coinjection of human SRP54 RNA, expressing either the p.T115A, the p.G226E, or the p.T117del mutation, did not rescue the neutrophil or the pancreatic phenotypes caused by knocking down zebrafish srp54, and in all cases showed phenotypes statistically indistinguishable from those observed in srp54 morphants (Figure 7 and Supplemental Figure 9, A and B). Of note, we found that expression levels of the hematopoietic stem cell genes runx1 and c-myb and the erythrocyte marker globin were not altered by srp54 knockdown (Supplemental Figure 11, A and B), consistent with the observation that quantitative and qualitative defects in neutrophils were the most prominent hematologic defects. Finally, srp54 knockdown did not affect embryo body length in assays performed up until 5 days after fertilization (Supplemental Figure 11C).

Discussion

IBMFSs are a heterogeneous group of disorders that share complex overlapping phenotypes, including dysmorphogenesis, a partial or total lack of mono- or multilineage blood cell production, and an increased risk of malignant transformation. Although traditionally classified according to characteristic clinical symptoms, a new disease nosology based on molecular etiology and pathway biology is emerging. Surprisingly, however, most, if not all, underlying mutations associated with IBMFS affect genes that are not specifically expressed in hematopoietic cells but that are instead engaged in fundamental and ubiquitous cellular processes, such as ribosome biogenesis, DNA repair, or telomere maintenance. Here, we add a key cellular entity to the genes known as mutated in IBMFS. We show that de novo mutations in SRP54 cause syndromic congenital neutropenia with a Shwachman-Diamond-like phenotype, a phenotype that was completely reproduced by knocking down the zebrafish homolog of this gene. In the cases presented here, several clinical and biological characteristics differ from those typically observed in classical SDS (summarized in Supplemental Table 1). Among them is the cell morphology of myeloid precursors that contain cytoplasmic vacuoles. This unusual feature indicates that the mechanism leading to neutropenia may be different in patients with SRP54 mutations compared with that in typical SDS patients.

Several other IBMFSs are also caused by an abnormal translational machinery. These include Diamond-Blackfan anemia and the canonical SDS (29). While all these disorders (and others) are associated with mutations that affect 4OS or 6OS ribosomal subunits and/or their assembly (with the exception of 2 sporadic cases of aplastic anemia that were mutated in another SRP subunit [SRP72] [ref. 30]), the SRP complex has thus far remained unaffected by any disease-causing mutations. SRP54 is therefore the second SRP component to be associated with a monogenic disease. Although several potentially pathogenic CNVs encompassing SRP54 have been reported in studies referring to unrelated phenotypes (Supplemental Table 3), to our knowledge, no pathogenic single nucleotide variant or indel has previously been reported in this gene. Several heterozygous variants predicted by bioinformatics tools to be damaging have been reported in public databases (Supplemental Table 4) (as is the case for many genes), but in contrast to the 3 de novo mutations reported here, none of them affects the conserved domains of the protein. Of note, with...
srp 54 gene expression in vivo. Interestingly, impairs protein translocation in at least a quantitative manner. It is therefore highly probable that modification of chain, to the SR on the ER membrane in a GTP-regulated manner it links 3 key elements of the SRP complex, i.e., the 7SL RNA and over one-third of the human proteome (see below). Indeed, SRP54 tral role played by SRP54 in the cotranslational process that affects molecules. This high level of conservation is explained by the cen-

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Figure 7. Mutated versions of SRP54 identified in patients cannot rescue neutropenia or exocrine pancreas development in srp54-deficient zebra-

Figure 7. Mutated versions of SRP54 identified in patients cannot rescue neutropenia or exocrine pancreas development in srp54-deficient zebra-

patients, including profound neutropenia and defective exocrine pancreas development. Neutrophils of SDS patients have been reported to show impaired chemotaxis and migration deficiency (34, 35), which lead to repeated infections, as was also observed in all 3 patients reported here. In accordance with the results of these studies, we observed a decrease in the capacity of neutrophils to migrate toward the injury site in the zebrafish model (Figure 5).

Given our results, the underlying disease mechanism could be, at this point, either haploinsufficiency or a dominant negative effect. Haploinsufficiency is supported by: (a) decreased expression of SRP54 in the bone marrow of patients AII.1 and BII.2 as compared with that seen in healthy controls (Figure 3A); (b) the phenotype of the SRP54-knockdown zebrafish (Figures 4–6); and (c) the intolerance of the SRP54 gene to loss-of-function muta-

the exception of the SRP RNA, SRP54 is the only one of the six SRP polypeptides that is universally conserved throughout evolution (31, 32), with 96% sequence identity between human and zebrafish molecules. This high level of conservation is explained by the cen-

tral role played by SRP54 in the cotranslational process that affects over one-third of the human proteome (see below). Indeed, SRP54 defines the business end of the signal recognition particle, because it links 3 key elements of the SRP complex, i.e., the 7SL RNA and the signal sequence of the nascent ribosome-bound polypeptide chain, to the SR on the ER membrane in a GTP-regulated manner (Figure 8) (33). It is therefore highly probable that modification of SRP54 function, as suggested by 3D modeling and enzymatic activity assessments and confirmed in zebrafish rescue experiments, impairs protein translocation in at least a quantitative manner.

We used the zebrafish model to explore the relevance of srp54 gene expression in vivo. Interestingly, srp54-knockdown fish faithfully phenocopied the hallmarks of SDS that are observed in

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respect to the age of first clinical manifestations) harbored an equally less severe mutation — on the basis of structural and functional analyses (including a lower impact on GTPase activity and an observed milder phenotype in zebrafish rescue experiments as compared with the other 2 mutations) — hints that SRP54 mutations could lead to diverse clinical phenotypes that may be based on the status of each patient’s specific mutation.

In conclusion, we report that heterozygous mutations in SRP54, a key component of the cotranslational protein machinery, lead to syndromic neutropenia with SDS-like features. This is directly relevant not only to patients with molecularly undiagnosed IBMFS, including SBDS-negative SDS patients, but also to the molecular nosology of IBMFS. These findings also open a window into the pathophysiology of the SRP.

Methods

Subjects and exome sequencing

The subjects reported in this study were members of 2 unrelated families of European descent (families A and B; Figure 1A) and 1 North American family of Hispanic (Mexican) origin (family C; Figure 1A). In all 3 families, the parents were nonconsanguineous and healthy, as were other siblings. Exome sequencing was performed for the parents and probands of all 3 families. Genomic DNA was extracted and purified from peripheral blood using standard protocols. For WES of family A, sequencing libraries were prepared using the Ion Ampliseq Exome Kit (Thermo Fisher Scientific) according to the manufacturer’s recommendations and sequenced on an Ion Proton Sequencer (Thermo Fisher Scientific). Sequencing libraries for families B and C were prepared using the TruSeq Exome Kit (Illumina) and NimbleGen SeqCap EZ probes (VCRome; Roche Sequencing), respectively, and WES was performed on Illumina NextSeq 500 and HiSeq 2500 platforms, respectively. A minimum of 6.5 Gb of sequence data per individual were obtained and mapped to the hg19 reference genome using the Ion Torrent Server (Thermo Fisher Scientific) for family A and the Isaac Genome Alignment Software 2.1.0 (Illumina) for family B. The mean coverage was at least 60-fold, and more than 95 % of target sequences were covered a minimum of 10 times. CNVs were identified using Conifer software (41). Other variants were called using the Torrent Variant Caller 5.0 with low-stringency calling settings (family A) or the Isaac Variant Caller 2.1.0 (family B). Only variants covered by more than 5 variant reads were considered. Annotation was performed using the KGGSeq software package (42). Bioinformatics analysis of family C was performed as previously described (43). We focused only on protein-altering variants (i.e., missense, nonsense, and splice-site variants and coding indels) with fewer than 0.01 alternative allele

Figure 8. Schematic positioning of SRP54 in the eukaryotic translational machinery. (A) Ribosomal and SRP cycles. Proteins involved in SDS are depicted: SBDS and EFL1 are involved in the assembly of the 60S and 40S subunits, and DNAJC21 is involved in maturation of the 60S subunit. (B) Close-up of the ribosomal complex bound to the SR on the ER membrane. SRP54 (blue) is part of the SRP composed of 6 proteins (SRP9, SRP14, SRP19, SRP68, SRP72) and of the approximately 300-nucleotide-long 7SL RNA molecule. The signal peptide of the emerging nascent peptide chain is recognized by the M domain of SRP54 and the 7SL RNA. The N and G domains of SRP54 are interacting with the homologous N and G domains of SRα (FtsY). The mutated residues are shown in red.
frequencies in dbSNP (build 135), 1000 Genomes Project, ExAC, an in-house database, and databases at Institut Imagine (Necker Hospital, Paris, France), Baylor College of Medicine, and Radboud University Medical Center (Nijmegen, Netherlands). To identify potential causal mutations, we focused on de novo mutations in the index cases of each family. Other modes of transmission — recessive and X-linked — were considered and excluded for all 3 families. The complete variant filtering strategy is presented in Supplemental Figure 4. Sequence variants reported here were deposited in the ClinVar (www.ncbi.nlm.nih.gov/clinvar/) public database under accession numbers SCV000583969, SCV000583970, and SCV000583971.

Targeted sequencing of SRP54

The candidate SRP54 variant identified by WES was validated using conventional capillary Sanger sequencing in the same families including the proband and his or her parents. Additional patients were screened using Sanger sequencing of all 15 exons of SRP54. Primers used for PCR amplification and sequencing are presented in Supplemental Table 5. Briefly, SRP54 exons were amplified using the Expand Long Template PCR System (Roche Diagnostics) according to the manufacturer’s recommendations. After purification with the Exostar Kit (GE Healthcare Life Sciences), PCR products were bidirectionally sequenced using the same amplification primers — except for exon 14 (Supplemental Table 5) — using the Big Dye Terminator Kit v3.1 (Thermo Fisher Scientific). Sequencing reactions were run on an ABI PRISM 3730xl sequencer (Thermo Fisher Scientific). Sanger sequencing for patient CII.1 was performed at Baylor Genetics clinical laboratories, and the primer sequences are listed in Supplemental Table 5.

3D modeling of SRP54 mutations

The structure of the SRP54 protein was modeled using PDB 2j37 chain W as a template (44). The GTP-binding site was modeled according to the superimposition of the SRP54 model and PDB 1rj9 (45). The mutant side-chain structures were modeled using the mutagenesis wizard of the PyMOL program (46). Electrostatic surfaces were calculated using PDB2PQR (47) and APBS (48). All figures were generated using PyMOL (46).

Quantification of SRP54 transcripts

Total RNA was extracted from mononuclear bone marrow cells (cells from 5 healthy bone marrow donors were obtained from Caltag Medsystems Ltd.) using TRizol Reagent (Invitrogen, Thermo Fisher Scientific) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Real-time quantitative PCR was performed in a total volume of 20 μl using the SsoAdvanced SYBR Green Supermix Kit (Bio-Rad) and the following gene-specific primers: SRP54, 5′-TGGATGCTTTAGCGGTGTCTTTTG-3′ and 5′-GCCGGCTCGAGATGTTCTTACGAGCAGCTTTGGAAGAAATATTAC-3′ and 5′-GCCGGGTCTGATATTAGTATATTGATGGAATATTGCCAATGGCGTAAC-3′. The GC content of the primers is 68%. The primer pair was designed using the Primer3 software (IDT). PCR products were sequenced on an ABI 3730xl DNA sequencer using Big Dye Terminator Kit (version 3.1; Applied Biosystems, Foster City, CA). Results were obtained using the ABI Prism Sequence Analysis Software version 3.1.1. Melting-curve analysis was performed using the ABI PRISM 7000 real-time PCR machine (60). Melting curve analysis was performed using the ABI PRISM 7000 real-time PCR machine (60). Amplification was carried out in triplicates.

Cloning and site-directed mutagenesis of SRP54 cDNA

Human SRP54 cDNA was first cloned into the pcDNA3- expression vector (RZPD). For this purpose, the human SRP54 gene (GenBank U51920.1) was amplified by PCR on cDNA synthesized from RNA of peripheral blood mononuclear cells obtained from a healthy donor, using the following primer pairs: 5′-GCCGGTCGAGATGTTCTTACGAGCAGCTTTGGAAGAAATATTAC-3′ and 5′-GCCGGGTCTGATATTAGTATATTGATGGAATATTGCCAATGGCGTAAC-3′. The PCR fragment was then cloned with the restriction enzymes Xhol and XbaI into pcDNA3- for zebrafish experiments (see next paragraph). This construct was then used to generate the mutants G226E (c.677G>A), T115A (c.343A>G), and T117del (c.349_351del) by site-directed mutagenesis according to the manufacturer’s instructions (QuikChange II XL Site-Directed Mutagenesis Kit; Agilent Technologies). The primers used to generate the G226E mutant were: 5′-TGGATGCTTTAGCGGTGTCTTTTG-3′ and 5′-GCCGGCTCGAGATGTTCTTACGAGCAGCTTTGGAAGAAATATTAC-3′. The GC content of the primers is 68%. The primer pair was designed using the Primer3 software (IDT). Sequencing reactions were run on an ABI PRISM 3730xl DNA sequencer (Thermo Fisher Scientific). Sanger sequencing was performed using the Big Dye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA). Results were obtained using the ABI Prism Sequence Analysis Software version 3.1.1. Melting-curve analysis was performed using the ABI PRISM 7000 real-time PCR machine (60). Amplification was carried out in triplicates.
MO design and validation. Human and zebrafish SRP54 share a high (96%) degree of sequence homology (Supplemental Figure 8). Two SRP54 splice MOs to prevent pre-mRNA splicing and a standard control MO were synthesized by Gene Tools (Gene Tools LLC): MO1, GTTTAACCTTGTTACTTCTGGAC; MO2, ACTACCCAAATAAGGCTCACCCTTATTAC, and the control MO, CCTTCTCCTAGTCTTACAATTTATA. Embryos were injected at the single-cell stage. Phenol red (0.05%) (Sigma-Aldrich) was added as an injection tracer. Embryos were raised to appropriate stages and fixed in 4% paraformaldehyde (PFA) in 1× PBS for gene expression analysis. For validation, control-injected and MO-injected embryos were collected, and mRNA was isolated according to the RNeasy Mini Kit (QIAGEN). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s protocol. Real-time PCR (RT-PCR) was performed using the following primers to verify splice modification on agarose gels: MO1, TAGCTGATCTGGAAGGAAA and GTTCTCTTGGAAACGGCATGT; MO2, CATCATGTCTTGTGGGTCTG and ACCACCTTGTCCAGGATGA. For rescue experiments, human WT and mutated SRP54 cDNAs cloned into the pCS2− expression vector (RP2D) were used (refer to the previous paragraph on cloning and site-directed mutagenesis of SRP54 cDNA). Capped RNA from each of the 4 constructs (WT, G226E, T115A, and T117del) was synthesized using the mMessage mMachine SP6 Kit (Ambion, Life Technologies, Thermo Fisher Scientific). RNA (50–100 pg) was injected into the yolk of single-cell-stage embryos together with the srp54 MO to rescue the MO phenotype.

WISH, flow cytometry, and tail fin wounding. WISH was performed as described in Konantz et al. (54). Briefly, c-myc riboprobes were generated using pBK-CMV as a plasmid template (a gift of Caroline E. Burns, Massachusetts General Hospital, Boston, Massachusetts, USA), EcoRI for DNA linearization, and T7 RNA polymerase for further antisense probe production. The insa probe was generated by PCR using the following primers: forward, 5′-ATGGCAGTGTGCTTTAGGC-3′ and reverse, 5′-GAATTCCTCAATGTTTAGTGT-3′. After PCR products were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN), the products were subcloned using the Dual Promoter TA Cloning Kit (Invitrogen, Thermo Fisher Scientific), and the plasmid was then linearized using the appropriate enzyme. RT-PCR was used to generate riboprobes for globin, mpx, srp54, and trypsin using the following gene-specific primers: srp54 (forward, GTTTTGCTGTGCAAAACCACAT, and reverse, GGGAGACATTGAGGAGGTAG); mpx (forward, CGTGGAAGGCCATGTGAGGTAAGG, and reverse, CAGTGTCTGAGTCCTAG); trypsin (forward, CAGGCGCAGAACATGTCTAG); and globin (forward, CCACGACTTGGTGGTATG, and reverse, GCCCTCTTGGACAGGTTG). Each antisense primer was designed with the T7-promoter sequence tagged to its 5′ end. Riboprobes were labeled with digoxigenin- or fluorescein 12–labeled (intra-labeled) UTP (Roche). Stained embryos mounted in 89% glycerol were photographed using a Leica digital MC170 HD camera attached to a Leica DM2000 LED (Leica Microsystems). For flow cytometry, dechorionated transgenic embryos were dissociated into single cells by incubating them with Liberase enzyme (Roche) at 30°C and at 200 g as previously described (55). After dissociation was complete, an equal amount of 5% BSA was added to inactivate the enzyme. Cells were washed once in PBS and passed through a 35-μM filter. The number of fluorescence-labeled cells was then determined using flow cytometry on a BD Accuri C6 Flow Cytometer (BD Biosciences), and SYTOX Blue (Thermo Fisher Scientific) was used to identify dead cells. For the tail fin wounding experiments, larvae were anesthetized using tricaine. The tail fins were cut at the end of the spinal cord using a sterile scalpel blade, and the embryos were then allowed to recover at 29°C in embryo medium for 6 to 8 hours. Embryos were then either fixed overnight in 4% PFA with PBS or mounted in 0.8% low-melting agarose on glass-bottomed slides for live imaging. Immunostaining was performed to detect neutrophils at the wound site according to standard protocols, using a rabbit polyclonal antibody against mpx (GTX128379; GeneTex International Corp.). Stained embryos were then mounted in 0.8% low-melting agarose. Images and videos were obtained using a Zeiss LSM 720 (Carl Zeiss) or a Leica SP5 II-Matrix (Leica Microsystems) confocal microscope. Mpx-positive cells from both WISH and immunostaining were semiautomatically counted using Fiji software (56). Finally, confocal time-lapse live imaging was performed in Tg(mpx:eGFP lyz:DsRed) embryos for 9 hours starting 30 minutes after the tail fin was amputated at 48 hpf.

Additional details regarding Methods can be found in the supplemental material.

Statistics

Data shown represent the mean ± SEM or the percentage of expression. P values were derived using a 1-way ANOVA for multiple comparisons, followed by a Newman–Keuls test for pairwise comparisons, a Mann-Whitney U test, a 2-tailed t test, or Fisher’s exact test for comparison of 2 groups. A P value of less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 6.

Study approval

All participants (and their parents) gave written informed consent for genetic analyses in this study, which was approved by the IRBs of Strasbourg University Hospitals (protocol DC-2013-1911) and Baylor College of Medicine (protocol H-22769).

Author contributions

RC and SB designed the study, analyzed the data, and wrote the manuscript. MK and CL designed zebrafish experiments, performed the experiments, and analyzed the data. JS, EA, and LS performed zebrafish experiments. ZM and EW designed, performed, and analyzed the 3D modeling data. VR, AM, NO, OL, and JGG performed protein experiments. AP, LN, AH, AS, MDF, SU, and NAA performed sequencing experiments. AK, NP, and YY performed NGS data analyses. MSL analyzed the exome-sequencing data and discussed the results. MPC and MDF performed and analyzed the pathology results. CSS interpreted radiological, CT-scan, and ultrasound images. PAB and VP performed and analyzed mitochondrial DNA sequencing by NGS. CM, AM, GA, NO, OL, and JGG performed the cloning and expression of WT and mutant proteins. ALMB and NM performed the CGH array experiments. CP, EBL, BI, AS, MF, NAA, and SU identified patients, provided samples, and analyzed and interpreted clinical and experimental data. MR, FR, AG, MCB, ME, MV, JG, LF, CTR, JT, KM, KLB, DHM, and DLS interpreted clinical data and discussed the results. All authors contributed to the writing and approved the final version of the manuscript.
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