Discovery of an unusual high number of *de novo* mutations in sperm of older men using duplex sequencing

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Running Title: High mutation frequencies in *FGFR3*

Abstract

De novo mutations (DNMs) are an important player in heritable diseases and evolution, yet little is known about the different mutagenic processes in our germline given the difficulty to reliably identify ultra-low frequency variants. Of particular interest are highly recurrent DNMs associated with congenital disorders that have been described as selfish mutations expanding in the male germline, thus becoming more frequent with age. Here, we have adapted duplex sequencing (DS), an ultra-deep sequencing method that renders sequence information on both DNA strands; thus, one mutation can be reliably called in millions of sequenced bases. With DS, we examined ~4.5 kb of the *FGFR3* coding region in sperm DNA from older and younger donors. We identified highly mutable sites with mutation frequencies 4-5 orders of magnitude higher than the genome average. Multiple mutations were found at a higher frequency, or exclusively, in older donors, suggesting that these mutations are testis exclusive mosaics expanding in the male germline with age. Also, older donors harbored more mutations associated with congenital disorders. Some mutations were found in both age groups with no significant difference, suggesting that these might result from a different mechanism (e.g., post-zygotic mosaicism). We also observed that independently of age, the frequency and deleteriousness of the mutations in sperm were elevated compared to reports in the population. Our approach is an important strategy to identify mutations that could be associated with aberrant receptor tyrosine kinase activity, with unexplored consequences in a society with delayed fatherhood.

Keywords

Duplex sequencing, ultra-rare mutation, *de novo* mutation, driver mutations, male germline, receptor tyrosine kinases.

Introduction

There are certain *de novo* mutations (DNMs) that are highly recurrent with mutation rates orders of magnitude higher than genome average. These mutations have been discovered because of their association with congenital disorders. Moreover, these mutations have several other associated characteristics (reviewed in Arnheim and Calabrese 2009; Goriely et al. 2009; Goriely and Wilkie 2012; Arnheim and Calabrese 2016): they encode missense substitutions with gain-of-function properties (activating mutations); they occur exclusively in the male germline; and older men have a higher probability of having an affected child than younger males, known as the paternal age-effect (PAE) described already decades ago (Risch et al. 1987; Crow 2000; Crow 2012). In the literature, these mutations have been termed as PAE-mutations, selfish, or RAMP (recurrent, autosomal dominant, male biased, and paternal age effect) mutations, as reviewed in Arnheim and Calabrese 2009; Goriely and Wilkie 2012; Arnheim and Calabrese 2016.

Over the last decades, it was shown that the high incidence levels and steep increase with age of these PAE-mutations are not solely due to errors occurring during the continuous replicative process of spermatogenesis (Risch et al. 1987; Crow 2000; Crow 2012). Instead, it was suggested that these behave like driver mutations, well known in cancer, which are mutations that promote their own propagation. In particular, PAE-mutations have been described in genes such as *FGFR2*, *FGFR3*, *HRAS*, *PTPN11*, *KRAS* and *RET* (Qin et al. 2007; Choi et al. 2012; Shinde et al. 2013; Maher et al. 2016), and more recently in six new genes (*BRAF*, *CBL*, *MAPK1*, *MAPK2*, *RAF1*, and *SOS1*) (Maher et

al. 2018), all acting in the receptor tyrosine kinase (RTK)-RAS signaling pathway and expressed in spermatogonial stem cells (SSCs). The mutations modify the signal modulation of the RTK-RAS pathway by an activating effect of the mutant protein. This dysregulation of the RTK-RAS pathway drives the preferential expansion of mutant SSCs, observed in the testis as mutant clusters that become larger with age (Qin et al. 2007; Choi et al. 2008; Choi et al. 2012; Shinde et al. 2013; Yoon et al. 2013; Maher et al. 2016; Maher et al. 2018). Therefore, as men age, the germline becomes a mosaic for multiple PAE-mutations, all in different anatomical locations of the testes (Shinde et al. 2013; Arnheim and Calabrese 2016; Maher et al. 2018), suggesting that each mutation arises and expands independently.

The clonal expansion of some PAE-mutations in the testis with age also results in more mutant sperm in older individuals, and an increased incidence of the associated congenital disorder with paternal age, as observed for example for achondroplasia (ACH), a growth disorder with a defect in FGFR3 signaling (Risch et al. 1987; Tiemann-Boege et al. 2002; Shinde et al. 2013). Interestingly, the transmission of mutations in SSCs to sperm and hence to the offspring does not always correlate positively, especially mutations associated with tumors hardly overlap with spontaneous germline mutations (Goriely et al. 2009; Aoki and Matsubara 2013; Giannoulatou et al. 2013).

In spite of the importance of PAE-mutations in the male germline which is highlighted by their high incidence, increased frequency with paternal age, and phenotypes potentially leading to early or late-onset disorders in children of older men, we know very little about this mutagenic mechanism. There are still many open questions on how and to what extent specific PAE-mutations affect cell growth and spermatogonial stem cell differentiation, and the role of apoptosis or cell-death counterbalancing clonally expanding cells. So far, studies have focused on well-characterized mutations associated with a disorder (reviewed in Arnheim and Calabrese 2009; Goriely et al. 2009; Goriely and Wilkie 2012; Arnheim and Calabrese 2016) or on the most prevalent mutations observed at late oncogenic stages in somatic tumors, which were shown to be unrelated to the selfish expansion of mutant spermatogonial stem cells (Goriely et al. 2009; Giannoulatou et al. 2013; Maher

et al. 2016). However, many genes in the RTK-RAS signaling pathway, or in other pathways, could harbor as yet unknown mutations that could be expanding with paternal age but have gone undetected so far, though, with important health consequences in children of older fathers. To overcome this, a more recent study used a sequencing strategy to discover prospective driver mutations in genes of the RTK-RAS pathway by enriching for mutant SSCs identified in testes biopsies by a high RTK activity that identified 61 mutations at a frequency of 0.06% or larger (Maher et al. 2018).

In this work, we further developed the discovery of prospective RTK mutations in the male germline to include ultra-low frequency mutants (<2 mutants in 100,000 wild type molecules or a mutation frequency of $<2x10^{-5}$). For this purpose, we used sperm since this biological material is easy to sample and provides information on whether mutations are passed on throughout the different maturation and differentiated stages of the male germline. Accurately identifying ultra-low frequency mutations by sequencing is still technically very challenging, especially if no enrichment of mutants (e.g., high RTK signaling) is possible. To date, only a few methods can achieve the required sensitivity and accuracy. One of these methods is duplex sequencing (DS), a strategy that organizes sequence reads derived from the same DNA molecule into families with information on the forward and reverse strands (Schmitt et al. 2012). Given the excessive sequencing depth required in DS, in this work we focused on the coding region of *FGFR3* because (1) this gene has well characterized PAE-mutations; (2) various reported DNMs associated with congenital disorders have incidence levels that fall within the sensitivity of DS (reviewed in Goriely et al. 2009; Goriely and Wilkie 2012; Shinde et al. 2013; Maher et al. 2018); and (3) *FGFR3* has been categorized as an oncogene with many missense mutations and high oncogenic score (Tomasetti and Vogelstein 2015).

We sequenced the coding regions of the *FGFR3* gene at an average coverage depth of ~11,000x (up to ~60,000x) in a young and an old donor pool. We identified 161 variants at 105 genomic positions in the exonic *FGFR3* sequence (exon 3-15 without exon 11); of those 29, have been reported in other databases to be associated with congenital disorders (Human Gene Mutation database (HGMD))

and/or tumors (Catalogue Of Somatic Mutations In Cancer (COSMIC)); 54 DNMs have never been reported. Some DNMs were found exclusively or at a higher frequency in the older donor set. Interestingly, we also identified many high frequency mutations independent of the age of the donors, suggesting that these might not be testis-exclusive mutations, but could have potentially happened earlier in development from a post-zygotic event. Overall, our method is a powerful approach to identify PAE-mutations that can be then further characterized to better understand this important type of mutagenesis in the male germline.

Results

DS detects ultra-low mutation frequencies

Short-read next-generation sequencing (NGS) is a widely used method for prospective screening of mutations, but given its high error rate of ~0.1-2%, it cannot be used for detecting low or ultra-low frequency variants or mutations (reviewed in Salk et al. 2018). In the past years, different approaches for library preparation have been published to distinguish technical errors from real DNA variants, decreasing error rates to ~10^{-5} (depending on the method) (Ebner et al. 2011; Jabara et al. 2011; O'Roak et al. 2012; Schmitt et al. 2012; Hiatt et al. 2013; Lou et al. 2013). This is achieved by individually tagging each DNA molecule before amplification and sequencing with an Illumina platform. The tags are then used for grouping reads into a consensus sequence (family). A true mutation is present in the majority of the reads within a family, whereas PCR and sequencing errors are only present in a subset of reads.

The sequencing method with the lowest reported error rate using such a tagging approach is DS, which uses a double barcode strategy to retrieve information from both strands of the original DNA molecule. In DS, reads are first organized into families of either the forward or the reverse strand,

known as single-stranded consensus sequence (SSCS). Then, the complement SSCS are further grouped into the duplex consensus sequence (DCS) representing both DNA strands of one initial DNA molecule (Figure 1A). A variant is considered true, if present in the majority of reads of both strands in the DCS, allowing for an extremely low error rate of $<10^{-7}$, which is a major improvement of at least 1-2 orders of magnitude compared to other ultra-deep sequencing methods (Schmitt et al. 2012; Fox et al. 2014; Kennedy et al. 2014). Most ultra-deep sequencing methods use information of only one strand and are limited mainly by DNA lesions (Lou et al. 2013; Arbeithuber et al. 2016). These DNA lesions can be amplified during the library preparation resulting in false positives. DS greatly reduces errors resulting from DNA damage since a true variant is present in both DNA strands, while a DNA lesion is only found in one DNA strand (Arbeithuber et al. 2016).

Here, we illustrate the higher accuracy of a duplex over a single-stranded consensus sequence (DCS vs. SSCS), the latter being equivalent to ultra-deep sequencing approaches examining only one DNA strand. We compared the mutation frequency obtained when grouping reads into a family from either the forward or the reverse strand (SSCS) with the mutations obtained from the DCS (Figure 1B). The average mutation frequency using SSCS was calculated to be 9.13×10^{-5} substitutions/sequenced base pairs and it is reduced to 6.46×10^{-7} in DCS (Figure 1B). Note that insertion-deletions were not considered here. Some variants within SSCS could be PCR jackpots (errors occurring during the initial PCR cycles that are exponentially amplified (Lou et al. 2013)) or alternatively the product of DNA lesions as observed previously (Arbeithuber et al. 2016). In fact, the majority (86%) of the called SSCS were C>A transversions (the product of oxo-G lesions) or G>A / C>T transitions (the product of cytosine deamination) as shown in Supplemental Figure S1.

The high accuracy of DS is tied with the disadvantage that this approach is very data expensive and only a fraction of the input molecules ends up in a DCS. For this reason, we only focused on exon 3 to 15 of the *FGFR3* gene (~4.5 kb). As shown in Figure 1C, each of our sequencing libraries targeted one of two ~2.5 kb regions of *FGFR3*. The two regions were divided further into 5 sub-regions (Up 1-5 or Down 1-5) each ~500 bp in size that could be fully covered by the two paired-end (PE)-reads

with an Illumina MiSeq v3 600-cycles sequencing kit. Our DS strategy also included a new approach that enriched for these sub-regions using restriction enzymes followed by the removal of bulk DNA via bead size selection (see Methods). With this DS strategy, we achieved a median DCS coverage for all merged libraries of 11,087 (range from 6,222 to 38,421 per site calculated using the median coverage of each position across all libraries). Thus, we accurately detected mutations with a variant allele frequency (VAF) as low as 1.5×10^{-5} (Figure 1D and Supplemental Table S1).

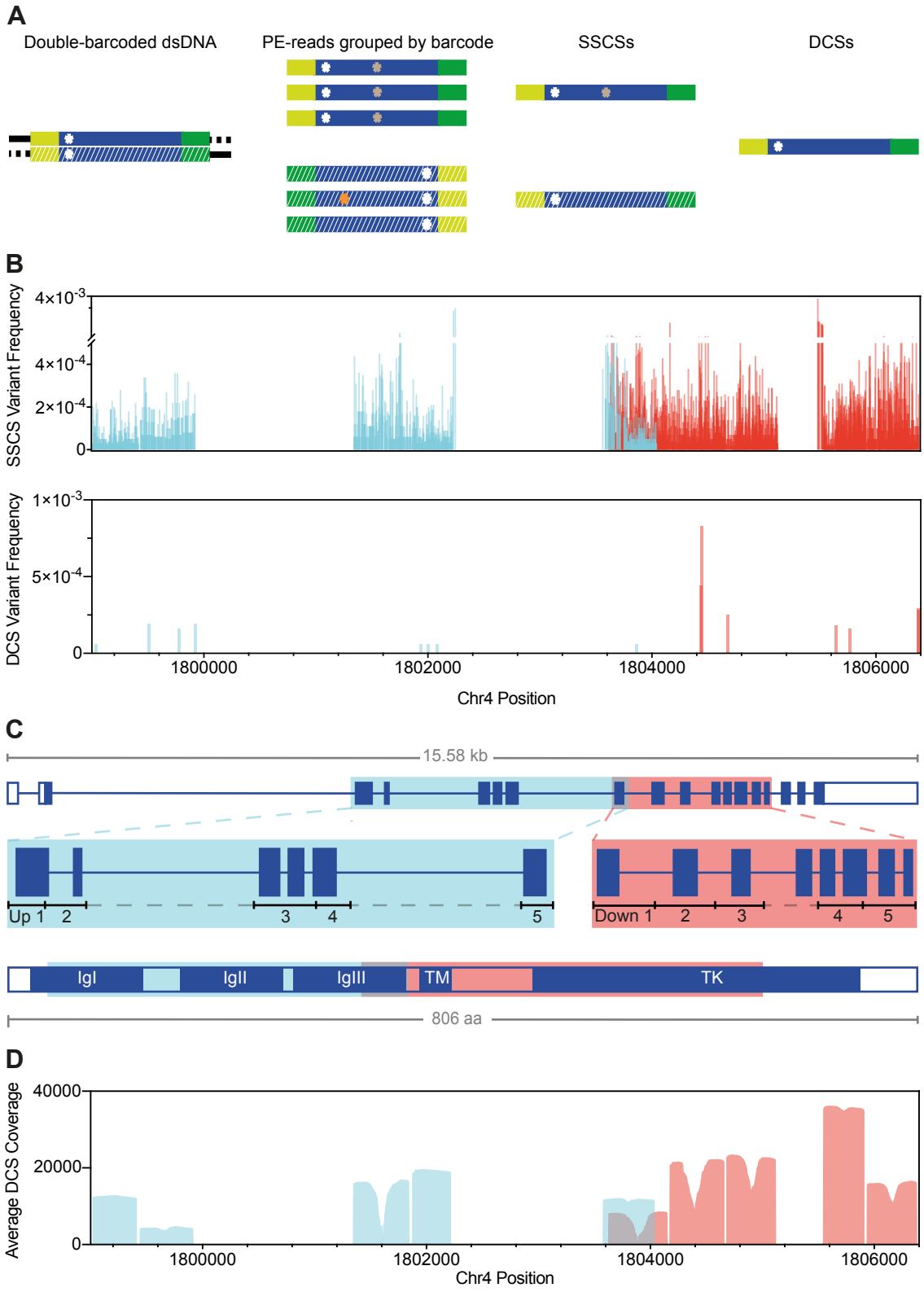

Figure 1. DS using *FGFR3* targeted sequencing strategy with enzymatic digestion. (*A*) DS overview: barcoded adapters (yellow and green) were ligated at both ends of size-selected restriction enzyme digested genomic fragments (blue). After several rounds of PCR amplification and hybridization captures, libraries were sequenced on the Illumina MiSeq (v3 600-cycles); for sequence analysis, paired-end (PE)-reads were grouped into Single Strand Consensus Sequences (SSCS) and complementary SSCS were joined into a Duplex Consensus Sequence

(DCS). Only DCS with mutations in both complementary SSCS (white asterisk) were considered true variants. Colored asterisks represent artefacts (e.g., sequencing and PCR errors). (B) Variants detected at 2,881 different positions of the total 4,405 sequenced positions identified in SSCS (n=14,552 total mutations; VAF = 1.0×10^{-5} to 3.8×10^{-3}) or at 15 positions in DCS (n=15 total mutations; VAF=6.0×10^{-5} to 8.3×10^{-4}) of two *FGFR3* libraries, each targeting either the Up 1-5 (blue) or the Down 1-5 (red) regions/subregions. (*C*) Exonic structure of *FGFR3*. Shown are the five upstream (blue) or downstream (red) regions targeted via restriction enzyme fragmentation that include exons 3 to 8 or 8 to 15 (except exon 11), respectively; restriction digests rendered fragments ~370-550 bp in size. Acronyms of the FGFR3 structure: Immunoglobulin-like domain I-III (IgI-III); Transmembrane domain (TM); Tyrosine kinase domain (TK); amino acids (aa). (*D*) Average DCS coverage per position of all sequenced libraries.

In order to confirm that variants called by our pipeline represent true mutations, eight of the mutations identified with DS were measured with other ultra-sensitive detection methods, namely bead emulsion amplification (BEA) as described in (Shinde et al. 2013) or droplet digital PCR (ddPCR); see Supplemental Table S2. BEA is an in-house digital PCR method based on the amplification of single molecules on magnetic beads within an emulsion (Boulanger et al. 2012; Shinde et al. 2013). The ddPCR technology (Bio-Rad Laboratories) is based on the partitioning of PCR reaction into thousands of individual reactions within water-in-oil droplets that are read out in an automated droplet flow-cytometer after the PCR reaction is concluded (Hindson et al. 2011); see Methods for further information. Our DS mutation measurements were very similar among methods (e.g., c.1118A>G was detected in 3 DCS for one of the libraries with a frequency of 3.23×10^{-4}, comparable to the 1.11×10^{-4} obtained with BEA). A Fischer's exact test for all possible comparisons did not reveal any significant difference between the measured mutation frequencies. However, we also observed larger deviations that could be due to sampling differences between methods. In particular, the low number of mutant counts in DS (in most cases one event) was prone to large variations because of the Poisson distribution of single events. In comparison, in BEA and ddPCR more starting molecules were used (~300,000 genomes) rendering also a larger number of mutants; thus, these two methods were more accurate for the quantification of very low mutation frequencies compared to DS.

As a further demonstration of the high sensitivity and accuracy of DS, we carried out a mixing experiment. For this purpose, different genomic DNA obtained from Coriell Cell Repositories (Camden, NJ) each carrying a point mutation (c.742C>T, c.746C>G, c.749C>G, and c.1620C>A) were spiked into DNA from a younger donor at ratios from 1/10 to 1/10,000 in steps of one order of magnitude (Figure 2). With this orthogonal assay, we demonstrated that the variant frequency detected with DS was equivalent to the expected input of the 10-fold dilutions. Note that the starting input amounts varied due to pipetting errors and the occasional inaccuracy of spectrophotometric measurements used to establish the initial DNA concentration. Fourteen out of 16 variants (four variants per library) were identified. Two replicates of the 1:10,000 dilutions were not detected likely due to sampling drop-out at the Poisson distribution level. The Pearson's correlation coefficient between observed and expected input dilutions of the 14 measurements was very high ($R^2 = 0.96$, p-value = 9.8×10^{-10}) demonstrating the power of DS to measure ultra-rare variants (Figure 2).

A

CDS Sub	1:10		1:100		1:1000		1:10000	
	DCS Cvrg	n	DCS Cvrg	n	DCS Cvrg	n	DCS Cvrg	n
c.742C>T	9245	896	11272	84	12891	17	14664	0
c.746C>G	9201	608	11182	48	12795	7	14548	2
c.749C>G	9181	1217	11193	122	12786	20	14552	3
c.1620C>A	11159	912	14174	72	15339	7	17861	0

B

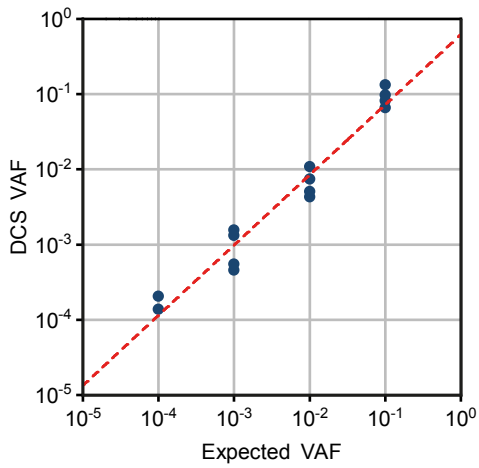

Figure 2. DS spike-in test. (*A*) DNA from 4 different cell lines (Coriell Cell Repositories; Ref: CD00002, NA00711, NA08909, GM18666) was serially diluted into sperm DNA from a young donor in order to prepare four DS libraries ("FGFR3 Y C 1:10", "FGFR3 Y C 1:100", "FGFR3 Y C 1:1000", "FGFR3 Y C 1:10000") with expected mutant frequencies of 1:10, 1:100, 1:1000, and 1:10000, respectively. DCS coverage and the number of times the mutants were found in each library are shown. (*B*)

Expected VAF vs. measured VAF with DS; 14 out of the 16 mutations were detected; $R^2 = 0.96$ (Pearson's correlation coefficient).

High frequency mutations detected in the coding region of* FGFR3 *in sperm DNA

In total, we sequenced 13 libraries, each prepared from sperm DNA pooled from five healthy individuals, most of which with European ancestry. Seven libraries featured younger donors with ages ranging from 26 to 30 years, and the remaining six featured older donors with ages ranging from 49 to 59 years; see Supplemental Table S3 and Supplemental Table S4. Consensus sequences were first formed using Du Novo (Stoler et al. 2016; Stoler et al. 2020) and variants were called using the Naive Variant Caller (Blankenberg et al. 2014), followed by a more thorough screening with our Variant Analyzer software (Povysil et al. 2021). The Variant Analyzer re-examines raw reads from Du Novo variant calls and provides different summary data that categorizes the confidence level of a variant by a tier-based system. This tier classification is based mainly on the number of reads per family and the proportion of the alternative allele in the consensus sequence (e.g., Tier 1 is composed by families with 3 or more members for both forward and/or reverse strands). With this tier classification (see Supplemental Table S5), we can distinguish true mutants from bad quality data or biases leading likely to false positives. Moreover, our Variant Analyzer supports the analysis of reads without a family as demonstrated in (Povysil et al. 2021), which allowed maximizing the data output.

In total, we identified 689 mutations classified as Tier 1 or Tier 2 located at 551 different genomic positions of the 4,405 sequenced positions of *FGFR3*. This list does not include single-nucleotide polymorphisms (SNPs) (variants with a frequency of ~10% in the donor pool, likely to be a heterozygous position in one of the individuals) or variants with DCS coverage below 1000. We shortlisted the 689 mutations to those located in exons. We only included Tier 2 variants when detected at least twice with a Tier 1 and/or Tier 2 in the same or different libraries. Multiple hits

increase the likelihood for these to be real. We retained 161 high-confidence variants at 105 genomic positions (Figure 3 and Supplemental Table S1).

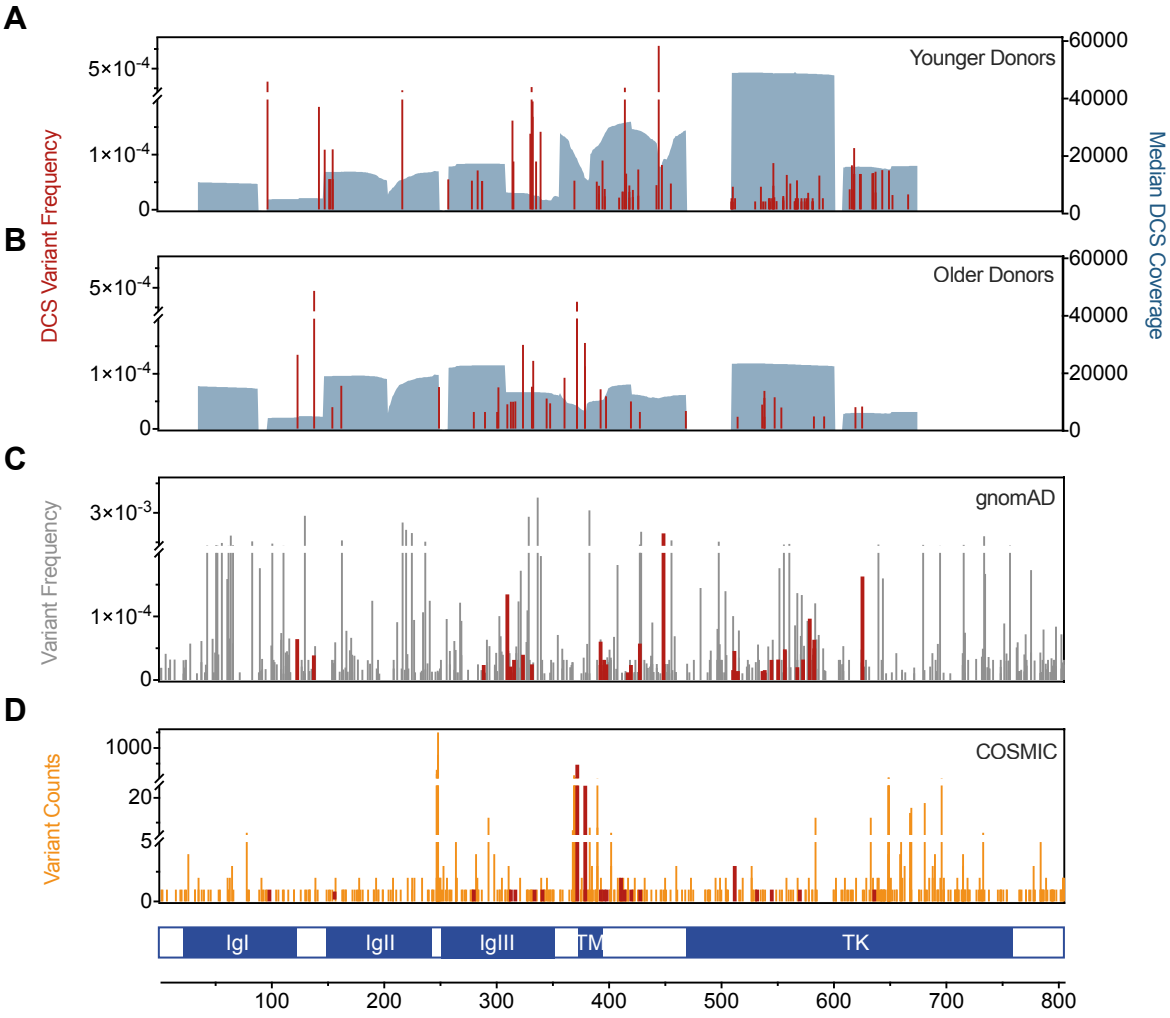

Figure 3. Variant frequency and DCS coverage. In red, the variant allele frequency (VAF) of the 161 exonic mutations found in all libraries from younger (118 mutations, (*A*)) and older donors (43 mutations, (*B*)) listed in detail in Supplemental Table S1. DCS coverage (grey shaded area) is the median DCS coverage at exonic positions of all libraries within the same age group. (*C*) Exonic gnomAD variants for *FGFR3* with frequencies between 1 x 10^{-5} and 1 x 10^{-2}. (*D*) Counts of exonic variants associated with tumors (orange) were retrieved from the COSMIC database. The gnomAD and COSMIC variants detected also by DS are shown in red. Immunoglobulin-like domain I-III (IgI-III); Transmembrane domain (TM); Tyrosine kinase domain (TK) of FGFR3.

All mutants detected in this study had a DCS frequency between 1 x 10^{-5} to 8 x 10^{-4} mutations per nucleotide (Figure 3 and Supplemental Table S1), which is 1000 – 10,000-fold higher than the human

germline mutation frequency, reported as low as ~1 x 10^{-8} mutations per nucleotide (Kong et al. 2012; Campbell and Eichler 2013; Francioli et al. 2015). These high mutation frequencies suggest that a mutational mechanism that confers a selective advantage might be responsible for the observed high mutant numbers accumulated in sperm. The young donor pool had a higher overall mutation frequency than the older donor pool (2.0 x 10^{-6} vs 5.2 x 10^{-7}, respectively) calculated by summing all mutants within all libraries of an age group and dividing it by the sum of the DCS coverage for the same libraries. The difference between donor pools might be explained by the higher coverage in three of the young libraries rendering more mutant counts.

Out of the 161 exonic mutations detected (all libraries combined), half were detected in the tyrosine kinase (TK) domain (Figure 3 and Supplemental Table S1). This larger number might be explained by the higher coverage for a part of this domain in the younger donors (71 of the 82 mutants found in the TK belong to libraries from younger donors). In fact, regardless of the larger absolute number of mutants, the mutation frequency in the TK domain was lower (median 2.23 x 10^{-5}) than in other domains of FGFR3 (see Supplemental Table S1). Why mutation frequencies are lower in the TK is not known, but it might be that mutations in this domain are highly deleterious and not tolerated in spermatogenesis. This was suggested for mutations at the active site of the TK domain (codon p.K650) analyzed in sperm: highly activating substitutions mainly associated with cancer (e.g., seborrheic keratoses) were underrepresented in sperm compared to less “oncogenic” substitutions (e.g., p.K650E and p.K650T resulting in thanatophoric dysplasia type II (TDII) and familial acanthosis nigricans, respectively) (Goriely et al. 2009).

In contrast, the high frequency observed in or around the transmembrane (TM) domain suggests that amino acid substitutions might be tolerated better in this part of the protein during sperm production. With DS, we detected 8 different high frequency mutations within or surrounding the TM domain. Interestingly, two of those mutations (p.Y373C and p.G380R) were found only in the older donor pools and have been reported to be associated with congenital disorders such as thanatophoric dysplasia type I (TDI) or ACH. We suspect that the TM domain could be an important

harbor for PAE-mutations expanding in the male germline with age, causing late-onset tumors and/or germline disorders. Moreover, in this domain or in the surrounding inter-domains, we captured some of the mutations with the highest frequencies (e.g., p.Y373C, p.G380R, and p.E447K); almost 50% higher in magnitude than the median of the other mutations (~$4x10^{-5}$) measured in *FGFR3*. Note that a higher number of COSMIC hits have also been reported in this domain; but not in the general population (Genome Aggregation Database (gnomAD)).

The Immunoglobulin-like (Ig)III domain is reported to have the highest number of mutations associated with congenital disorders according to the HGMD database (Figure 4). Our DS data also partially reflect this; however, of the 20 mutations associated with congenital disorders in this domain (Figure 4), we detected only c.1000G>A (p.A334T) and c.749C>G (p.P250R) causing craniosynostosis and Muenke syndrome, respectively. Additionally, we discovered 20 new mutations in this domain, 5 and 10 times more than found in IgII and IgI, respectively. These are domains with similar size and comparable median coverage, especially IgII and IgIII.

Mutational mechanisms

Looking at the entire targeted *FGFR3*, 54 of the 161 mutations have not been reported before in any public databases, 21 were unique mutations associated with tumors (mainly bladder, skin, and multiple myeloma, which are the most common cancer associations within *FGFR3*), and 45 were mutations reported in the gnomAD database (likely viable mutants). Whether these are associated with a paternal-age effect and might have a deleterious effect like a congenital disorder, a late-onset disease (e.g., cancer), or instead a normal phenotype, is not known.

Aiming to explore the possible mutational mechanisms behind our measured mutations (Supplemental Table S1), we further characterized 17 mutations (15 amino acid substitutions) that either (1) co-occurred in both younger and older donor pools or (2) that occurred exclusively in older donors at frequencies in the upper range (>1 x 10^{-4}); see Table 1. These latter mutations, c.1118A>G and c.1138G>A, with a frequency of ~3 x 10^{-4} and ~1 x 10^{-4}, were captured in multiple libraries from

older donors with frequencies >1x 10^{-4}, but not in young libraries in spite of the high coverage at the same position in younger donors (e.g., >10,000 DCS coverage). However, we cannot exclude that in younger pools these were missed given the Poisson probability of single events. Interestingly, both of those mutations are also associated with congenital disorders: variant c.1118A>G (p.Y373C) causes TDI and variant c.1138G>A (p.G380R) is linked to ACH (Figure 4). The latter has been described as a PAE-disorder with older men fathering more affected offspring (revised in Tiemann-Boege et al. 2002).

It is possible that variants occurring only in the older donor pool might be testis-exclusive mosaics growing larger over time. Other interesting variants are c.1620C>A and c.1620C>G. Both are missense mutations that result in the same amino acid substitution (p.N540K) and were found 3-times in 2 different older donors libraries (frequency of 2.2 x 10^{-5} and 7.0 x 10^{-5}). Whereas, in younger donors libraries, they were found only once (frequency of 1.6 x 10^{-5}), albeit the average DCS coverage was higher in the latter. This amino acid substitution is known to be associated with hypochondroplasia, which also suggests that this variant could expand in the male germline with age. A more in-depth analysis of more sperm samples or the distribution within testis with a less expensive approach will provide more details in this regard.

			Younger Donors				Older Donors					
CDS	**AA**	**FGFR3 Domain**	**n**	**DCS Coverage**	**Frequency**	**Avg DCS Coverage**	**n**	**DCS Coverage**	**Frequency**	**Avg DCS Coverage**	**Previously Described**	**Category**
c.464G>T	p.R155L	IgII	1	17840	**5.61×10^{-5}**	13627	1	25141	3.98×10^{-5}	19100	-	PZM-Candidate
c.841G>T	p.A281S	IgIII	1	18935	**5.28×10^{-5}**	16488	1	32262	3.10×10^{-5}	22784	2	PZM-Candidate
c.952G>A	p.D318N	IgIII	1	11412	**8.76×10^{-5}**	6563	1	20014	5.00×10^{-5}	12857	1, 2	PZM-Candidate
c.999C>T	p.D333D	IgIII	1	7241	**1.38×10^{-4}**	6206	1	12984	7.70×10^{-5}	12839	1	PZM-Candidate
c.1000G>A	p.A334T	IgIII	1	3750	**2.67×10^{-4}**	6198	1	8045	1.24×10^{-4}	12835	1, 3	PZM-Candidate
c.1118A>G	p.Y373C	Inter-domain				15225	1 3	3169 9277	**3.16×10^{-4}** **3.23×10^{-4}**	8134	2, 3	PAE-Candidate
c.1138G>A	p.G380R	TM				12808	1 1	6391 8790	**1.56×10^{-4}** **1.14×10^{-4}**	6586	2, 3	PAE-Candidate
c.1182G>A	p.T394T	TM	1 1	22756 34880	4.39×10^{-5} 2.87×10^{-5}	17554				10974	1, 2	PAE-Candidate
c.1182G>T							1	13852	**7.22×10^{-5}**			
c.1196G>A	p.R399H	Inter-domain	1 1	40263 26444	2.48×10^{-5} 3.78×10^{-5}	19747	1	16741	**5.97×10^{-5}**	12945	1, 2	PAE-Candidate
c.1262G>A	p.R421Q	Inter-domain	1 1	30509 45305	3.28×10^{-5} 2.21×10^{-5}	25999	1	19931	**5.02×10^{-5}**	16941	1, 2	PAE-Candidate
c.1286C>A	p.A429E	IDM	1	38608	2.59×10^{-5}	26152	1	31914	**3.13×10^{-5}**	16573	-	PAE-Candidate
c.1620C>A	p.N540K	TK	1	64874	1.54×10^{-5}	43207	1	44819	**2.23×10^{-5}**	27389	3	PAE-Candidate
c.1620C>G							2	28809	**6.94×10^{-5}**			
c.1647G>T	p.G549G	TK	1 2 1	11762 45522 61836	**8.50×10^{-5}** 4.39×10^{-5} 1.62×10^{-5}	41905	1	17424	5.74×10^{-5}	27067	1, 3	PZM-Candidate
c.1862G>T	p.R621L	TK	1	15309	**6.53×10^{-5}**	20141	1	25137	3.98×10^{-5}	10612	-	PZM-Candidate
c.1879G>A	p.E627K	TK	1	15299	**6.54×10^{-5}**	19781	1	24588	4.07×10^{-5}	10385	1, 3	PZM-Candidate

Table 1. Comparison of mutations found in younger and older donor pools. Mutations found in both older and younger donors are displayed together with mutations found in older pools with a frequency higher than 1×10^{-4}. For the latter, DCS coverage in younger pools of donors is >10,000 for the corresponding position. The number of times the variant is found within a library and its corresponding DCS coverage is shown together with the average DCS coverage obtained for each variant across all libraries of the same age group. Variants with a higher frequency in older donors and variants exclusively found in older pools at a higher frequency were labelled as Paternal Age Effect (PAE) candidates; the remaining variants were considered post-zygotic mosaic (PZM) candidates. Previously described in gnomAD (1); in COSMIC (2), as congenital disorder (3), or newly discovered mutation (-); e.g., 1,3 or 2, or –. Immunoglobulin-like domain I-III (IgI-III); Transmembrane domain (TM); Tyrosine kinase domain (TK) of FGFR3.

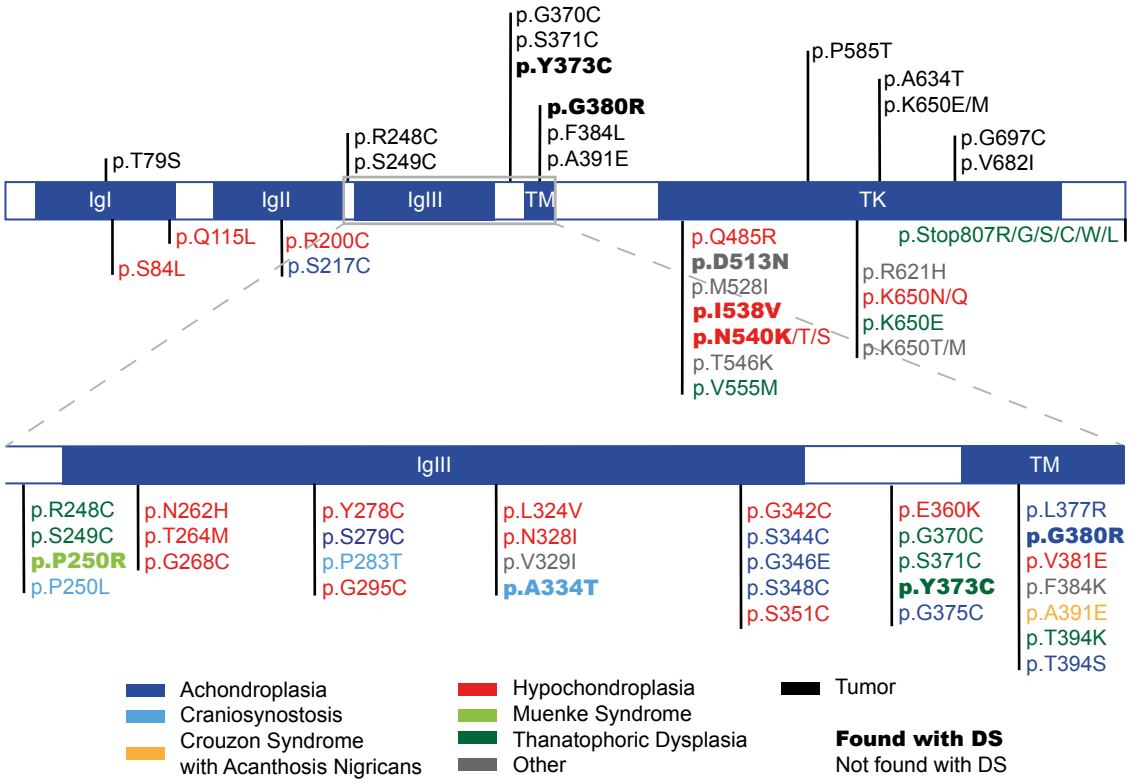

Figure 4. *FGFR3* mutations associated with germline disorders and/or tumors**.** Shown are FGFR3 protein domains and associated mutations (top) documented to cause germline disorders (color-coded like the respective disorder) according to the HGMD database and the variants (bottom) with the most counts in the COSMIC database. Mutations captured with our DS approach are highlighted in bold. Immunoglobulin-like domain I-III (IgI-III); Transmembrane domain (TM); Tyrosine kinase domain (TK).

In comparison, eight mutations were found only once in both older and younger pool libraries. The variable coverage between libraries makes it difficult to assess if these are driver mutations expanding in the germline with paternal age forming testis-exclusive mosaics or whether other mutational mechanisms can explain their high frequency. One possible mutational mechanism could be post-zygotic mosaicism (PZM). DNMs occurring post-zygotically, but still early in development, will potentially be present in different cell populations (the earlier in development, the wider is the range of affected tissues), consequently originating a so-called mosaic state (Rahbari et al. 2016). Especially, mutations in genes of the RTK pathway have been documented as PZMs (reviewed in Tiemann-Boege et al. 2020).

Albeit challenging, PZM might be distinguished from testis-specific mosaics (documented as PAE-candidates) (Goriely et al. 2003; Qin et al. 2007; Maher et al. 2018; Tiemann-Boege et al. 2020). In Table 1, we categorized variants as PAE-candidates if detected at a higher frequency or exclusively in older donors, and as PZM-candidates if variants co-occurred in both donor pools at similar frequencies. Note that there are some variants found exclusively in younger donors. These could also be PZM-candidates missed in the older donor pool due to sampling.

For those selected mutations, we further investigated if we see functional or biological differences between the PZM and PAE candidates (Table 1). Specifically, we compared the predicted deleteriousness annotated for each variant by the Combined Annotation Dependent Depletion (CADD) scores. CADD translates multiple genome annotations into a single score obtained through machine learning used as a measure of deleteriousness of single-nucleotide variants or small insertion-deletions (Rentzsch et al. 2018). We analyzed this parameter for all the mutations detected in younger pools, older pools, *FGFR3* exonic variants reported in the gnomAD database, and in the COSMIC database. As shown in Figure 5A, the different mutation categories found in sperm (younger or older donors, PZM or PAE) have similar CADD scores as COSMIC variants, implying that mutations found in sperm can be as deleterious as tumor-associated variants. Interestingly, gnomAD variants had the lowest CADD scores, whereas scores of the sperm categories and COSMIC variants were significantly higher. This suggests that at least part of these substitutions might have a stronger functional impact than most of the variants currently segregating in the population.

Using the same grouping of mutations, we investigated the association with germline disorders (Figure 5B) and tumors (Figure 5C). PAE-candidates contained the largest proportion of mutants associated with germline disorders and tumors. Also, the percentage of association with germline disorders was higher for mutations found in older donors when compared to the younger pools. In comparison, PZM-candidates were not associated with as many germline disorder or tumor variants, highlighting further differences between these two groups. As expected, variants found in the general population (gnomAD) had the lowest percentage for both associations.

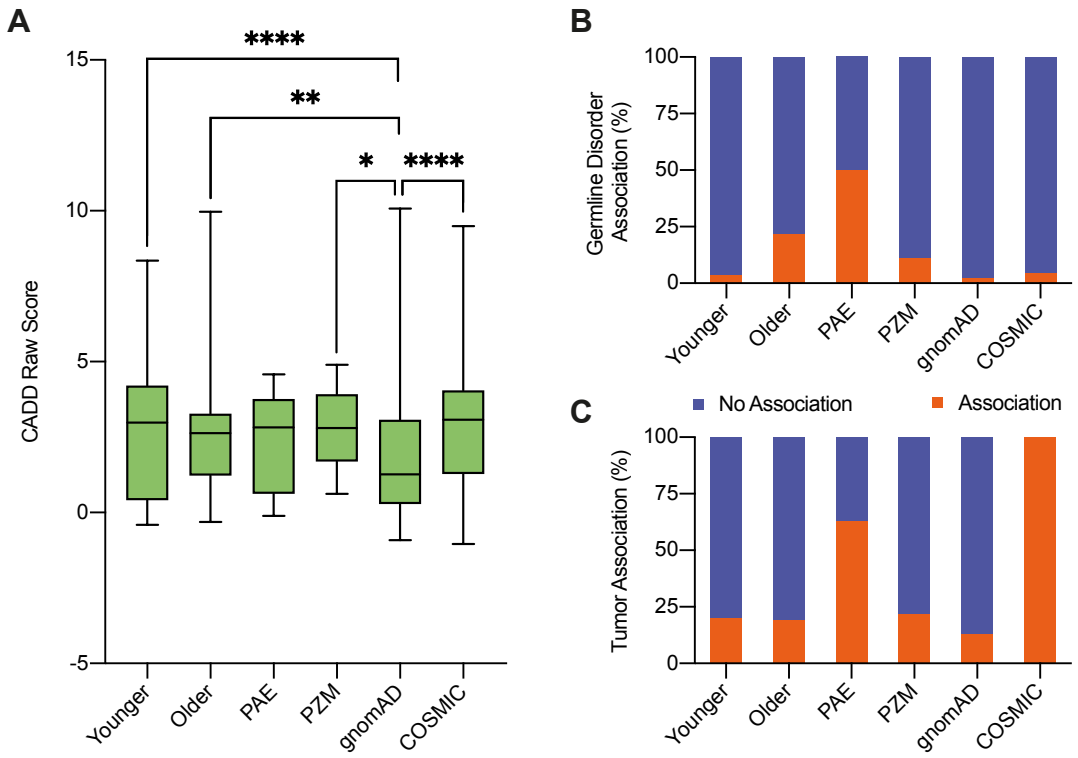

Figure 5. Predictive deleteriousness of variants and association with germline disorders and tumors. (*A*) Six groups of exonic single-nucleotide substitutions were analyzed: Variants found in libraries from younger donors (Younger, n=87, Supplemental Table S1); variants found in libraries from older donors (Older, n=37, Supplemental Table S1); mutations selected from all sequenced libraries as potential paternal age effect (PAE, n=9); mutations selected from all sequenced libraries as potential post-zygotic mosaics (PZM, n=8); exonic single-nucleotide substitutions from gnomAD v2.1.1 database (gnomAD, n=876); exonic single-nucleotide substitutions from COSMIC v90 database (COSMIC, n=397). Boxplot comparison of the CADD raw scores obtained for each variant group. A higher score reflects a higher probability of a deleterious effect. Pairwise testing was performed using Wilcoxon-Mann-Whitney test and only significant differences are marked ((*) p-value < 0.05; (**) p-value < 0.01; (****) p-value < 0.0001). (*B*) Percentage of variants associated with germline disorders according to ClinVar. (*C*) Percentage of variants associated with tumors according to the COSMIC database.

Discussion

Targeted DS of a human gene

The establishment of DS opened exciting new possibilities in ultra-rare variant detection with an unprecedented sensitivity for a sequencing-based method (Schmitt et al. 2012; Salk et al. 2018).

However, the need for consensus sequences for each DNA strand requires the sequencing of multiple PCR replicates, which translates into extremely high costs if the targeted region is large (Kennedy et al. 2014). Studies comprising the screening of mitochondrial DNA (mtDNA) have greatly benefited from the application of DS. With a length of 16,569bp, mtDNA can capitalize on the high accuracy and sensitivity of DS and still be targeted in its entirety (Kennedy et al. 2013; Ahn et al. 2015). Furthermore, in comparison with nuclear DNA, mtDNA is technically less demanding since it does not require a targeted capture strategy considering the input DNA is almost exclusively mtDNA (Kennedy et al. 2014).

The most commonly used strategy to target genomic regions for DS is based on consecutive rounds of hybridization captures and enrichment (Schmitt et al. 2015; Krimmel et al. 2016; Loeb et al. 2019; Salk et al. 2019; Shen et al. 2019). Typically, the input genomic DNA is sonicated according to the chosen read length. After this step, targeted and untargeted DNA is still present. The hybridization capture strategy, although commonly used, is not efficient when dealing with small targets, with most DNA molecules being lost in the process (Winters et al. 2017; Nachmanson et al. 2018). Our DS library preparation approach used restriction enzymes to fragment DNA, followed by size selection. Like the CRISPR/Cas9 approach (Nachmanson et al. 2018), this also enables the selection of pre-determined target fragments, achieving also a low number of off-targets. Further enrichment of targeted regions is performed by two rounds of hybridization capture followed by PCR, as previously described (Schmitt et al. 2015). The DCS coverage obtained for every library (Supplemental Figure S2) shows variation across different libraries, which can be explained by the different parameters used (e.g., input DNA, PCR cycles, sequencing read number). We also observed variable DCS coverage values between different targeted regions within each library that can be attributed to differences in performance of each restriction enzyme, amplification efficiency of each amplicon, and capture oligo efficiency. The lower DCS coverage obtained in the central parts of the restriction fragments is originated by the trimming of low quality read ends. However, with our DS approach we

achieved a median DCS coverage of ~11,000 (up to ~60,000) allowing to call mutations at a frequency of 1/10,000 to 1/100,000.

***Mutation accumulation in* FGFR3**

The very high frequency of DNMs in *FGFR3* increasing with age in the paternal germline was first described for ACH (c.1138G>A) by screening a panel of sperm donors of different ages (Tiemann-Boege et al. 2002). Another high-resolution study that analyzed the frequency of all 9 possible mutations in the active site of the TK domain p.K650 observed that the mutation prevalence in this particular codon is dependent on the activating effect of the mutation on receptor signaling (Goriely et al. 2009). Several DNMs in receptor kinase receptors (e.g., *FGFR2* and *FGFR3*) expressed in the male germline have been classified as driver mutations that accumulate over time because the resulting mutated protein confers a selective advantage to the SSCs, leading to a clonal expansion of the mutant. These mutations have reported frequencies of up to 1,000-fold higher than the germline average (reviewed in Arnheim and Calabrese 2009; Goriely and Wilkie 2012; Arnheim and Calabrese 2016).

In this study, we were able to detect 9 mutations (7 amino acid substitutions) that are PAE-candidates and are likely expanding in the male germline given the higher frequencies in older donor pools (Table 1). Three out of seven of these amino acid substitutions have been associated with PAE disorders and are suspected to increase with paternal age. One of these PAE-candidates is the c.1118A>G (p.Y373C) mutation measured at a frequency of ~3 x 10^{-4}; one of the highest frequencies measured in our dataset. This mutation is causal for the autosomal dominant congenital disorder TDI, also described as a PAE-disorder (Rousseau et al. 1996b; Wilcox et al. 1998). Similarly, the c.1138G>A (p.G380R) mutation measured at a frequency of ~1 x 10^{-4} (very close to the frequency reported in other studies (Tiemann-Boege et al. 2002)) is associated with the autosomal dominant congenital disorder ACH, another PAE-disorder (Rousseau et al. 1994). This mutation is also known to increase in frequency in sperm of older donors (Tiemann-Boege et al. 2002) and it was reported to

confer an advantage to the SSCs and to form clustering in the testis of an older man (Shinde et al. 2013). Another potential driver mutation rendering the amino acid substitution p.N540K was also enriched in the older donor pool. Note that for this missense mutation we detected two different nucleotide substitutions (c.1620C>A/G). The C>A substitution was found in an older donors library with a frequency of 2.23×10^{-5} and the C>G substitution was detected at a frequency of 6.94×10^{-5}. These substitutions were either lower (1.54×10^{-5}) or absent in the younger donors pool, respectively. These two mutations were also identified in a study measuring prospective mutations enriched in testis biopsies with a high RTK activity (Maher et al. 2018). The p.N540K amino acid substitution is related to the skeletal dysplasia associated with a paternal age effect, the autosomal dominant congenital disorder hypochondroplasia (Bellus et al. 1995; Rousseau et al. 1996a). The higher frequency detected in older donors coupled with an association to a PAE-disorder suggests that the p.N540K substitution might be also a testis-specific mosaic expanding with age.

Apart from the few characterized PAE-candidates, our study identified further mutations that are potentially expanding with paternal age and have not been described before. These occur mainly within or downstream of the TM domain (e.g., p.T394T, p.R399H, p. R421Q, p.A429E) and are associated with a high deleteriousness and tumors (Figure 5). Moreover, they are viable since they have been detected in the general population. Thus, our approach is an important tool to identify prospective mutations expanding with paternal age, but more evidence is required to demonstrate a selective advantage of these variants in the male germline and whether these form testis-specific mosaics with age.

Testis-exclusive mosaics versus post-zygotic mosaicism

Driver mutations and their association with congenital disorders gain a special importance in industrial societies in which parenthood is delayed. The full understanding of driver mutations accumulating in the male germline is key to assess the risks and the impact on future generations. DNMs can occur in adult spermatogonia that are positively selected and are therefore restricted to

the seminiferous tubules forming testis-exclusive mosaics. These are often associated with PAE-disorders; the numbers of mutant sperm increase with paternal age and they can be transmitted and become constitutional mutations in the offspring (Goriely et al. 2003; Qin et al. 2007; Maher et al. 2018; Tiemann-Boege et al. 2020).

In contrast to these PAE-mutations discussed in the previous paragraphs, we also detected 45 mutations with frequencies over 1 x 10^{-5}. These are viable variants found in the human population (reported in gnomAD) and 12 of them have also been associated with tumors (reported in COSMIC). Since they were not detected in multiple libraries and/or their frequency was not higher in older donors, we considered that these might come from a different source: DNMs occurring post-zygotically, but early in development lead to widespread tissue mosaicism where different tissues have different genotypes (Rahbari et al. 2016). A few variants detected in this study could be potential post-zygotic mutations that arose early in development, although we did not have further somatic tissue of the same donors to inspect the presence of the same variant in other tissues (Table 1). Moreover, we noticed a difference in the association with germline disorders and tumors between PZM-candidates and PAE-candidates; although, our mutation deleteriousness analysis did not show any significant differences.

The expansion of PZM variants with age is not yet fully understood. In theory, the fitness of these variants conferred to the host cell should dictate their development over time. Therefore, some mosaic variants might expand clonally, and others might remain at low frequencies. But similar to gonadal-exclusive mosaics, these would also be associated with a high recurrence risk in the offspring. Depending on how early in development they occur, the range and expansion might vary (reviewed in Tiemann-Boege et al. 2020), but a post-zygotic mutation might be widespread and also increase with age (Salk et al. 2019)and (Acuna-Hidalgo et al. 2017). More importantly, if a DNM occurred before the separation of the somatic and gonadal lineages, then these would be equally likely to be found at high levels in younger sperm donor pools or in more pieces of testis biopsies (see such an example in Maher et al. 2018). Yet, in blood, the higher number of aberrant clonal

expansions observed in older individuals is not fully explained by this theory. In the germline, driver mutations expand clonally with age and its frequency is higher in older individuals, but in the case of post-zygotic mutations occurring early in development, this is currently unknown. As reviewed in (Tiemann-Boege et al. 2020), different lineages of a PZM variant might have distinct fates during development. An expansion over time is observed in some tissues, but the opposite can occur in others due to a deleterious effect and subsequent decrease in relative frequency in that particular tissue.

Effect of mutations on the RTK

To date the majority of known PAE mutations affect the RTK or components of its downstream RAS-ERK signaling pathway (FGFR3, HRAS, PTPN11, KRAS, RET, RAF1, PTPN11, BRAF, CBL, MAPK1, MAPK2) (Tiemann-Boege et al. 2002; Qin et al. 2007; Goriely et al. 2009; Choi et al. 2012; Shinde et al. 2013; Maher et al. 2016; Maher et al. 2018). Dysregulation of the FGFR3 pathway drives the preferential expansion of mutant SSCs resulting in mutant micro-clusters in testis observed with DNA-based assays (Qin et al. 2007; Choi et al. 2008; Choi et al. 2012; Shinde et al. 2013; Yoon et al. 2013) or protein markers (Goriely et al. 2003; Giannoulatou et al. 2013; Maher et al. 2016; Maher et al. 2018). The clonal expansion of PAE-mutations with age in the testis results in more mutant sperm in older individuals (Tiemann-Boege et al. 2002; Goriely et al. 2009; Maher et al. 2018), and increased incidence of the PAE-mutation.

PAE-mutations have been called “selfish” since the mutation itself, by a change in the gene product, increases its self-propagation (Goriely and Wilkie 2012). It has been postulated that the expansion of driver mutations in the testis is proportional to the hyper-reactivity of the mutant protein, with larger clusters and more mutant sperm forming with strongly activating mutations compared to mild ones (Goriely et al. 2009; Goriely and Wilkie 2012). However, the transmission of driver mutations to sperm does not always correlate with the *in vitro* activity of the RTK-RAS signaling pathway by the mutation (Giannoulatou et al. 2013), so there is no simple relationship between the activation of the

mutation and the conferred strength of the selective advantage. Moreover, even though the molecular and cellular events caused by driver mutations in the male germline might parallel the events in tumorigenesis in somatic tissue, the mutational spectrum observed in tumors hardly overlaps with symptomatic spontaneous germline mutations (Aoki and Matsubara 2013; Giannoulatou et al. 2013). This difference could be explained by a tissue-specific increase in RTK-RAS signaling pathway activity. Alternatively, the mutations observed in late oncogenic stages in metastatic tissue are usually different from the initial "mild" driver mutations and change dramatically during the course of the tumor development (Sottoriva et al. 2015; Tomasetti and Vogelstein 2015). Or yet, very strong driver mutations could be detrimental in development, as it was shown for the most prevalent cancer mutation in *HRAS,* which was diminished in sperm and completely absent in birth data (Giannoulatou et al. 2013).

Accordingly, of the discovered 21 distinct variants associated with tumors, only 4 might be potential PAE-candidates. Whether the remaining 17 might expand with gonadal age and contribute to viable offspring with a certain disorder or late-onset tumor remains to be tested. Interestingly, 3 out of these 4 mutations are located within or nearby the TM domain of the FGFR3 protein, indicating that mutations affecting this particular domain might have a considerable impact on the protein's activity, even in different tissues.

Not only PAE-mutations causing early-onset diseases (e.g., rasopathies or skeletal dysplasias) have been described to increase with paternal age (reviewed in Arnheim and Calabrese 2009; Goriely et al. 2009; Goriely and Wilkie 2012; Arnheim and Calabrese 2016). Reports have also documented an increased frequency with paternal age of late-onset disorders like breast cancer or cancers in the nervous system (Hemminki and Kyyronen 1999; Hemminki et al. 1999), or neurological and behavioral disorders, including autism (Kong et al. 2012) (Hultman et al. 2011; Frans et al. 2013), bipolar disorders (Frans et al. 2008; Grigoroiu-Serbanescu et al. 2012) or schizophrenia (Svensson et al. 2012), also reviewed in Sharma et al. 2015; Acuna-Hidalgo et al. 2016; Paul and Robaire 2013. Thus, the observed PAE with consequences in neurological, heart, or cancer development, might

also be partially the result of DNMs in driver genes that lead to abnormalities in the signaling pathway (e.g., RTK-RAS). However, in order to better understand these effects, we need to further investigate DNMs in driver genes in the male germline, how these DNMs affect the self-propagation of the mutation, and the nature of their phenotypic consequences.

Conclusions

With the DS approach demonstrated here we detected high frequency mutations in *FGFR3*, enabling an unprecedented view of the mutational landscape of the human male germline. Out of the 161 *FGFR3* exonic variants detected, 9 were found at higher frequency or even solely in older donors, and are distinct from the variants detected in both younger and older donors in terms of origin and phenotypic consequences. Four of them represent newly identified candidates for PAE-mutations, but further characterization is necessary. The analysis of the discovered *FGFR3* variants revealed that these mutations have a high deleteriousness compared to variants segregating in the population. Finally, our work showcases a new and accurate approach for the detection and study of DNMs in the human male germline and sheds light into different mutational mechanisms potentially affecting the receptor kinase activity and the potential risk of fathering a child at an increased age.

Methods

Collection of sperm and testes samples

Sperm DNA from anonymous donors mainly of Central European ancestry aged between 26 to 30 and 49-59 years were pooled using 5 donors per pool; the exact pool composition is shown in Supplemental Table S4. Samples were collected from the Kinderwunsch Klinik, MedCampus IV, Kepler Universitätsklinikum, Linz, following the protocol approved by the ethics commission of

Upper Austria (Approval F1-11). Three human genomic DNA samples encoding the *FGFR3* mutations c.742C>T (NA00711), c.746C>G (NA08909), and c.749C>G (CD00002) were purchased from Coriell Cell Repositories (Camden, NJ). Similarly, genomic DNA with the *FGFR3* c.1620C>A transversion (p.540N>K) was extracted from the B-lymphocyte cell line purchased from the Coriell Cell Repositories (GM18666).

Sample and library preparation

Sperm genomic DNA (gDNA) was extracted using the Gentra Puregene Cell kit (QIAGEN). In short, DNA of 25 µL of semen was extracted as detailed in Supplemental Materials (SM) and prepared further for the library preparation steps. DS was based on previous protocols (Kennedy et al. 2014) with substantial modifications including an initial restriction enzyme digest to pre-select the target regions. We used in the different libraries some modifications to our main protocol either starting with distinct input DNA amounts, different adapters or amplification strategies. Supplemental Table S3 summarizes the differences between each of our library preparation protocols. Genomic DNA (500-5000 ng, Supplemental Table S3) was subject to an overnight restriction enzyme digest that targeted 5 regions of the *FGFR3* gene (Supplemental Table S6). Resulting fragments are expected to be between ~300 to ~600 bp. A double size selection was performed using SPRIselect beads (Beckman Coulter) in order to exclude fragments outside of this size range. We used 3 different adapter synthesis protocols to produce Adapter 1, Adapter 2 and Adapter 3. The details of the protocols used to produce these adapters can be found in SM. Supplemental Figure S3 shows the structural differences between adapters: Adapter 1 is identical to the original open-hairpin structure, whereas adapters 2 and 3 have a closed loop hairpin structure that is opened up after the ligation step by the cleavage of the uracil with the USER enzyme mix (New England Biolabs). It is suggested that closed loops adapters reduce adapter dimers during ligation (New England Biolabs). Structurally, adapter 3 is similar to adapter 2, but is 9 bp longer and contains a phosphorothioate bond before the T-overhang on the 3’ end such that removal of this overhang is reduced (Supplemental Figure S3).

Size selected genomic DNA was end-repaired and A-tailed using the NEBNext® Ultra™ II End Repair/dA-Tailing Module (New England Biolabs) according to the manufacturer's instructions described in SM. DNA fragments with A-3' end overhangs were then ligated to the DS adaptors with T-3' overhangs using the NEBNext® Ultra™ II Ligation Module (New England Biolabs) following the manufacturer's instructions and then purified by 0.8 volumes of AMPure XP beads (Beckman Coulter).

Amplification of ligated DNA was executed using KAPA HiFi HotStart ReadyMix (KAPA Biosystems). Except for 4 libraries, a strategy of 12 cycles of single primer extensions was adopted before the PCR reaction in order to get multiple copies of the initial genomic template to improve the representation of both forward and reverse SSCS and prevent the exponential amplification of templates resulting in very large family sizes. Reaction volumes and PCR conditions are described in SM and Supplemental Table S7. Primer sequences are shown in Supplemental Table S8. After the extension/PCR step, PCR products were purified with AMPure XP beads followed by 2-3 rounds of targeted capture steps to enrich further for templates of interest. A third targeted capture was done for 3 of the libraries using the same procedure as the second one. The number of cycles of both post-capture PCRs varied across libraries (Supplemental Table S3). Pools were verified using Fragment Analyzer HS NGS (Agilent) as described in more detail in SM.

Libraries were diluted and pooled for sequencing according to the concentration of dsDNA measured with DeNovix® dsDNA High Sensitivity. Finally, the libraries were subject to further quantification with the KAPA Library Quantification kit (KAPA Biosystems). Sequencing reactions were performed on the MiSeq Illumina platform using the kit MiSeq Reagent v3 600 cycles (Illumina) at the Center for Medical Research of the Johannes Kepler University and at the VBCF NGS Unit.

Data processing and variant filtering

FastQ files were analyzed in Galaxy (in both public (usegalaxy.org) and private (zusie.jku.at) servers)) according to a DS specific pipeline that includes an error correction tool (Stoler et al. 2020). Variants

(substitutions only) were further inspected and assigned to tiers using the Variant Analyzer (Povysil et al. 2021). Every variant was categorized as a SNP if detected in all libraries that shared the same donor pool/target region combination with a frequency ~10%. Variants with DCS coverage below 1000, intronic variants and SNPs were discarded from our analysis and only exonic variants of Tier 1 were kept, together with Tier 2 that were detected more than once. For more details on this analysis see (Povysil et al. 2021). Selected variants were annotated using Variant Effect Predictor (VEP) (McLaren et al. 2016) and wANNOVAR (Yang and Wang 2015). The variant frequency was calculated by dividing the number of DCS calling the variant by the DCS coverage at the position of the variant within the library it was detected.

Available data on the *FGFR3* variants were extracted from gnomAD v2.1.1 (transcript ENST00000440486.2, https://gnomad.broadinstitute.org) (Karczewski et al. 2020) and COSMIC v90 (transcript ENST00000440486.7, https://cancer.sanger.ac.uk) (Tate et al. 2018) databases on January 23rd 2020 and January 21st 2020, respectively. Variants from gnomAD were remapped from GRCh37/hg19 to GRCh38/hg38 using NCBI Genome Remapping Service (https://www.ncbi.nlm.nih.gov/genome/tools/remap). Exonic single-nucleotide substitutions retrieved from gnomAD and COSMIC were annotated with VEP and wANNOVAR. Deleteriousness analysis was performed using CADD raw scores (Rentzsch et al. 2018) extracted from VEP annotation. Pairwise comparison between every group of variants was done with the pairwise Mann-Whitney U test. Germline association was investigated using ClinVar data extracted from wANNOVAR output. Tumor association was investigated by consulting the presence or absence of variants in the extracted COSMIC data.

Data Access

Raw sequencing data is available at the NCBI Sequence Read Archive (BioProject ID: PRJNA684907).

No Competing interests

The authors declare no competing interests.

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Authors' contributions: I.T.-B. conceived the research; R.S., B.A, M.I, I.H., and I.T.-B. designed the experiments; R.S, S.M, T.M, A.H. and M.I performed the experiments; T.E., O.S, J.P. and I.T.-B. contributed samples, new reagents/analytic tools/data; R.S. and M.H. analyzed the data; and R.S. and I.T.-B. wrote the paper. All authors read and approved the final manuscript.

References

Acuna-Hidalgo R, Sengul H, Steehouwer M, van de Vorst M, Vermeulen SH, Kiemeney L, Veltman JA, Gilissen C, Hoischen A. 2017. Ultra-sensitive Sequencing Identifies High Prevalence of Clonal Hematopoiesis-Associated Mutations throughout Adult Life. *Am J Hum Genet* **101**: 50-64.

Acuna-Hidalgo R, Veltman JA, Hoischen A. 2016. New insights into the generation and role of de novo mutations in health and disease. *Genome Biology* **17**.

1 Ahn EH, Hirohata K, Kohrn BF, Fox EJ, Chang CC, Loeb LA. 2015. Detection of Ultra-Rare
2 Mitochondrial Mutations in Breast Stem Cells by Duplex Sequencing. *PLoS One* **10**:
3 e0136216.
4 Aoki Y, Matsubara Y. 2013. Ras/MAPK syndromes and childhood hemato-oncological diseases. *Int J*
5 *Hematol* **97**: 30-36.
6 Arbeithuber B, Makova KD, Tiemann-Boege I. 2016. Artifactual mutations resulting from DNA lesions
7 limit detection levels in ultrasensitive sequencing applications. *DNA Res* **23**: 547-559.
8 Arnheim N, Calabrese P. 2009. Understanding what determines the frequency and pattern of human
9 germline mutations. *Nat Rev Genet* **10**: 478-488.
10 Arnheim N, Calabrese P. 2016. Germline Stem Cell Competition, Mutation Hot Spots, Genetic
11 Disorders, and Older Fathers. *Annu Rev Genomics Hum Genet* **17**: 219-243.
12 Bellus GA, McIntosh I, Smith EA, Aylsworth AS, Kaitila I, Horton WA, Greenhaw GA, Hecht JT,
13 Francomano CA. 1995. A recurrent mutation in the tyrosine kinase domain of fibroblast
14 growth factor receptor 3 causes hypochondroplasia. *Nature Genetics* **10**: 357-359.
15 Blankenberg D, Von Kuster G, Bouvier E, Baker D, Afgan E, Stoler N, Taylor J, Nekrutenko A, Galaxy T.
16 2014. Dissemination of scientific software with Galaxy ToolShed. *Genome Biology* **15**: 403.
17 Boulanger J, Muresan L, Tiemann-Boege I. 2012. Massively parallel haplotyping on microscopic beads
18 for the high-throughput phase analysis of single molecules. *PLoS One* **7**: e36064.
19 Campbell CD, Eichler EE. 2013. Properties and rates of germline mutations in humans. *Trends Genet*
20 **29**: 575-584.
21 Choi SK, Yoon SR, Calabrese P, Arnheim N. 2008. A germ-line-selective advantage rather than an
22 increased mutation rate can explain some unexpectedly common human disease mutations.
23 *Proceedings of the National Academy of Sciences of the United States of America* **105**:
24 10143-10148.

Choi SK, Yoon SR, Calabrese P, Arnheim N. 2012. Positive selection for new disease mutations in the human germline: evidence from the heritable cancer syndrome multiple endocrine neoplasia type 2B. *PLoS genetics* **8**: e1002420.

Crow JF. 2000. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* **1**: 40-47.

Crow JF. 2012. Upsetting the dogma: germline selection in human males. *PLoS Genet* **8**: e1002535.

Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. 2011. Easy sperm processing technique allowing exclusive accumulation and later usage of DNA-strandbreak-free spermatozoa. *Reprod Biomed Online* **22**: 37-43.

Fox EJ, Reid-Bayliss KS, Emond MJ, Loeb LA. 2014. Accuracy of Next Generation Sequencing Platforms. *Next generation, sequencing & applications* **1**: 1000106.

Francioli LC, Polak PP, Koren A, Menelaou A, Chun S, Renkens I, van Duijn CM, Swertz M, Wijmenga C, van Ommen G et al. 2015. Genome-wide patterns and properties of de novo mutations in humans. *Nature Genetics* **47**: 822-+.

Frans EM, Sandin S, Reichenberg A, Langstrom N, Lichtenstein P, McGrath JJ, Hultman CM. 2013. Autism risk across generations: a population-based study of advancing grandpaternal and paternal age. *JAMA Psychiatry* **70**: 516-521.

Frans EM, Sandin S, Reichenberg A, Lichtenstein P, Långström N, Hultman CM. 2008. Advancing paternal age and bipolar disorder. *Arch Gen Psychiatry* **65**: 1034-1040.

Giannoulatou E, McVean G, Taylor IB, McGowan SJ, Maher GJ, Iqbal Z, Pfeifer SP, Turner I, Burkitt Wright EM, Shorto J et al. 2013. Contributions of intrinsic mutation rate and selfish selection to levels of de novo HRAS mutations in the paternal germline. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 20152-20157.

Goriely A, Hansen RM, Taylor IB, Olesen IA, Jacobsen GK, McGowan SJ, Pfeifer SP, McVean GA, Rajpert-De Meyts E, Wilkie AO. 2009. Activating mutations in FGFR3 and HRAS reveal a

shared genetic origin for congenital disorders and testicular tumors. *Nat Genet* **41**: 1247-1252.

Goriely A, McVean GA, Röjmyr M, Ingemarsson B, Wilkie AO. 2003. Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. *Science* **301**: 643-646.

Goriely A, Wilkie AO. 2012. Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. *Am J Hum Genet* **90**: 175-200.

Grigoroiu-Serbanescu M, Wickramaratne PJ, Mihailescu R, Prelipceanu D, Sima D, Codreanu M, Grimberg M, Elston RC. 2012. Paternal age effect on age of onset in bipolar I disorder is mediated by sex and family history. *Am J Med Genet B Neuropsychiatr Genet* **159B**: 567-579.

Hemminki K, Kyyronen P. 1999. Parental age and risk of sporadic and familial cancer in offspring: implications for germ cell mutagenesis. *Epidemiology* **10**: 747-751.

Hemminki K, Kyyronen P, Vaittinen P. 1999. Parental age as a risk factor of childhood leukemia and brain cancer in offspring. *Epidemiology* **10**: 271-275.

Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. 2013. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. *Genome Res* **23**: 843-854.

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC et al. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical chemistry* **83**: 8604-8610.

Hultman CM, Sandin S, Levine SZ, Lichtenstein P, Reichenberg A. 2011. Advancing paternal age and risk of autism: new evidence from a population-based study and a meta-analysis of epidemiological studies. *Mol Psychiatry* **16**: 1203-1212.

Jabara CB, Jones CD, Roach J, Anderson JA, Swanstrom R. 2011. Accurate sampling and deep sequencing of the HIV-1 protease gene using a Primer ID. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 20166-20171.

Karczewski KJ Francioli LC Tiao G Cummings BB Alföldi J Wang Q Collins RL Laricchia KM Ganna A Birnbaum DP et al. 2020. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* **581**: 434-443.

Kennedy SR, Salk JJ, Schmitt MW, Loeb LA. 2013. Ultra-sensitive sequencing reveals an age-related increase in somatic mitochondrial mutations that are inconsistent with oxidative damage. *PLoS genetics* **9**: e1003794-e1003794.

Kennedy SR, Schmitt MW, Fox EJ, Kohrn BF, Salk JJ, Ahn EH, Prindle MJ, Kuong KJ, Shen JC, Risques RA et al. 2014. Detecting ultralow-frequency mutations by Duplex Sequencing. *Nat Protoc* **9**: 2586-2606.

Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, Gudjonsson SA, Sigurdsson A, Jonasdottir A, Jonasdottir A et al. 2012. Rate of de novo mutations and the importance of father's age to disease risk. *Nature* **488**: 471-475.

Krimmel JD, Schmitt MW, Harrell MI, Agnew KJ, Kennedy SR, Emond MJ, Loeb LA, Swisher EM, Risques RA. 2016. Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic TP53 mutations in noncancerous tissues. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 6005-6010.

Loeb LA, Kohrn BF, Loubet-Senear KJ, Dunn YJ, Ahn EH, O'Sullivan JN, Salk JJ, Bronner MP, Beckman RA. 2019. Extensive subclonal mutational diversity in human colorectal cancer and its significance. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 26863-26872.

Lou DI, Hussmann JA, McBee RM, Acevedo A, Andino R, Press WH, Sawyer SL. 2013. High-throughput DNA sequencing errors are reduced by orders of magnitude using circle sequencing. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 19872-19877.

Maher GJ, McGowan SJ, Giannoulatou E, Verrill C, Goriely A, Wilkie AO. 2016. Visualizing the origins of selfish de novo mutations in individual seminiferous tubules of human testes. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 2454-2459.

Maher GJ, Ralph HK, Ding Z, Koelling N, Mlcochova H, Giannoulatou E, Dhami P, Paul DS, Stricker SH, Beck S et al. 2018. Selfish mutations dysregulating RAS-MAPK signaling are pervasive in aged human testes. *Genome Res* **28**: 1779-1790.

McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, Flicek P, Cunningham F. 2016. The Ensembl Variant Effect Predictor. *Genome Biology* **17**.

Nachmanson D, Lian S, Schmidt EK, Hipp MJ, Baker KT, Zhang Y, Tretiakova M, Loubet-Senear K, Kohrn BF, Salk JJ et al. 2018. Targeted genome fragmentation with CRISPR/Cas9 enables fast and efficient enrichment of small genomic regions and ultra-accurate sequencing with low DNA input (CRISPR-DS). *Genome Res* **28**: 1589-1599.

O'Roak BJ, Vives L, Fu W, Egertson JD, Stanaway IB, Phelps IG, Carvill G, Kumar A, Lee C, Ankenman K et al. 2012. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. *Science* **338**: 1619-1622.

Paul C, Robaire B. 2013. Ageing of the male germ line. *Nat Rev Urol* **10**: 227-234.

Povysil G, Heinzl M, Salazar R, Stoler N, Nekrutenko A, Tiemann-Boege I. 2021. Increased yields of duplex sequencing data by a series of quality control tools. *NAR Genomics and Bioinformatics* **3**.

Qin J, Calabrese P, Tiemann-Boege I, Shinde DN, Yoon SR, Gelfand D, Bauer K, Arnheim N. 2007. The molecular anatomy of spontaneous germline mutations in human testes. *PLoS biology* **5**: e224.

Rahbari R, Wuster A, Lindsay SJ, Hardwick RJ, Alexandrov LB, Turki SA, Dominiczak A, Morris A, Porteous D, Smith B et al. 2016. Timing, rates and spectra of human germline mutation. *Nature genetics* **48**: 126-133.

1 Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. 2018. CADD: predicting the
2 deleteriousness of variants throughout the human genome. *Nucleic Acids Research* **47**:
3 D886-D894.
4 Risch N, Reich EW, Wishnick MM, McCarthy JG. 1987. Spontaneous mutation and parental age in
5 humans. *Am J Hum Genet* **41**: 218-248.
6 Rousseau F, Bonaventure J, Legeai-Mallet L, Pelet A, Rozet J-M, Maroteaux P, Merrer ML, Munnich A.
7 1994. Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia.
8 *Nature* **371**: 252-254.
9 Rousseau F, Bonaventure J, Legeai-Mallet L, Schmidt H, Weissenbach J, Maroteaux P, Munnich A, Le
10 Merrer M. 1996a. Clinical and genetic heterogeneity of hypochondroplasia. *Journal of*
11 *Medical Genetics* **33**: 749-752.
12 Rousseau F, El Ghouzzi V, Delezoide AL, Legeai-Mallet L, Le Merrer M, Munnich A, Bonaventure J.
13 1996b. Missense FGFR3 Mutations Create Cysteine Residues in Thanatophoric Dwarfism
14 Type I (TD1). *Human Molecular Genetics* **5**: 509-512.
15 Salk JJ, Loubet-Senear K, Maritschnegg E, Valentine CC, Williams LN, Higgins JE, Horvat R,
16 Vanderstichele A, Nachmanson D, Baker KT et al. 2019. Ultra-Sensitive TP53 Sequencing for
17 Cancer Detection Reveals Progressive Clonal Selection in Normal Tissue over a Century of
18 Human Lifespan. *Cell Reports* **28**: 132-144.e133.
19 Salk JJ, Schmitt MW, Loeb LA. 2018. Enhancing the accuracy of next-generation sequencing for
20 detecting rare and subclonal mutations. *Nature Reviews Genetics* **19**: 269-285.
21 Schmitt MW, Fox EJ, Prindle MJ, Reid-Bayliss KS, True LD, Radich JP, Loeb LA. 2015. Sequencing small
22 genomic targets with high efficiency and extreme accuracy. *Nat Methods* **12**: 423-425.
23 Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA. 2012. Detection of ultra-rare mutations
24 by next-generation sequencing. *Proceedings of the National Academy of Sciences of the*
25 *United States of America* **109**: 14508-14513.

Sharma R, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF. 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol* **13**: 35.

Shen JC, Kamath-Loeb AS, Kohrn BF, Loeb KR, Preston BD, Loeb LA. 2019. A high-resolution landscape of mutations in the BCL6 super-enhancer in normal human B cells. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 24779-24785.

Shinde DN, Elmer DP, Calabrese P, Boulanger J, Arnheim N, Tiemann-Boege I. 2013. New evidence for positive selection helps explain the paternal age effect observed in achondroplasia. *Human molecular genetics* **22**: 4117-4126.

Sottoriva A, Kang H, Ma Z, Graham TA, Salomon MP, Zhao J, Marjoram P, Siegmund K, Press MF, Shibata D et al. 2015. A Big Bang model of human colorectal tumor growth. *Nat Genet* **47**: 209-216.

Stoler N, Arbeithuber B, Guiblet W, Makova KD, Nekrutenko A. 2016. Streamlined analysis of duplex sequencing data with Du Novo. *Genome Biol* **17**: 180.

Stoler N, Arbeithuber B, Povysil G, Heinzl M, Salazar R, Makova KD, Tiemann-Boege I, Nekrutenko A. 2020. Family reunion via error correction: an efficient analysis of duplex sequencing data. *Bmc Bioinformatics* **21**.

Svensson AC, Lichtenstein P, Sandin S, Öberg S, Sullivan PF, Hultman CM. 2012. Familial aggregation of schizophrenia: the moderating effect of age at onset, parental immigration, paternal age and season of birth. *Scand J Public Health* **40**: 43-50.

Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, Boutselakis H, Cole CG, Creatore C, Dawson E et al. 2018. COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Research* **47**: D941-D947.

Tiemann-Boege I, Mair T, Yasari A, Zurovec M. 2020. Pathogenic postzygotic mosaicism in the tyrosine receptor kinase pathway: potential unidentified human disease hidden away in a few cells. *The FEBS Journal* **n/a**.

Tiemann-Boege I, Navidi W, Grewal R, Cohn D, Eskenazi B, Wyrobek AJ, Arnheim N. 2002. The observed human sperm mutation frequency cannot explain the achondroplasia paternal age effect. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 14952-14957.

Tomasetti C, Vogelstein B. 2015. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **347**: 78-81.

Wilcox WR, Tavormina PL, Krakow D, Kitoh H, Lachman RS, Wasmuth JJ, Thompson LM, Rimoin DL. 1998. Molecular, radiologic, and histopathologic correlations in thanatophoric dysplasia. *Am J Med Genet* **78**: 274-281.

Winters M, Monroe C, Barta JL, Kemp BM. 2017. Are we fishing or catching? Evaluating the efficiency of bait capture of CODIS fragments. *Forensic Science International-Genetics* **29**: 61-70.

Yang H, Wang K. 2015. Genomic variant annotation and prioritization with ANNOVAR and wANNOVAR. *Nature Protocols* **10**: 1556-1566.

Yoon SR, Choi SK, Eboreime J, Gelb BD, Calabrese P, Arnheim N. 2013. Age-dependent germline mosaicism of the most common noonan syndrome mutation shows the signature of germline selection. *Am J Hum Genet* **92**: 917-926.