Frequency of mosaicism points towards mutation-prone early cleavage cell divisions.

As much as 30% and 50% of de novo mutations (DNM) may occur during the early cleavage cell divisions in males and females, respectively, causing frequent mosaicism and a high sibling recurrence risk of DNM-dependent diseases.

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It has recently become possible to directly estimate the germ-line de novo mutation (DNM) rate by sequencing the whole genome of father-mother-offspring trios, and this has been conducted in human[1-5], chimpanzee[6], birds[7] and fish[8]. In these studies DNMs are defined as variants that are heterozygous in the offspring while being absent in both parents. They are assumed to have occurred in the germ-line of a parent and to have been transmitted to the offspring via the sperm or oocyte. This definition assumes that detectable mosaïcism in the individual in which the mutation occurred is negligible. However, instances of mosaïcism are well-

documented in humans and other organisms, including ruminants[9,10]. We herein take advantage of the unique pedigree structure of cattle to show that mosaïcism associated with DNMs is a common occurrence, and that this should be taken into account to accurately estimate the mutation rate in this and possibly other species. It suggests that early cleavage cell divisions are particularly mutation-prone, and that the recurrence risk of DNM-dependent disorders in sibs may be higher than generally assumed.

To study the process of DNMs in the cattle germ-line, we sequenced the whole genome of 46 members of three four-generation pedigrees (Figure 1). The source of the sequenced DNA was venous blood for females and sperm for males. Grand-parents, parents and offspring (referred to as probands) were sequenced at average 26-fold depth (min = 23), and grand-offspring at average 21-fold depth (min = 10).

We identified 151 candidate DNMs as variants that were (i) detected in a proband, (ii) absent in both parents (and grand-parents when available), (iii) transmitted to at least one grand-offspring, and (iv) not previously reported in unrelated individuals from the 1,000 Bulls project[11] (Suppl. Table 1 and Suppl. Figure 1). We developed amplicons spanning 94 candidate DNMs and sequenced them at average depth of ~1,250 in the 46 animals plus 57 relatives (Figure 1). This confirmed the genuine nature of 91/94 variants, demonstrating the excellent specificity of our pipeline. The three remaining ones were also detected in one of the parents (although not in the grand-parents) in the confirmation, and momentarily ignored.

We examined what proportion of DNMs detected in a proband occurred during its development rather than inherited via the sperm or oocyte. An unambiguous distinction between the two types of mutations is that the former will, upon transmission to the next generation, show partial linkage with one of the homologues (i.e. never transmitted with homologue A, sometimes transmitted with homologue B), while the latter will show complete linkage (i.e. never transmitted with homologue A, always transmitted with homologue B). We first examined the pedigree with 11 grand-offspring as it provided the best opportunity to distinguish partial from complete linkage (Suppl. Fig. 2). 21 variants showed complete linkage with the paternal homologue, 13 complete linkage with the maternal homologue, and 16 partial linkage with either the paternal or maternal homologue. Thus 21 DNMs appeared to have been inherited from the father, 13 from the mother, while 16 would have occurred early enough during the development of the proband to generate detectable levels of mosaïcism in sperm DNA. Three additional predictions can be made if these 16 mutations occurred during the development of the proband. (i) They should a priori have equal chance to affect the paternal and maternal homologues. Five of the 16 variants affected the paternal and 11 the maternal homologue ($p=0.21$). (ii) The allelic ratio should be inferior to 50% in the proband but equal to 50% in the grand-offspring. The mean allelic ratio was 0.28 in the proband, and 0.50 in the grand-offspring, and this difference was highly significant ($p < 10^{-6}$). The corresponding means were 0.47 and 0.48 ($p = 0.40$) for the 34 mutations showing complete linkage with either the paternal or maternal homologue (Figure 2, Suppl. Table 1). (iii) The proportion of grand-offspring inheriting the mutation and the strength of linkage should increase

with the allelic ratio in the proband, and this was indeed the case ($p_{transmission}$ = 0.02; $p_{linkage}$ = 0.008) (Suppl. Fig. 3). We conclude that, in this pedigree, at least 32% of the DNMs detected in bulk sperm DNA of the proband using standard criteria did not occur in the germ-line of either the sire or dam but rather during the development of the proband. We will refer to this type of DNMs as Proband-Mosaic (PM), while the DNMs that are absent in the sire and showing complete linkage with the paternal homologue in the grand-offspring will be referred to as Sperm-Non-Mosaic (SNM) (meaning that the sire is not mosaic for the mutation transmitted via his sperm), and those that are absent in the dam yet showing complete linkage with the maternal homologue as Oocyte-Non-Mosaic (ONM) (meaning that the dam is not mosaic for the mutation transmitted via her oocyte).

One could argue that the observed high level of mosaicism is due to the fact that we analyzed sperm DNA rather than somatic DNA. We therefore analyzed the three female probands (blood DNA), using the same approach. It is noteworthy that with only five grand-offspring, the probability to detect a mosaic DNM and demonstrate incomplete linkage is reduced (Suppl. Fig. 2). We detected 72 mutations transmitted in complete linkage with the paternal homologue, 14 in complete linkage with the maternal homologue, and 12 in partial linkage with either of these. Three of 12 were partially linked to the paternal and nine to the maternal homologue (p=0.14). Their allelic ratio in the probands was 0.29, while being 0.50 in the grand-offspring ($p < 10^{-6}$). The corresponding means were 0.50 and 0.49 (p = 0.66) for the 86 mutations showing complete linkage with either the paternal or maternal homologue (Figure 2, Suppl. Table 1). Thus at least 12% of DNMs detected in blood DNA of female probands using standard

95 procedures occurred during their development rather than being inherited from
96 the parents, hence being of "PM" type. We did not observe a positive correlation
97 between the level of somatic mosaicism in the female probands and the
98 proportion of grand-offspring inheriting the mutation or the strength of linkage
99 in the grand-offspring ($p_{transmission}$ = 0.85; $p_{linkage}$ = 0.94), suggesting that the
100 degree of mosaicism in the female soma is a poor indicator of the degree of
101 mosaicism in the germ line (Suppl. Fig. 3).

102 If detectable mosaicism for DNMs is common, requiring their absence from the
103 parental DNA (as typically done) will eliminate genuine DNMs. We attempted to
104 recover such events as variants that were (i) absent in the grant-parents, (ii)
105 detected in either sire or dam with a fraction of mutant reads significantly < 50%
106 (Suppl. Table 1), (iii) transmitted to the proband with an allelic ratio of ~50%,
107 (iv) transmitted to at least one grand-offspring with an allelic ratio of ~50%, and
108 (v) not previously reported in unrelated individuals[11]. We detected 61
109 candidates, including the 3/89 variants mentioned above (Suppl. Table 1 and
110 Suppl. Fig. 1). We developed amplicons for 37, and sequenced (average 1,083-
111 fold depth) all 46 individuals plus 57 relatives including ≥ five half-sibs for each
112 proband (Figure 1). For 11 of the 37 tested candidates, the sire's or dam's allelic
113 ratio in the confirmation was ~1:1, and linkage to either the paternal or maternal
114 homologue (in the proband's half-sibs) perfect. These 11 events were thus
115 genuine DNMs, yet were more likely to have occurred in the grand-parents than
116 in the parents. For 25/26 remaining candidates, we observed partial linkage in
117 the half-sibs of the corresponding probands, confirming that these DNMs
118 occurred during the development of the (hence mosaic) parent. For the last

1/26, although linkage in the half-sibs of the proband appeared complete, the allelic ratio in the parent remained very significantly skewed when compared to descendants ($p < 10^{-5}$), strongly suggesting that this mutation also occurred during the development of the (hence mosaic) parent. For 20/26 the DNMs were transmitted to at least one of the analyzed half-sibs (Suppl. Table 1 and Suppl. Fig. 1). Following this targeted confirmation, we took advantage of whole genome sequence information that became available for 17 of the probands' half-sibs, to trace the inheritance of the 24 non-confirmed candidate variants. We demonstrated partial linkage for 23/24 variants, hence confirming the mosaicism of the corresponding parent. For 15/24 the DNMs were transmitted to at least one of the analyzed half-sibs (Suppl. Table 1 and Suppl. Fig. 1). Taken together, confirmation data provided (i) strong evidence against parental mosaicism for 11/61 variants, (ii) strong evidence in favor of parental mosaicism for 49/61, and (iii) unconfirmed evidence for parental mosaicism for 1/61. Twenty-seven of the 50 (49+1) non-excluded variants were detected in a sire, and 23 in a dam (despite the fact that the sire could not be studied in the 11-grand-offspring pedigree). Twenty-three occurred on the paternal and 27 on the maternal chromosome ($p = 0.34$). The allelic ratio in the parents was 0.13, while being 0.50 in the proband and grand-offspring ($p < 10^{-6}$) (Figure 2, Suppl. Table 1). We did observe a significant positive correlation between the level of somatic mosaicism in the sire and the strength of linkage in half-sibs ($p_{transmission} = 0.002$), and between the level of somatic mosaicism in the dam and the proportion of half-sibs inheriting the mutation ($p_{linkage} = 0.0008$) (Suppl. Fig. 3). These data suggest that – in cattle – paternal bulk sperm DNA may be mosaic for at least 24% of DNMs present in a sperm cell, while maternal blood DNA may be

mosaic for at least 51% of DNMs present in an oocyte. We will refer to these types of mutations as Sperm-Mosaic (SM) and Oocyte-Mosaic (OM), respectively (meaning that the sire/dam is mosaic for the mutation transmitted via the sperm/oocyte).

When analyzing the transmission patterns of SM and OM mutations to the half-sibs of the probands, we were struck by the fact that (i) very few half-sibs inherited none of the DNMs detected in the proband, while more than 50% would be expected (23/60; p = 0.05), and (ii) half-sibs sharing multiple DNMs with the proband appeared surprisingly common (Suppl. Fig. 4).

In mammals, after fertilization, cleavage, and segregation of (i) the inner cell mass from the trophoblast, (ii) the epiblast from the hypoblast, (iii) the embryonic epiblast from the amniotic ectoderm, a small number of epiblast-derived cells located in the wall of the yolk sac in the vicinity of the allantois are induced to become primordial germ cells (PGC). These migrate to the primitive gonad where they expand and produce >1 million gametogonia. Oogonia initiate meiosis prior to birth in females. Spermatogonia will resume mitotic divisions at puberty allowing (i) the maintenance of a pool of stem cell like spermatogonia, and (ii) sustained spermiogenesis involving ~2 additional mitotic divisions followed by meiosis (Suppl. Fig. 5). We simulated the process of de novo mutagenesis in the male and female germ cell lineages assuming (i) uniform pre- and post-natal mutation rates per cell divisions, and (ii) 40 unrelated PGCs (i.e. sampled at random from the epiblast). Pre- and post-natal mutation rates were adjusted to match the observed number of mutations per gamete (31 in sperm, 14 in oocytes). Under these conditions, we virtually never observed the level of

mosaicism, nor the sharing between sibs characterizing the real data (Figure 3). We (i) increased the relative mutation rate during the early cell divisions (keeping the mutation rate per gamete constant)(10 and 20-fold increase during the first 2, 4 or 6 cell divisions), (ii) reduced the number of induced PGCs (40 or 4), and (iii) varied the relatedness between PGCs (unrelated or related)(Suppl. Fig. 5). Increasing the mutation rate during the very first cell divisions, and increasing the relatedness between induced PGCs in the simulations matched the real data much better (Figure 3). To quantitatively evaluate model fitting we used (i) the proportion of PM, SM and OM mutations with corresponding rate of mosaicism in sperm and soma, and (ii) the proportion of sibs sharing 0, 1, 2, ... DNMs with a proband, to compute the likelihood of the data under different scenarios (see M&M). The data were at least $10^{5.8}$ times more likely when assuming an increased mutation rate during the very first cell divisions than not (whichever the values of the other parameters). Assuming a higher mutation rate during the first cell divisions, the best model with 40 related PGCs was $10^{6.1}$ times more likely than the best model with 40 unrelated PGCs, and $10^{18.3}$ times more likely than with 4 PGCs. The data were $10^{3.2}$ more likely when the mutation rate was increased during the 4 or 6 first cell divisions rather than only the first two cell divisions (Table 1 and Supplemental Table 2).

We estimated the numbers of the five types of mutations for the four studied probands, accounting for estimated genome coverage (Figure 4A). The estimated number of DNMs per gamete (SNM+SM, ONM+OM) averaged 44.5 for sperm cells and 18.1 for oocytes (male/female ratio of 2.5), corresponding to an average mutation rate of $\sim 1.25 \times 10^{-8}$ per base pair per gamete. These rates are

likely to be slightly underestimated as a number (~ 1-5) of SM and OM mutations (with highest degree of mosaicism in the parents) would have remained undetected. The standard approach of ascertaining DNMs (i.e. erroneously considering PM mutations, ignoring SM and OM mutations) would have yielded a mutation rate of 0.97×10^{-8} per bp per gamete, with a 2.2-fold higher mutation rate in bulls than in cows. 195 of the 209 identified DNMs were nucleotide substitutions, 14 small insertion-deletions. Amongst substitutions, the transition (Ti) – transversion (Tv) ratio was unexpectedly low (1.14). This was shown to be due to a significant ~8-fold enrichment of C>A and/or G>T transversions in the mosaic classes of mutations (PM, SM and OM). The non-mosaic classes of mutations (SNM and ONM) were ~30-fold enriched in CpG>TpG transitions as expected, while this signature was less pronounced for mosaic mutations (Figure 4B&C). There was no obvious difference between the profile of DNMs in the male and female germ line (data not shown). DNMs were uniformly scattered across the genome (Suppl. Fig. 6).

Estimates of the DNM rate per generation from sequencing human families are ~2-fold lower than estimates from primate sequence divergence and possible reasons for this discrepancy have been discussed[12,13]. We wondered whether the rate of mosaicism might affect the rate of nucleotide substitution per generation. We simulated the fixation of DNM per generation in populations of varying effective population size, with constant mutation rate per gamete but varying levels of mosaicism. There was no evidence for an effect of mosaicism on fixation rate (which was always ~ μ) nor on fixation time (which was always ~4Ne)(Suppl. Figure 7).

Our work strongly suggests that – in the bovine - early cleavage cell divisions are particularly mutation prone, possibly accounting for as many as ~50% of DNM detected in oocyte, and ~30% of DNM detected in a sperm cell. These findings are consistent with recent evidence reported in humans[14]. The corresponding early mutations were characterized by a distinct signature dominated by C>A and/or G>T transversions, in contrast with DNM occurring during later stages of gametogenesis which are dominated by the expected methyl-CpG to TpG transversions. It is worth nothing that mutations in the proofreading domain of polymerase epsilon, synthesizing the leading strand during DNA replication, cause a mutator phenotype dominated by C>A in tumors[15]. This suggests that the observed early DNM might primarily result from replication errors. The predominance of early C>A transversions causes the overall Ti/Tv ratio to ~1.14, well below expectations. This was unlikely to be artifactual, as the expected Ti/Tv was obtained when applying the same bioinformatics pipeline on simulated DNM in the same pedigrees (M&M). Paradoxically the Ti/Tv ratio is ~2 when considering DNA sequence polymorphisms segregating in the domestic cattle population (Suppl. Figure 8). This suggests either sampling variation (meaning that Ti/Tv ratios might differ between families and that we by chance sampled families at the low end) or that the mechanisms underlying the observed excess of C>A (or G>T) transitions emerged recently. It is worth noting in this regard that most analyzed animals were bred by artificial insemination and/or in vitro embryo production.

Our results support the notion that the population of induced PGCs is ontogenetically related. This is not unexpected given their physical proximity at

the base of the allantois. This may - in combination with a high incidence of early cleavage cell divisions – be medically extremely relevant. Indeed, if applicable to human, it implies that the recurrence risk of DNM-dependent disorders in sibs may be higher than generally assumed[9,13].

DNM occurring during the development of an individual, should a priori affect the maternal and paternal chromosome with equal probability. Indeed, this null hypothesis could not be disproved within specific individuals for the PM, OM and SM type of mutations detected in this work. When repeating the test across individuals, however, we note that 47 mosaic mutations occurred on the maternal chromosome versus 29 on the paternal chromosome ($p = 0.05$). This suggests that the maternal and paternal chromosomes might be epigenetically distinct during early development and that this may affect their mutability.

Our work points towards the fact that direct estimates of mutation rates from sequencing families may have to be revisited, taken PM, OM and SM status into account, to obtain more accurate estimates of the mutation rate per gamete and per generation which have been raising some questions[12,13]. This may affect both the overall mutation rate as well as its male/female ratio. However, our analyses suggest that the effect is likely to be modest and insufficient to explain the present discrepancy between direct and indirect estimates in human studies.

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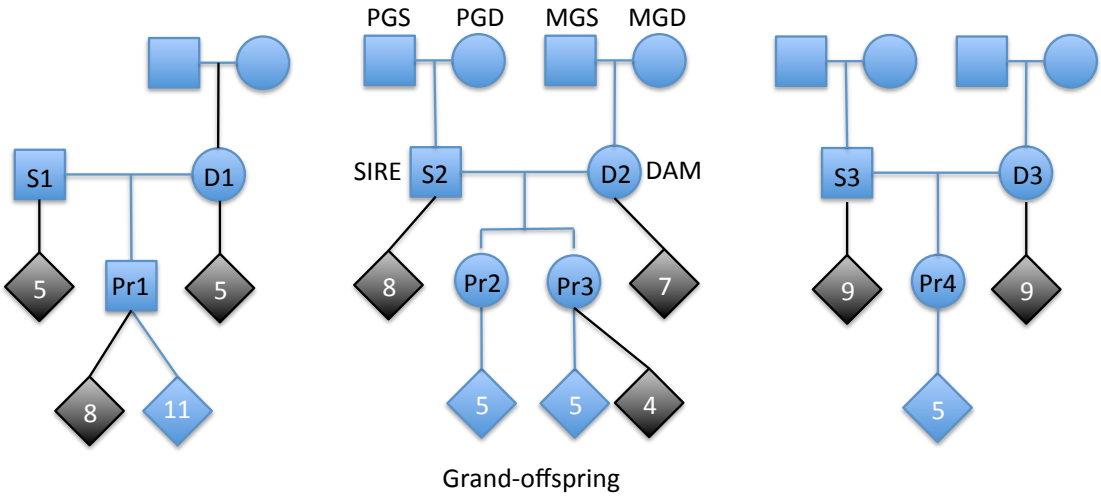
Authors contributions

306 MG, CH, CC: designed the experiments. EM: provided samples. LK, NC, MD, WC:

307 performed the sequencing. CH, MG, CC: analyzed data. MG, CH, CC: wrote the

308 paper.



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310 **Figure 1: Pedigrees used for the detection of DNMs.** PGS: paternal grand-sire,
311 PGD, paternal grand-dam, MGS: maternal grand-sire, MGD: maternal grand-dam,
312 Pr: probands. S: sires. D: dams. Animals in gray were used for confirmation.

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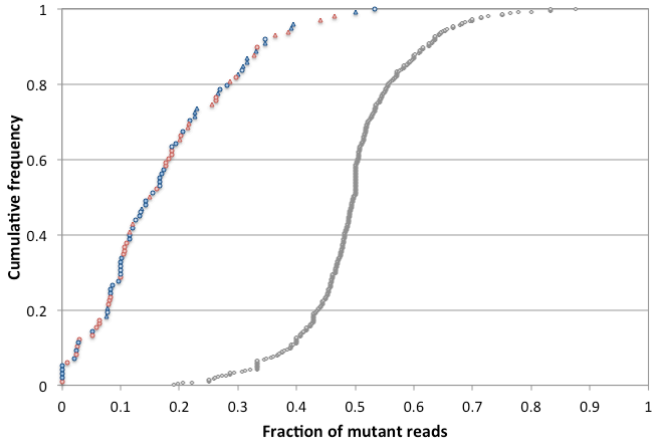


Figure 2A

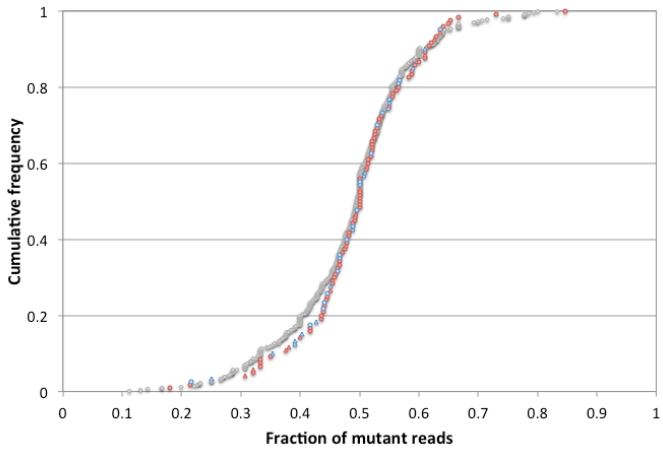
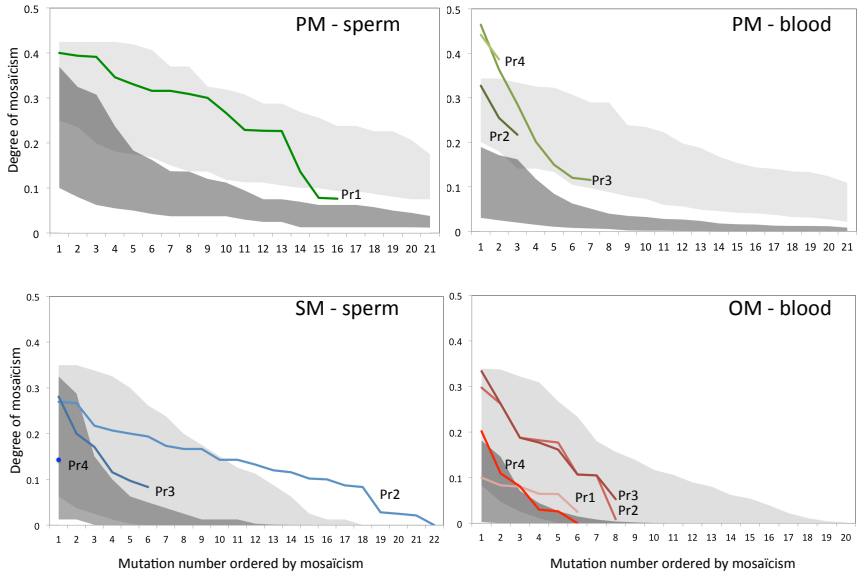


Figure 2B

Figure 2: Cumulative frequency distribution of the proportion of mutant reads. (A) DNMs with detectable mosaicism in the individual (proband or parent) in which the mutation occurred: Blue triangles: P(roband)M(osaic) mutations having occurred during the development of the male proband (Pr1) and for which the proband's sperm is mosaic. Red triangles: P(roband)M(osaic) mutations having occurred during the development of the three female probands (Pr2, Pr3, Pr4) and for which the proband's blood is mosaic. Blue circles: S(perm)M(osaic) mutations having occurred during the development of two sires (S2, S3) and for which the sires' sperm is mosaic. Red circles: O(oocyte)M(osaic) mutations having occurred during the development of three dams (D1, D2, D3) and for which the dams' blood is mosaic. Grey diamonds: same mutations in the descendants of the mosaic animal. **(B) DNMs without detectable mosaicism in the individual in which the mutation occurred:** Blue triangles: S(perm)N(on)M(osaic) mutations transmitted by the (non mosaic) sire to the male proband (Pr1) in whom the mutation was detected. Red triangles: S(perm)N(on)M(osaic) mutations transmitted by the (non mosaic) sire to the three female probands (Pr2, Pr3, Pr4) in whom the mutation was detected. Blue circles: O(oocyte)N(on)M(osaic) mutations transmitted by the (non mosaic) dam to the male proband (Pr1) in whom the mutation was detected. Red circles:

O(oocyte)N(on)M(osaic) mutations transmitted by the (non mosaic) dam to the three female probands (Pr2, Pr3, Pr4) in whom the mutation was detected.



338 Figure 3A

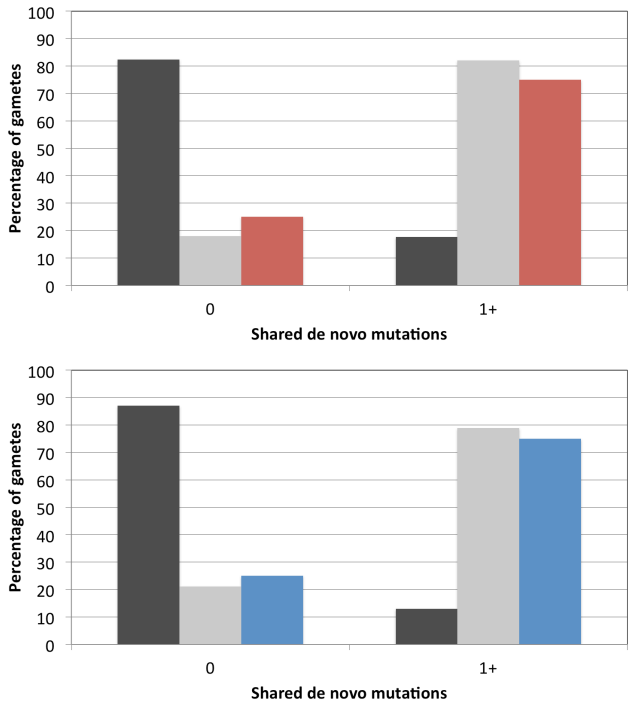


Figure 3B

340 **Figure 3: (A)** DNMs mosaic in sperm DNA of the male proband (PM – sperm) or
341 sires (SM – sperm), or in blood DNA of the female probands (PM – blood) or
342 dams (OM – blood) ranked by rate of mosaicism. Colored lines: real data. Pr1-4:
343 proband 1-4. Dark gray shaded area: 95% confidence interval obtained from
344 simulations assuming uniform mutation rate per cell division and 40 unrelated
345 PGCs. Light gray shaded area: 95% confidence interval obtained from
346 simulations assuming 10-fold higher mutation rate during the first 4 cell
347 divisions, and 40 related PGCs (Table 1). **(B)** Distribution of the proportion of
348 half-sibs of the four probands that share 0, or at least 1 (1+) of the DNMs
349 detected in the corresponding proband. Red bars: real observations for DNMs

transmitted by the sire (SM+SNM). Blue bars: real observations for DNMs transmitted by the dam (OM+ONM). Dark grey bars: expectation under the null hypothesis of uniform prenatal mutation rate per cell division and 40 unrelated PGCs. Light grey bars: expectation under the best alternative model assuming a 10x increased mutation rate during the first 4 cell division and 40 related PGCs (Table 1).

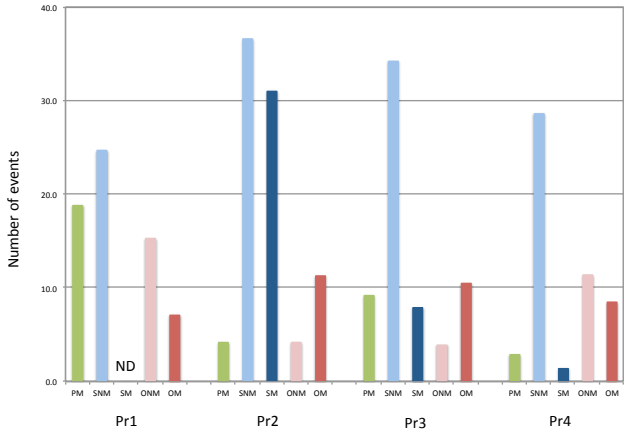


Figure 4A

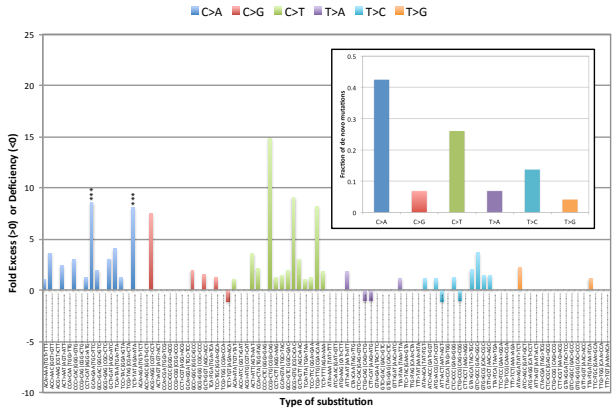


Figure 4C

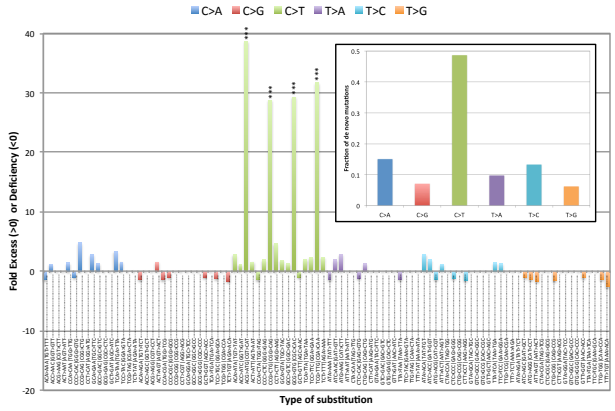


Figure 4B

Figure 4: (A) Types and number of DNMs detected in sperm DNA of male proband Pr1, and in blood DNA of female probands Pr2, Pr3 and Pr4. The overall numbers were estimated based on the observed DNMs, their rate of confirmation, and the estimated degree of genome coverage. **(B)** SNM and ONM (i.e. DNMs assumed to have occurred in the later stages of gametogenesis): fold excess or deficiency over expected for specific nucleotide substitutions accounting for trinucleotide context; ***: p< 0.002 (accounting for multiple

testing by Sidak correction). Inset: Proportion of DNMs corresponding to the six possible types of nucleotide substitutions. **(C)** Idem for PM, SM and OM (i.e. DNMs assumed to have occurred in the early stages of gametogenesis).

Table 1: Relative likelihood of the observations under different models of gametogenesis

Fold increase mutation rate	During first x divisions	Number of PGC	Related PGCs or not	Log(LR)
10x	**4**	**40**	**T**	0.00
10x	**6**	40	T	-0.16
10x	**2**	40	T	-3.31
20x	2	40	T	-4.90
10x	4	40	**F**	-5.01
10x	2	**4**	F	-18.33
1x	6	40	T	-38.83

The first four columns correspond to the parameters that were tested in the model: (i) the fold increase of the mutation rate (1x, 10x, 20x), (ii) during the x first cell divisions (2, 4, 6), (iii) the number of PGCs (4, 40), and (iv) the ontogenetic relatedness of the PGCs (F(alse) or T(rue)). Log(LR) corresponds to the logarithm (base 10) of the likelihood of the data relative to the best model (first line). Parameters are in bold when the corresponding model is the best given that parameter value. We only show results for models that are the best given at least one parameter value. Likelihoods of all models are given in Suppl. Table 2.