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**Homozygous deletion of *SUN5* in three men with decapitated spermatozoa**

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**Abstract**

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5 A recent study of 17 men with decapitated spermatozoa found that 8 carried two rare *SUN5*  
6 alleles, and concluded that loss of *SUN5* function causes acephalic spermatozoa syndrome.  
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8 Consistent with this, the *SUN5* protein localises to the head-tail junction in normal  
9 spermatozoa, and *SUN* proteins are known to form links between the cytoskeleton and the  
10 nucleus. However, six of the ten *SUN5* variants reported were missense with an unknown  
11 effect on function, and only one man carried two certain loss-of-function (LOF) alleles:  
12 p.Ser284\* homozygosity. A potential exonic splice mutation, homozygous variant  
13 p.Gly114Arg, was not tested experimentally. Thus definitive proof that loss of *SUN5* function  
14 causes acephalic spermatozoa syndrome is still lacking. Based on these findings, we  
15 determined the sequence of the *SUN5* gene in three related men of North African origin with  
16 decapitated spermatozoa. We found all three men to be homozygous for a deletion-insertion  
17 variant (GRCh38 - chr20:32995761\_32990672delinsTGGT) that removes 5090 base pairs  
18 including exon 8 of *SUN5*, predicting the frameshift, p.(Leu143Serfs\*30), and the inactivation  
19 of *SUN5*. Thus we present the second case where acephalic spermatozoa syndrome is  
20 associated with two LOF alleles of *SUN5*. We also show that the p.Gly114Arg variant has a  
21 strong inhibitory effect on splicing in HeLa cells, evidence that homozygosity for  
22 p.Gly114Arg is causal of acephalic spermatozoa syndrome through loss of *SUN5* function.  
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24 Our results and those of the previous study together show that *SUN5* is required for the  
25 formation of the sperm head-tail junction and male fertility.  
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## Introduction

It has been estimated that 14% of couples fail to conceive a child naturally after one year, and compromised male fertility is identified in 60% of these couples (1). Assisted reproduction technology (ART) will provide a therapeutic solution for half of these couples, and today accounts for 1-3% of live births in developed countries (2). Despite this the genetic causes of male infertility and their pathophysiological consequences for gamete quality are known in few cases. The identification of genetic causes will be imperative for the development of personalised treatments, the extension of treatment to more infertile couples, and informed risk assessment for individuals conceived by ART and their descendants. Presently, the most solid progress has been made in uncovering the genetic basis of teratozoospermia.

Monomorphic teratozoospermia is a group of rare conditions in which almost all the spermatozoa share a specific malformation that renders them non-functional. Over the last decade, homozygous loss-of-function (LOF) mutations have been identified in four genes for three teratozoospermia phenotypes: *AURKC* (MIM 603495) in large-headed multiflagellar polyploid spermatozoa (MIM 243060) (3), *DPY19L2* (MIM 613893) (4, 5) in globozoospermia (MIM 613958), *SPATA16* (MIM 609856) (6) in globozoospermia (MIM 102530), and *DNAH1* (MIM 603332) in MMAF (multiple morphological abnormalities of the flagellum) (7).

In a recent study of seventeen Chinese men with acephalic spermatozoa syndrome (MIM 617187), rare coding variants in the spermatid-specific *SUN5* gene (MIM 613942) were found homozygous or compound heterozygous in eight cases (8). In this syndrome the sperm head frequently separates from the flagellum, because the sperm head-tail junction is absent (9, 10). *SUN5* is an attractive candidate for a gene involved in the attachment of the flagellum to the sperm head because the protein localises to the vicinity of the head-tail

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3 junction in spermatozoa (8, 11), and SUN family proteins are known to be part of links  
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5 between the inner nuclear membrane and the cytoskeleton (12).  
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7 Six of the ten *SUN5* variants reported are missense with an unknown effect on the  
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9 protein. Although one variant, p.Gly114Arg (c.340G>A), was predicted to affect splicing, this  
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11 was not confirmed experimentally. The other four variants were certainly LOF variants:  
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13 intronic splice site (c.425+1G>A), frameshift (p.Val128Serfs\*7) and two nonsense mutations  
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15 (p.Trp72\* and p.Ser284\*). However, only one man was homozygous for a certain LOF  
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17 variant, p.Ser284\*, and the variants identified in *SUN5* were designated as causal based on  
18  
19 the high frequency of men carrying two rare *SUN5* alleles in the study group. In three cases  
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21 with a missense variant, the *SUN5* protein was not detected at the head-tail junction, although  
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23 it was present in spermatozoa from two control patients with acephalic spermatozoa syndrome  
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25 but without a variant in *SUN5* (8). This is consistent with these missense variants being LOF,  
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27 but the possibility that *SUN5* absence is secondary to junction loss cannot be completely  
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29 excluded, because the pathological mechanism in the acephalic controls is unknown, and  
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31 could differ from that in the *SUN5* variant cases. Furthermore six of the *SUN5* variants are  
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33 present at a low frequency in the gnomAD database (13), and four predominate in, or are  
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35 exclusive to, the East Asian group, including the most frequent, the p.Val128Serfs\*7 LOF  
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37 variant, which is present in 1:140 individuals. Indeed overall in gnomAD approximately  
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39 1:100 individuals from the East Asian group are heterozygous for one of the rare variants  
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41 designated as causal of acephalic spermatozoa syndrome. If all these variants were LOF, this  
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43 sperm phenotype would be expected to have a high incidence among infertile men in East  
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45 Asia, but this has never been reported, raising a doubt about the pathogenicity of some *SUN5*  
46  
47 missense variants.  
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53 Thus although the case is persuasive that the loss of *SUN5* function causes a failure of  
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55 the sperm head-tail junction, definitive proof requires a second case where two certain LOF  
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3 *SUN5* alleles are found associated with acephalic spermatozoa syndrome. We therefore  
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5 searched for pathogenic variants in the *SUN5* gene in three men with decapitated  
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7 spermatozoa.  
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## 10 11 12 **Results**

### 13 14 **Homozygous deletion of exon 8 of *SUN5***

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16 Two brothers (13-1662 and 13-2016) and their cousin (13-4335) were diagnosed with  
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18 acephalic spermatozoa syndrome following consultation for primary couple infertility at our  
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20 clinic. They are members of a consanguineous family from Algeria (Fig. 1). We have  
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22 previously described the phenotype of the two brothers (14), and clinical details for all three  
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24 men are summarized in Table 1. Based on the recent report of the association of *SUN5*  
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26 variants with acephalic spermatozoa (8), we sequenced the coding region of *SUN5* in two of  
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28 the affected individuals (brother 13-1662 and cousin 13-4335) to determine if mutation in  
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30 *SUN5* was responsible for their infertility. We found no single nucleotide variants, but we  
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32 failed to amplify exon 8. An additional marker tested in the middle of intron 8 was also  
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34 negative in the three men, confirming the existence of a homozygous deletion in the three  
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36 affected men (Fig. 2A). To further define the deletion, we amplified from upstream of exon 7  
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38 to downstream of exon 9 (7 kb) and upstream of exon 8 to downstream of exon 9 (6.2 kb) and  
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40 obtained shorter than expected bands of, respectively, 2 kb (not shown) and 1.2 kb (Fig 2A).  
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42 The 1.2 kb fragment was sequenced across the deletion junction revealing the deleted segment  
43  
44 to cover 5090 bp that include the entirety of exon 8 (Fig. 2 and Fig. S1). There is an insertion  
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46 of four bases, TGGT, at the junction of the breakpoint. There are no blocks of homology at  
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48 the breakpoints, showing that the deletion was not mobilised by unequal crossing-over and is  
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50 unlikely to be a recurrent event. The deletion of exon 8 is predicted to inactivate *SUN5* as it  
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52 will induce a frameshift, p.(Leu143Serfs\*30), making the transcript lacking exon 8 a substrate  
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3 for NMD (nonsense mediated decay). Using a PCR test that amplifies the deletion allele  
4 specifically, we did not detect the *SUN5* deletion in 150 infertile men without the acephalic  
5 sperm phenotype recruited from our clinic, showing it to be a rare variant.  
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### 8 9 ***SUN5* exonic variant c.340G>A (p.Gly114Arg) inhibits splicing of exon 5**

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11 To further strengthen the evidence that loss of *SUN5* function is a cause of acephalic  
12 spermatozoa syndrome, we investigated the effect of the c.340G>A (p.Gly114Arg) variant on  
13 splicing. This rare variant affects the last base of exon 5 and was previously reported in the  
14 homozygous state in a man with decapitated spermatozoa (8). We made two expression  
15 constructs, one with the variant (SUN5-G114R) and the other without (SUN5-WT), in which  
16 the genomic region from exon 4 to exon 6 of *SUN5* is under the transcriptional control of the  
17 CMV promoter. We transfected SUN5-G114R and SUN5-WT into HeLa cells and assessed  
18 the splicing of exons 4, 5 and 6 by RT-PCR with primers in exon 4 and in exon 6. Our results  
19 show that both constructs produce transcripts lacking exon 5, but that only SUN5-WT  
20 produces transcripts that include exon 5 (Fig 3). The transcripts without exon 5 from the  
21 SUN5-WT construct are certainly artefacts of our cellular assay system, since the same  
22 transcript was not amplified from human testis cDNA (Fig. 3). The absence of transcripts that  
23 include exon 5 from the SUN5-G114R construct shows that p.Gly114Arg greatly diminishes  
24 splicing efficiency, and will lead to exclusion of exon 5 (62 bases) from the mature *SUN5*  
25 transcript. This will cause a frameshift preventing expression of the *SUN5* protein.  
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### 47 **Discussion**

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49 Here we present the second homozygous LOF mutation in *SUN5*, in three related men of  
50 North African origin who produce decapitated spermatozoa. We also provide functional  
51 evidence that the previously published variant p.Gly114Arg strongly affects splicing and will  
52 result in a reduction of *SUN5* protein levels. Taken together with the recent report of biallelic  
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3 *SUN5* variants in Chinese men with acephalic sperm syndrome, our findings establish that  
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5 loss of *SUN5* function causes a failure of head-tail junction formation during spermiogenesis,  
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7 and show that LOF mutation of *SUN5* is also a cause of acephalic sperm phenotype in non-  
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9 Asian populations.  
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12 The two brothers in our study have had treatment of their infertility by ICSI (14),  
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14 using spermatozoa with the head and tail still joined. Despite the severity of the sperm  
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16 phenotype in these men in whom the proximal centriole is not anchored to the nucleus (14),  
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18 ICSI resulted in the development of good quality embryos in both cases. Following embryo  
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20 transfer, pregnancy was obtained for each brother, resulting in the birth of a healthy girl and  
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22 triplets, including identical twins. Thus for infertile men who produce decapitated  
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24 spermatozoa consequent to the loss of *SUN5* function, ICSI can be envisaged as a therapeutic  
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26 solution. However a full evaluation of the efficiency of ICSI with *SUN5*-deficient  
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28 spermatozoa, and the associated risks, will require additional cases and the long-term follow-  
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30 up of the children conceived.  
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34 Several knockout mouse models share key features with acephalic spermatozoa  
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36 syndrome in human: isolated male infertility, the presence of epididymal spermatozoa and an  
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38 extreme fragility of the head-tail junction. The mouse genes inactivated in these models are  
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40 *Hook1* (15), *Oaz3* (16), *Odf1* (17) and *Spata6* (18). Like *SUN5*, all are expressed specifically  
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42 in the testis, and represent good candidates for genes that could carry causal mutations in  
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44 unresolved cases of human male infertility with decapitated spermatozoa.  
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48 In conclusion, our results establish that *SUN5* plays a key role in the attachment of the  
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50 flagellum to the sperm head and demonstrate the prognostic value of testing for *SUN5*  
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52 mutations in men with decapitated spermatozoa seeking to father a child.  
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## 55 56 **Materials and methods** 57 58 59 60



## Patients

Patient 13-4335 and his wife were 34 and 31 years old, respectively, and had been trying to have a child for three years without success. Both had a normal karyotype, the patient was 46,XY,21ps+ and his wife's was 46, XX. Both were healthy with no history of significant illness. His wife had regular menses, and her hysterosalpingography and hormone assessment was normal. Two semen samples from patient 13-4335, collected after three days of abstinence, were analysed and revealed severe oligozoospermia and complete teratozoospermia (Table 1). While there were only  $2.3 \times 10^6$  intact spermatozoa/ml in each sample, there were, respectively,  $41 \times 10^6$  and  $64 \times 10^6$  headless flagella/ml. These characteristics are similar to those of his cousins (13-1662 and 13-2016) and are typical of acephalic spermatozoa syndrome.

The clinical details of the two brothers, 13-1662 et 13-2016, have been described elsewhere (14), but are summarised for comparison with those of 13-4335 in Table 1. The three men gave their informed consent for their samples to be used in the search for the genetic cause of their infertility.

## *SUN5* sequencing and deletion interval characterisation

The *SUN5* gene was sequenced with the BigDye v1.1 terminator cycle sequencing kit (Applied Biosystems) following amplification of the coding exons with specific flanking primers (Table S1). PCR products for sequencing *SUN5* and deletion mapping were amplified with Q5 Taq polymerase (New England Biolabs). The allele-specific PCR assay used to screen controls was performed as a duplex PCR with primers o5226 and o5340 that flank the deleted segment around exon 8 (deletion allele = 490 base pairs) and o5216 and o5217 that flank exons 2 and 3 of *SUN5* (positive control = 802 bp).

## Transfection of *SUN5*-WT and *SUN5*-G114R constructs

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3 The segment from exon 4 to exon 6 of the *SUN5* gene was amplified from genomic DNA  
4 from a fertile man using primers o5336 (exon 4) and o5337 (exon 6) with, respectively *NheI*  
5 and *XhoI* sites added at their 5' end. To obtain the SUN5-WT construct the genomic fragment  
6 was cloned into the *NheI* and *XhoI* sites of pcDNA3.1-cGFP (pcDNA3.1+ - Life  
7 Technologies – with a C-terminal GFP cassette cloned between *XhoI* and *ApaI* sites). For the  
8 SUN5-G114R construct, a SUN5-G114R insert was cloned into pcDNA3.1-cGFP, following  
9 its amplification from SUN5-WT using two-step PCR-fusion with flanking primers o5336  
10 and o5337 and central primers o5338 and o5339 specifying the p.Gly114Arg variant. The  
11 sequence of both constructs was determined and showed that neither contained any errors. All  
12 PCR steps used the Q5 DNA polymerase (New England Biolabs).

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Constructs were transfected into HeLa cells using JetPrime and the RNAs harvested after 24  
h. RNA was reverse-transcribed to cDNA using the GFP-specific primer o5374 and the PCR  
performed using primers o5336 and o5337.

### Acknowledgements

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### Conflict of interest

All authors declare no conflict of interest

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## 20 21 22 23 24 25 26 27 28 29 30 31 32 **Figure Titles and Legends**

33  
34 **Figure 1:** Family pedigree of the three infertile men presenting with acephalic spermatozoa  
35 syndrome (MIM: 617187). Infertile men: 1) 13-1662; 2) 13-2016; 3) 13-4335.

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41 **Figure 2: Homozygous deletion of exon 8 from *SUN5* in two brothers and a cousin with**  
42 **acephalic sperm syndrome.** A. Mapping of the *SUN5* deletion using locus-specific PCR.

43 Results are shown for the three affected men, a fertile man and H<sub>2</sub>O, following  
44 electrophoresis on ethidium bromide-stained agarose gels. Sample order for all gels is as  
45 specified on the gel at the left. Affected men: 1) - 13-1662, 2) - 13-2016, 3) - 13-4335.

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51 Primers are shown for each test (oNNNN) and their approximate position in *SUN5* is  
52 indicated by a white or black shape. B. Schematic representation of the *SUN5* deletion and  
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3 the sequence of the deletion junction. Deletion coordinates are based on the NCBI assembly  
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5 GRCh38. The corresponding sequence electropherogram is available in Fig. S1.  
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10 **Figure 3: The *SUN5* p.Gly114Arg variant reduces splicing efficiency in HeLa cells.**

11 HeLa cells were transfected with two constructs in which the genomic region of *SUN5* from  
12 exon 4 to exon 6 (3.6 kb) was under the control of the CMV promoter in pcDNA3.1+. There  
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14 is one nucleotide difference between these constructs: *SUN5*-G114R carries the p.Gly114Arg  
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16 variant, and *SUN5*-WT has the wild-type sequence. 24 h after transfection, RNA was  
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18 extracted from cells, treated with DNase-I and RT-PCR performed with primers o5336 and  
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20 o5337 from exon 4 and exon 6, respectively. Normal testis RNA (Testis-WT) and H<sub>2</sub>O were  
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22 included as controls. The expected sizes are indicated to the right of the gel: 206 bp for  
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24 normal splicing including exon 5, and 143 bp if exon 5 is excluded. The asterisk indicates the  
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26 200 bp band of the size marker (M) (1 kb plus - Life Technologies).  
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34 **Figure S1: Schematic representation of the *SUN5* deletion with the electropherogram of**  
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36 **the sequence across the deletion junction.** The position of the primers used to amplify the  
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38 junction fragment are shown – square = o5226 and triangle = o5229.  
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Table

| Patients   | 13-1662 |         | 13-2016 |         | 13-4335 |         |
|--|---------|---------|---------|---------|---------|---------|
| Age  | 36      |         | 25      |         | 34      |         |
| Spermogram   | A       | B       | A       | B       | A       | B       |
| Sperm concentration (10 <sup>6</sup> /ml)              | 4.5     | 0.5     | 1.7     | 1.0     | 2.3     | 2.3     |
| Motility a/b/c <sup>a</sup> (%)                        | 0/20/80 | 0/10/90 | 0/15/85 | 0/30/70 | 5/5/90  | 5/20/75 |
| Acephalic sperm (sperm flagella) (10 <sup>6</sup> /ml) | 45      | 83      | 7.9     | 16.8    | 41      | 64      |
| Normal forms <sup>b</sup>                              | 0       | 2       | 0       | 0       | 0       | 0       |
| Vitality (%)   | 40      | 40      | 53      | 53      | 72      | 40      |

<sup>a</sup>: WHO criteria (2010). <sup>b</sup>: David criteria.

**Table 1:** Sperm parameters of the three related infertile men carrying a homozygous deletion within the SUN5 gene.

#### Abbreviations

ART: Assisted reproduction technology

AURKC: Aurora kinase C

bp: base pair

CMV: cytomegalovirus

delins: deletion/insertion

DNAH1: Dynein axonemal heavy chain 1

DPY19L2: Dpy-19 like 2

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fs: frameshift

GFP: Green fluorescent protein

Hook1: Hook microtubule tethering protein 1

ICSI: Intra cytoplasmic sperm injection

ins: insertion

kb: kilobase

LOF: Loss-of-function

MMAF: multiple morphological abnormalities of the flagellum

ml: millilitre

NMD: nonsense mediated decay

Oaz3: Ornithine decarboxylase antizyme 3

Odf1: Outer dense fiber of sperm tails 1

PCR: Polymerase chain reaction

Spata6: Spermatogenesis associated protein 6

SPATA16: Spermatogenesis associated protein 16

SUN5: Sad1 and UNC84 Domain Containing 5

WT: Wild type



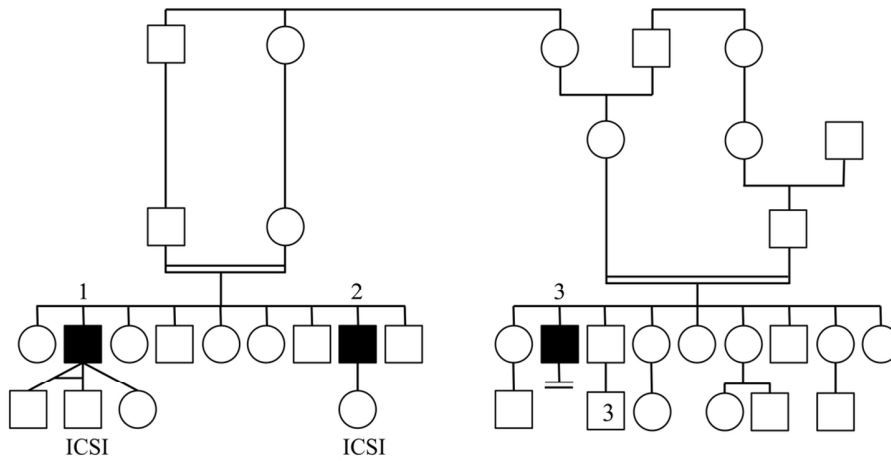


Figure 1: Family pedigree of the three infertile men presenting with acephalic spermatozoa syndrome (MIM: 617187). Infertile men: 1) 13-1662; 2) 13-2016; 3) 13-4335.

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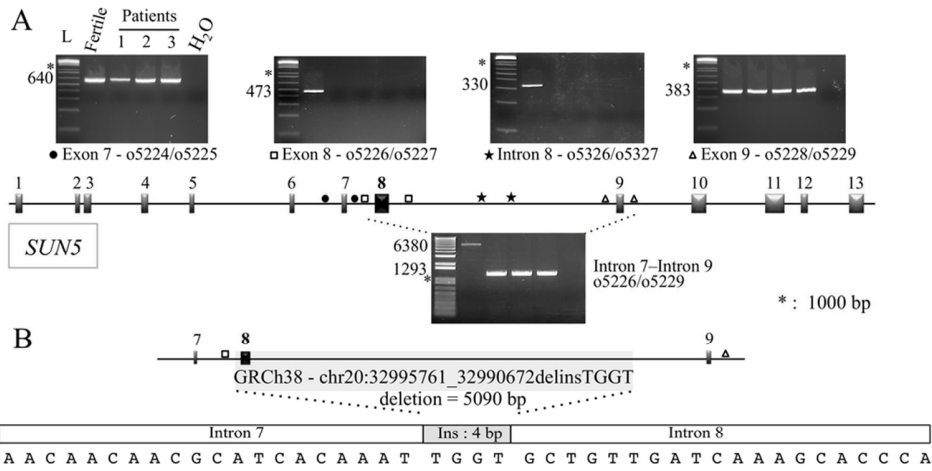
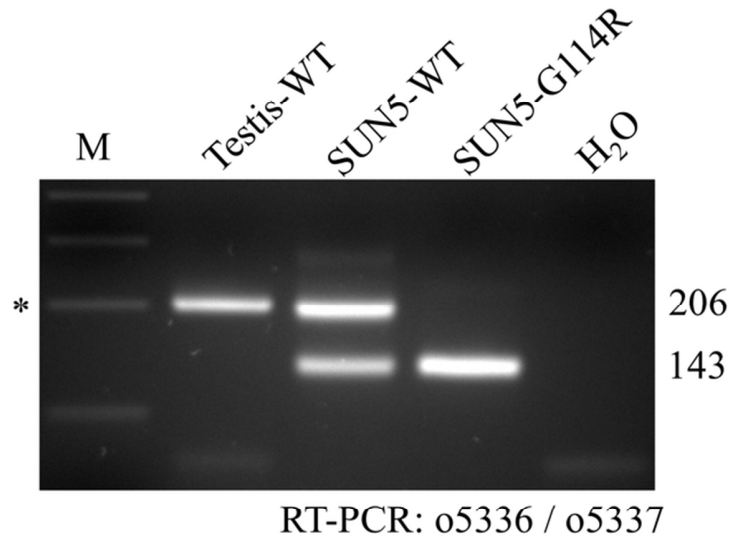


Figure 2: Homozygous deletion of exon 8 from SUN5 in two brothers and a cousin with acephalic sperm syndrome. A. Mapping of the SUN5 deletion using locus-specific PCR. Results are shown for the three affected men, a fertile man and H<sub>2</sub>O, following electrophoresis on ethidium bromide-stained agarose gels. Sample order for all gels is as specified on the gel at the left. Affected men: 1) - 13-1662, 2) - 13-2016, 3) - 13-4335. Primers are shown for each test (oNNNN) and their approximate position in SUN5 is indicated by a white or black shape. B. Schematic representation of the SUN5 deletion and the sequence of the deletion junction. Deletion coordinates are based on the NCBI assembly GRCh38. The corresponding sequence electropherogram is available in Fig. S1.

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Figure 3: The SUN5 p.Gly114Arg variant reduces splicing efficiency in HeLa cells. HeLa cells were transfected with two constructs in which the genomic region of SUN5 from exon 4 to exon 6 (3.6 kb) was under the control of the CMV promoter in pcDNA3.1+. There is one nucleotide difference between these constructs: SUN5-G114R carries the p.Gly114Arg variant, and SUN5-WT has the wild-type sequence. 24 h after transfection, RNA was extracted from cells, treated with DNase-I and RT-PCR performed with primers o5336 and o5337 from exon 4 and exon 6, respectively. Normal testis RNA (Testis-WT) and H<sub>2</sub>O were included as controls. The expected sizes are indicated to the right of the gel: 206 bp for normal splicing including exon 5, and 143 bp if exon 5 is excluded. The asterisk indicates the 200 bp band of the size marker (M) (1 kb plus - Life Technologies).

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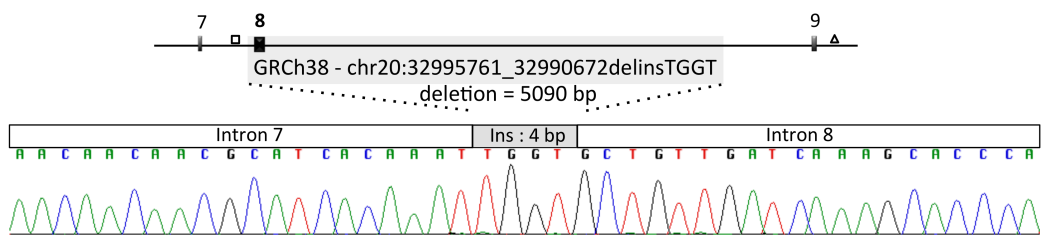


Figure S1: Schematic representation of the SUN5 deletion with the electropherogram of the sequence across the deletion junction. The position of the primers used to amplify the junction fragment are shown – square = o5226 and triangle = o5229.

| Primer Number  | Target                              | Primer sequence                                |
|--|-------------------------------------|--|
| <b><i>SUN5</i> gene sequencing</b>   |                                     |  |
| o5214  | Exon 1f                             | CACCAGCTCCCAGAGTTCC                            |
| o5215  | Exon 1r                             | GTGCCCAGAGATAGGCATCA                           |
| o5216  | Exon 2-3f                           | GGTAGGAGCAGACAAAGGAAC                          |
| o5217  | Exon 2-3r                           | CCTCTCCAGCCACCAAAGA                            |
| o5218  | Exon 4f                             | GTGCCACCTGTAGTGACCT                            |
| o5219  | Exon 4r                             | GTGCAAATGTGTGGAGGTGG                           |
| o5220  | Exon 5f                             | CTGCCACTCATGAGCTCCCA                           |
| o5221  | Exon 5r                             | GGAGGGAGGAGATTCTTTG                            |
| o5222  | Exon 6f                             | CAGCCAGCATGGAGATCATA                           |
| o5223  | Exon 6r                             | TCTGGGTCAAGTCAGGAACTG                          |
| o5224  | Exon 7f                             | GAGATAGGAGGCTAGGTGAA                           |
| o5225  | Exon 7r                             | TGACTTGCCTAAGGTCAGCC                           |
| o5226  | Exon 8f                             | CTGATGAATGGGTCCAGGGATG                         |
| o5227  | Exon 8r                             | CCCAGCCAAACTATCAACCA                           |
| o5228  | Exon 9f                             | GGTCAGGATGGCTTTGTGTC                           |
| o5229  | Exon 9r                             | CCAGCTTGGAACAGAGGATG                           |
| o5230  | Exon 10f                            | ACATGGCTGTAATCACATTTCC                         |
| o5231  | Exon 10r                            | GCCCATGCCAGTGTAGTC                             |
| o5232  | Exon 11f                            | GAAC TGAAGTAAGGCTTACC                          |
| o5233  | Exon 11r                            | GCCTTAACCAAGCTGCATTC                           |
| o5234  | Exon 12f                            | CTCTGACTCCCAGGCCAGTG                           |
| o5235  | Exon 12r                            | GCCACCCACTACGGAGACTCT                          |
| o5236  | Exon 13f                            | GTCTGTCTTCTGGCCTCAG                            |
| o5237  | Exon 13r                            | ACCCGGAATGGGATCAACC                            |
| <b><i>SUN5</i> deletion mapping</b>  |                                     |  |
| o5326  | Intron 8f                           | GGAGTCCCCAGGGAATATGCAG                         |
| o5327  | Intron 8r                           | GTGGCCCTGGAGACAGCAG                            |
| o5340  | Intron 8r                           | TCTGCTGCCAGATGCGGATG                           |
| <b>p.G114R expression construct and reverse transcription (RT) primers</b> |                                     |  |
| o5336  | Exon 4f_ <i>Nhe</i> I               | <u>AGCCTGGCTAGCATGGCCTGGTTACCTGTTTTGCCTG</u>   |
| o5337  | Exon 6r_ <i>Xho</i> I               | <u>AGGTTCTCGAGCTGCCAGACTTTCATTTTCGATGGTAAG</u> |
| o5338  | Exon 5f_ G>A to add p.G114R variant | CTGTGCTTTCAGTGTGTCCCAG                         |
| o5339  | Exon 5r_ C>T to add p.G114R variant | CTGGGACACACTGAAAGCACAG                         |
| o5374  | eGFPr_RT                            | TGGTGCAGATGAACTTCA                             |

**Table S1:** Primer sequences.